# MOLECULAR BIOLOGY OF MUSCLE DEVELOPMENT

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April 11 - 17, 1994; Snowbird, Utah

*Co-Sponsored by The Muscular Dystrophy Association and The Director's Sponsors Fund*


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*Somitogenesis*

**W 001** MYOGENIC LINEAGES IN MOUSE SOMITES AND LIMB BUDS. G. Cossu[1,2] S. Tajbakhsh[2], G. Cusella-De Angelis[1], R. Kelly[2], E. Vivarelli[1] and M. Buckingham[2]. [1]Istituto di Istologia, Università di Roma, [2]Dépt. Biologie Moléculaire, Institut Pasteur, Paris.

Myotome formation in mammals is still poorly understood both in terms of commitment of mesodermal cells and lineage relationships among different myoblasts. We have investigated the role of the neural tube in regulating commitment of these early myogenic cells in relation to possible "community effects". To this end we have isolated cells from embryos carrying an MLC3F-nlsLacZ transgene (which is expressed in all differentiated muscle cells since 9.5 d.p.c.) and have challenged their differentiation potential under different experimental conditions. The results obtained show that at the onset of somitogenesis, mesodermal cells need to be surrounded by sister cells in order to activate a myogenic program. They fail to differentiate if surrounded by other cell types, including neural tube cells. At later stages (somites V to XV) single paraxial mesodermal cells can give rise to myogenic clones, but only on a feeder layer of other mesodermal derivatives; interestingly the frequency and the size of myogenic clones is higher on muscle cell feeder layers than on fibroblasts, while the opposite is true for myogenic cells isolated from early limb buds. It is only at this stage that the neural tube exerts its effect on medial but not on lateral somitic cells. These data suggest the existence of several distinct steps in the specification of a paraxial mesodermal cell to the myogenic lineage. After commitment, myogenic cells migrate ventrally from the dermamyotome and differentiate into mononucleated post-mitotic myocytes. These early myotomal cells do not express detectable levels of MyoD or myogenin proteins, in contrast to all successive generations of myoblasts. Since *Myf*-5, the first of the myogenic factors to appear during development, is expressed in cells lying along the dorso-medial edge of the dermamyotome (adjacent to the neural tube), we hypothesized that these cells are the direct precursors of myotomal cells. Experiments will be described aimed at establishing the relationships between different populations of myoblasts located in the medial half of the early somite which will likely contribute to epaxial muscles. Furthermore we observed that a very small but reproducible fraction of cells derived from the neural tube undergoes muscle differentiation *in vitro*, suggesting lineage promiscuity among early neuroectoderm and paraxial mesoderm.

**W 002** CONTROL OF SOMITIC DIFFERENTIATION BY AXIAL STRUCTURES, Olivier Pourquié, Monique Coltey, Marie-Aimée Teillet, Charles P. Ordahl[1], and Nicole M. Le Douarin, Institut d'Embryologie du CNRS et du Collège de France, 49 bis avenue de la Belle Gabrielle, 94736 Nogent sur Marne Cedex, France, [1]Department of Anatomy, School of Medicine, University of California at San Francisco, CA 94116, USA.

In the vertebrate embryo, one manifestation of anteroposterior polarity is the segmentation of the mesenchymal paraxial mesoderm into epithelial balls, the somites. In higher vertebrates such as birds or mammals, the somites become secondarily polarized along the dorso-ventral axis into a dorsal epithelial component : the dermomyotome, and a ventral mesenchymal component : the sclerotome. During its differentiation, the paraxial mesoderm is in close contact with the notochord, an axial mesodermal structure playing a critical role in establishing the dorso-ventral polarity of the neural tube. We have examined the inductive properties of the notochord and the floor plate on the differentiation of the paraxial mesoderm in the chick embryo. We have shown that implantation of a notochord between the neural tube and the unsegmented paraxial mesoderm does not alter somite segmentation along the rostrocaudal axis, nor does it prevent the formation of its anterior and posterior compartments. In contrast, grafted notochord and floor plate were able to profoundly modify the segregation of the somite in its dorsal and ventral components. In the operated embryos, the dorsal derivatives of the somites (dermomyotome, axial muscles and dermis) had completely disappeared and the somite develops exclusively into ventral derivatives, i.e., cartilage (1). Moreover, when the notochord is removed prior to somite segmentation, the somites develop exclusively into dorsal derivatives such as muscles. We infer from these results that notochord acts as a primary ventral inducer controlling the dorsoventral polarization of the somite and that the dorsal differentiation constitutes a default pathway taken by somitic cells that escape the influence of the ventral axial structures.

(1) Pourquié, O., Coltey, M., Teillet, M.-A., Ordahl, C.P., and Le Douarin N.M., Control of dorso-ventral patterning of somitic derivatives by notochord and floor plate. (1993). Proc. Natl. Acad. Sci. USA, (90) 5242-5246.

**W 003** PROGRAMMED DEATH OF AXIAL MUSCLE PRIMORDIA IN SOMITES, Kathryn W. Tosney, Department of Biology, The University of Michigan, Ann Arbor.

Lateral dermatomes and myotomes are the primordia of lateral body wall muscles which develop in thoracic but not in limb segments. To analyze segment-specific fate and interactions controlling fate of these primordia, I used serial plastic sections, electron microscopy, and embryonic surgery in chick embryos.

Surprisingly, I found that lateral muscle primordia develop in both thoracic and limb segments, but then die at limb levels. The segment-specific fate is first signaled at stages 21-23 by different activities in the dermatomes. In thoracic segments, each dermatome forms a specialized structure, the "cap", by curving inward to surround the lateral edge of the myotome. Within a cap, myocytes increase in number, and the primordium grows into the lateral body wall. In contrast, in limb segments, lateral dermatomes fail to form a cap. Instead, the dermatome cells die and are phagocytized. New myocytes cease to be generated and remaining myocytes are later phagocytized. Since alterations in dermatomes presage both fates (death or maturation), the dermatome (rather than the myotome) appears to control morphogenesis of these muscle primordia.

Embryonic surgeries identify an interaction that specifies segment-specific fate: death requires an interaction with limb tissue. Deleting a hindlimb at stage 17 saves the primordia. When the limb is absent, limb-level dermatomes fail to die; instead they form caps and myocytes increase. Even reducing proximal limb tissues will partially save primordia. In contrast, when a limb is transplanted to thoracic levels, the thoracic primordia die. Surprisingly, primordia do not require a trophic interaction with body wall, neural tube, or nerves, since fate is unchanged when these structures are surgically removed at stage 17. Thus, differential access to trophic interactions fails to explain the differential fate of these primordia. Instead, a "killing" interaction with limb tissue programs the death of adjacent primordia.

**W 004** *Abstract Withdrawn*

*Myogenic Cell Lineages*

**W 005** MYOGENIC FACTORS DURING MOUSE EMBRYOGENESIS: EXPRESSION OF *MYF*-5 IN THE NEURONAL CELL LINEAGE.

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Each of the four members of the MyoD family of myogenic factors has a distinct pattern of expression during the formation and maturation of skeletal muscle (1). *Myf*-5 is expressed first and the introduction of LacZ into the *myf*-5 locus by homologous recombination has permitted us to follow with greater cellular resolution the expression of this marker (2) in somitic and presomitic precursor cells of axial and peripheral musculature (3). Examination of the *Myf*-5/LacZ mice also indicated unexpected expression in cells of the central nervous system. In the embryonic brain, the *myf*-5 gene is transcribed in areas of both the mesencephalon and diencephalon, expression becoming restricted to a specific region of the hypothalamus later in development. Occasional cells in the neural tube are also positive. In embryoid bodies formed by differentiating embryonic stem cells in culture, cells positive for neuronal markers and for *myf*-5 are present from an early stage. When embryonic cells are cultured *in vitro*, neural tube cells, co-expressing the neuronal marker ß-tubulin III and *myf*-5 will differentiate into muscle whereas similarly marked cells from the embryonic midbrain do not. The expression of other myogenic factors in these cells, together with the general question of the interplay between myogenic and neurogenic lineages will be discussed.

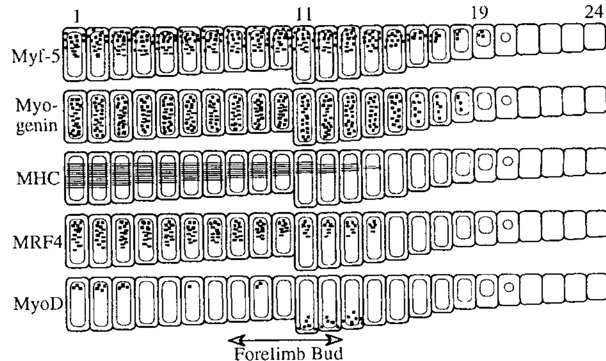
1) Buckingham, M. (1992) TIG **8**, 144-149.
2) Buckingham, M. & Tajbakhsh, S. (1993) C.R. Acad. Sci. Paris Life Sciences **316**, 1040-1046.
3) Tajbakhsh, S. & Buckingham, M. (1993) Proc. Natl. Acad. Sci. USA in press (January 94).

**W 006** TEMPORAL PATTERNS OF MUSCLE DEVELOPMENT IN THE CHICK LIMB, Cynthia Lance-Jones, Department of Neurobiology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261

Cell tracking studies and *in ovo* manipulation experiments have been used to elucidate mechanisms of muscle pattern formation in the hindlimb of chick embryos. Prior studies have shown that the precursors of hindlimb muscle cells migrate from the lumbosacral (LS) somites into the early limb bud beginning on embryonic day 2 (E2). By E8, these cells have formed primary myotubes arranged in adult-like patterns of muscles and muscle fiber types. We have examined the relationship between the time that a muscle cell precursor leaves an LS somite and that cell's fate with respect to limb position and fiber type. If cells with different fates have different temporal patterns of migration and/or settling in the limb, then one can focus more sharply on when and where cells may be responding to patterning information. To characterize early patterns of migration, we injected the lipophilic dye, DiI, into individual LS somites just prior to the onset of migration. At E2-4, maps were made of the positions of labeled cells. There was a clear somatotopic relationship between a labeled cell's anteroposterior position in the limb bud and its somitic level of origin, but no obvious temporal pattern of migration from different LS somites. Cells originating from both anterior and posterior somites appeared to reach different regions of the limb at roughly the same time. To examine temporal patterns of actual muscle formation, we removed chick limb buds at different stages during the migration period and placed them within the coelomic cavity to prevent further population by somitic cells. Analysis of these limb buds at E8 with antibodies to fast and slow isoforms of myosin heavy chain (kindly provided by Crow and Stockdale) indicated that early migrating muscle cell precursors contribute predominantly to muscles that normally contain large numbers of slow fibers. Compatible results were obtained in quail-chick limb bud chimeras. One possible explanation for the above findings is that there is a temporal change in the limb environment leading to a change in the development of different types of muscle cells. Alternatively, there may be a change in the population of muscle cell precursors prior to the entrance of these cells into the limb bud. The results of three different experimental manipulations suggest the latter possibility. When a young limb bud is placed adjacent to older somites, either by transplanting a young limb bud onto an older embryo or by shifting the limb bud anteriorly, a specific depletion of slow muscle fibers is seen at E8. In contrast when an older pair of somites is replaced with a younger pair, some muscles in the adjacent limb show an apparent increase in slow fibers. These findings suggest that the population of precursor cells giving rise to limb muscle is heterogeneous prior to the time that cells migrate into the limb bud. Supported by NIH HD25676.

**W 007** SOMITES, LINEAGES, AND DOMAINS OF MRF PROTEIN EXPRESSION. Jeffrey Boone Miller and Timothy H. Smith. Neuromuscular Laboratory, Massachusetts Gen. Hosp., Charlestown MA 02129, and Neuroscience Program, Harvard Med. Sch.

We show by immunohistology that distinct expression patterns of the four muscle regulatory factor (MRF) proteins identify subdomains of mouse somites. Myf-5 and MyoD are, at specific stages, each expressed in myotome and dermatome cells. Myf-5 is initially expressed in dorsal cells in all somites, as is MyoD in neck somites, whereas MyoD is initially expressed in ventral cells in trunk somites. A few sclerotome cells in forelimb somites also express Myf-5. Myogenin and MRF4 are limited to myotome cells, though myogenin⁺ cells are always found throughout the myotome, whereas MRF4⁺ cells are initially less widely distributed. The figure summarizes MRF and myosin heavy chain (MHC) protein expression patterns in a 24 somite (E9.5) embryo. At this stage, each MRF protein has a distinct expression domain in every somite. Note that, in the most caudal somites, Myf-5 expression is limited to the dorsal-anterior quadrant, whereas myogenin, though also limited to the anterior half, is found throughout the dorsal-ventral extent of the myotome. By E10.5 (35 somites), Myf-5 is down-regulated in rostral somites, and differences in MRF expression patterns are limited to the 2 or 3 most caudal MRF-expressing somites. The distinct expression patterns of the four MRF proteins may distinguish multiple myogenic cell lineages in the somites and suggest that skeletal muscle cells in somites originate at multiple sites and via multiple molecular pathways. (Support by NIH, AHA, MDA)




**W 008** **BOTH THE NEURAL TUBE AND THE NOTOCHORD ESTABLISH DIVERSE LINEAGES WHEN THEY INDUCE MYOGENESIS WITHIN SOMITES OF THE VERTEBRATE EMBRYO** Frank E. Stockdale and Nicholas Buffinger, Stanford University, School of Medicine, Stanford CA 94305-5306

The skeletal myogenic cell lineage is first established in the somites of the early vertebrate embryo. There is a spacial and temporal pattern to myogenesis within somites along the axis of the embryo. Individual somites, the neural tube, and notochord can be assessed in explant cultures to determine the mechanisms that establish these patterns. We find that specification of unspecified somites (somites that do not autonomously form muscle fibers when cultured alone) to a myogenic fate is dependent upon their interaction with either the notochord or neural tube. Contact between somites and notochord or neural tube is not required for initiation of somitic myogenesis. Both these axial structures produce diffusible factors that positively facilitate muscle cell differentiation within somites which without this interaction express myogenic regulatory factors, but do not form muscle fibers. The notochord, as well as the neural tube diffusible factor can initiate muscle fiber-type diversity, characterized by the formation of both fast, and fast and slow myosin-expressing muscle fibers. There are both positive and negative factors that modulate the myogenic lineage in somites as neural tube, unlike the notochord, has a myogenic inhibitory as well as an inducing action on somites. Thus it appears that the skeletal myogenic lineage begins within cells of the axial mesoderm that express myogenic regulatory factors prior to segmentation of the mesoderm into somites; the neural tube and notochord sustain and modulate their commitment to myogenesis as these mesodermal cells are segmented into somites; and in so doing establish both a fast fiber and a fast/slow fiber lineage among the first muscles of the embryo.

*Patterning*

**W 009** THE ROLE OF PAX GENES IN MAMMALIAN DEVELOPMENT Peter Gruss[1], Patrick Tremblay[1], Eva Bober[2], Hans-Henning Arnold[2] and Ahmed Mansouri[1], [1]Max Planck Institute of Biophysical Chemistry, Dept. of Mol. Cell Biol., Goettingen Germany. [2]Dept. of Cell and Mol. Biology, Inst. Biochem. and Biotech., Braunschweig, Germany.

In order to study the role of Pax genes, we have examined loss of function mutations. *Pax-3* could be correlated with a pre-existing mutant, *splotch* (*Sp*). This mutant exhibits e.g. exencephaly, spina bifida and partial lack of spinal ganglia. Furthermore, our data indicate that *Pax-3* interferes with migrating myoblasts required to colonize forelimbs and hindlimbs. These data explain the muscle deficiency observed in *splotch* mice and indicate that *Pax-3* could not only be a good marker for migrating myoblasts, but in addition is a prerequisite for the proper specification, proliferation and/or migration of these precursor cells. For *Pax-3* a human homologue has been identified and individuals with mutations in this gene exhibit the Waardenburg Syndrome. Consequently, this gene allows not only the study of developmental processes, it also enables us to gain insights into the molecular pathology of human syndromes. Besides *Pax-3*, loss of function phenotypes of *Pax-6* (*Small-eye*) and *Pax-7* will be reported. *Small-eye* is a naturally occurring mutant and data will be presented analysing in particular the dysgenesis of certain brain structures. *Pax-7* has been inactivated by homologous recombination. The phenotype of *Pax-7* homozygous mice will be reported.

**W 010** **THE NEURAL TUBE PROMOTES MYOTOME FORMATION IN EARLY SOMITES *IN VITRO*.** **Andrea Münsterberg and Andrew Lassar, Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, 240 Longwood Ave., Boston, MA 02115.**

In vertebrates, all skeletal muscle cells arise from an embryonic structure termed the somite . We are interested in cell specification and commitment in the developing somite. Inductive interactions of the developing chick somite with neighboring axial tissues were monitored after *in vitro* co-culture of the somite with adjacent neural tube/notochord complex. Somites cultured in the absence of these axial tissues express neither the myogenic HLH regulators (MyoD and myogenin) nor myosin heavy chain. In contrast, somites cultured in the presence of the neural tube/notochord complex activate both the expression of the myogenic HLH genes and myosin heavy chain. We propose that the axial tissues play an inductive role in myogenic determination in embryogenesis. We have found that skeletal muscle inducing activity is localized to the neural tube. In addition we will present evidence that there are regional differences in the neural tube with respect to its capacity to induce skeletal muscle.

**W 011** WNT-GENES IN THE REGULATION OF MESODERMAL DEVELOPMENT, S. Takada[1], J. McMahon[1], K. Stark[2] and A. McMahon[1] [1]Harvard University, Biological Laboratories, 16 Divinity Avenue, Cambridge, Massachusetts 02138, [2] Amgen, Inc. 1840 De Havilland Drive, Thousand Oaks, California 91320

Ectopic expression of several members of the *Wnt*-family of putative signaling molecules in the early Xenopus embryo has implicated *Wnt*-signaling in the regulation of mesodermal patterning. To address this issue in the mouse, we have examined twelve members of the *Wnt*-gene family for expression in the gastrulating embryo. Three of these *Wnt-3a, Wnt-5a* and *Wnt-5b* - are expressed in different regions of the gastrulation stage egg-cylinder. Expression of *Wnt-5a* and *Wnt-5b* is large confined to cells fated to generate extraembryonic mesoderm of the amnion and yolk sac. In contrast, *Wnt-3a* is expressed in cells fated to generate embryonic, principally somitic, mesoderm. Inactivation of the *Wnt-3a* locus by gene targeting leads to a loss of somites at a precise axial level, whereas notochordal and lateral mesoderm is largely unaffected. Thus, *Wnt-3a* may regulate somite cell fate. In addition, *Wnt-3a* is required for establishment of the tail bud. In mutant embryos the entire axis is truncated anterior to the hindlimbs, due to a loss of the entire mesodermal precursor population. Progress on the analysis of *Wnt-3a, Wnt-5a* and *Wnt-5b* functions will be discussed.

**W 012** ROLE OF HOMEOBOX GENES IN MUSCLE DEVELOPMENT-EARLY AND LATE EVENTS, John Lincecum,[1] Karl Degenhardt[1,2], Kening Song[1], Yaoqi Wang[1], Anna Pavlova[1,2] and David Sassoon[1,2], 1 Department of Biochemistry, Boston University School of Medicine, Boston MA 02118, 2 currently at Brookdale Center for Molecular Biology, Mount Sinai Medical Center, New York, NY 10029

We have been pursuing the role that homeobox genes play in guiding the development of skeletal muscle during mouse embryogenesis. We can divide skeletal muscle development into two phases during development. The Early Phase is characterized by mesodermal cells that are uniquely committed to the skeletal muscle lineage. This compartment is marked by no overt accumulation of transcripts for any of the currently identified myogenic regulatory factors (MRFs) but strong expression of the Id gene [1]. In this compartment, myoblasts are highly proliferative, migratory, and have yet to assume a positional value within the embryo. A Late Phase follows and is characterized by overt accumulation of transcripts for at least one of the MRFs and, although still proliferative, are nearer to terminal differentiation and no longer positionally plastic. We have examined in detail early phase myoblasts in the initial mouse limb bud stages and find that at least one homeobox containing gene, *Msx1* (formerly Hox-7), which is expressed by all mesodermal components prior to differentiation and fate assignment, can block or delay terminal differentiation of myogenic cells when forced expressed [2]. We have extended this analysis to determine how differentiation may be regulated at the levels of the MRFs and what other potential targets of *Msx1* may be. Analysis of myoblasts that force-express *Msx1* and *Msx2* reveals that although the two gene products share high homology, they result in very different cell behaviors. In particular, we observe that myoblasts that express *Msx1* and *Msx2* will differentially sort and achieve final positions in relation to each other that reflects their normal distribution in vivo. This sorting behavior may be due in part to regulation of cell adhesion molecules including the Cadherins. We propose that these homeobox genes, in concert with other Hox or pax genes guide the development and progression of skeletal myogenesis in vivo allowing the proper migration, timing of differentiation and final patterning of muscle tissue. We are currently investigating how these genes are regulated in vivo. These and other data will be discussed.

**1** Wang, Y., Benezra, R., and Sassoon, D.A. (1992). Id expression during mouse development: a role in morphogenesis. Devel. Dynamics. 194(2):222-230.
**2** Song, K., Wang, Y. and Sassoon, D.A. (1992) Expression of Hox-7.1 in myoblasts results in inhibition of differentiation and induces cell transformation. Nature 360:477-481

**W 013** HOX GENES AND THE CONTROL OF LIMB PATTERNING,
Cliff Tabin[1], Bruce Morgan[1], Craig Nelson[1], Ann Burke[1], Randy Johnson[1], Bob Riddle[1], Phil Ingham[2], and Andy McMahon[3], 1. Harvard Medical School, Boston, MA 02115, 2. Harvard University, Cambridge, MA 02138, 3. ICRF, Developmental Biology Unit, Oxford, England.

Various Hox genes have been implicated in limb patterning by their expression patterns. Consistent with such a hypothesis, some of their expression domains are ectopically duplicated by surgical manipulations which cause morphological mirror-image limb pattern duplications. In spite of much speculation, however, such experiments do not address whether the Hox genes have a causal role in the morphological changes. Moreover the exact role of each individual Hox gene is not elucidated.

We have used a replication-competent retroviral vector to ectopically express individual Hox genes specifically in the developing limb primordia (but not in the rest of the embryo). Such an approach is very quick allowing many animals and genes to be tested. Moreover a "transgenic limb" avoids the pleiotropic problems and lethality problems potentially associated with transgenic animals. Using this approach we have obtained clear homeotic transformations of regions of the limb including changing of one digit morphology to that of another. These transformations are consistent with models of positional control by specific combinations of Hox genes.

We have also begun investigations into molecules possibly involved in controlling the expression of the Hox genes and hence in initiating the pattern of the limb.

*Myogenic Determination I*

**W 014** MYOGENESIS IN VERTEBRATES: REGULATORY CIRCUITS INVOLVING THE MYOGENIC bHLH PROTEINS,
Hans-H. Arnold, Department of Cell- and Molecular Biology, University of Braunschweig, F.R.G.

The myogenic capacity of the muscle regulatory proteins Myf-5, myogenin, MyoD, and MRF-4 has been discovered in 10 T1/2 fibroblasts. In this and other in vitro systems each of the four factors have similar effects, partly due to their ability to activate their own and each others' gene expression. However, different spatio-temporal expression patterns of individual members of this gene family during embryonic and fetal development have suggested that auto- and cross-regulatory circuits may not be effective in vivo and each factor may serve distinct aspects of myogenesis. To begin to study individual functions, Myf-5 and MyoD have been inactivated in mice by gene knock-out. Both homozygous mutants are born and develop apparently normal skeletal muscle suggesting that neither gene individually is absolutely essential for myogenesis. Interestingly, Myf-5 which is normally down-regulated during fetal life remains highly expressed in MyoD mutants. In Myf-5 minus mutants development of the somitic myotome and myogenin expression are considerably delayed, until MyoD starts to accumulate. In addition, these mice have severe defects in rib formation but no other axial skeletal structures. Taken together, these results argue for partially redundant fuctions of Myf-5 and MyoD in myogenesis. Convincing evidence for this comes from double knock-out mutants lacking both genes. These animals fail to develop myoblasts and consequently contain no skeletal muscles. This establishes a myogenic determining role for both genes and distinguishes them from myogenin, since myogenin gene inactivation results in apparently normal numbers of myoblasts that fail to differentiate. Thus, Myf-5 and MyoD appear to act upstream of myogenin with little functional overlap. A direct role of Myf-5 may be the activation of the myogenin gene. Using Myf-5-ER, a hormone regulated hybrid protein, we demonstrate that Myf-5 directly induces myogenin transcription without the need for de novo protein synthesis. As this process critically depends on the transcription factor MEF-2, we discuss a posttranslational mechanism of activation.

*Myogenic Determination II*

**W 015 COMPARTMENTALIZATION OF MRFs EXPRESSION IN THE SOMITE.**
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Transfection experiments revealed a complex pattern of interactions among the four myogenic regulatory genes (*MyoD1*, *myogenin*, *myf5* and *MRF4*). Yet, at early stages of mammalian and avian development each MRF display a distinct temporal activation, suggesting a very limited cross talk. Targeted mutagenesis indicated some but not absolute functional redundancy within this gene family. It was therefore of interest to examine whether despite the structural and functional similarities each MRFs is expressed in different cell lineages within the developing embryo and in particular in the somite. To this end we have analyzed their mode of expression at the early stages of myotome formation, using transgenesis and whole mount in situ hybridization methodologies.

The enhancer-promoter sequences of the human *MyoD1* gene (Goldhamer et al., 1992) and the quail homolog to the mammalian *MyoD1* gene (Pinney et al., in preparation), were employed to target expression of the bacterial $\beta$-galactosidase reporter gene to skeletal muscle precursor cells. At the onset of lacZ expression driven by the mammalian regulatory elements the pattern differed significantly in cervical and more posterior somites. In cervical somites stained cells are dispersed throughout the myotome, whereas in more posterior somites staining is confined to the ventral part of the myotome. Later, the ventral staining spreads dorsally, never reaching the dorsal group of stained cells. The gap of non-stained myotomal cells persists throughout the myotomal stage. In contrast, the expression of lacZ driven by the avian regulatory sequences is restricted to spindle-shaped cells in the very middle part of the myotome in both rostral and caudal somites. Similar experiments using the *myogenin* regulatory elements revealed a third pattern of expression (Cheng et al., 1993; Fujisawa-Schara et al., 1993; Yee and Rigby; 1993). To determine the relevance of transgene expression to the endogenous mRNAs, we have analyzed early stage embryos by whole mount in situ hybridization. These studies revealed a distinct spatial pattern of expression of these regulators. Thus, *myf5* transcripts first appeared in the dorso-medial lip of the myotome and subsequently also in the anterial, but not in the middle or posterior, part of the myotome. *Myogenin* expressing cells were evenly distributed throughout the myotome at all stages, whereas the pattern for the *MyoD1* transcripts was indistinguishable from that of the lacZ transgenics. These results demonstrate the existence of further heterogeneity in the population of myogenic precursor cells within the myotomal compartment, that could reflect different cell lineages or different local microenvironments.

**W 016** MECHANISMS REGULATING MUSCLE CELL FATE DURING EMBRYOGENESIS, Eric N. Olson, The University of Texas M. D. Anderson Cancer Center, Houston, Texas.

Establishment of skeletal muscle during embryogenesis involves commitment of mesodermal progenitors to the myogenic lineage and subsequent activation of muscle-specific genes. The muscle-specific bHLH protein myogenin acts as a genetic switch to activate muscle-specific gene expression and is an essential component of the regulatory pathway leading to skeletal muscle formation during mouse embryogenesis. Transcriptional activation of the myogenin gene in the somites and limb buds of mouse embryos is dependent on a MEF2 binding site within the myogenin promoter. During mouse embryogenesis, MEF2 genes are expressed in the somite myotome, as well as within the precardiac mesoderm, the primitive heart tube, and the embryonic heart. The role of MEF2 in myogenic lineage commitment is also being examined in *Drosophila* (in collaboration with R. Schultz), which contains a single MEF2 gene, termed D-MEF2. D-MEF2 expression is first detected within uncommitted mesoderm prior to separation into the somatic and visceral muscle lineages, which give rise to skeletal muscle and muscles of the heart and gut, respectively. During late gastrulation and germ band extension, the expression pattern of D-MEF2 is similar to that of *twist*, which regulates mesoderm formation, and precedes expression of the myogenic bHLH protein *nautilus*. In homozygous *twist* and *snail* embryos, which fail to make mesoderm, D-MEF2 is not expressed. The temporal and spatial pattern of expression of D-MEF2 make it a likely candidate for a regulator of cell fate within the myogenic lineage. Together, our studies demonstrate that myogenic HLH proteins such as myogenin act in conjunction with members of the MEF2 family to regulate cell determination and differentiation within the myogenic lineage. The finding that MEF2 is expressed in precursors for cardiac as well as skeletal muscle also raises the possibility that it may control aspects of the myogenic program common to both lineages.

**W 017** MUSCLE PATTERN FORMATION IN THE *DROSOPHILA* EMBRYO, Alan M. Michelson and Laurel A. Mezitt, Department of Medicine, Division of Genetics, Brigham and Women's Hospital, Howard Hughes Medical Institute and Harvard Medical School, Boston, MA 02115.

The body wall muscles of the *Drosophila* embryo form a stereotyped array that exhibits distinct segmental differences. This diversity is first apparent early in myogenesis when the complete set of muscle precursors has formed. Thus, myogenic diversification occurs prior to innervation and to the formation of epidermal attachments. We have now established that the identities of abdominal muscles are specified at the precursor stage of development by members of the Bithorax complex of homeotic genes. *Ultrabithorax* (*Ubx*) and *abdominal-A* (*abd-A*) have equivalent functions in promoting the formation of particular muscle precursors in the first seven abdominal segments. In contrast, *Abdominal-B* (*Abd-B*) suppresses the development of these same precursors in the most posterior region of the abdomen. When ectopically expressed in the same mesodermal cells, either UBX or ABD-A is capable of overriding the inhibitory influence of ABD-B. This suggests that these homeotic factors may compete in the differential regulation of a common set of downstream genes. Furthermore, targeted embryonic misexpression of *Ubx* or *abd-A* achieved under the control of a yeast transcriptional activator expressed in a tissue-specific pattern indicated that these genes exert their effects on muscle diversification by acting autonomously in mesodermal cells. These findings illustrate how positional information that is generated early in embryogenesis can influence a morphogenetic process at a much later developmental stage.

*Workshop: Fiber Type Structure, Function and Regulation*

**W 018** CRITICAL TRANSITIONS OF METABOLISM AND ASSEMBLY IN EARLY MUSCLE DEVELOPMENT OF *C. ELEGANS*, Henry F. Epstein, Feizhou Liu, Philip R. Deitiker, Irving Ortiz, and Douglas L. Casey, Baylor College of Medicine, Houston, TX 77030.

Body-wall muscle cells are born at specific times from well-demarcated progenitor cells in early embryos and larvae of *Caenorhaditis elegans*. 81 of these muscle cells are generated and differentiate during embryogenesis and 14 muscle cells are born in the L1 larval stage. Embryonic muscle cells first begin to express the thick filament proteins myosin heavy chains A and B and paramyosin at about 300 min (400 cell stage) as detected by immunofluorescence microscopy. By about 360 min, these proteins are organized into linear nascent structures which genetic and double staining experiments show are distinct from mature thick filaments, stress fiber-like structures, or the multifilament assemblages seen in thick filament mutants. Between 420 and 450 min, premyofibrillar structures appear which share the same sensitivity to the neomorphic paramyosin mutant *e73* as the later recognizable myofibrils. Biochemical and structural studies indicate that additional proteins P20, P28, and P30 associate with paramyosin in the core structures of mature thick filaments. The core proteins may play a critical role in the transition from nascent to premyofibrillar assembly. A new muscle-specific antigen has been found which is expressed in a single or a few cells as early as the 28 cell stage (D and C lineages), in other muscle precursor cells (MS and AB lineages) before myosin expression, and then appears most strongly in developing embryonic and larval muscle cells which exhibit the nascent linear structures. The protein disappears in mature body-wall muscle cells. Molecular cloning and sequencing of a 3.3 kb cDNA reveals that it encodes a bifunctional enzyme, isocitrate lyase - malate synthase, whose glyoxylate cycle activities have been found by previous workers to be highest in embryonic stages of nematodes and plants. These enzyme activities convert the products of yolk fatty acid degradation to carbohydrate. The single genomic locus is on LGV, Northern blots indicate a single mRNA of 3.4 kb, and immunoblots with the monoclonal antibody F11 reveal a single polypeptide of 120 kDa which represents 1-3% of total embryonic protein. The expression of this bifunctional enzyme at or just after commitment to a body-wall muscle fate may represent a critical metabolic transition during muscle development. This research was supported by grants from the NIGMS and MDA.

**W 019** NEURAL CONTROL OF ACCUMULATION OF MYOD AND MYOGENIN mRNA IN ADULT FAST AND SLOW SKELETAL MUSCLE.
Simon M. Hughes[1], Alison M. Maggs[1], Jonathon Blake[1] and Charlotte A. Peterson[2], [1] MRC Muscle and Cell Motility Unit, 26-29 Drury Lane, London WC2B 5RL, UK [44 71 836 8851 ph 44 71 497 9078 fax] [2] Dept of Biochemistry, University of Arkansas Medical School, Little Rock, AR72205, USA.

In skeletal muscle MyoD, Myogenin, MRF4, and Myf-5, the myogenic helix-loop-helix family of proteins, are candidate regulators for the control of muscle gene expression. Distinct functions have not been ascribed to the individual myogenic regulatory proteins, but one possibility is that their differential expression or activity may contribute to the distinct phenotypes characteristic of different muscles of the limb. We find that MyoD and Myogenin mRNAs are differentially expressed in adult rat hindlimb muscles that differ in metabolic and contractile activity, and in myosin heavy chain expression. Furthermore, within a single muscle, in situ hybridization shows that MyoD and Myogenin transcripts are differentially expressed in regions that differ in their proportions of fast and slow muscle fibers: MyoD is prevalent in the fastest muscle regions and Myogenin in slow regions. The analysis is also being extended to the protein level. Alteration of the phenotype of soleus muscle by cross-reinnervation or treatment with thyroid hormone and clenbuterol alters the Myogenin/MyoD mRNA expression pattern. Myogenin is decreased in those regions of the slow soleus muscle that convert to express fast myosin heavy chain isoforms. Based on these correlations we propose that nerve-dependent changes in the fast/slow phenotype of adult muscle may be affected by the array of myogenic helix-loop-helix molecules induced. Further studies of the effects of electrical activity per se upon muscle fibre phenotpye and MyoD and Myogenin expression and the effects of forced MyoD or Myogenin over-expression on adult muscle fibre phenotype will be discussed.

Hughes, S.M., J.M. Taylor, S.J. Tapscott, C.M. Gurley, W.J. Carter and C.A. Peterson (1993) Selective accumulation of MyoD an Myogenin mRNAs in fast and slow adult skeletal muscle is controlled by innervation and hormones. Development 118: 1137-1147.

*Transcriptional Control Mechs I*

**W 020** **REGULATION OF Id1 ACTIVITY IN MAMMALIAN CELLS,**
Olivia Tournay, Chengua Gu, and Robert Benezra, Memorial Sloan-Kettering Cancer Center, New York 10021

We have been studying post-translational and transcriptional control of Id activity in mammalian cells. Id is a member of the helix-loop-helix (HLH) family of proteins which, unlike other members, lacks a DNA binding domain adjacent to the HLH dimerization motif. Id can associate with other members of the HLH family and prevent them from binding DNA or other HLH proteins. Id was first shown to antagonize the activity of the muscle regulatory factor MyoD by competing with MyoD for binding to the ubiquitously expressed products of the E2A gene (called E proteins) thereby preventing the formation of active MyoD/E heterodimers. Recent evidence suggests that Id's activity is not confined to the muscle lineage since overexpression of Id blocks tissue specific gene expression in cardiac muscle, pancreatic b cells, B cells, T cells and osteoblasts. We have identified an activity present in muscle cell extracts which may regulate the association of Id with the E proteins. This activity is required in order for purified E12 (one product of the E2A gene) to associate with purified Id in vitro. We have begun to purify this activity by standard chromatographic techniques. Our partially purified fraction is active at 37oC but not 25oC, is heat labile, sensitive to low levels of SDS and works independent of the presence of ATP. Preliminary results suggest that the activity may destabilize E12 homodimers and thereby facilitate the interaction of E12 with Id monomers. A second level of control of Id activity that we are examining is transcriptional. We and others have shown that Id is dramatically down-regulated when cells in culture are induced to differentiate under a variety of conditions. In addition, Id expression in the mouse embryo is ubiquitous, peaks at about 8.5-9 days post-coitum and decreases dramatically thereafter as differentiation proceeds in multiple cell lineages. We have first focused on the regulation of Id transcription in C2C12 muscle cells in culture. In this system, Id transcription is repressed within 1 hour of induction by mitogen depletion. We have shown that a 1.5 kB promoter/enhancer element upstream of the Id gene can confer this response to a heterologous reporter gene. This element has a high density of transcription factor binding sites including 9 E-boxes (bHLH factor binding sites), 16 AP1 sites (fos/jun binding sites), 6 VDRE 1/2 sites (vitamin D receptor binding sites), 2 perfect NF-kB sites. Deletion analysis has allowed us to define a 200 bp element that is necessary and sufficient for proper Id regulation in C2C12 cells. A model for the regulation of Id by serum factors will be discussed. Finally, we have begun to investigate the potential role of Id in the cell-cycle. Data from our laboratory suggests that Id1 must be down-regulated before mammalian cells can enter a state of quiescence. Forced expression of Id1 in both muscle cells and myeloid cells prevents these cells from entering a state of quiescence prior to the onset of differentiation in response to growth factor withdrawal. In addition, we have observed that there are no detectable post-mitotic cells in the mouse embryo that are Id1 positive. These observations indicate that the down-regulation of Id is necessary for cell cycle withdrawal and have prompted us to investigate what factors bind DNA in response to Id down-regulation during mitogen depletion. Using a target site-selection protocol, we have identified E-box binding activities that appear in fibroblasts after 2 and 24 hours of mitogen depletion, a subset of which are disrupted upon addition of purified Id. We have initiated a search for the factors present in these complexes and the target genes which may be controlled by these elements.

**W 021** THE FUNCTIONS OF RSRF/MEF2 PROTEINS IN XENOPUS EMBRYOS,
Tim Mohun, Malcolm Logan and Anne Chambers, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA.

The RSRF or MEF2 family of transcription factors are thought to play an important role in skeletal myogenesis. Binding to A/T rich motifs in the regulatory regions of muscle-specific genes, these proteins regulate both E box-dependent and E box-independent branches of the myogenic programme. We have previously identified two RSRF/MEF2 genes (SL1 and SL2) that are expressed in *Xenopus* embryos (Chambers et al. 1992). Transcripts of both are present in the maternal RNA inherited by the egg, but zygotic transcription commences during gastrulation. The SL1 gene is activated in dorsal, presomitic mesoderm in the early gastrula whilst the SL2 gene is only expressed during terminal differentiation of muscle cells. In the tailbud embryo, transcription of both genes switches from being somite-specific to ubiquitous. We have now used polyclonal antibodies specific for SL1 and SL2 to examine the distribution of RSRF/MEF2 binding activities and find that both proteins are present in a wide variety of adult tissues and cultured cell types, consistent with multiple functions for this family of transcription factors. To examine their role in muscle differentiation, we have tested the effects of ectopic expression of SL1 and SL2 in fertilised eggs and compared their effects with those obtained with XMyoD and XMyf5. Both RSRF/MEF2 genes can be activated by proteins of the MyoD family in cultured animal pole explants and this is consistent with their temporal order of expression in the embryo. Neither SL1 nor SL2 induces muscle formation in explants, nor do they act synergistically with the MyoD proteins to activate muscle-specific genes. SL1 can be distinguished from SL2 by its expression in the cardiac mesoderm of the late neurula embryo suggesting that this protein may play a role in cardiac myogenesis. This conclusion is supported by ectopic expression studies which demonstrate that SL1 is capable of activating a heart muscle differentiation marker in cultured animal pole explants.

Chambers, AE, Kotecha, S, Towers, N and Mohun, TJ: 1992. Muscle-specific expression of SRF-related genes in the early embryo of *Xenopus laevis*. EMBO J. 11: 4981-4991.

*Transcriptional Control Mechs II*

**W 022** MOLECULAR CONTROL MECHANISMS REGULATING MRF4 ACTIVITY.
Serge Hardy, Sally E. Johnson, Yanfeng Kong, Lea C. Longcor, Kam-Leung Mak, Padma Naidu, Robert Q. To and Stephen F. Konieczny, Department of Biological Sciences, Purdue University, West Lafayette, IN.

MRF4 is a member of the muscle-specific basic helix-loop-helix (bHLH) transcription factor family that also includes MyoD, myogenin and Myf-5. Expression of MRF4 in a variety of fibroblasts converts the cells to differentiated myotubes that express several skeletal muscle-specific genes. During embryogenesis, MRF4 is expressed transiently in myotomes and later in the developing limb musculature. In culture, MRF4 transcription occurs late, appearing after MyoD, myogenin and Myf-5 transcripts and restricted solely to fully differentiated muscle fibers. The late appearance of MRF4 suggests that other members of the bHLH family may control MRF4 expression. In support of this hypothesis, we find that the MRF4 promoter is *trans*-activated by myogenin as well as by the myocyte-specific enhancer factor 2 (MEF2). Whether these muscle-specific transcription factors are the primary activators for MRF4 expression remains to be addressed. An additional aspect of MRF4 regulation involves controlling MRF4 dimerization, DNA binding and transcriptional activities in muscle cells. Although MRF4 functions as a positive transcriptional regulator, the MRF4 protein is ineffective in activating the myogenic program in cells exposed to fetal bovine serum, basic fibroblast growth factor (bFGF) or to transforming growth factor-β. A similar inhibition in MRF4 activity is observed in 10T1/2-*ras* cells, suggesting that growth factors and oncogenes may inhibit MRF4 activity through common signal transduction pathways that negatively regulate myogenesis. As a first step in understanding how diverse signalling networks interact, the phosphorylation status of MRF4 has been examined. Our results indicate that MRF4 exists *in vivo* as a phosphoprotein and serves as an efficient substrate for several serine/threonine protein kinases including the cyclic AMP-dependent protein kinase (PKA), protein kinase C (PKC), and casein kinase II. Overexpression of PKA and PKC dramatically inhibits MRF4 activity in a fashion analogous to inhibition by bFGF. *In vitro* PKC-directed phosphorylation of a conserved threonine residue (T-99) also abolishes transcriptional activity by inhibiting the protein from interacting with an E-box target. Mutational analysis, however, has failed to reveal a direct *in vivo* role for either PKA or PKC phosphorylation in regulating MRF4 activity. Likewise, exposure of cells to bFGF does not lead to a change in the PKA- or PKC-dependent phosphorylation status of the protein, suggesting that the negative regulation imposed by bFGF, PKA and PKC does not involve a direct modification of MRF4 at these kinase sites but instead may involve the modification of specific coregulators that interact with this muscle regulatory factor. Several experimental systems are currently being developed to decipher how the MRF4 activation, DNA binding and dimerization domains function and how additional protein partners may influence MRF4 activity during myogenesis.

**W 023** **Myogenin gene disruption leads to developmental abnormalities in myogenic lineage cells**
Yoko Nabeshima, Eisaku Esumi, Kiichi Arahata, Ikuya Nonaka, Michiko Hayasaka, Kazunori Hanaoka, and Yo-ichi Nabeshima, National Institute of Neuroscience, 4-1-1 Ogawa-higashi Kodaira 187 Tokyo, Japan

Skeletal muscle originates from progenitor cells that arise in the early somite. In maturing somite, these stem cells become the dermomyotome, from which myoblasts migrate into the embryo. Myoblasts differentiate into multinucleated myotubes, in which muscle specific genes are activated to form highly organized muscle contractile apparatus. Throughout this developmental program each myogenic gene follows a unique pattern of expression. This distinct spatio-temporal expression pattern for each of the four MyoD family members has suggested that they may have unique functions during myogenesis. To understand the role of myogenin in myogenesis, targeted inactivation of myogenin gene was performed. Homologous mutants were perinatal lethal owing to a skeletal muscle defect. The extent of muscle disorganization differed in three regions of the embryo. In latero-ventral, body wall regions, there was a paucity of most cell-types, including the myogenic population, and a form of hydrops rapidly developed. In the limbs, cell masses containing myogenic precursors were observed, but these were severely disorganized and many of the myogenic cells remained mononucleated, with myoblast-like features. In contrast, considerable amounts of axial, intercostal and back muscle was observed, although most of the myofibers were disorganized and Z-lines failed to assemble normally in the nascent myofibrils. These findings offer the first evidence that myogenin plays a crucial role(s) in muscle development, and that other members of the myoD family cannot compensate for the absence of this transcription factor.

**W 024** STUDIES ON THE DIMERIZATION OF THE MYOGENIC HELIX-LOOP-HELIX DNA-BINDING FACTORS,
Bruce M. Paterson, Masaki Shrakata, Qin Wei, Ilene Karsch-Mizrachi, and Jian-Min Zhang, Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892.

Very little is known about the factors that modulate dimerization or determine dimerization specificity of the myogenic DNA-binding factors in vivo. All are nuclear phosphoproteins that are thought to form heterodimers with other positive and negative regulatory factors through a conserved region called the helix-loop-helix (HLH) domain. Here we report that phosphorylated chicken MyoD, called CMD1, produced in sf9 cells using the baculovirus system, is qualitatively similar to CMD1 isolated by immunoaffinity purification from primary cultures of embryonic breast muscle. Functional analysis of phosphorylated and dephosphorylated CMD1 indicates that phosphorylation inhibits the DNA-binding activity of MyoD homodimers but not MyoD-E12 heterodimers. Our results suggest that cellular phosphorylation changes the homodimer-heterodimer equilibrium which, in turn, modulates and/or eliminates binding site competition between MyoD homodimers and MyoD/E-protein heterodimers in the cell. Among the dimerizing combinations of E12 and the four avian myogenic factors, dimerization specificity is directed by nonconserved hydrophylic residues in the HLH domain. Using a non-DNA-binding MyoD mutant with a normal HLH domain as a dimerization competitor in gel mobility shift assays in conjunction with various MyoD HLH mutants, nonhydrophobic amino acids were identified in the HLH domain that contribute to dimerization specificity with E12. The assay detected subtitle differences in dimerization activity among the mutant MyoD proteins that correlated with their ability to activate transcription in vivo, but this correlation was not apparent in the absence of competitor. The identification of such nonhydrophobic residues enabled us to predict the differences in dimerization affinity among the four vertebrate myogenic factors and E12. The experiments confirmed the prediction. Furthermore, a high-affinity homodimerizing analog of MyoD was designed by a single substitution at one of these residue positions. HLH domain swaps between the avian and Drosophila MyoD proteins confirm that the activity of MyoD depends upon the formation of the proper heterodimer combination as specified by the nonconserved hydrophilic residues in the HLH domain.

**W 025** REGULATORY ELEMENTS OF THE CHICKEN α-SKELETAL ACTIN GENE DIRECT HIGH LEVEL AND TISSUE-SPECIFIC EXPRESSION OF A hIGF-I cDNA IN CULTURED MYOGENIC CELLS AND TRANSGENIC MICE RESULTING IN ENHANCED MYOGENESIS AND PRONOUNCED MUSCLE HYPERTROPHY, Michael E. Coleman, Kuo Chung Yin, Francisco DeMayo, and Robert J. Schwartz, Department of Cell Biology, Baylor College of Medicine, Houston, TX 77030.

Insulin like growth factors play an important role in the development of skeletal muscle by stimulating myoblast replication, enhancing myogenesis, and promoting myofiber hypertrophy and protein accretion. We report here that the proximal 411 bp promoter region, together with the first intron and 2.3 kb of 3 prime flanking sequence of the chicken α-skeletal actin gene direct high level and tissue-specific expression of a hIGF-I cDNA in stably-transfected C2C12 myoblasts and transgenic mice. Transgene expression in stably-transfected C2C12 cells was negligible in replicating myoblasts and up-regulated significantly with the onset of myogenesis as determined by Northern blotting and RIA analysis of conditioned media. This resulted in increased levels of myogenic factor and contractile protein mRNAs and myotube hypertrophy. The 3 prime UTR of the chicken α-skeletal actin gene was found to be necessary for restricting expression of the transgene to skeletal and cardiac muscle in mice. Expression of the single copy transgene in mice resulted in concentrations of IGF-I in skeletal muscle being 10 to 20 fold higher for transgenic animals as compared to littermate controls. This resulted in pronounced hypertrophy (i.e., an approximate 70 to 80 % increase in cross-sectional diameter) of both type I and type II myofibers. IGF-I concentrations in serum and body weights of transgenic mice were not different from controls suggesting that the effects of transgene expression are local. In addition, we have observed that IGF-I up-regulates α-skeletal actin promoter activity in vitro through the multiple SREs suggesting that expression of the α-skeletal actin/IGF-I transgene may be subject to autoregulation. In summary, it is clear that enhanced expression of IGF-I in this murine model system leads to long term muscle hypertrophy, and it is our perspective that this model will facilitate our understanding of the mechanisms that govern muscle hypertrophy in vivo.

**W 026** MULTICOMPONENT COMPLEXES INVOLVING MYOGENIN, MRF4, MYF5 AND MYOD, Woodring E. Wright[1], Karen J. Farmer[1], Marina Bouché[2] and Taichi Uestuki[3], [1]Dept. of Cell Biol. and Neurosci., U.T. Southwestern Med. Ctr., 5323 Harry Hines Blvd., Dallas, TX, 75235, [2]Universita di Roma, Rome, Italy,[3]Tokyo Metropolitan Institute for Neuroscience, Tokyo, Japan.

DNA binding sites can be isolated by reiterative selection techniques (CASTing) in which a degenerate oligonucleotide flanked by PCR amplifiable sequences is mixed with the protein of interest. After the protein has bound to sequences it can recognize, protein-DNA complexes are separated from unbound oligonucleotides, the bound DNA is reamplified, and the procedure repeated until most of the DNA actually contains binding sites. Most transcriptional complexes appear to function as multicomponent assemblies, in which the binding of one factor stabilizes the binding of adjacent factors (cooperative binding). Since each cycle of selection using CASTing preferentially enriches for the binding sites with the highest affinity, binding sites for such multicomponent complexes can be isolated if an oligonucleotide with a degenerate core large enough to accommodate multiple binding sites is used together with a crude nuclear extract as a source of multiple factors. All four of the muscle bHLH factors were tagged with a 9 amino acid epitope from the *Hemophilus influenza* agglutination antigen, then these *in vitro* translated proteins were mixed with myotube nuclear extracts and a 75-mer oligonucleotide composed of PCR primable sequences flanking a totally degenerate stretch of 35 random bases. Bound protein-DNA complexes were isolated using a monoclonal antibody against the HA antigen. Binding sites for NF1 and COMP1 were found with all four factors. In addition, four-six additional sequences were found uniquely associated with each of the factors. Some of these CASTing-derived sequences lacked E-boxes, but gave specific gel shifts with nuclear extracts but not with *in vitro* translated bHLH factors alone, suggesting that the muscle bHLH factors in these complexes were not contacting the DNA directly. Issues of specificity and implications for the different functional roles of each of the four muscle bHLH factors will be discussed.

*Isoform Families: Structure-Function Analysis*

**W 027** FUNCTIONAL ANALYSIS OF CHICKEN MYOSIN HEAVY CHAIN ISOFORMS, Everett Bandman, Laurie A. Moore, Maria Arrizubieta, Ta-Hsiang Chao, Macdonald Wick, and Fakhrieh Vojdani, Department of Food Science and Technology, University of California, Davis, CA 95616.

Myosin heavy chain (MyHC) isoform diversity is a ubiquitous property of vertebrate sarcomeric myosins. Multigene families such as MyHC may encode isoforms that have unique functions and/or provide a selective advantage by maintaining functionally equivalent proteins encoded in redundant genes. In the chicken, 9 isoforms have been cloned and their patterns of expression in developing and mature muscle characterized. The isoforms are classed as fast, slow, or cardiac based upon their expression in adult chicken muscles and the observation that they are organized in the chicken genome as separate linkage groups. We have recently focussed on the chicken fast MyHC class. This group of 5 isoforms exhibit more sequence homology than the corresponding fast MyHCs of rodents and primates. Furthermore, the chicken fast MyHCs display characteristics of a recently expanded gene family. Analysis of their primary sequence has identified highly conserved and more divergent regions. We have been studying the molecular basis for the structural and functional characteristics associated with highly conserved and diverging sequences within the chicken MyHC rod. Studies on the functional properties of recombinant full length chicken MyHCs and on expressed rod domains will be presented. The role of sequence diversity in providing unique properties associated with different chicken MyHC isoforms will be discussed. (Supported by NIH grant AG08573 and USDA grant 91-37206-6715 to EB).

**W 028** UNDER-, OVER- AND MIS-EXPRESSION OF MYOSIN ISOFORMS, Sanford I. Bernstein, Richard M. Cripps, Linda Wells, William A. Kronert, Patrick T. O'Donnell, K. David Becker, Michelle Mardahl, Dianne Hodges, Venetia Collier, Norbert Hess and Kevin Edwards, Department of Biology and Molecular Biology Institute, San Diego State University, San Diego, California 92182-0057.

*Drosophila melanogaster* has a single muscle myosin heavy chain (MHC) gene which gives rise to multiple isoforms of the protein by alternative RNA splicing. In collaboration with Ron Milligan (Scripps Research Institute) we mapped the locations of the alternatively encoded regions onto the three-dimensional structure of the myosin head. Two of these areas are near the active site of the molecule, one is at the "lip" of the pocket which permits ATP entry into the active site, and one is close to an α-helical region near the myosin light chain binding site. In addition, alternative isoforms differ in the hinge region of the rod and at the C-terminus of the protein.

We have characterized two types of mutations in the *Mhc* gene: homozygous-viable alleles affecting MHC expression in subsets of muscles (defects in alternative exons) and homozygous-lethal alleles affecting all muscle types (defects in constitutive exons). Reduction by 50% in the level of MHC severely affects the function of the highly-ordered indirect flight muscles (IFM), but does not drastically alter the function of muscles required for viability.

To develop the *Drosophila* MHC system for transgenic studies, we isolated a 45 kb cosmid clone which contains the entire gene and used it for germline transformation. We obtained three stably transformed lines, each of which contains a single insert. Each insert rescues the dominant flightless phenotypes and myofibrillar defects associated with myosin null mutations. These transgenes also complement recessive lethal phenotypes, indicating that they functionally rescue muscles other than the flight muscles. Approximately wild-type levels of MHC are produced from each transgene.

We used these transformed genes to study the effects of overexpressing MHC. Three copies of *Mhc* have little effect upon flight ability, and no defects in the IFM ultrastructure are evident. However, four copies result in flies which are severely flight-impaired, and have aberrant myofibrillar structure, with mis-aligned thick filaments at the periphery of the myofibril. Additional increases in gene dosage to six copies are lethal, demonstrating that the effect of gene copy-number upon muscle function can also be detected in the other muscles of the organism, not only in the structurally regular IFM. Thus stoichiometry of the contractile proteins is important in determining myofibril structure and muscle function.

To analyze whether MHC protein isoforms differ functionally, we cloned a cDNA encoding the major embryonic isoform downstream of the *Mhc* promoter, and obtained germline transformants expressing this construct in all muscles. These transformed lines are flightless, have reduced jumping capability, and show ultrastructural abnormalities in their IFM, including fusion and fraying of the myofibrils. As indicated above, these phenotypes are not seen in flies with three copies of the wild-type *Mhc* gene. The ultrastructural abnormalities increase in severity as the fly ages. Co-expression of the IFM and embryonic forms of MHC is not compatible with normal muscle structure and function, clearly indicating that the isoforms are functionally distinct. Expressing the embryonic form in the absence of wild-type MHC permits approximately normal levels of accumulation of the protein in the IFM, but flies are flightless. Assembly of defective sarcomere-like structures occurs, and we are currently examining these in more detail. Overall our studies show that both quantitative and qualitative differences in myosin accumulation can dramatically alter the structure and function of *Drosophila* muscles.

**W 029** TRANSGENIC AND CELL CULTURE MODELS OF MUSCLE DEVELOPMENT AND FUNCTION, Leslie A. Leinwand[1], Karen Vikstrom[1], Alyson Kass-Eisler[1], David Elfenbein[1], Erik Falck-Pederson[2], and Anthony J Straceski[1], [1]Albert Einstein College of Medicine, New York, [2]Cornell Medical Center, New York.

Muscle contraction requires the orderly assembly and interaction of numerous proteins, many of which are represented by multiple isoforms. One of the most intensively studied proteins of muscle is myosin, which is encoded by a large multigene family in mammals. Although purified myosin can self-assemble into filaments, their size and composition do not reflect the native thick filament of the sarcomere. We have developed a cell culture model system for defining the determinants of myosin thick filament formation and for studying how the process might be perturbed by myosin mutations. Striated muscle myosin heavy chain (MHC), when transfected into nonmuscle (COS) cells assembles into orderly bundles of thick filaments which possess a 40-50nm periodicity. Through deletion analysis, we have defined 29 amino acids near the carboxyl terminus of the molecule as necessary for the formation of these structures. Cardiac MHC gene mutations have been linked to an autosomal dominant genetic heart disease, familial hypertrophic cardiomyopathy (FHC). This disease is characterized by asymmetric hypertrophy, myocyte disarray and sudden death. The identification of dominant, disease-causing mutations in a structural component of muscle has led us to begin to assess the impact of myosin mutations on muscle development and function. We have examined the ability of myosin with different FHC missense mutations to assemble into filaments. Up to 29% of COS cells transfected with FHC mutations fail to demonstrate filamentous structures compared with 2% of cells transfected with wild type MHC. Electron microscopic analysis suggests that the mutant MHC still assembles, but frequently into smaller structures. In collaboration with Dr. L. Sweeney (Univ. of Penn.), we have demonstrated that one these mutations (arg 403 gln) results in a molecule with lowered actin-activated ATPase activity, due to impaired actin binding. To create an animal model for FHC, we have made transgenic mice expressing a cardiac MHC in which the actin-binding domain has been mutated. These mice have a cardiac pathology similar to that seen in FHC, and should serve as a useful model for assessing the development of cardiac disease. We have recently initiated a program of virus-mediated *in vivo* gene transfer into muscle. Extremely efficient widespread gene transfer results from injection of recombinant adenoviruses into the thoracic cavity of neonates or into the adult myocardium. The duration of gene expression in adults is limited, while neonates exhibit prolonged expression. Adenoviruses therefore seem likely to be useful for the introduction of mutant contractile protein genes into muscle *in vivo*.

*Growth Factors/Oncogenes*

**W 030** *ski* AND FIBER TYPE HYPERTROPHY, Pramod Sutrave[1], Dolores P. Lana[1], John Leferovich[2], Mark J. Federspiel[1], James C. Engert[3], Lisa Shelton[1], Nadia Rosenthal[3], Alan Kelly[2], and Stephen H. Hughes[1], [1]ABL-Basic Research Program, NCI-Frederick Cancer Research and Development Center, Frederick, MD 21702, [2]Department of Pathobiology, University of Pennsylvania School of Veterinary Medicine, Philadelphia, [3]Massachusetts General Hospital East, Harvard Medical School, Charlestown.

We showed several years ago that a c-*ski* transgene could cause substantial hypertrophy in the skeletal muscles of transgenic animals. The effect is selective; only type IIb fibers are hypertrophied. In the initial experiments we used a truncated version of c-*ski*; we have done additional experiments to show that full-length versions of c-*ski* can also induce hypertrophy. In the animals that develop pronounced hypertrophy, the *ski* cDNAs are linked to an MSV LTR. We have not been able to induce hypertrophy with other promoters. In the muscular mice, the c-*ski* transgene is expressed at high levels in muscle and bone; the other tissues we have examined contain significantly lower levels of *ski* RNA and protein. At birth the *ski* transgenic mice appear normal; hypertrophy can be observed both macroscopically and microscopically about 10-12 days postpartum. This correlates with a substantial increase in *ski* expression. Expression of the *ski* transgene is under both neurological and thyroid control; this could explain part or all of the increase seen about 10-12 days after birth.

We are interested in determining how *ski*, which is a nuclear protein, induces hypertrophy. We have identified two promoters that respond (either directly or indirectly) to *ski*. It should be possible to use these promoters to determine what factors *ski* interacts with and how it can regulate gene expression. Research sponsored in part by the National Cancer Institute, DHHS, under contract No. N01-CO-74101 with ABL.

**W 031** FIBROBLAST GROWTH FACTOR SIGNALING PATHWAYS INVOLVED IN THE REPRESSION OF MYOGENESIS, Bradley B.Olwin[1], Arthur J. Kudla[1], Kevin Hannon[1], Kirstin M. Arthur[1], Alan C. Rapraeger[2], and Daniel F. Bowen-Pope[3], [1]Department of Biochemistry, Purdue University, West Lafayette, IN 47907, [2]Department of Pathology and Laboratory Medicine, University of Wisconsin-Madison, and [3]Department of Pathology, University of Washington, Seattle, WA.

Fibroblast growth factors repress myogenesis in skeletal muscle cell lines and primary cultures. High affinity FGF binding and signaling exhibits an absolute requirement for heparan sulfate. We have proposed a model for a FGF receptor signaling complex that is comprised of at least two FGF-binding proteins--an FGF receptor tyrosine kinase (FGFR) and a cell surface heparan sulfate proteoglycan (HSPG). As the growth factor and the receptor both bind heparan sulfate, these heparin binding site(s) may play critical roles in FGF signaling. To determine sites critical for signaling, we have characterized cell surface FGF binding proteins in the MM14 skeletal muscle cell line. MM14 myoblasts express FGFR-1, a cysteine-rich FGF receptor, and two classes of cell surface HSPGs including syndecan-1 and a member of the glypican family. Removal of FGF-2 from the growth medium initiates terminal differentiation and a decline of the mRNAs for FGFR-1 and syndecan-1 to undetectable levels. Transfection of FGFR-1 into MM14 cells increased by ~10-fold $^{125}$I-FGF-2 binding in both proliferating and terminally differentiated cells. As no syndecan is present in the differentiated cells, glypican is likely to participate in formation of the high affinity FGF binding complex. Experiments are underway to determine if glypican is the only proteoglycan capable of participating in formation of this binding complex or if syndecan also participates. To begin delineating the signaling pathway(s) used by the high affinity FGF binding complex, we transfected MM14 cells with the PDGF-ß receptor (PDGFR-ß). MM14 cells do not bind PDGFAA, or PDGFBB, express detectable PDGFR, or respond to PDGFAA, or PDGFBB. If FGFR-1 and the PDGFR-ß use similar or identical intracellular signaling pathways, both FGF-2 and PDGFBB should repress differentiation. Although addition of PDGFBB to MM14 cells expressing PDGFR-ß elicited autophosphorylation of the receptor and tyrosine phosphorylation of intracellular substrates, PDGFBB was incapable of repressing myogenesis or acting as an MM14 cell mitogen. Thus, stimulation of the signaling pathway(s) utilized by the PDGFR-ß are not sufficient to repress differentiation or stimulate mitogenesis in MM14 cells. Interestingly, PDGFBB addition to differentiation-defective MM14 cells derived from myoblasts expressing the PDGF-ß receptor do respond to PDGFBB as a mitogen. We propose that the intracellular FGF signaling pathway involved in repression of terminal differentiation requires additional signaling elements distinct from the PDGFBB signaling pathway. Experiments are in progress to identify signaling proteins involved in the repression of myogenesis.

**W 032** GROWTH FACTOR REGULATION OF MYOGENIC TRANSCRIPTION FACTORS, Kenneth Walsh[1] and David H. Gorski[2], [1]St. Elizabeth's Medical Center, Boston, MA 02135, [2]Case Western Reserve University School of Medicine, Cleveland, Ohio 44106.

Homeobox transcription factors control differentiation, proliferation and migration in many cell types. We have isolated and characterized a cell type-specific transcription factor gene dubbed *Gax* for Growth arrest-specific homeobox. *Gax* is predominantly expressed in adult cardiomyocytes and in vascular smooth muscle cells (VSMCs). VSMCs differ from skeletal and cardiac myocytes in that they de-differentiate and re-enter the cell cycle in response to growth factor stimulation, and these events contribute to the pathology of blood vessel diseases. In VSMCs, *Gax* expression is rapidly down-regulated when quiescent cells are stimulated with growth factors. *Gax* encodes a sequence-specific DNA-binding protein, and it contains transcriptional activation and repression domains in the N- and C-terminal portions of the *Gax* peptide, respectively. Microinjected *Gax* peptide significantly inhibits mitogen-induced entry into S-phase indicating that the *Gax* transcription factor is a negative regulator of cell growth. This inhibition is specific to *Gax*, dose-dependent, and can be reversed by the co-injection of a highly oncogenic *ras* mutant protein. We propose that *Gax* functions to promote differentiation and to negatively regulate cell proliferation, and thus may represent a potential target for genetic therapy of diseases that result from abnormal myocyte proliferation.

*Neuromuscular Diseases*

**W 033** CORRECTION OF MUSCULAR DYSTROPHY IN *mdx* MICE, Jeffrey S. Chamberlain[1,2], Gregory A. Cox[1], Jill A. Rafael[1], Kathleen Corrado[1], Michael A. Hauser[1], and Stephanie A. Phelps[1]. [1]Department of Human Genetics, and [2]Human Genome Center, University of Michigan Medical School, Ann Arbor, Michigan 48109.
Several strains of *mdx* mice have been described that contain mutations in the dystrophin gene and which are animal models for Duchenne/Becker muscular dystrophy (DMD/BMD). We have been characterizing the murine and human dystrophin genes to study structure-function relationships and expression patterns of the protein dystrophin and to explore the feasibility of gene therapy of DMD. Expression of full-length murine dystrophin cDNA clones under the control of the mouse muscle creatine kinase (MCK) enhancer plus promoter in transgenic *mdx* mice prevents development of the dystrophic pathophysiology in skeletal and cardiac muscle. Fifty-fold overexpression of dystrophin in skeletal muscles of *mdx* mice leads to normal muscle morphology as well as normal levels of force and power generation without detectable toxicity. We have generated additional transgenic lines using various combinations of dystrophin isoforms to analyze the levels of dystrophin needed to eliminate symptoms and to explore functional domains of the protein. Low level expression of dystrophin produces a dramatic but incomplete elimination of muscle pathology, but significantly higher levels of expression are required to achieve normal morphology and force generation. Expression vectors encoding dystrophin molecules truncated at either the amino or carboxy terminal portions of the protein are able to prevent the appearance of dystrophic symptoms in mice, suggesting that sequences from several domains of the protein are not critical for function in skeletal muscle. These and other expression vectors are being used to identify sequences required for binding to proteins associated with dystrophin at the sarcolemma. We have also cloned truncated mouse and human dystrophin BMD-like cDNAs deleted for exons 17-48, which corresponds to a genomic deletion observed in a patient with an extremely mild case of BMD. Transgenic mice expressing high levels of the BMD-like protein are being analyzed to determine whether such truncated constructs might be of therapeutic value. Identification of subdomains of dystrophin that are not absolutely required for dystrophin function could lead to the development of compact expression cassettes suitable for introduction into viral delivery vectors. We are currently testing methods for the delivery of such constructs to muscles of *mdx* mice, which if successful could provide an approach for gene therapy of Duchenne muscular dystrophy.

**W 034** GENETICALLY MODIFIED MYOBLASTS TO DELIVER RECOMBINANT PROTEINS TO THE SYSTEMIC CIRCULATION *IN VIVO*, Sandeep Tripathy[1], Eliav Barr[1], Christopher Sullivan[1], Marion Verp[1], Eugene Goldwasser[1], and Jeffrey M. Leiden[1], [1]University of Chicago, Chicago, IL 60637.



A number of serum protein deficiencies including diabetes mellitus, hemophilias A & B, and the erythropoietin-responsive anemias are currently treated by repeated subcutaneous or intravenous infusions of purified or recombinant proteins. The development of a cell-based system which could produce stable and physiological levels of recombinant proteins in the systemic circulation would represent an important advance in our ability to treat these diseases. Previous approaches using genetically-modified keratinocytes, hepatocytes, bone marrow, and fibroblasts have each failed to stably produce physiological levels of recombinant proteins in the serum. Skeletal muscle stem cells (myoblasts) display a number of properties which make them attractive candidates for such a serum protein delivery system: (i) these cells are readily obtained from routine skeletal muscle biopsies, (ii) they have a large proliferative capacity *in vitro*, (iii) they can be transduced with a variety of viral and plasmid-based vectors, (iv) cultured myoblasts can be implanted into host muscle by intramuscular injection, and subsequently fuse with each other and endogenous myocytes to become stably incorporated into host muscle, and (v) such implanted myoblasts remain localized at the site of injection and can therefore be surgically removed if it is necessary to terminate therapy. We have demonstrated previously that murine C2C12 myoblasts transfected with a human growth hormone (hGH) expression vector synthesize and secrete relatively large amounts of hGH *in vitro* (12 ng/$10^6$ cells/hour). More importantly, following implantation into syngeneic C3H mice, these genetically modified C2C12 cells produced stable and physiological levels of hGH (0.3-1 ng/ml) in the systemic circulation. To demonstrate the feasibility of this approach for human therapy, it was necessary to show that primary human myoblasts can secrete high levels of recombinant proteins *in vitro*, and, more importantly that such cells can be used to produce physiological levels of recombinant proteins and the appropriate biological effects *in vivo*. Accordingly, we have developed methods for the isolation and growth of pure populations of primary human myoblasts. These cells have been transfected *in vitro* with a human erythropoietin (hEpo) expression vector using lipofectamine and clones secreting high levels of hEpo *in vitro* (14-16 U/$10^6$ cells/day) have been isolated from these transfections. The hEpo secreted by these genetically modified myoblasts is appropriately glycosylated and possesses full biological activity *in vitro*. Implantation of these hEpo-producing primary human myoblasts into *Scid* mice by intramuscular injection resulted in significant elevations in hematocrits (47±1.4 in control mice vs. 59±1 in mice receiving hEpo-producing myoblasts; $p \leq 0.005$). Thus, genetically modified primary human myoblasts represent a feasible approach for the treatment of acquired and inherited human serum protein deficiencies.



**W 035** THE DUCHENNE MUSCULAR DYSTROPHY GENE: PRODUCTS AND REGULATION OF EXPRESSION. U. Nudel, D. Yaffe, S. Bar, E. Barnea, D. Greenberg, D. Lederfein, A., Makover, F. Marquez, P. Pizzo, H. Prigojin, D. Rapaport and D. Zuk. Dept. of Cell Biology, The Weizmann Institute of Science, Rehovot 76100, Israel.



Duchenne muscular dystrophy (DMD) is an X-linked recessive disorder which manifests as progressive degeneration of muscles and results in death. A less severe disorder, Becker's muscular dystrophy (BMD), is allelic to DMD. Some 30% of DMD patients also have mild to moderate mental retardation. The DMD gene is the largest gene known to date, consisting of almost 0.1% of the entire human genome. The product of the gene in the muscle, dystrophin, is a 427 kDa protein, translated from a 14 kb mRNA. Dystrophin is a rod-shaped protein containing an actin-binding N-terminal domain, a large domain of spectrin-like repeats, a cysteine-rich domain with potential $Ca^{2+}$ binding sites and a C-terminal domain. We and others found that a very similar isoform of dystrophin, encoded by the same gene, is found in the brain. The expression of the two isoforms is regulated by two promoters. One is active in muscle cells and glia cells. The other is active mainly in neurons. A third dystrophin isoform, which is expressed specifically in Purkinje cells, has been described very recently. A 70.8 kDa protein called Dp71 is the product of a promoter located between exons 62 and 63 of the DMD gene. This protein consists of the cysteine-rich and C-terminal domains of dystrophin, but lacks the actin-binding domain and the spectrin-like repeats. Dp71 is the main product of the DMD gene in brain, liver and many other nonmuscle tissues. An analysis of the expression of the DMD gene products during embryonic development has shown that Dp71 is already expressed in the embryonic stem cells. The known dystrophins and their mRNAs are detected only after differentiation of specialized cell types. An additional promoter located between exons 55 and 56 of the DMD gene encodes a 116 kDa protein, which is expressed specifically in peripheral nerve cells. The data may have interesting implications with respect to the ontogenic activation of the huge DMD gene during development, as well as regarding its possible evolution.

*Patterning; Embryogenesis; Muscle Development*

**W 100** GENETIC ANALYSIS OF MUSCLE FORMATION IN *DROSOPHILA*, Susan M. Abmayr, Mary R. Erickson Barbara A. Bour and Michelle Kulp, Department of Molecular and Cell Biology, The Pennsylvania State University, University Park PA, 16802

In an effort to understand genes and cell-fate decisions regulating muscle development in *Drosophila*, we have focused our efforts, in part, on *nautilus*, the *Drosophila* homolog of the family of myogenic regulatory genes characterized by MyoD. We have conducted a genetic analysis of the genomic region encoding *nautilus* to identify mutations that disrupt *nautilus* function *in vivo*. Several overlapping genetic deficiencies that remove *nautilus* as well as several surrounding genes have been generated. Analysis of these deficiencies demonstrates that *nautilus* is not required for expression of muscle-specific structural proteins such as myosin heavy chain. Moreover, *nautilus* does not appear to be essential for formation of all multinucleate syncitia since at least a subset of the embryonic muscle fibers are formed in its absence. The mutant phenotypes of these alleles and their relationship to *nautilus* will be discussed.

We have also focused attention on a second gene that is essential for muscle development. This new mutation (A3) was isolated as a second hit during an EMS mutagenesis of the 3rd chromosome (above), but was found to reside on the 2nd chromosome. Embryos homozygous for the A3 mutation exhibit a dramatic absence of muscle fibers, and a large number of unfused myosin-expressing cells. Two additional mutant alleles of the A3 locus have been isolated. These exhibit intermediate phenotypes, having fused but disorganized muscle fibers. Genetically, all three mutations can be mapped to a region of the second chromosome between genetic position 54.8 and 72.0. Precise genetic mapping of all three alleles is in progress.

**W 101** SPECIFICATION OF PRECARDIAC MESODERM OCCURS DURING GASTRULATION IN QUAIL. Parker B. Antin and Tatiana Yatskievych. Dept. of Animal Sciences, University of Arizona, Tucson, AZ 85721

A classical view of heart development, derived primarily from experiments in several species of urodeles, suggests that mesoderm with the capacity to form heart muscle arises in response to an inductive influence of anterior endoderm during neurula stages of development. Recent studies in Xenopus, however, have shown that specification of precardiac mesoderm (PCM) occurs during gastrulation and that removal of endoderm from early gastrulae has no effect on the appearance of a beating heart. It is therefore not clear whether endoderm-dependent induction of PCM is a general pattern of vertebrate development. We have undertaken a series of explant experiments in quail to determine whether anterolateral endoderm is involved in the specification of PCM in a higher vertebrate species. Mesoderm from precardiac regions of stage $4^{+}$-6 embryos was explanted alone or in combination with adjacent endoderm or ectoderm, cultured for 12 to 72 hours in several types of culture media and then assayed by morphological and immunocytochemical criteria for the presence of differentiated cardiac myocytes. Using a defined medium (75:25 mixture of DMEM and McCoy's basal medium plus 1ug/ml linoleic acid, 250µg/ml BSA and 100nM dexamethasone) we have found that mesoderm from heart forming regions is capable of differentiating into beating cardiac myocytes by stage $4^{+}$, the earliest time at which we could isolate mesoderm from adjacent cell layers. Although an interaction with anterolateral endoderm from stage $4^{+}$ onward is therefore not required for the specification of PCM, the addition of endoderm to explants enhanced the rate of myocyte differentiation and shortened the delay between expression of myosin heavy chain and the onset of beating. Endoderm also played a central role in early heart morphogenesis since beating heart tubes formed only in explants that contained both mesoderm and endoderm. In contrast, ectoderm from stage $4^{+}$-$5^{+}$ embryos did not support development of precardiac mesoderm. Our findings indicate that specification of PCM in quail occurs prior to or during gastrulation, precluding a role for anterolateral endoderm in this process. The timing of PCM specification in quail and Xenopus are therefore similar and may reflect a more general pattern of heart development than the later, endoderm-dependent specification observed in urodeles.

**W 102** TRANS-DIFFERENTIATION OF EMBRYONIC QUAIL PINEAL-BODY CELLS TO MYOBLASTIC CELLS AND MYOTUBES, Akira Asano, Tatsuya Yoshimi and Masahiko Nakamura, Institute for Protein Research, Osaka University, Suita, Osaka 565, JAPAN

Embryonic pineal-body cells of quail have been known to differentiate to lens cells, nerve cells, muscle cells under appropriate conditions. We improved the method and established very efficient conditions for trans-differentiation of the pineal cells to skeletal muscle cells by step-wise increase of NaCl concentrations. During the course of differentiation, desmin was detected first followed by myosin by specific antibodies. Extensive cell fusion was observed between these two events. Actually, myosin was found only in fused cells (myotubes). Among muscle-specific regulatory factors surveyed by Northern bloting, qmf-1 (MyoD) appeared first, but after about 10 days of the detection of desmin. Qmf-2 (myogenin) was found next, and qmf-3 (Myf-5) can not be detected in this system so far, but was detected in myoblasts of embryonic thigh muscles. Creatine kinase activity was increased in parallel with qmf-1. Several molecular species of NCAM was found at early phase of the differentiation, but a molecular species containing muscle-specific domain was detected with a specific antibody almost at the same time of qmf-1 detection. This seems to be excellent system for the study of early phase of myogenic differentiation.

**W 103 GENOTYPE SPECIFIC DIFFERENCES IN THE MYOGENICITY OF PRIMARY CULTURES FROM ADULT SKELETAL MUSCLE,** Manfred W. Beilharz*, Moira A.L. Maley, Ying Fan and Miranda D. Grounds, Departments of Pathology and Microbiology*, University of Western Australia, Queen Elizabeth II Medical Centre, Nedlands, WA, 6009, Australia.

The superior muscle regeneration seen in the tibialis anterior muscle of adult SJL/J mice, in comparison to age and sex matched BALB/c mice, has been extensively studied *in vivo* [1]. Observations have associated this superior myogenicity with earlier muscle precursor cell (mpc) replication and larger numbers of inflammatory cells in the SJL/J mice. However, bone marrow transplantation experiments have indicated that the genotype of the inflammatory cells does not influence thepattern of muscle regeneration [2]. In order to separate the relative roles of host environment and the myogenic potential of the mpc themselves, the present study has examined primary cultures of skeletal muscle from both strains of mice. The time course of expression of the skeletal specific regulatory genes MyoD and myogenin was monitored for 72 hrs after culturing skeletal muscle on either matrigel and gelatin. *In situ* detection of expression with MyoD and myogenin riboprobes and myogenin antibody showed that the onset of expression of these genes occurred earlier in cells from SJL/J mice. Probe positive cells and myotubes were also more frequent in cultures from SJL/J mice than in BALB/c. The onset of expression of MyoD and myogenin was delayed in cultured cells relative to the time course seen following injury *in vivo*. Myogenin protein was demonstrated in replicating cells and all myogenin positive cells expressed desmin. The observed strain-specific differences in these cultures infers a greater intrinsic myogenicity of cells in SJL/J muscle *in vitro* and reflects the superior capacity for new muscle formation seen in SJL/J mice *in vivo*.

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**W 104 A MURINE PARAXIAL MESODERM-SPECIFIC B-HLH PROTEIN CAN INDUCE AXIAL DEFECTS IN XENOPUS,**
Michael A. Blanar[1,6], Tony Muslin[2], Kevin Peters[2,5], Philip Crossley[3], Eirikur Steingrimsson[4], Nancy Jenkins[4], William J. Rutter[1], [1]Hormone Research Institute, [2]Cardiovascular Research Institute, [3]Dept. of Anatomy and Program in Developmental Biology, University of California San Francisco, CA 94143. [4]Mammalian Genetics Laboratory, NCI-FCRDC, Frederick MD 21702-1201. [5]Current Address: Dept. Cardiology, Duke University Medical Center, Durham, NC 27710-3623. [6]Current Address: Dept. Cardiovascular Drug Discovery, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton NJ 08543-4000.

We have isolated a novel member of the basic-helix-loop-helix (bHLH) family of transcription factors, Meso-1, that is expressed in the presomitic (paraxial) mesoderm of the developing mouse embryo. Meso-1 was identified by the 'interaction cloning' method [Blanar and Rutter (1992) *Science* **256**: 1014] in which the bHLH protein interaction domain of the ubiquitously expressed E2A gene product was radiolabeled and used to probe a mammalian cDNA λgt11 expression library. No significant similarity to any other gene/protein could be identified outside of the bHLH region. Meso-1 is expressed initially in the developing mouse between embyonic day 7 and 7.5 in mid-gastrulation. Remarkably, expression at this early time point is restricted solely to paraxial mesoderm. Segmentation of the paraxial mesoderm into somites occurs in an anterior-to-posterior gradient on both sides of the developing neural tube beginning at about embryonic day 8. Expression of Meso-1 continues uniquely in all cells of these coalescing somites. Upon differentiation of the somites, Meso-1 expression is significantly reduced in the myotomal compartment concomitant with the appearance of the first myogenic regulatory protein, *myf-5*. Meso-1 continues to be expressed in cells of the dermatome and sclerotome. Consistent with the observed expression pattern of Meso-1, the murine chromosomal map position of Meso-1 places it in a location involved with various developmental abnormalities.

This specific pattern of expression suggests that Meso-1 is likely to be involved in the early critical events that commit cells to the somitic lineage. If so, it might be possible in early development to reprogram non-paraxial mesoderm cells into somitic precursors. To test this, synthetic Meso-1 mRNA was micro-injected into 1-cell stage frog embryos. Whereas embryos develop normally when micro-injected with buffer or with mRNA encoding a mutant Meso-1 protein, expression of wild-type Meso-1 results in various axial defects. Immunohistochemical analyses of these embryos suggests that Meso-1 may be capable of directing cell-fate into the somitic lineage.

**W 105 INDUCTION OF SOMITIC MYOGENESIS BY FACTORS FROM THE NEURAL TUBE AND NOTOCHORD**
Nicholas Buffinger and Frank E. Stockdale, Stanford University, School of Medicine, Stanford CA 94305-5306

The first evidence of myogenesis in vertebrate embryos is found in the somites during early embryonic development. We systematically tested the twelve most newly formed somites from stages 10 to 15 (approximately 2 days of incubation) chick embryos for specification to myogenesis. We divided these somites into four groups, comprising the three most newly formed pairs of somites, the three next newly formed pair of somites, and so on. We find the first evidence of myogenic specification within somites is in the stage 11 (13 somite) embryo. Explants made from the rostral 3 groups of somites form muscle fibers autonomously, while explants from the most caudal group (and therefore most recently segmented somites) and all somites from stage 10 embryos do not form muscle fibers. These somites are designated as unspecified. The segmental plate from stages 10 to 15 was also tested, and found not to form muscle fibers in explant culture. We found that the notochord is able to induce myogenesis in somite explants which would not form muscle fibers when cultured alone. The notochord was able to induce myogenesis in all unspecified somites from stage 11 to 15 embryos and in all but the three most newly formed pairs of somites from stage 10 embryos. The notochord is not able to induce myogenesis in explants of the segmental plate. The neural tube is able to induce myogenesis in all unspecified somites and segmental plate from all stages tested (stage 10 to 15). The difference between the myogenic inducing factors from the neural tube and notochord appears to be qualitative rather than quantitative, as increased amounts of notochord (approximately 6 fold increase) are unable to induce myogenesis in the segmental plate. Both signals appear diffusible. We have placed explants of unspecified somites across nucleopore filters from inducing tissues. We find that both the neural tube and notochord can induce muscle fiber formation in somites from across the filter. The signal from the neural tube acts quickly - less than 18 hours transfilter exposure is required for induction of myogenesis. Interestingly, we also find evidence of a factor from the neural tube which inhibits myogenesis. The dorsal half of the neural tube is a poor inducer of myogenesis in unspecified somites, and appears to actively inhibit myogenesis in somites which have already been specified to myogenesis.

**W 106 REGULATION OF SLOW MYOSIN HEAVY CHAIN EXPRESSION BY INNERVATION IN VITRO**
Joseph X. DiMario and Frank E. Stockdale, Stanford University, School of Medicine, Stanford CA 94305-5306

Although much research has established a causal relationship between innervation and expression of myosin heavy chain (MHC) genes in vivo, the mechanisms of nerve dependent MHC gene expression have not been described. We have developed a model system in which the mechanism of the regulation of slow MHC genes by innervation can be studied in vitro. Fetal avian myoblasts, isolated from fast or fast/slow MHC containing muscles, form myotubes in vitro which do not independently express slow MHC2 or slow MHC3. However, when myoblasts isolated from the slow contracting medial adductor muscle are cultured in the presence of avian spinal cord cells, slow MHC gene expression is induced as assessed by immunoreactivity of myotubes in these cultures with the anti-slow MHC monoclonal antibody S58. Myoblasts isolated from the fast contracting pectoralis major muscle form myotubes in vitro that do not express S58 reactive MHC(s) in the presence or absence of spinal cord cells. These results indicate that a mechanism exists in myoblasts from the medial adductor muscle, and not the pectoralis major, which responds to the presence of innervation and results in expression of select MHC genes.

**W 107 DO DIFFERENT CLASSES OF HUMAN SATELLITE CELLS EXIST ?**
Frederique Edom, Vincent Mouly, J. Patrick Barbet*, Gillian S. Butler-Browne. C.N.R.S. 1448, Faculte de Medecine, Paris 75006, FRANCE * Hôpital St-Vincent de Paul, Paris 75014, FRANCE.

Satellite cells were isolated from human quadriceps (mixed) and masseter (predominantly slow) muscles. The growth kinetics, morphological features and programmes of differentiation of these cells were examined in vitro.

The expression of the different myosin heavy (embryonic, fetal, fast and slow) and light chain isoforms was used to assay myotube diversification.

In addition to embryonic and fetal isoforms we found that fused cultures of human satellite cells express both adult fast and slow MHCs. Only the four fast type light chains (MLC1emb, MLC1f, MLC2f and MLC3f) were synthesized and slow MLCs were never detected in these cultures. In order to determine if the human satellite cells were commited to distinct fast and slow cell lineages a clonal analysis was also carried out on both cell populations. This analysis was first carried out on clonal populations and was confirmed by the analysis of isolated clones. All myogenic clones expressed both fast and slow MHCs suggesting that there is no evidence for different fast and slow satellite cell lineages in human skeletal muscle. This is in contrast to the developing muscle where different classes of myogenic cells can be identified.

**W 108** RELATED MYOBLASTS FUSE WITH BOTH PRIMARY AND SECONDARY FIBERS DURING FETAL MUSCLE DEVELOPMENT, Darrell J.R.Evans and Peter M.C.Wigmore, Department of Biomedical Sciences, Marischal College, University of Aberdeen, Aberdeen AB9 1AS United Kingdom.

Different populations of myogenic cells are thought to be present at different stages of myogenesis and to contribute to different types of fiber. During the later stages of myogenesis primary fibers are growing by the incorporation of new cells and secondary fibers are forming by cell fusion. It is unclear whether the same population of cells contributes to both types of fiber at this age. A laparotomy was performed on pregnant anaesthetised mice on embryonic day (E)15 or E17 and two replication deficient retroviruses were simultaneously injected into the hind limb muscles of the fetuses. Both viruses carry the lac Z gene for beta-galactosidase. This is localised to the nucleus of cells infected by one virus and the cytoplasm of cells infected by the other. All animals were killed at E19 when primary and secondary fibers were still distinguishable. At both ages widely separated clusters of 2 to 5 marked fibers were present. A total of 24 clusters of fibers were scored between both ages. All clusters contained fibers marked by only one type of virus. These clusters are assumed to result from the fusion of single clones of cells with surrounding fibers. Most (83%) clusters contained both primary and secondary fibers as identified under low power transmission electron microscopy. This would indicate that related cells have contributed both to the formation of new secondaries and to the continued growth of primaries. These results suggest that at E15 and E17 a single population of cells contributes to both types of fiber (Evans et al. Dev. Biol.submitted).

**W 109** CARDIAC MESODERM SPECIFICATION/DETERMINATION IN *XENOPUS LAEVIS*, Sylvia M. Evans, Jeanette Ponce, and M. Patricia Murillo, Department of Medicine, University of California San Diego, La Jolla, Ca, 92093

Not much is known about the early signalling events in cardiac mesoderm specification/determination, although recent work by other authors indicates that relatively high doses of the growth factor activin can induce expression of a cardiac specific gene in *Xenopus laevis* animal cap explants (1). In *Drosophila*, a homeobox gene, *tinman*, appears to be essential for cardiac mesoderm formation (2). A mouse homologue of *tinman*, Csx, is specifically expressed in the developing heart (3), suggesting that this homeobox family may be essential for cardiac mesoderm formation in vertebrates. To examine the possible role that the *tinman* family might play in cardiac specification/determination, we have cloned several members of this family from a *Xenopus laevis* neurula stage cDNA library. The mRNAs encoded by several of these clones are expressed in adult heart, in addition to other tissues. Whole mount mRNA *in situ* analysis of several embryonic stages indicates that some of these genes are expressed in early cardiac progenitors and in the early heart tube. In addition to *tinman* homologues, we have also cloned several markers of cardiac determination, partial cDNAs for *Xenopus* α-myosin heavy chain (XMHC) and *Xenopus* atrial light chain (XALC). We are currently sequencing and further characterizing our *tinman* homologues, comparing their expression patterns to those of XMHC and XALC. Future studies will involve mesoderm induction assays and microinjection assays to examine the role that the *tinman* homologues play in cardiac mesoderm specification/determination in vertebrates.

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**W 110** **Dorsoventral patterning of mammalian somites: a role for epidermis in patterning the dermomyotome.** Chen-Ming Fan and Marc Tessier-Lavigne, Department of Anatomy, UCSF, 513 Parnassus Avenue, San Francisco, CA 94143-0452

When somites first pinch off from the segmental plate, they are undifferentiated epithelial balls, but become rapidly patterned into sclerotome (ventromedially) and dermomyotome (dorsolaterally). Grafting experiments in chick embryos (Pourquie et al., PNAS **90**, 5242-5246, 1993) have shown that the sclerotome is induced by two axial structures, the notochord and the floor plate. To study dorsoventral patterning of somites in the mouse, we have developed an *in vitro* assay in which segmental plate explants isolated from embryonic day (E) 9.5 mice are cultured in three-dimensional collagen matrices in the presence of embryonic tissues that are sources of inductive and patterning signals. To assay for patterning, we have used several marker genes encoding transcription factors with restricted domains of expression within somites: To identify dermomyotome, we have followed the expression of *Pax*-3 and *Sim*-1 (a mammalian homologue of the *Drosophila single-minded* gene), which are initially expressed throughout the segmental plate but rapidly become restricted to the dermomyotome in the somite. To identify sclerotome, we have followed the expression of *Pax*-1, which is not expressed in segmental plate but is expressed in sclerotome as it forms. Finally, we have used *Mtwist* as the fourth marker for all somitic cells: *Mtwist* is expressed in the segmental plate and, although it is more highly expressed in sclerotome, it nonetheless remains expressed in dermomyotome.

To examine the effects of the notochord on segmental plate, we cocultured these tissues and assayed for gene expression by RT-PCR. Prior to culture, *Pax*-3, *Sim*-1 and *Mtwist* are all expressed in segmental plate. After 24hr in culture with notochord, *Pax*-1 expression is detected, whereas *Pax*-3 and *Sim*-1 are downregulated. This result is consistent with previous studies showing that the notochord can induce sclerotome *in vivo*. This induction occurs even when the tissues are cultured at a distance or separated by a porous membrane, indicating that it is due to a diffusible factor(s) secreted by notochord. Surprisingly, when segmental plate is cultured alone, *Pax*-3 and *Sim*-1 expression are downregulated, while *Mtwist* expression is maintained. When cocultured with dorsal epidermis, however, segmental plate continues to express *Pax*-3 and *Sim*-1, suggesting that interaction of segmental plate with epidermis is required for formation of the dermomyotome. In contrast to the effect of the notochord, this effect of the epidermis requires close contact between the tissues. These results suggest that dorsoventral patterning of the segmental plate requires interaction not just with axial structures but also with the epidermis.

**W 111** THE FORMATION OF VISCERAL MUSCULATURE AND HEART IN DROSOPHILA DEPENDS ON INDUCTIVE EVENTS THAT INVOLVE dpp (TGFβ) AND wingless (Dwnt), Manfred Frasch, Natalia Azpiazu, and Zhizhang Yin, Brookdale Center for Molecular Biology, Mount Sinai School of Medicine, New York, NY 10029

The homeo box genes tinman and bagpipe function during early Drosophila embryogenesis to determine the formation of the visceral mesoderm and heart from dorsal mesodermal cells. We found that bagpipe is expressed in segmental clusters of dorsal mesodermal cells that correspond to the primordia of the visceral mesoderm. In bagpipe mutant embryos, gut muscle formation is severely reduced, whereas upon ectopic expression of bagpipe, ventral mesodermal cells that normally give rise to somatic mesoderm are transformed into visceral mesoderm. Therefore, in wild type embryos, bagpipe appears to specify segmental repeats of dorsal mesodermal cells to adopt visceral mesoderm fates. The expression of bagpipe depends on the function of the tinman gene. tinman is initially expressed in the whole mesoderm. In a process that reflects one of the first steps of spatial subdivisions of the mesoderm, tinman expression becomes restricted to dorsal domains that appear to define the dorso-ventral extent of bagpipe expression. Since bagpipe expression is absent in tinman mutants, dorsal mesodermal cells fail to form gut musculature in these embryos. In subsequent stages, tinman becomes further restricted to cells at the dorsal crest of the mesoderm and functions to specify these cells as heart progenitors.

These results indicate that the proper spatial regulation of tinman and bagpipe expression ultimately defines the subdivision of the mesoderm into the primordia of the visceral and the somatic mesoderm. We present evidence that dpp, a Drosophila homolog of TGFβ that is expressed in dorsal ectodermal cells, may act as an inductive signal to activate tinman expression in the dorsal mesoderm. The signalling molecule wingless that is expressed in ectodermal stripes is required, in addition to tinman, for the segmental expression of bagpipe in the dorsal mesoderm. Therefore, the primordia of the visceral mesoderm appear to be defined by a combination of dorso-ventral and anterior-posterior signalling molecules. We identified enhancer elements of tinman and bagpipe that could be targets of these signals.

N. Azpiazu & M. Frasch, Genes & Dev. 7, 1325-1340 (1993).

**W 112 MATURATION OF MYOGENIC AND CHONDROGENIC PRECURSORS WITHIN THE PRESOMITIC MESODERM,** Mindy George-Weinstein, Jacqueline Gerhart, Gerard Foti, Joseph Flynn, Carolyn Miehle and Beth Callihan, Dept. of Anatomy, Philadelphia College of Osteopathic Medicine, Phila., PA 19131

The establishment of cells with myogenic or chondrogenic potential is temporally and spatially separated from terminal differentiation. Both cell types arise from the paraxial mesoderm which consists of somites and caudally, the segmental plate (sp). Chick embryo somite and sp cells were cultured as monolayers on a substrate of gelatin and fibronectin in serum containing medium. More skeletal muscle and chondroblasts arose in cultures prepared from progressively more rostral regions of the paraxial mesoderm. Sp cells from 48 hour embryos differentiated to a greater extent than 36 hour somite cells. Somites and the rostral two thirds of the sp from 48 hour embryos contained cells which differentiated in vitro without replicating. Only the progeny of cells from the caudal third of the 48 hour sp, or from 36 hour somites and sp, underwent differentiation. These results suggest that some myogenic and chondrogenic cells obtain the ability to differentiate under these conditions after 36 hours of development, and as they occupy more rostral positions within the sp relative to the addition of cells at its caudal end. Epidermal growth factor (EGF) and prostaglandin E1 produced an increase in the percentage of skeletal muscle cells in cultures of sp cells grown in serum free medium. The maturation of sp cells is subject to environmental regulation.

**W 113 TARGETED MUTAGENESIS OF THE HEART-EXPRESSED HOMEOBOX GENE *NKX-2.5* RESULTS IN ABNORMAL HEART DEVELOPMENT AND EMBRYONIC LETHALITY**
Richard P. Harvey, Ian Lyons, Ruili Li, Linda M. Parsons, Lynne Hartley, Jane Andrews and Matthijs Smith, The Walter and Eliza Hall Institute of Medical Research, Post Office, Royal Melbourne Hospital, Victoria 3050 Australia

We have cloned and characterized a murine homeobox gene, *Nkx-2.5*, that is expressed in early myocardiogenic progenitor cells and cardiomyocytes of embryonic, foetal and adult hearts. At early developmental stages *Nkx-2.5* is also expressed in primitive pharyngeal endoderm, a tissue believed to be the source of factors that induce myogenesis and, later, to advance heart morphogenesis. These expression characteristics suggest that *Nkx-2.5* plays an important role in heart development and perhaps in induction events. To test this we have disrupted the *Nkx-2.5* gene in ES cells by gene targeting and have created a new mouse strain via the blastocyst injection, chimaera production route. Although a transcript is expressed from the disrupted allele, it does not have the capacity to code for an intact homeodomain. Heterozygous mutant animals are healthy and fecund. On the other hand, homozygous embryos arrest growth after 9 days of *in utero* development and ultimately die. The hearts tubes of these embryos beat, but develop abnormally and at best progress little beyond early stages of looping morphogenesis. Macroscopically, there is poor discrimination, if any, between future atrial and ventricular regions and the truncus arteriosus is uncharacteristically narrow.

The data to date shows that the defect is inherited in a Mendelian fashion, thus demonstrating an essential role for the *Nkx-2.5* gene in heart development. At present we assume that the cause of growth arrest and lethality is an inability of defective hearts to effectively perfuse embryos with growth factor-rich serum. Experiments are under-way to examine histological, growth and molecular parameters of mutant myocardial tissue with the aim of assessing whether the mutant phenotype is attributable to a defect in establishing the cardiac myogenic lineage, or in an essential pathway that runs parallel to otherwise normal myogenesis. Our current findings will be presented.

**W 114 A HOX SITE WITHIN THE MYOSIN LIGHT CHAIN 1/3 ENHANCER BINDS DIFFERENT PROTEINS OF MUSCLES DERIVED FROM DISTINCT SOMITIC LINEAGES IN THE MOUSE,** Leslie A. Houghton[1,2] and Nadia Rosenthal[2], [1]Department of Biology, Boston University, 5 Cummington Street, Boston, MA 02215 and [2]Cardiovascular Research Center, Massachusetts General Hospital-East, 149 13th Street, Charlestown, MA 02129.

Distinct populations of myogenic precursor cells arise in the vertebrate somite. These cells are influenced by different embryonic environments including the neural tube and the somatopleural mesoderm. Further distinctions amongst these cells may result from their initial location along the anterio-posterior axis of the embryo, and the possible expression of homeotic genes within the somite, particularly the myotome. The myosin light chain 1/3 enhancer contains a suspected Hox binding site which has been used as a probe in gel mobility shift assays. We have observed that cellular extracts prepared from segmental muscles, such as intercostals, exhibit a different pattern of band shifts compared with extracts of laterally derived muscles such as those of the leg. Gel shifts have also been performed with extracts of somites and limb buds from mouse embryos at early embryonic stages in an effort to define these distinctions in binding affinity to the Hox site during development, as well as with *in vitro* translated homeotic proteins of known identity. The localization of the homeotic protein Hox B9 (2.5) in the somite and its colocalization with myogenic factors restricted to the mytotome has also been analyzed by *in situ* hybridization of embryos transgenic for a *Hox B9 - LacZ* construct.

**W 115 ASSOCIATION OF A *PAX 7* RFLP WITH INCREASED EFFICIENCY OF SKELETAL MUSCLE REGENERATION IN ADULT MICE,** Peter H. Kay, Emma Pereira, Chris A. Mitchell, Annik Pannicker and John M. Papadimitriou, Molecular Pathology Laboratory, Department of Pathology, Queen Elizabeth II Medical Centre, Nedlands, Western Australia, 6009

Mechanically injured skeletal muscle is regenerated more efficiently in SJL/J mice compared to other laboratory strains. Consequently, we hypothesised that variation in efficiency of skeletal muscle regeneration would be influenced by only a small number of genes which differ between SJL/J and other laboratory mice. In an attempt to identify relevant genes, we have previously shown that SJL/J mice have a unique form of *Myo-D1*. Segregation studies have indicated that efficiency of skeletal muscle regeneration is partly influenced by the unusual form of *Myo-D1* found in SJL/J mice, and that other genes are also involved. Thus far we have been unable to implicate other myogenic genes such as the equivalent of *Myf-4, Myf-5* and *Myf-6* in variation in efficiency of tissue regeneration in the mature animal since no structural differences in these genes have been found between SJL/J and other mouse strains. By contrast, we have recently shown that compared to most laboratory mice, SJL/J have an unusual structural form of the myogenic paired box/homeobox gene *Pax7* (Jostes *et al.*, 1991) which is also shared by Quackenbush mice. Functional studies have shown that Quackenbush mice also have skeletal muscle regenerative properties similar to SJL/J mice. Thus, by association, these studies provide evidence that the rate of regeneration of damaged skeletal muscle in the mature animal may also be influenced by structural variation of the *Pax7* gene and provide new approaches to understanding the molecular genetic control of myogenesis.

Jostes, B., Walther, C. and Gruss, P. *Mechanisms of Development,* 33, 27-38 (1991).

**W 116** A NEW MUTATION CAUSING MUSCLE LOSS IN *DROSOPHILA MELANOGASTER*, S. Knirr, S. Ohlmeier, A. Paululat and R. Renkawitz-Pohl, Faculty of Biology / University of Marburg, Germany, Fax: 49 6421 287077

Using a combined genetic and molecular approach we want to isolate and characterize genes involved in the muscle differentiation process of *Drosophila melanogaster*.

Here we present a genetic, cytological and molecular characterization of a P-element induced mutant, named *not enough muscles (nem)*. This mutant shows disturbances in the architecture of somatic musculature as well as the deletion of parts of the musculature. The development of the visceral musculature and of the heart is not markedly affected, showing a specific effect of this mutation on the differentiation program of the somatic musculature. Using "plasmid-rescue" procedure and subsequent screening of genomic libraries we cloned the genomic region surrounding the P-insertion site. With these genomic clones we isolated one cDNA with an open reading frame of about 1.4 kb. Our first sequence comparison, based on the deduced protein sequences, shows no significant homologies to genes of the vertebrate MyoD family or to muscle structural genes. More information about the function of the affected gene will be given by embryonic whole mount in situ hybridization experiments to determine the cytological distribution of the transcript. To gain more insight into the function of the encoded protein, we isolated three EMS-induced alleles. With the combination of cytological and molecular genetic methods we hope to get an insight in the function of this new gene and its developmental relevance.

**W 117** The Intracellular Portion of Mouse Notch Functions as a Constitutively Activated Repressor of Myogenesis: A Requirement for Nuclear Localization. Raphael Kopan[1]*, Jeffrey S. Nye[2] and Harold Weintraub[1] 1: The Fred Hutchinson Cancer Research Center, Howard Hughes Medical Institute, Seattle WA 98104. 2: Columbia University College of Physicians and Surgeons, Department of Biochemistry. New York, New York 10032.

We show that Myf-5 and mNotch mRNA are both present in the presomitic mesoderm (PSM) before muscle cell commitment and before muscle structural gene activation. The failure of presomitic mesoderm to respond to Myf-5 and express myogenic properties implies that some mechanism must exist to suppress its differentiation. Here we show that ectopic expression of the intracellular domain of mNotch (mNotchIC) functions as a constitutively activated repressor of myogenesis both in cultured cells and in frog embryos. mNotchIC contains a putative nuclear localization signal (NLS) and localizes to the nucleus. Removal of the NLS reduces nuclear localization and diminishes the inhibition of myogenesis caused by Myf-5 or MyoD. Both nuclear localization and inhibition of myogenesis are restored by adding a NLS sequence from SV40, suggesting that nuclear localization is required for efficient inhibition of myogenesis. We suggest a speculative model of transcriptional regulation whereby a membrane receptor is cleaved and the cytoplasmic domain translocates to the nucleus to inhibit a key transcription factor.

**W 118** AN E-BOX IN THE DESMIN PROMOTER COOP - ERATES WITH THE E-BOX AND MEF-2 SITES OF A DISTAL ENHANCER TO DIRECT MUSCLE-SPECIFIC TRANSCRIPTION, Hui Li and Yassemi Capetanaki, Department of Cell Biology, Baylor College of Medicine, Houston, Texas, 77030, USA.

The unique pattern of desmin expression serves as a model to investigate the regulatory mechanisms involved in the early stages of myogenesis. In constrast to most muscle genes, desmin is expressed in replicating myoblasts and satellite cells. Upon terminal differentiation desmin expression is increased several fold. In addition, desmin is one of the first muscle specific proteins to appear during mammalian embryonic development. Our in vitro studies during skeletal myogenesis so far have shown the first 81nt upstream of the transcription initiation site of the mouse desmin gene, which contain an E-box (E1), the binding site of the HLH myogenic regulators, are sufficient to confer low but muscle-specific expression. High levels of desmin expression, however, are due to an enhancer , located between -798nt to -976nt, which contains an additional E box (E2) and a muscle-specific enhancer factor-2 (MEF-2) binding site. We have previously shown that both myoD and myogenin can bind to the proximal E1 and distal E2 boxes. Here we demonstrate that MEF-2C, a myocyte specific member of the MEF-2 family, can bind to the desmin MEF-2 site. Site-directed mutagenesis showed that functional units for the enhancer activity require intact E2 and MEF-2 elements. Mutations at both sites reduced the CAT activity to the level comparable with that of the proximal promoter. Mutations at either E2 or MEF-2 site alone, caused only 25% reduction in the transcriptional activity. Thus, the desmin enhancer can fuction relatively well with either the E2 box or the MEF-2 site and only mutation of both of them eliminates transcriptional enhancement. The presence of both of these elements is required for maximum enhancer activity. On the other hand, mutagenesis of just the proximal E1 box showed that this element is essential for desmin gene expression. Double mutations of E1 with E2 or MEF-2 sites suggested that for high expression of the desmin gene, E1 seems to serve as a bridge for either E2 or MEF-2 enhancer elements to function. The location of the E1 site relative to the TATA box is crucial. Its activity is DNA-turn and distance dependent. It was further demonstrated that maximum transactivation of desmin by myoD and myogenin in 10T1/2 fibroblasts, can be achieved mainly through the E1 box and does not necessarily require the enhancer. Inactivation of the E1 site diminished the transactivation by these factors but at least 35% of the activity was retained when MRF4 and Myf5 were used, indicating that the latter two factors might use a somehow different mechanism for transactivation in these cells.

**W 119** D-MEF2: A MADS BOX PROTEIN THAT DEFINES THE SOMATIC MUSCLE CELL LINEAGE DURING DROSOPHILA EMBRYOGENESIS, Brenda Lilly, Samuel Galewsky, Anthony Firulli, Robert Schulz and Eric N. Olson, Department of Biochemistry and Molecular Biology, The University of Texas M. D. Anderson Cancer Center, Houston, TX 77030

The myocyte enhancer factor (MEF)-2 family of transcription factors has been implicated in the regulation of muscle transcription in vertebrates, but the precise position of these regulators within the genetic hierarchy leading to myogenesis is unclear. MEF2 proteins belong to the MADS superfamily of regulatory proteins, which control specific programs of gene expression in species ranging from yeast to humans. We have isolated a cDNA clone encoding a protein from *Drosophila*, termed D-MEF2, that shares extensive amino acid homology with the MADS domains of the vertebrate MEF2 proteins. The D-MEF2 protein binds the same DNA sequence as its vertebrate homologs and can activate transcription through tandem copies of the MEF2 binding site. D-MEF2 expression is first detected during *Drosophila* embryogenesis within mesodermal precursor cells prior to separation into the somatic and visceral muscle lineages. During late gastrulation and germ band extension, the expression pattern of D-MEF2 is similar to that of *twist*, which is required for the establishment of mesoderm, and precedes that of the MyoD homolog, *nautilus*. In contrast to *nautilus*, which is expressed in only a subset of somatic muscle cell precursors, D-MEF2 is expressed in all somatic and visceral muscle cell precursors and is maintained in all somatic muscle cells. In homozygous *twist* and *snail* mutant embryos, which fail to make mesoderm, D-MEF2 is not expressed. D-MEF2 is the earliest marker for the myogenic lineage to be identified in *Drosophila*; its temporal and spatial pattern of expression and its transcriptional activity make it a likely candidate for a regulator of cell fate within myogenic cell lineages. The phenotypes of *Drosophila* lines harboring heat shock regulated D-MEF2 and dominant negative D-MEF2 mutant genes will be presented.

**W 120** CARDIOGENESIS IN *XENOPUS LAEVIS*.

Malcolm Logan and Tim Mohun, Department of Developmental Biochemistry, National Institute for Medical Research, Mill Hill, London, NW7 1AA. U.K.

In order to facilitate a molecular study of heart development in the amphibian, *Xenopus laevis*, we have isolated a cDNA fragment encoding a portion of the myosin heavy chain a-isoform (XMHCa)( Logan and Mohun 1993).The XMHCa transcript is expressed exclusively in embryonic heart tissue and therefore provides a tissue-specific marker for cardiac muscle differentiation during early *Xenopus* embryogenesis. XMHCa gene expression can be induced in explants of *Xenopus* animal pole cells by treatment with the growth factor, activin A. This response is dose dependant. High doses of activin induce both cardiac and skeletal muscle whilst lower doses induce only skeletal muscle. XnKX2.5, the Xenopus homologue of the Drosophila gene Tinman as well as the endocardial marker GATA 4 are also induced in these explants. Together these results suggest that this system faithfully recapitulates many of the events of cardiogenesis.

Logan, M. and Mohun, T.1993. Induction of cardiac muscle differentiation in isolated animal pole explants of *Xenopus laevis* embryos. Development 118,865-875.

**W 121** *Nkx*-3.1: A NOVEL HOMEOBOX GENE EXPRESSED IN DEVELOPING SOMITES,

Kathleen A. Mahon, Lawrence S. Amesse, Aradhana Venkatesan, and Lidia Gleizer, Laboratory of Mammalian Genes and Development, NICHD, NIH, Bethesda, MD 20892.

We have isolated a new gene from the mouse, *Nkx*-3.1, that contains a homeobox similar to that of the *Drosophila Nk*-3 gene. This gene encodes a 2.2 kb transcript that is dynamically expressed during somitogenesis. *Nkx*-3.1 is specifically expressed in the youngest, most newly formed somites at the caudal end of the embryo. The localization of *Nkx*-3.1 transcripts within each somite varies depending upon its position along the rostrocaudal axis. For example, in the most caudal (and most recently formed) somite at 9.5 days, expression is concentrated near the neural tube and notochord. In the adjacent somite, the expression appears to diffuse away from the notochord, and is most pronounced in the ventral portion of the somite corresponding to the prospective sclerotome. *Nkx*-3.1 is expressed most strongly in the 5-6 most caudal somites. In more rostral, and more well-differentiated somites, the gene is sharply downregulated, and is expressed only in the most posterior third of the somite. This dynamic expression pattern suggests that *Nkx*-3.1 may play a role in the early determination of the somite and in the specification of somitic cell lineages.

**W 122** MHox MUTANT MICE SHOW MULTIPLE CRANIOFACIAL DEFECTS,

James F. Martin*, Allan Bradley+ and Eric N. Olson*, *The Department of Biochemistry and Molecular Biology, The University of Texas M. D. Anderson Cancer Center, and The +Institute for Molecular Genetics, Howard Hughes Medical Institute, Baylor College of Medicine, Houston, TX 77030

Homeobox-containing genes have been implicated in morphogenesis and pattern formation during development. Characterization of the mouse Hox genes has demonstrated that these genes are important for anterior-posterior axial patterning. Recently, a subfamily of homeobox-containing genes has been identified, which are unlinked and show unique mesenchymal restricted expression patterns. One of these genes, MHox, was identified because of its ability to bind an AT-rich element in the muscle creatine kinase enhancer. During mouse development, MHox is first expressed at 9 days post-coitum. High levels of MHox expression are detectable in the forming limb bud as well as the lateral mesoderm and cranial ectomesenchyme. Later, MHox is expressed in the pharyngeal arches, the maxillary processes, the mandible and the frontonasal mass. In adult mice, MHox is expressed at high levels in the heart and skeletal muscle. To begin to further define the function of MHox during development, the MHox gene has been inactivated in mice through homologous recombination. Mutant mice die within 12 hours of birth due to asphyxiation and show multiple abnormalities of craniofacial development. MHox mice have mandibular hypoplasia, posteriorly displaced ears, and a cleft palate. Skull formation is also abnormal. The temporal and supraoccipital bones are absent. The middle ear ossicles show abnormalities ranging from fusion of the incus and malleus to complete absence of the ossicles. In summary, the MHox mutation primarily affects the development of the chondrocranium, resulting in the absence of structures derived from separate mesenchymal cell lineages.

**W 123** CELL DEATH IN EMBRYONIC CHICK HEAD MUSCLES

Deedra McClearn and Drew Noden, Section of Ecology & Systematics and Department of Anatomy, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853

Degenerating myofibers have been reported in embryonic avian and mammalian muscles, but neither the pervasiveness of the phenomenon nor the spatio-temporal patterns of degeneration have been examined in detail. Using TEM we observed large numbers of degenerating fibers in all head and neck muscles examined, including the biventer cervicis, depressor mandibulae, branchiomandibularis, and complexus. Degenerating myofibers are found on days 9-18 of incubation, but are most abundant during days 12-15, which is several days after the period of motor neuron apoptosis. Muscle cell death occurs predominantly in primary myocytes, and is evident in both clustered and scattered myocytes. By day 21 there is little or no evidence of muscle cell degeneration. Degenerating muscle cells are characterized by densely-staining, highly contracted myofibrils, numerous cytoplasmic vacuoles, nuclear abnormalities including blistering of the nuclear envelope, disrupted cell membranes, and proximity of extracellular macrophages. Serial reconstructions of 15-day embryonic biventer muscles using light and electron microscopy reveal that degeneration is not always uniform along the length of a single fiber. However, these necrotic foci are not restricted to particular regions of the developing muscle. We hypothesize that programmed myofiber degeneration in embryonic chick head muscles represents a normal and biologically significant phase of remodeling that affects the spatial patterning of the muscle and, most likely, fiber type distribution. These data indicate that motor nerves change their targets as a normal part of craniofacial muscle maturation. Supported by NIH grant DE06632 from the NIDR.

**W 124** SKELETAL MUSCLE CONTRACTILE PHENOTYPE IN MYOD-GENE INACTIVATED MICE, Joseph M. Metzger*, Michael A. Rudnicki+, Margaret V. Westfall*, Wan-In Lin*, and Rudolf Jaenisch+. *Dept. of Physiology, University of Michigan, Ann Arbor Michigan, +Whitehead Institute, MIT, Cambridge MA.

MyoD belongs to a small family of myogenic transcription factors that are thought to play an important role in the differentiation of skeletal muscle during development. However, recent experiments have demonstrated apparent normal skeletal muscle development following the inactivation of the MyoD gene in mice (MyoD -/-). In the present study we used the permeabilized single fiber preparation to determine whether MyoD gene-inactivation has an effect to alter regulatory protein structure and function at the cellular level. In adult fast skeletal muscle fibers from MyoD (-/-) mice the sensitivity of the contractile apparatus to activation by $Ca^{2+}$ was highly variable among individual fibers. This cellular variation in $Ca^{2+}$ sensitivity was significantly different from that obtained from wild-type controls (Balb\c; 129 ter\sv) in which the $Ca^{2+}$ responsivity of the contractile apparatus was relatively similar from fiber to fiber. The basis of altered $Ca^{2+}$ regulation of contraction in skeletal muscle fibers from MyoD (-/-) mice is unknown but may relate to a disruption in the normal tightly controlled expression of thin filament regulatory proteins in these fibers. Analysis of protein composition by SDS-PAGE showed that the phenotype of the myosin heavy chain, myosin light chains and troponin I and troponin C was not altered in single fibers from MyoD (-/-) mice. Western Blot analysis of troponin T and tropomyosin isoforms is currently in progress to determine whether the phenotype of these important regulatory proteins is altered in skeletal muscle fibers lacking MyoD. An alteration in the expression of troponin T and/or tropomyosin isoforms could account for the observed contractile dysfunction in fast skeletal muscle fibers from MyoD (-/-) mice.

**W 125** MYOGENESIS AND THE INTERMEDIATE FILAMENT PROTEIN, NESTIN. Jeffrey Boone Miller and Amy M. Kachinsky, Neuromuscular Laboratory, Massachusetts General Hospital, Charlestown, MA 02129.

Nestin is a large intermediate filament (IF) protein which is related to the neurofilament proteins but is sufficiently different to be placed in a distinct class. Most studies of nestin have focused on its expression in neuroepithelial stem cells and neural tumors. Nestin expression is, however, not limited to neural tissue cells; an additional major site of nestin expression is in somitic myotomes (Hockfield and McKay, J. Neurosci. 5, 3310-29, 1985). We have now further examined nestin expression in myogenic cells using a combination of immunofluorescence and immunoblotting assays of myogenic cells in vitro and in vivo. The results include: (i) Nestin is expressed by myoblasts in culture. Both cell line myoblasts (C2C12 and L6) and primary myoblasts from embryonic (E11) and fetal (E16) mouse limbs express nestin. (ii) In the early stages of primary cultures, many cells express nestin in the absence of detectable amounts of MyoD, desmin, and myosin heavy chain. As differentiation proceeds in fetal cultures, nestin and MyoD are transiently coexpressed, and then nestin expression diminishes so that many cells express MyoD, but not nestin. (iii) Unconverted 10T1/2 cells express nestin. 3T3-L1 cells, however, do not normally express nestin unless converted to myogenesis by exogenous MyoD or myogenin. (iv) Nestin is not expressed in the somites of E8.5 embryos (7 somite stage), but is expressed by somitic myocytes in E9 embryos (15 somite stage). Some nestin expression is also seen in the columnar epithelial cells of the dermatome. These observations suggest that nestin is expressed in the early stages of myogenic cell lineage commitment, in some cases perhaps, prior to the bHLH muscle regulatory factors. (Support by NIH, AHA, MDA)

**W 126** A NOTOCHORD-DEPENDENT SIGNAL FROM THE NEURAL TUBE INDUCES MYOGENESIS IN CHICK EMBRYONIC SOMITES. Andrea E. Münsterberg and Andrew B. Lassar, Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, 240 Longwood Avenue, Boston MA 02115

We are interested in cell fate specification and commitment in the developing somite. In particular we have focused on the differentiation of the myogenic lineage, with the aim to characterize possible inducers of skeletal muscle differentiation in vertebrate development. It has previously been demonstrated that the axial structures of the vertebrate embryo, neural tube and notochord, play a role in somite morphogenesis. Using in vitro co-culture of tissue explants followed by RT-PCR, we have monitored inductive interactions of the neural tube and notochord with the adjacent somitic mesoderm in chick embryos. This approach has allowed us to determine when myogenic precursor cells are present in the embryo and when they become independent of extrinsic signals. The data indicate that the activation of myogenic basic helix-loop-helix (bHLH) genes (MyoD and myogenin) and subsequent myogenic differentiation of somitic cells in vitro requires interaction between the neural tube and the adjacent paraxial mesoderm. We show that autonomous differentiation of somitic cells into skeletal muscle, independent of the neural tube, correlates with stable expression of the myogenic bHLH genes. Presumably inductive interactions between the somite and neural tube initiate high level expression of myogenic regulators whose expression is subsequently maintained in the absence of the neural tube. Both myogenic lineages in the immature somite (i.e. muscle precursors in both the medial and lateral somite) apparently require an extrinsic signal to activate the muscle differentiation program in vitro. Interestingly, we found that the ability of the neural tube to activate myogenic regulatory genes in vitro requires either prior or concomitant interaction with the notochord. The muscle inducing activity within the neural tube can itself be induced by cultivating immature neural tube in the presence of notochord. A complex extracellular matrix, "Matrigel", containing growth factors as well as ECM components can substitute for the presence of the notochord in this context. Interactions between the notochord and the neural tube are known to be important for neural tube patterning, in particular, it has been demonstrated that the notochord induces floor plate and motor neurons. Therefore, we propose that a notochord-dependent neural tube cell type is necessary for the induction of skeletal muscle precursors in the developing somite.

**W 127** DEVELOPMENTAL REGULATION OF A RAT MYOSIN LIGHT CHAIN 1 TRANSGENE. Craig Neville, Jiang Ping, and Nadia Rosenthal, Cardiovascular Research Center, Massachusetts General Hospital, Charlestown MA 02129

The myosin light chain (MLC) 1/3 locus is an excellent marker for studying the mechanisms by which gene expression is regulated in muscle development. The two MLC proteins, referred to as MLC1 and MLC3, are produced via an alternative splicing mechanism and controlled by two widely spaced promoters and a common downstream enhancer. The enhancer contains multiple recognition sites for several transcription factors that direct myogenesis. They are sufficient to drive reporter genes in both tissue culture and transgenic mice in an expression profile that reflects the fast-fiber specific nature of the endogenous MLC 1/3 gene. In addition, an MLC1-chloramphenicol acetyl transferase (CAT) transgene in mouse shows an unexpected gradient of expression during development. It is established along the anteroposterior axis of the embryo by regulators of regional specification in myogenic precursors. This gradient persists in the segmented muscle groups in the adult animal. Developmentally-regulated demethylation of the transgene enhancer is simultaneous with the activation of the reporter gene, whereas demethylation of the promoter occurs three days later. Current studies are utilizing transgenic mice to determine whether the methylation gradient causes or results from the rostrocaudal position-dependent expression of the MLC1-CAT transgene. Transgenic animals are also being used to identify the precise cis-acting sequences in the transgene that confer the rostrocaudal transcriptional gradient.

**W 128** A NOVEL MYOGENIC REGULATORY CIRCUIT CONTROLS SLOW/CARDIAC TROPONIN C TRANSCRIPTION IN SKELETAL MUSCLE. Michael S. Parmacek, Hon Ip, Frank Jung, Eric N. Olson and Jeffrey M. Leiden. Dept. of Medicine, University of Chicago, Chicago, IL 60637 and Dept. of Biochemistry and Molecular Biology, MD Anderson Cancer Center.
The slow/cardiac troponin C (cTnC) gene is expressed in both cardiac myocytes and embryonic fast skeletal myotubes. cTnC gene expression in cardiac muscle is regulated by a 124 base pair (bp) promoter/enhancer located in the 5' flanking region of the gene. In contrast, cTnC gene expression in embryonic skeletal myotubes is regulated by a distinct and independent 145 bp transcriptional enhancer located within the first intron of the gene. This transcriptional enhancer is skeletal myotube-specific, and developmentally regulated during the differentiation of myoblasts to myotubes. DNase I footprint, electrophoretic mobility shift, and mutational analyses revealed that the cTnC skeletal muscle-specific enhancer contains three functionally important nuclear protein binding sites: a CACCC box, a MEF-2 binding site, and a previously undescribed nuclear protein binding site, designated MEF-3, which is also present in other skeletal muscle-specific transcriptional regulatory elements. Unlike most previously characterized skeletal muscle-specific transcriptional regulatory elements, the cTnC enhancer does not contain a consensus binding site (CANNTG) for the basic helix-loop-helix (bHLH) family of transcription factors, and does not bind *in vitro* translated MyoD/E12 protein complexes. Despite these findings, the cTnC enhancer can be trans-activated by over-expression of MyoD or myogenin in C3H10T1/2 cells. Taken together, these results demonstrate that bHLH transcription factors can indirectly trans-activate the expression of a subset of muscle-specific genes and, thereby, define a novel downstream regulatory pathway in bHLH-induced skeletal myogenesis.

**W 129** DEFECTS IN THE FUSION OF MYOBLASTS TO MYOTUBES IN THE DROSOPHILA *ROLLING STONE* MUTANT
Achim Paululat, Susanne Ohlmeier, Anette Goubeaud and Renate Renkawitz-Pohl, Faculty of Biology, University of Marburg, 35032 Marburg, Germany, Fax: 49 6421 287077
The Drosophila P-element induced mutant *rolling stone* shows disturbances in the fusion of myoblasts to myotubes in the somatic musculature, sometimes parts of the musculature are deleted. The development of the visceral musculature as well as of the heart is not markedly affected, showing a specific effect of this mutation on the differentiation program of the somatic musculature. Using "plasmid-rescue" procedure we cloned the genomic region surrounding the P-insertion site. With these genomic clones we isolated cDNAs. Our first sequence comparison, based on the deduced protein sequences, shows no significant homologies to genes of the vertebrate MyoD family or any other gene. Parallel to the sequence analysis we performed a further cytological characterization of the P-element induced mutant and our new generated EMS induced alleles. More information about the function of the affected gene will be given by embryonic whole mount in situ hybridization experiments to determine the cytological distribution of the transcript in the wildtype situation as well as in *rolling stone* and its EMS induced alleles. With the combination of cytological and molecular genetic methods we hope to get an insight in the function of this new gene and its developmental relevance.

**W 130** RAT MYOBLASTS INJECTED INTO ADULT MUSCLES MAINTAIN THEIR *IN VITRO* PHENOTYPE. EVIDENCE FOR INTRINSIC PROGRAMMING OF MYOBLAST FATE. Christopher L. Pin and Peter A. Merrifield, Department of Anatomy, University of Western Ontario, London, ONT, CANADA N6A 5C1.

Muscle is composed of multinucleated fibres, which can be classified as slow (Type I) or fast (Type IIA,IIB,IIX) based upon their contractile properties and their pattern of myosin heavy chain (MyHC) expression. One theory to explain the development of these different muscle fibre types is that they arise from genetically distinct populations of muscle precursor cells (or myoblasts). In support of this theory, we have recently shown that myoblasts derived from embryonic day 14 (ED14) and ED20 rat hindlimbs express different patterns of MyHC isoforms following differentiation in culture (Pin and Merrifield, Devel. Genetics 14, 356-368. 1993). To further investigate the role of the environment on fibre type development, rat myoblasts were retrovirally infected with a LacZ reporter gene and injected into various muscles (ie. soleus, EDL,TA) of immunosuppressed, adult rats in order to follow their fate in vivo. Upon differentiation these myoblasts fused to form both homotypic (donor-donor) and heterotypic (host-donor) muscle fibres. In all muscles examined, the homotypic fibres proceeded through a transition of MyHC isoforms characteristic of their *in vitro* phenotype. Heterotypic fibres also maintained the expression of MyHC isoforms characteristic of their muscle of origin, but these were expressed in the form of nuclear domains around donor nuclei. These results suggest that the fate of different myoblast populations may be the result of genetic programming inherent to the cells, rather than environmental cues such as innervation patterns. Similar results have recently been reported for quail myoblasts from different lineages injected into embryonic chicks (DiMario et al, Nature 362,165-167.1993). We are currently examining the stability and molecular basis of such genetic programs. (Supported by operating grants from the Medical Research Council of Canada (MRCC) and the Muscular Dystrophy Association of Canada (MDAC) to PAM. CLP is the recipient of an MDAC Predoctoral Fellowship).

**W 131** Dissection of the Roles of MLC1 Promoter and Downstream Enhancer in Gene Regulation during Muscle Development by Transgenic Mice, Jiang Ping and Nadia Rosenthal, Cardiovascular Reseach Center, Massachusetts General Hospital-East, Charlestown, MA 02129
To elucidate how the rat myosin light chain 1 (MLC1) promoter region and downstream enhancer induce chloramphenical acetyltransferase (CAT) reporter gene expression in a fast-fiber-specific pattern and at the correct developmental stage in transgenic mice, we began to dissect the roles of the MLC1 promoter region and the downstream enhancer. We are introducing the MLC1 promoter region-CAT with no downstream enhancer into transgenic mice, although such a construct was demonstrated to be unable to drive CAT expression in primary culture test. We are also carrying out experiments to produce transgenic mice carrying the downstream MLC enhancer coupled to a heterologous promoter, to answer the question whether or not the enhancer regulates CAT expression in a promoter-dependent or -independent manner. These studies should identify sequences in MLC1/3 locus which are responsible both for the developmental timing of MLC1 gene activation, and for the fiber-restricted expression pattern of the gene.

**W 132** Myogenin Is Required For Normal Muscle Development *In Vivo* But Not For Myogenesis *In Vitro*. J. Alan Rawls, Diane Edmondson, William Klein, and Eric N. Olson. Department of Biochemistry and Molecular Biology, University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

Myogenin is a muscle-specific transcription factor that belongs to the basic-helix-loop-helix family of DNA-binding proteins which includes MyoD, myf-5, and MRF4. The evidence for these genes' role in myogenesis is based primarily on the gain-of-function experiments in tissue culture, where they act as potent myogenic factors. Converted cells behave like myoblasts in their responses to growth factors, genes they express, and ability to fuse into multinucleated myotubes. To test myogenin's role in embryonic development directly, mice homozygous for a targeted mutation in the myogenin gene were generated (Hasty et al., *Nature* 364:501-535). These mice display a severe reduction of differentiated skeletal muscle throughout their bodies, despite normal levels of MyoD. This suggests myogenin plays an essential role in skeletal muscle formation.

Myoblast cell lines were generated from myogenin-null mice for the purpose of further studying myogenin's role in muscle differentiation. We observed that myogenic cells from myogenin-null mice, which are unable to differentiate *in vivo*, undergo normal myogenesis in culture. This result suggests the dependence of myoblasts *in vivo* on myogenin for differentiation is due to modulation of the function of the other myogenic bHLH proteins by local influences in the embryo. Changes in the regulation of expression of muscle-specific and proliferation-specific genes will be presented for myogenin-null myoblasts from embryos and those grown in tissue culture.

**W 133** THE TIMING FOR THE RESPECIFICATION OF THE MUSCLE PATTERN IN MANIPULATED CHICK WINGS, Lesley Robson, Anne Crawley and Cheryll Tickle. Department of Anatomy and Developmental Biology, University College London, London, U.K.

The musculature of the chick embryonic wing is highly patterned at both the tissue and cellular levels. At the tissue level, the individual muscles of the forearm region each occupies a specific location with respect to the other muscles and skeletal elements. At the cellular level, each muscle is not only composed of a mixture of fibre types, but these specific fibre types are arranged into specific areas of the muscle. We have used this highly patterned collection of muscles in the chick forearm to follow the timing of the patterning of the individual muscles, at both the tissue and the cellular levels. Manipulations of the chick forearm were carried out which cause changes in the pattern of the limbs, such as grafting cells from the polarising region to the anterior margin of the early chick wing, or the local application of low doses of retinoic acid also to the anterior margin. Both manipulations lead to a respecification of the anterior cells to form posterior structures. When the musculature of these manipulated wings is analysed there appears to be a mirror image arrangement of the muscles, with the muscles in the anterior of the limb taking on the characteristics of posterior muscles. However, when the timing of the respecification of the anterior musculature is analysed, there appears to be a difference in the time that the tissue and cellular patterns are altered. The Hox D expression pattern is irreversibly altered along with the skeletal elements, 24 hrs after the manipulations. From the analysis of the muscle masses which divide to give the individual muscles, there is a respecification at least 48 hrs after the manipulation. This is most evident, with a new anterior muscle mass that mirrors the posterior muscle mass, ventrally. However, there appears to be no change in the composition of the muscle masses until 90 hrs after the manipulation has been performed. It therefore appears that the connective tissue may specify the muscle pattern at the tissue level but that the cellular composition of the muscles is at first predetermined, and is later changed once the tissue pattern is altered, and the identity of the muscles established.

**W 134** THE DETERMINATION OF SKELETAL MUSCLE DURING EMBRYONIC DEVELOPMENT Michael A. Rudnicki[1], Patrick N.J. Schnegelsberg[2], Ronald H. Stead[1], Thomas Braun[3], Hans-Henning Arnold[3], and Rudolf Jaenisch[2]. [1]Institute for Molecular Biology and Biotechnology, McMaster University, Canada. [2]Whitehead Institute, U.S.A.. [3]Institut für Biochemie und Biotechnologie, Germany. The myogenic basic helix-loop-helix (bHLH) family of transcription factors is believed to play an important regulatory role in the development of skeletal muscle. This group includes MyoD, myogenin, Myf-5, and MRF4. Previously, we demonstrated that mice lacking a functional MyoD gene are viable and fertile, and exhibit no morphological or physiological abnormalities in skeletal muscle. Mutant MyoD mice did, however, exhibit a 3.5 fold increase in the amount of Myf-5 mRNA. Similarly, newborn mice lacking a functional Myf-5 gene display no obvious defects in skeletal muscle, but die perinatally due to severe rib abnormalities. The sustained induction of Myf-5 mRNA levels in mice lacking MyoD, and the delay in the onset of muscle-specific gene expression in mice lacking Myf-5, raised the possibility that Myf-5 and MyoD functionally substitute for one another in muscle development. To further investigate the role of Myf-5 and MyoD in myogenesis, we have generated mice lacking both myogenic factors by crossing Myf-5 and MyoD mutant mice. Strikingly, newborn mice deficient for the two myogenic bHLH factors Myf-5 and MyoD were totally devoid of all skeletal muscle. Mutant pups were born alive, but were completely immobile and died within minutes. Northern and S1 nuclease analysis demonstrated the absence of markers for skeletal muscle, and skeletal myoblasts. Histological examination revealed that these animals lacked all skeletal muscle, and the spaces normally occupied by muscle were filled with amorphous loose-connective and adipose tissues. Our results have important implications for the understanding of the molecular control of skeletal muscle development by the myogenic bHLH factors. The lack of myoblasts in mice lacking both Myf-5 and MyoD suggests that either Myf-5 or MyoD is required for the determination and/or propagation of myoblasts, and indicates that Myf-5 and MyoD are, for the most part, functionally-redundant in myogenesis.

**W 135** FROG EMBRYOS LOCALLY SUPPRESS XMYOD PROTEIN ACTIVITY BY BLOCKING ITS NUCLEAR TRANSPORT IN THE ANIMAL HEMISPHERE, Ralph A.W. Rupp*, Laurie Snider, and Harold Weintraub, Fred Hutchinson Cancer Research Center, 1124 Columbia Street, Seattle, WA 98104 (*current address: Friedrich Miescher Laboratorium der Max-Planck-Gesellschaft, Spemannstr. 37-39, D-72076 Tübingen, FRG).

Zygotic transcription of XMyoD first occurs transiently and throughout the Xenopus embryo at the midblastula transition (MBT). This finding is somewhat paradoxical, since it is known that MyoD can autoactivate its own transcription. To understand why "MBT-XMyoD mRNA" does not apparently activate stable MyoD expression throughout the embryo, we have ectopically overexpressed MyoD by injection of synthetic RNAs into embryos. Here we show that the transcriptional activity of exogenous XMyoD protein is under negative control in cells of the animal hemisphere, a region competent to form muscle in response to mesoderm induction. The heterologous MyoD protein from mouse ("mMyoD"), in contrast, strongly activates muscle specific genes upon overexpression in these cells, thus behaving like a positive control mutant of XMyoD. By concentrating on this qualitative difference we have found that overexpressed XMyoD protein is nuclear in the presumptive mesoderm, but accumulates in the cytoplasm of cells of the animal hemisphere. Overexpressed mouse MyoD protein is constitutively nuclear. We present evidence that the cytoplasmic retention of XMyoD protein can be saturated at non-physiological concentrations, and that in general nuclear MyoD protein - either frog or mouse - is sufficient to activate myogenesis ectopically in the embryo. Our results lead us to suggest that frog embryos regulate the intracellular localization of XMyoD protein to control its activity outside the mesoderm.

**W 136 PAX7: MEMBER OF THE MYOGENIC REGULATORY NETWORK?**

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In search for novel regulators potentially involved in myogenesis, we identified a homeobox of the paired type in human muscle. Subsequent cDNA cloning revealed that we had cloned the full length coding region of human PAX7. Our human sequence extends the known mouse cDNA both on the 5' and the 3' end. It shows high homology to another paired box gene, PAX3, with paired box and homeobox being almost identical. As a first step in order to test for a potential involvement of PAX7 in myogenesis, we analyzed its expression pattern in chicken. This allowed to corroborate the already known mouse data as well as a direct comparison between the expression of Chpax7 and the myogenic bHLH genes. The chicken probe which was cloned for this purpose, is 87% homologue to the human sequence. Chicken Pax7 transcripts are present in the neural tube and in the dermamyotome of the developing somites. Our results indicate that the pattern of expression of Pax7 is conserved between mouse and chicken. We also assessed the expression of Pax7 in different mouse and human cell lines. We found Pax7 transcripts specifically in myogenic cells and not in any other cell types looked at including fibroblasts, hepatocytes and neuroblasts. Interestingly, Pax7 is already present at the myoblast stage. Moreover, 10T1/2 fibroblasts converted to myoblasts by either transfection of myoD or treatment with 5-aza-cytidine expressed Pax7, whereas the parental cells were negative. While myoD was able to activate Pax7 in 10T1/2 cells, expression of Pax7 in 10T1/2 cells was not sufficient to induce the myogenic phenotype. However, our expression data are consistent with a role of Pax7 during the commitment to the myogenic lineage.

W 137 MULTIPLE ROLES FOR α4 INTEGRIN IN MYOGENESIS: EMERGENCE OF THE MYOGENIC LINEAGE FROM THE SOMITE AND SECONDARY MYOGENESIS.

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We have presented evidence previously that binding of α4 integrin, on primary myotubes, to one of its ligands, VCAM-1, on secondary myoblasts and myotubes, mediates interactions between primary and secondary generations of cells during skeletal myogenesis. Here, we provide evidence of a second role for α4 integrin and its extracellular matrix ligand, fibronectin, during early somitogensis. The pattern of expression of α4 integrin was compared to that of myf-5, the earliest known determinant of the skeletal myogenic lineage, during somitogensis in the mouse. Although neither was apparent by immunostaining on the early somite, both appeared prior to myotome formation. These results suggest that α4 integrin is rapidly activated by myf-5, and thus is the earliest known determinant of skeletal muscle lineage produced in response to myf-5. We have identified multiple E boxes in the promoter for the α4 gene that appear to be targets of activation by myf-5 and other muscle transcription factors. During early somitogenesis, α4 integrin appears to interact with its extracellular matrix ligand, fibronectin, which surrounds the somite and the myotome. As the myotome developed, the expression of myf-5 declined, whereas α4 integrin expression increased both on the myotome and on premuscle cells in the developing limb. α4 integrin expression may persist in muscle cells as a result of expression of myogenin and myoD, which appear as myf-5 expression subsides. Our results then suggest roles for α4 integrins throughout skeletal muscle differentiation. Early in somitogenesis, α4 integrin appears to interact with its extracellular matrix ligand fibronectin. Such interactions have been shown previously in other cell types to control the pattern of gene expression and to mediate cell migration. Therefore, we propose that α4 integrins are important either for signaling events that are involved in muscle cell lineage commitment and/or for the subsequent migration of the cells once they are committed to the muscle lineage. Later in muscle differentiation, α4 integrins interact with VCAM-1 to mediate interactions between primary and secondary generations of cells that appear critical for secondary myogenesis.

W 138 ZEBRAFISH HEART DEVELOPMENT: A GENETIC APPROACH,

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We are interested in understanding the cellular and molecular mechanisms underlying heart development. We have chosen to approach this question in the zebrafish. The transparency and accessibility of the zebrafish embryo, its rapid development, the prominence of the heart, and the potential for genetic analysis make it the ideal model system in which to study the development of heart form and function.

In order to study the earliest events in cardiac morphogenesis, we have begun to analyze the cell lineage of heart progenitors. We also undertook a detailed descriptive study of wild-type heart development.

Genetic analysis of heart development was initiated by studying two recessive lethal mutations. The *silent heart (sih)* mutation (isolated in Oregon by Walker and Kimmel) is cardiac specific and, as the name implies, the mutant heart never starts beating. Despite this lack of function, the heart develops normally. Mutant hearts show normal electrical excitation, as visualized by $Ca^{2+}$ influx, yet there is no contraction. This is a classic example of excitation-contraction (EC) uncoupling. We surveyed various proteins known to be involved in EC coupling in the cardiac muscle and found that cardiac troponin T, a key link, is not present in the *sih* heart.

The *cloche (clo)* mutation affects the basic morphogenesis of the heart tube by deleting the endocardium, the endothelial layer of the heart. Further analysis, including cell transplantation studies, suggests that the *clo* mutation affects the generation and/or migration of the endocardial progenitor cells in a cell-autonomous manner.

Finally, in collaboration with Dr. Wolfgang Driever and his colleagues, we are isolating further mutations that affect cardiac form and function. A progress report of this screen will be presented.

W 139 IN VITRO SOMITE MYOGENESIS IS DEPENDENT ON NEURAL TUBE,

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Previous in ovo neural tube and notochord extirpation experiments suggest that one of these axial structures is required for muscle formation in the somites [Rong et al., Development 115, 657-672 (1992)]. The molecular nature of this tissue interaction is unknown. To facilitate analysis of neural tube-somite communication, we developed a cell culture system whereby one somite of a somite pair is explanted from a chicken embryo and cultured alone while the contralateral somite is co-cultured with a piece of neural tube. When 48 hour culture experiments are performed with caudal somites from E2 chick embryos (stages 9 to 15), staining with the muscle specific myosin antibody, MF20, reveals an increased number of myosin-positive cells in the somite/neural tube co-cultures versus the control somites cultured alone. Neural tube cultured by itself does not give rise to myosin-positive cells. These results are consistent with the hypothesis that the neural tube either actively induces or passively supports myogenesis in caudal somites. In contrast, rostral somite cultures exhibit significant muscle in both neural tube co-culture and control somites, indicating that beyond a certain developmental time point, the somite no longer requires the neural tube to support in vitro myogenesis. For the stages tested here, the number of caudal somites dependent on neural tube for myogenesis decreases as embryo age increases. To examine the specificity of the neural tube effect, somites were co-cultured with neural retina from E6 chick embryos or lateral plate mesoderm from E2 embryos. Neither tissue caused an enhancement of muscle differentiation in the co-cultures versus the controls. Preliminary data suggest that the notochord also may promote in vitro somite myogenesis, though the effect is weaker than with neural tube. The neural tube effect is not demonstrable via known growth factors, neural tube extracts, or neural tube conditioned medium; contact or extremely close proximity is required. Correlation of somite myogenic induction with muscle determination factor gene expression is in progress.

**W 140** ISOLATION OF MATERNAL GENES INVOLVED IN ASCIDIAN LARVAL TAIL MUSCLE DEVELOPMENT.

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The larval muscle cells of ascidians are specified by factors localized in the myoplasm, a specialized cytoplasmic region of the egg inherited by presumptive muscle cells during cleavage. I have identified several maternal genes which may be involved in muscle determination during embryogenesis. NN18 stains the egg myoplasm and larval muscle cells of ascidian larvae. Western blot analysis of proteins separated by 2-D electrophoresis shows that NN18 antibody recognizes a 58,000 kDa protein (p58) with at least three isoforms ranging in pI from 6.0-6.5. P58 is enriched in the egg insoluble fraction after extraction with Triton-X 100, suggesting that it is linked to the cytoskeleton. P58 surrounds the germinal vesicle in young oocytes, but becomes progressively localized in the egg cortex, which becomes a part of the myoplasm. P58 appears to be a novel component of the myoplasmic cytoskeleton in all ascidian tailed larvae. *Molgula occulta* is a species that has an anural (tailless) larva. *In situ* and northern hybridization with muscle actin and myosin heavy chain cDNA probes has shown that *M. occulta* embryos and larvae do not express muscle contractile protein genes. A possible explanation for lack of muscle cell differentiation in *M. occulta* embryos is the elimination or modification of a maternal muscle determinant. To investigate this possibility, we used a subtractive hybridization procedure to isolate genes expressed in tailed species' oocytes and eggs, but not expressed or with modified expresssion in *M. occulta* oocytes or eggs. Three different clones were isolated that are expressed early in oogenesis in a tailed ascidian species. One of these genes, *manx*, has a zinc finger similiar to known transcription factors and a nuclear localization signal. *Manx* has been shown to be important in larval tail development by treatment of embryos with antisense oligodeoxynucleotides.

**W 141** NEGATIVE REGULATION OF MYOGENIC DIFFERENTIATION BY THE DNA-BINDING AGENT DISTAMYCIN-A,

Alan Taylor*, Gabriele Linden, Tom Gustafson, Keith Webster and Larry Kedes, *Department of Biological Sciences, Wichita State University, Wichita, KS 67208

Distamycin A, a naturally occurring antibiotic which binds selectively to A/T-rich regions of the DNA and alters DNA conformation, has been used to analyze the sequence-specific protein interactions on muscle-specific promoters. Previous results have shown that binding of the CArG-binding factor/SRF to its A/T-rich binding sites within the human cardiac actin promoter alters DNA conformation (Gustafson et al.1989. PNAS 86(7):2162-2166). Gel retardation assays performed in the presence of distamycin A indicate that this drug selectively inhibits the interaction of the CArG/SRF transcription factor with its DNA binding site while not affecting the binding of the Sp1 transcription factor to its G/C-rich binding site. Northern analyses of RNA from C2 myogenic cells, induced to differentiate in the presence or absence of distamycin A, indicate that distamycin A selectively inhibits muscle-specific gene transcripts such as cardiac $\alpha$-actin, skeletal $\alpha$-actin, myosin heavy chain, and myo-D. However, distamycin A did not alter the expression of non-muscle specific transcripts such as $\beta$-actin, glyceraldehyde-3-phosphate dehydrogenase, heat shock protein 83, and histone H3. The same pattern of expression was observed when distamycin A was added late in the differentiation time course and the effects were reversible upon removal of the drug. Transient transfection assays using muscle- and non-muscle specific promoters indicate that distamycin A selectively down-regulated the expression of the cardiac $\alpha$-actin and skeletal $\alpha$-actin promoters while not inhibiting the $\beta$-actin or c-*fos* promoters. Over expression of myo-D using a $\beta$-actin expression vector did not releave the inhibition exerted by distamycin A. Additionally, treatment with distamycin A of the pluripotential cell line TA1, which differentiates into both myocytes and adipocytes, indicated that this drug selectively inhibits myogenic differentiation in this system.

**W 142** MEF2 FACTORS ARE EXPRESSED IN PRESOMITIC MESODERM IN ZEBRAFISH EMBRYOS,

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The myocyte enhancer factor 2 (MEF2) is a DNA binding activity that recognizes a conserved A/T-rich sequence motif (the MEF2 site) on which multiple muscle-specific promoters and enhancers depend. Products of four distinct human MEF2 genes, and homologues from other species, have been cloned previously and all share a conserved domain homologous to the MADS family of transcription factors. MEF2 factors interact directly with the myogenic proteins of the MyoD family, and regulate myogenin transcription. In order to define the role of MEF2 in skeletal and cardiac muscle development we have analyzed MEF2 expression in zebrafish. Zebrafish was chosen as a model because of its rapid development, easily visualized embryos and potential for genetic manipulation. We isolated cDNAs from zebrafish for homologues of the human MEF2A, -C, and -D. There is extensive conservation of sequences between zebrafish and human MEF2 genes, especially in the critical MADS/MEF2 domain. The pattern of expression of MEF2 was determined by in situ hybridization using zebrafish embryos. MEF2D transcripts are detected earliest, and are found in paraxial presomitic mesoderm in 9 hour gastrulae (90% epiboly), appearing within one hour after MyoD and in a similar distribution. Expression of MEF2D in later embryos precedes overt somite formation in a rostral to caudal progression. MEF2A and C transcripts appear later than MEF2D in a similarly restricted distribution, and are also expressed in the heart. Transcripts for MEF2A were detected in brain. The high degree of conservation of MEF2 protein structure among different vertebrates and the pattern of expression of MEF2 genes during embryogenesis indicate an important role for MEF2 in myogenesis and cardiogenesis. We are now in a position to perturb MEF2 expression in the zebrafish embryo by somatic means in order to determine the role of MEF2 in the transcriptional mechanisms controlling muscle formation.

**W 143** MYOGENESIS IN MICE LACKING MYOGENIN-

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Mice homozygous null for myogenin exhibit a severe skeletal muscle deficiency that results in perinatal lethality. To determine when during embryogenesis the muscle defect is first apparent and the exact nature of the defect, we have begun a detailed analysis of myogenesis in these mice. Initially we examined myosin heavy chain (MHC) expression during different embryonic and fetal stages by indirect immunofluorescent labeling of sections. At the earliest stages examined, MHC protein levels and the numbers of cells expressing MHC were considerably reduced in mutants compared to nonmutant siblings. As development proceeds, the levels of MHC protein expression and the number of cells expressing MHC increased in both mutants and nonmutants. However, the number of cells expressing MHC in mutants never approached that observed in nonmutant siblings. During the fetal stages, there was a dramatic increase in the number of MHC positive cells in the nonmutants, due to the differentiation of secondary myotubes. In contrast, mutant embryos failed to generate significantly more MHC positive cells. Similarly, *in situ* hybridization using nucleic acid probes to embryonic, perinatal, and beta-cardiac MHCs as well as alpha-cardiac actin revealed that the levels of expression of these muscle specific markers were consistently reduced in mutant embryos and that as development proceeds the differences between mutant and nonmutants became more apparent. The dramatic difference between mutant and nonmutants after the onset of secondary fiber formation raises the interesting possibility that myogenin may be essential for secondary myogenesis. Since some fibers are present in mutant neonates, the question arises as to whether these residual fibers represent a subset of the array of muscle fiber types that would normally differentiate. In other words, is myogenin required for the differentiation of some muscle fiber types (fast vs slow) and other members of the MyoD family required for others? This was assessed by examining the expression of different isoforms of MHC in the mutant background.When sections through the hindlimbs of mutant and nonmutant siblings were examined with monoclonal antibodies to embryonic, fast, and slow isoforms of MHC we found that all fiber types were present albeit at reduced levels in mutants. These results suggest that myogenin does not play a role in muscle fiber type specification during development.

**W 144 GENES AFFECTING MUSCLE PATTERNING IN THE *DROSOPHILA* EMBRYO,** Talila Volk, Dan Strumpf and Yael Rosenberg-Hasson, Department of Molecular Genetics, Weizmann Institute of Science, Rehovot 76100, Israel.

The correct patterning of muscles in the *Drosophila* embryo depends on the migration of developing muscles over the ectoderm, and on the attachment of these muscles onto specific attachment sites. We investigate the molecular mechanisms implicated in embryonic muscle morphogenesis by the identification of key genes essential for that process.

Defects in the activity of MSP-300, a protein that exhibits homology to human dystrophin, induced by a point mutation in that gene, lead to abnormalities in the arrangement and attachment of the ventral oblique muscles in the embryo. The basis for this abnormality is currently being investigated.

Laminin, an extracellular matrix glycoprotein, has been implicated in muscle development. The absence of laminin-A chain, induced by imprecise excision of P-element in this gene, leads to derangement in the patterning of the somatic muscles. The involvement of laminin in muscle morphogenesis is currently being investigated. We also examine possible genetic interactions between the activity of both laminin and MSP-300 genes.

The genes *stripe* and groovin are expressed in the ectodermal cells which are responsible for somatic muscle attachment. Defects in the *stripe* gene, induced by P-element insertion, lead to abnormal organization of somatic muscles. *stripe* is currently cloned in our lab. Groovin is a protein identified by specific reactivity with a monoclonal antibody raised against membrane proteins from *Drosophila* embryo. We obtained several cDNA clones of this gene, and analyzed its structure. In addition, we are creating lines with P-element insertions in the groovin gene in order to obtain mutation and to dissect its role in muscle morphogenesis.

**W 145** TEMPORAL AND SPATIAL SPECIFICATION OF DEVELOPING CHICK EYE MUSCLES AND NERVES
Christina M. Wahl and Drew M. Noden, Department of Anatomy, Cornell University, Ithaca, NY 14853
The development of cranial muscles, including myoblast origins and movements within mesoderm, initial contact by motor axons, and integration with neural crest-derived connective tissues, presents many unresolved problems. We focused on the relations between the VIth nerve and the lateral rectus (LR) and pyramidalis/quadratus (P/Q) muscles. By injecting avian retroviruses containing the *Lac-Z* reporter deep into paraxial mesoderm at stage 9-10, the sites of origin of LR and P/Q myoblasts were mapped to partially overlapping foci in somitomeres 4 and 5; the dorsal oblique precursors are in somitomere 3. Jaw muscles were not labeled, confirming their more lateral origins. By stage 18, abducens (Abd) and accessory abducens (acAbd) axons were seen emerging from the hindbrain. Abd axons course rostrally from rhombomeres (Rh) 5 and 6 to the caudal part of the undifferentiated LR-P/Q condensation, located beneath Rh2. Accessory abducens (AcAbd) fibers emerge exclusively from Rh5, fasciculate with the Abd fibers, then separate and project around the LR-P/Q mass to enter the rostral (future P/Q) part of the condensation. The patterns of nerve branching are different for each eye muscle. We conclude that (1) specific myogenic precursors are delineated within predictable sites of head paraxial mesoderm, (2) specific nerve-target relations are established before myoblasts migrate to their sites of terminal differentiation, and (3) each eye muscle imposes a unique and specific pattern of terminal branching upon developing motor axons.
Supported by NIH grants EY06401, DE06632, and EY02007-16A2.

**W 146** EMBRYONIC EXPRESSION PATTERN OF THE AVIAN PAX-3 GENE IN MESODERM. Brian A. Williams and Charles P. Ordahl, Department of Anatomy and Cardiovascular Research Institute, University of California, San Francisco, CA 94143
Specification of the vertebrate myogenic lineage begins prior to gastrulation and culminates in the emergence of determined myogenic precursor cells from the somites. The myoD family (MDF) of transcriptional activators controls late step(s) in myogenic specification that are closely followed by terminal muscle differentiation. Genes expressed in myogenic specification at stages earlier than MDFs are unknown. Using in situ hybridization on staged chick embryos, we have found that the Pax-3 gene is expressed in all the cells of the caudal segmental plate, the early mesoderm compartment that contains the precursors of skeletal muscle. As somites form from the segmental plate and mature, Pax-3 expression is progressively modulated. Beginning at the time of segmentation, Pax-3 becomes repressed in the ventral half of the somite, leaving Pax-3 expression only in the dermomyotome. Subsequently, differential modulation of Pax-3 expression levels delineates the medial and lateral halves of the dermomyotome, which contain precursors of axial (back) muscle and limb muscle, respectively. Pax-3 expression is then repressed as dermomyotome-derived cells activate MDFs. Quail-chick chimera and ablation experiments confirmed that the migratory precursors of limb muscle continue to express Pax-3 during migration. Since limb muscle precursors do not activate MDFs until 2 days after they leave the somite, Pax-3 represents the first molecular marker for this migratory cell population. A null mutation of the Pax-3 gene, *Splotch*, produces major disruptions in early limb muscle development (Franz, et. al., [1993] Anat. Embryol. 187, 153-160). We conclude, therefore, that Pax-3 gene expression in the paraxial mesoderm marks earlier stages in myogenic specification than MDFs, and plays a crucial role in the specification and/or migration of limb myogenic precursors. (This work was supported by NIH grants HL43821 and GM32018 and by a grant from the Muscular Dystrophy Association).

**W 147** DEVELOPMENTAL REGULATION OF MUSCLE GENE REPRESSION IN PARABIOTIC CHICK-QUAIL EMBRYOS, Ted S. Wong and Charles P. Ordahl, Department of Anatomy and Cardiovascular Research Institute, University of California San Francisco, San Francisco, CA 94143-0452
Muscle protein genes are expressed in a developmentally regulated pattern from early embryonic through neonatal development. A subset of genes, including cardiac troponin T (cTNT), is expressed predominantly during the early embryonic through the mid-fetal stages of skeletal muscle development when their expression is transcriptionally repressed. Simultaneously, muscle protein isoforms present in mature muscle are upregulated. Although this highly conserved program of isoform switching is likely important for proper muscle development, little is presently known about the regulation of the repression of these early muscle isoforms. In the present study, we examined the possibility that repression of early muscle genes might be triggered by serum borne factors induced during specific periods of development. Earlier analysis has shown that cTNT repression begins earlier in Japanese quail embryos (embryonic day 10) compared to White Leghorn chick embryos (embryonic day 12). Based on this observation, two-day old quail and chick embryos were combined *in ovo* resulting in circulatory parabiosis of the two embryos via the formation of a common extra-embryonic vascular system. Using this model we tested whether the developing quail embryo serum was capable of inducing premature cTNT repression in chick embryos or if repression initiation was delayed in quails by chick serum. Pectoralis muscle from parabiotic and sham embryos were harvested and analyzed by northern blot. Preliminary results suggest that cTNT repression may be an intrinsic property of developing myocytes and is supported by results in primary skeletal muscle cultures where the cTNT induction/repression program proceeds despite constant high serum levels. We are currently performing chick-quail chimera segmental plate grafts to further examine whether early muscle gene repression is an inherent property of developing muscle cells or if extracellular influences play a regulatory role.
Supported by NIH grants HL35561 and GM32018 (C.P.O.) and NIH-National Research Service Award 08380 (T.S.W.)

**W 148** MYOGENESIS IN THE ADULT: EXPRESSION OF REGULATORY AND STRUCTURAL MYOGENIC PROTEINS DURING PROLIFERATION AND DIFFERENTIATION OF SATELLITE CELLS ON MUSCLE FIBERS, Zipora Yablonka-Reuveni, Susan M. Taylor and Anthony J. Rivera, Department of Biological Structure, School of Medicine, University of Washington, Seattle, WA 98195
Myogenic precursors in adult skeletal muscle (satellite cells) are mitotically quiescent but can proliferate in response to a variety of stresses. Several studies have indicated that adult myoblasts represent a distinct myogenic population which appears in late stages of embryogenesis. To gain further understanding of the adult lineage we analyzed myogenesis of satellite cells on intact fibers isolated from adult rat muscle. In this culture model satellite cells are maintained in their *in situ* position underneath the muscle fiber basement membrane. Patterns of satellite cell proliferation, expression of myogenic regulatory factor proteins, and expression of differentiation-specific, cytoskeletal proteins were quantitatively determined via immunohistochemistry. The temporal appearance and the numbers of cells positive for proliferating cell nuclear antigen (PCNA) or for MyoD were similar, suggesting that MyoD is present in detectable amounts in proliferating, but not quiescent satellite cells. Satellite cells positive for myogenin, α-smooth muscle actin (αSMactin), or developmental sarcomeric myosin (DEVmyosin) appeared following the decline in PCNA and MyoD expression. However, expression of myogenin and αSMactin was transient, while DEVmyosin expression was continuously maintained. Moreover, the number of DEVmyosin+ cells was only half of the number of myogenin+ or αSMactin+ cells -- indicating perhaps that only 50% of the satellite cell descendants entered the phase of terminal differentiation. These observations support the notion that following proliferation, only one of the two satellite cell progeny undergoes terminal differentiation. Our results additionally suggest that in this fiber model, the second daughter cell returns to quiescence but can be activated again. We further determined that more satellite cells became proliferative when basic FGF was added to the fiber cultures, but the overall schedule of cell cycle entry, proliferation and differentiation was unaffected. We thus conclude that satellite cells conform to a highly coordinated multistep program when undergoing myogenesis at their native position by the muscle fiber. This coordinated program, as we further determined, is not fully maintained in cultures of satellite cells released from the fibers. (Supported in parts by NIH, MDA, AHA and USDA)

## *DNA Binding Proteins*

**W 149** A NOVEL DNA-BINDING PROTEIN THAT INTERACTS WITH STRONG POSITIVE ELEMENT OF ß-MYOSIN HEAVY CHAIN GENE, Cynthia R. Adamson, Irwin L. Flink, Joseph J. Bahl and Eugene Morkin. Departments of Medicine, Pharmacology, Physiology, and University Heart Center, University of Arizona College of Medicine, Tucson, AZ 85724
The human ß-myosin heavy chain (ßMHC) gene contains a strong positive element (SPE) at positions -298/-277 that is required for high-level expression in cultured fetal rat cells. This element was used to screen a fetal cardiac expression library and a single positive isolate was obtained. The sequence of the 1.6 kb cDNA, Fetal ßMHC Binding Factor (FßMBF), was not similar to any <u>trans</u>-acting factors previously identified in muscle, but is closely homologous to the $NH_2$-terminal one-third of PO-GA, a 128 kDa DNA binding protein that may be involved in the regulation of the pituitary-specific pro-opiomelanocortin gene. In the region of overlap both proteins contain a helix-turn-turn helix (HTH) motif. Northern analysis to determine temporal expression of the FßMBF transcript revealed a 7.5 kb band in 15-, 17-, and 21-day rat placenta. No signal was detected in fetal rat heart or embryo at these stages. Expression also was not detected in adult ventricular tissue. Coupled transcription/translation in rabbit reticulocyte lysate produced two proteins of 41 and 38 kDa. When cotransfected in an expression vector with a ß-MHC reporter construct into fetal rat heart cell cultures, FßMBF caused a decrease in activity. FßMBF and PO-GA represent a newly described subgroup of eukaryotic HTH proteins that may play a role in developmental expression of cardiac genes.

**W 150** IN VITRO SELECTION OF OPTIMAL DNA TARGETS BY CARDIAC AND SKELETAL MUSCLE MEF2 ISOFORMS, Vicente Andrés, Sunjay Kaushal, Margarita Cervera, Bernardo Nadal-Ginard and Vijak Mahdavi, Department of Cardiology, Children's Hospital, and Department of Pediatrics, Harvard Medical School, Boston, Massachusetts 02115.

It is well established that commitment and differentiation of the skeletal muscle cell lineage is triggered by the basic-helix-loop-helix (bHLH) factors of the MyoD family. Although most of the skeletal muscle-specific genes that are regulated by bHLH factors are also expressed in cardiac muscle, MyoD-like factors have not been found in cardiac muscle. These observations suggest the involvement of other factors outside the bHLH group in skeletal and cardiac myogenic development. A candidate for such a factor is the myocyte-specific enhancer factor 2 (MEF2). Indeed, different alternatively spliced MEF2 isoforms are specific to skeletal and cardiac muscle and nerve cells. Remarkably, bHLH myogenic factors and MEF2 interact and cooperate to transactivate target genes, suggesting that cardiac-determining factors might also interact and cooperate with MEF2. To test this hypothesis, we have carried out an *in vitro* selection of optimal binding sites for MEF2 using cardiac and skeletal muscle extracts and anti-MEF2 antibodies. Although the MEF2 consensus target in both tissues is almost identical, this approach allowed the co-selection of other conserved sequences, some of which appear to be cardiac-specific. We are currently analyzing these sequences by electrophoretic mobility shift and transient transfection assays to gain some insights into their possible role as cardiac-determining factors.

**W 151** FORKHEAD DOMAIN CONTAINING TRANSCRIPTION FACTOR EXPRESSED IN PRESOMITIC MESODERM OF XENOPUS EMBRYOS, Naina Bhatia-Dey, Marie-Luise Dirksen, Robert. S. Adelstein, Milan Jamrich, Laboratory of Molecular Cardiology, N.H.L.B.I., NIH Bethesda, MD 20892 and Laboratory of Developmental Biology, FDA, Rockville, MD 20852

Transcription factors containing forkhead DNA binding domains have been shown to be involved in tissue specific expression as well as in the patterning of vertebrate and invertebrate embryos. Using degenerate primer PCR we have isolated a new forkhead gene which is preferentially expressed in the presomitic mesoderm. The gene encodes a protein of 483 amino acids. A 2.3 kb transcript is first detected in early gastrulae and RNA is also present in neurulae and tadpoles, but not in unfertilized eggs.The transcription of this gene seems to be induced by activin in animal caps. Whole mount in situ hybridization shows that in addition to the the presomitic mesoderm this gene is expressed in the head mesoderm. The transcription of this gene seems to be turned off when somites start to differentiate. Based on the sequence analysis and expression pattern we conclude that this gene belongs to the same gene subfamily as murine MF-1.

**W 152 IDENTIFICATION OF A CARDIAC-SPECIFIC ZINC FINGER PROTEIN GENE EXPRESSED IN ADULT HUMAN HEART.** Nigel Brand, Nina Dabhade, Penny Thomas, Magdi Yacoub and Paul Barton, Department of Cardiothoracic Surgery, National Heart & Lung Institute, London SW3 6LY, UK.
The zinc finger defines a well-conserved DNA-binding motif found in many transcriptional regulators from yeast to man. In order to investigate the potential role of zinc finger proteins (*zfp*) in cardiac gene regulation, we set out to clone cDNAs encoding novel *zfp*s from cardiac muscle. Hybridisation screening of an adult human atrial muscle cDNA library with a radiolabelled oligonucleotide probe specific for $C_2H_2$-type *zfp*s related to the *Drosophila* segmentation gene *krüppel* resulted in the isolation of several *zfp* cDNA clones. One of these, which we call *human zfp6* (*hzfp6*), is expressed in atrial and ventricular muscle from adult heart, but is not expressed in other tissues tested, including skeletal and smooth muscles, as well as a variety of non-muscle tissues. Furthermore, its pattern of expression during human fetal life appears to be restricted primarily to heart, where it is detected as early as 9 weeks of gestation. *In situ* hybridisation reveals that expression localizes predominantly to the myocardium in sections of fetal heart. This study paves the way for dissecting the function of *hzfp6* in relation to the regulation of cardiac gene expression.

**W 153** A NOVEL bHLH PROTEIN, C-HLH1, IS EXPRESSED IN MESENCHYME DURING MURINE EMBRYOGENESIS
Doris Brown, Keith L. Ligon, Gary Lyons*, Eric Olson, and Peter Cserjesi, Department of Biochemistry and Molecular Biology, M.D. Anderson Cancer Center, Houston,TX 77030, *Department of Anatomy, The University of Wisconsin Medical School, Madison, WI 53706

Basic-helix-loop-helix (bHLH) proteins have been shown to play important roles in the control of cell growth and differentiation. Cell-specific bHLH proteins typically bind DNA as heterodimers with ubiquitous bHLH proteins such as E12 and E47. The yeast two hybrid cloning system was utilized to screen a 14.5d embryonic mouse cDNA library for novel bHLH proteins that can dimerize with E12. Several clones were isolated and are presently being characterized by sequence, expression and functional studies. Sequence data reveals that one of the clones, C-HLH1, contains the conserved bHLH motif found in all identified bHLH proteins. Data base searches indicate that C-HLH1 does not correspond to any previously described bHLH proteins. *In situ* hybridization shows C-HLH1 is first expressed at 10.5 d.p.c. in sclerotomal regions of the embryo. C-HLH1 expression from 11.5 to 15.5 d.p.c. is restricted to cartilage precursor and dense mesenchymal regions such as the developing ribs and vertebrae. Northern analysis indicates that C-HLH1 is expressed in skeletal muscle, heart, brain, lung, testis, and kidney from adult mouse. Expression was not detected in liver or spleen. Electrophoretic mobility shift and binding site selection assays are being performed to determine the DNA sequence(s) to which the C-HLH1 protein binds. Preliminary data suggests that C-HLH1 binds to DNA as a heterodimer with E12. These data suggest that C-HLH1 is involved in chondroblast development in the mouse embryo.

**W 154** A NOVEL BASIC HELIX-LOOP-HELIX PROTEIN EXPRESSED IN PARAXIAL MESODERM AND IMMATURE SOMITES OF MOUSE EMBRYOS AND IN HEART AND SKELETAL MUSCLE OF THE ADULT MOUSE, Rob Burgess, Peter Cserjesi and Eric N. Olson, Department of Biochemistry and Molecular Biology, The University of Texas M.D. Anderson Cancer Center, Houston, Texas 77030
Basic helix-loop-helix transcription factors regulate cell-specific transcription in a variety of lineages. We have cloned a novel bHLH protein, tentatively referred to as meso-1, which is expressed in the paraxial mesoderm and immature somites of mouse embryos. The deduced open reading frame of meso-1 exhibits 80% homology within the bHLH region to c-HLH1, which is expressed in the sclerotome and developing axial skeleton and cartilage. Meso-1 transcripts were shown by in situ hybridization to be present within the neural crest, paraxial mesoderm and immature somites of day 7.5 p.c. mouse embryos. Expression dissipates through later stages of development and is no longer detected in the neural crest by day 10 p.c. and correspondingly in the somites by day 12 p.c. In adult mice, meso-1 transcripts of about 1.35 kb are abundant in the heart and skeletal muscle. Larger transcripts of 7.3 kb exhibit a similar pattern of expression though at much lower levels. Meso-1 transcripts are present in C2 myoblasts and myotubes but not in a variety of other cell types including 10T1/2, 3T3, Hela, Cos, HepG2 and HL60. The complete expression pattern and attempts to define the function of meso-1 will be presented.

**W 155** ZBU1, A *BRAHMA*-RELATED GENE, ENCODES A DNA-BINDING PROTEIN WITH A SITE IN THE MYOSIN LIGHT CHAIN 1/3 ENHANCER. Hilary Clark, Xiaohua Gong, and Nadia Rosenthal, Cardiovascular Research Center, Massachusetts General Hospital - East, Charlestown, MA 02129.

The human ZBU1 gene was previously identified by virtue of the ZBU1 protein binding to a region of the mouse myosin light chain 1/3 enhancer in an expression screen. Sequence homology with the *Drosophila brahma* and yeast SNF2/SWI2 helicase motif was found. Unlike *brahma* and SNF2/SWI2, however, ZBU1 also has a DNA binding domain. Here is reported the cloning of the mouse ZBU1 homolog. DNA binding studies are being conducted to study the selectivity of binding to the myosin light chain 1/3 enhancer. Adjoining the E-box site at which ZBU1 binds is a consensus Hox binding site. This suggests that homeodomain proteins may interact with ZBU1 in regulating transcription at the MLC1/3 enhancer. Indeed, *Drosophila brahma* was identified in a genetic screen as a suppressor of *Polycomb*, which represses transcription of homeotic genes in the Antennapedia and Bithorax complexes. Biochemical studies are being carried out to try to identify Hox genes which may interact with ZBU1 at the MLC1/3 enhancer. In addition, mutant forms of ZBU1, including either the helicase or the DNA binding domain alone, are being introduced into mice as transgenes in hopes of generating a dominant negative effect. Examination of the phenotype may suggest a role in muscle development for ZBU1.

**W 156** BINDING SITE SELECTION FOR THE *PAX* PAIRED-DOMAIN AND EVIDENCE FOR DNA DEPENDENT CONFORMATIONAL CHANGES. Jon Epstein, Jiexing Cai and Richard Maas. Divisions of Genetics and Cardiology, Howard Hughes Medical Institute, Brigham and Women's Hospital, Boston, MA 02115.

*Pax* genes encode a family of transcription factors that are expressed in developmentally regulated patterns. Mutations in *Pax* genes are responsible for several mutant mouse phenotypes (*undulated, Splotch, and Small eye)* as well as inherited human disorders (Waardenburg's syndrome and aniridia). In addition to their likely role in neural development, *Pax* genes have been implicated in diverse developmental processes. *Pax-3* is expressed in migrating neural crest cells and may play a role in cardiac conotruncal septation. The expression pattern of *Pax-7* suggests a role in myogenic differentiation. All *Pax* genes share a conserved 128 amino acid DNA binding domain, termed the paired domain. Mutational analysis has begun to identify DNA sequences recognized by many *Pax* proteins, though promoter sequences of the few target genes studied had appeared unrelated to one another.

We have selected the optimal binding sequence for the *Pax-2* and *Pax-6* paired domain proteins from amongst a pool of random sequences. The results identify a common sequence core, spanning at least 15 base pairs, that is recognized by many, if not all, *Pax* gene products. The paired domain protects 28 base pairs from DNaseI digestion, and makes numerous contacts over a wide range as shown by methylation interference assays. In addition, we have demonstrated that an alternatively spliced form of the *Pax-6* paired domain displays altered sequence recognition. Finally, circular dichroism spectroscopy demonstrates that the paired domain changes from a molecule that is nearly structureless in solution to one that displays a high alpha helical content upon binding its recognition sequence.

These results will aid further structural analysis of the paired domain and will be useful in identifying target genes regulated by *Pax* proteins.

**W 157 EXPRESSION ANALYSIS OF THE MUSCLE INHIBITOR GENE M-TWIST.** Ernst-Martin Füchtbauer and Bettina Engist, Max-Planck Institute of Immunobiology, Stübeweg 51, D79108 Freiburg

The *M-twist* gene belongs to a family of transcription factors with a conserved basic helix-loop-helix motif (bHLH). We have shown that it is an inhibitor of muscle differentiation in tissue culture cells (see Hebrok et al., this meeting). Expression data and mutant analysis in *Drosophila* suggest that *twist* has an early function for gastrulation but may also play a role in organogenesis, namely in muscle differentiation. Our expression data suggest that in the mouse *M-twist* gene also serves multiple functions. The early expression at about day 7.5 p.c. is strikingly similar to the expression of the *Brachyury* gene which is correlated with gastrulation in vertebrates. In later embryogenesis *M-twist* expression is mainly confined to somites, developing limbs and branchial arches. In all these areas there is a significant exclusion between the expression of *M-twist* and the early myogenic marker gene *myf5*. We will present a detailed comparison of *M-twist, Brachyury* and *myf 5* expression by whole mount and conventional *in situ* hybridization.

**W 158 MOUSE-TWIST IS AN INHIBITOR OF MUSCLE DIFFERENTIATION.** Matthias Hebrok, Karin Wertz and Ernst-Martin Füchtbauer, Max-Planck Institute of Immunobiology, Stübeweg 51, D79108 Freiburg

*M-twist* belongs to a family of transcription factors with a conserved basic helix-loop-helix motif (bHLH). In *Drosophila* twist plays an important role in gastrulation and is expressed in mesodermal derivatives. The mouse *twist* gene is also expressed in mesodermal cells as well as in neural crest cells that develop into mesoderm-like structures. In later mouse embryogenesis *twist* is expressed in the somite. During the process of somite differentiation into dermamyotome and sclerotome *twist* expression becomes excluded from the myotome where the myogenic HLH gene *myf5* is activated. Strikingly similar in *Drosophila twist* is expressed in muscle precursor cells and disappears at the time when *ß-tubulin,* an early myogenic marker, is first detected. Therefore a common function of *twist* might be to prevent premature muscle differentiation in cells that are already committed to form muscle. By stable transfections of myogenic mouse cells (C2C12) with a *twist* expression vector we show that *M-twist* can act as an inhibitor of muscle differentiation. About 65% of the *twist* expressing C2C12 clones were greatly impaired in their ability to differentiate and to form myotubes when transferred to differentiation medium (2% HS). Control transfections resulted in only 20% non-differentiating clones. The degree of 'differentiation incompetence' correlates with the level of *twist* expression. We can rescue *M-twist* expressing clones by incubation with twist-antisense oligos. This effect is concentration dependent, control oligos had no effect.

We are currently using these *twist* transfected cell lines as tools to search for positively or negatively regulated target genes of *twist*.

**W 159 TEF-1 ISOFORMS ARE MUSCLE-ENRICHED M-CAT BINDING FACTORS.**
Sarah B. Larkin*, Alexandre F. R. Stewart*[1], Iain K. G. Farrance, Janet H. Mar[2], Deborah E. Hall, Anthony Azakie and Charles P. Ordahl
Department of Anatomy and Cardiovascular Research Institute, University of California, San Francisco, CA 94143.

M-CAT elements mediate cardiac and embryonic skeletal muscle-specific expression of the cardiac troponin T gene and a number of other cardiac-specific genes. M-CAT binding factor was shown to be biochemically and immunologically related to cloned human TEF-1, a transcriptional regulator of the SV40 viral enhancer. Here we describe the cloning of TEF-1 from chick heart and the identification of several novel isoforms derived by alternative splicing. TEF-1 mRNA is considerably enriched in cardiac and skeletal muscle and expressed at lower levels in gizzard and liver, consistent with a proposed role in muscle gene transcription (see abstract by Farrance *et al.*). The predominant TEF-1 isoforms, TEF-1A and a novel isoform TEF-1B, bind M-CAT elements with high affinity and in a sequence-specific manner. The potential of these isoforms to transactivate is being tested by transfection into primary muscle and fibroblast cells. Preliminary data indicates a difference in transactivation ability between isoforms. These data will be presented.

This work was supported by a Neuromuscular Disease Research Fellowship from the Muscular Dystrophy Association (to AFRS), by a International Research Fellowship from the Fogarty International Center, National Institutes of Health (to SBL), by a postdoctoral fellowship from the National Institutes of Health (to IKGF) and by grants HL35561, HL43821 and GM32018 from the National Institutes of Health (to CPO).

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**W 160 Co-crystal structure of MyoD bound to DNA**

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**Abstract**

Myo D is a member of the family of muscle regulatory factors that includes myogenin, Myf-5, and MRF-4. It is present in the nuclei of proliferating myoblasts and differentiated myotubes, but is not expressed in fibroblasts or other non-muscle cell types. In addition to its role in myogenesis, the protein is interesting for its homology to the *myc*-family of proteins. The region of homology has been named the 'basic-helix-loop-helix' motif and it has been shown that it is an important motif in DNA-binding proteins. Myo D binds DNA specifically, and moreover, the expression of the bHLH region alone is necessary and sufficient to induce myogenesis in 10 T 1/2 fibroblasts. Myo D is also interesting for its interactions with other proteins. It can form homodimers, as well as heterodimers with the kappa enhancer binding proteins, E12 and E47. These heterodimers can bind to two regions of the mouse muscle creatine kinase gene upstream enhancer. In order to understand the structural basis for the formation of these heterodimers and homodimers, as well as to understand how the bHLH dimer recognizes its specific DNA site, we have recently crystallized and solved the X-ray diffraction structure of the Myo D bHLH peptide bound to a 14 bp DNA oligo containing the specific binding site of Myo D. The resolution of the structure is at 2.8 angstroms and shows Myo D bound to DNA as a homodimer. The structure of the homodimer is a parallel left-handed four helical bundle with an extended helix that sits in the major groove of the DNA. The overall fold of the protein shows a high degree of similarity to the helix-loop-helix domain of the recently solved Max structure (D'Amare-Ferre *et al*, Nature 363, 38-45). Unlike Max, Myo D does not contain a leucine zipper extension at the C-erminus of the HLH domain. In spite of this, plus the relatively low 25% sequence identity with Max in the bHLH domain, the Myo D structure has only a 1.17 Angstrom rms deviation in the peptide backbone of the helical regions of the bHLH domain. The bHLH domain thus appears to be a highly conserved protein fold. The wealth of biochemical data on the Myo D bHLH domain, together with a comparison of the Myo D structure with that of Max allow us to explain some aspects of the structural basis of DNA recognition by bHLH proteins as well as the mechanisms by which these proteins contribute to the activation of transcription.

**W 161** ANALYSIS OF GENE EXPRESSION PATTERNS IN THE EMBRYONIC MOUSE MYOTOME WITH THE GREEN FLUORESCENT PROTEIN, A NEW VITAL MARKER Jennifer Barnett Moss and Nadia Rosenthal, CVRC, MGH-East, Charlestown, MA 02129

We are probing the existence of positionally restricted patterns of gene expression along the anteroposterior axis through the construction of cDNA subtraction libraries. RNAs derived from newly formed somites from embryonic mice of different ages is the starting material. Differences in gene expression between somites at different developmental stages are suggested by the manifestation of chloramphenicol transferase (CAT) expression in a gradient of a myosin light chain transgene that increases posteriorly, opposite to the direction of somite maturation. Because the transcripts of the endogenous MLC 1/3 locus do not follow this pattern, we believe that the transgene contains a sensitive subset of recognition sequences responding to differential methylation and to other factors that may exist in discrete regions along the AP axis.

We have chosen to cast a wide net for the capture of cDNAs that may be differentially expressed by constructing a similar MLC transgene that replaces CAT with the green fluorescent protein (GFP). This vital marker requires no substrate other than light for visualization. In a separate GFP construct, the Myogenin promoter will be used to detect earlier time points due to its myotome-restricted expression. Initially, we will observe the fluorescence generated from these two transgenes in cell culture. Because a pure population of (green) myotomal cells can potentially be isolated intact from a fluorescence activated cell sorter (FACS), newly formed somites will be sorted and the resulting myotome-specific RNA converted to cDNA and subtracted. We expect to identify $10^3$ to $10^4$ different clones within subtraction libraries constructed from cDNAs produced and subtracted while attached to latex beads. The clones will be differentially screened using subtracted probes and positives confirmed via *in situ* analysis. Using this unbiased approach, we hope to find a variety of proteins expressed in young somites at a time when patterns are in the process of being established but have not yet been finalized.

**W 162** D-mef 2: A DROSOPHILA MESODERMAL GENE WITH A BI-PHASIC EXPRESSION PROFILE DURING EMBRYOGENESIS. Hanh T. Nguyen[1,4], Susan Abmayr[5], Rolf Bodmer[6] and Bernardo Nadal-Ginard[1,2,3,4], Dept. of Cardiology[1] and Howard Hughes Medical Institute[2], Children's Hospital, Dept.of Cellular and Molecular Physiology[3] and Dept. of Pediatrics[4], Harvard Medical School, Boston, MA 02115; Dept. of Molecular and Cellular Biology[5], Pennsylvania State University, University Park, PA 16802; Dept. of Biology[6], University of Michigan, Ann Arbor, MI 48109.

Regulated expression of muscle-specific genes is dependent upon the function of myogenic factors belonging to the *myoD* and MEF2 gene families.To explore the role of MEF2 in the control of mesoderm differentiation, in a system that is more amenable to genetic manipulations, we have isolated and characterized the *Drosophila* homologue of MEF2 (*D-mef2*). The single *D-mef2* gene codes for a 515 aa protein which contains the MADS and MEF2-specific domains that are characteristic of the MEF2 family of transcription factors. The encoded D-MEF2 protein has DNA binding and trans-activation properties that are similar to its vertebrate homologues. *D-mef2* exhibits a bi-phasic pattern of expression during embryogenesis. It is first expressed in the presumptive mesoderm at the late cellular blastoderm stage, much before the appearance of *nautilus* and *myosin* heary chain RNA. After invagination, *D-mef2* RNA is present in all mesoderm before being restricted to the dorsally-located mesoderm. Following the subdivision of the mesoderm, *D-mef2* is expressed in a new pattern which includes cardioblasts, visceral and somatic mesodermal cells. *Twist* activity is required for the initial *D-mef2* expression while *snail* function is needed for the maintenance and less for its initiation. The potential role of *D-mef2* in mesoderm differentiation and its relationship with other mesodermal genes will be discussed.

**W 163** ***E1A MEDIATED INHIBITION OF MUSCLE CELL DIFFERENTIATION AND MYOGENIC ACTIVITIY OF MYF-5,***

Andrea Sandmöller and Hans-Henning Arnold, Department of Cell- and Molecular Biology, TU of Braunschweig, Spielmannstrasse 7, 38106 Braunschweig, Germany

Oncogenic gene products, which act by association with cellular proteins and thereby alter their normal functions have been particulary useful in dissecting the regulatory processes of cell differentiation and proliferation. Myogenic differentiation is inhibited by the adenovirus E1a protein in L6 rat muscle cells. Constitutive expression of E1a specifically interferes with expression of the myogenin gene and trans-activating function of Myf-5, the two helix-loop-helix (HLH) proteins that are expressed in L6 cells. Repression of Myf-5 activity by E1a does not involve reduction of its synthesis or ability to bind DNA, but is mediated via its basic HLH and C-terminal regions. Certain mutations within the basic region of Myf-5 abolish trans-activating activity without affecting DNA binding similar to the E1a effect. Both observations could be explained by the interruption of Myf-5 interaction with an accessory protein serving as coactivator.

In the course of studies pertaining to the identification of a putative cofactor, we first defined the inhibitory domain of E1a and found it to reside to aa 38-62. Since a direct physical interaction between Myf-5 and E1a could not be demonstrated, we favour a model of competitive inhibition by cellular binding partner(s) common to both, possibly bridging the basic and carboxy-terminal activator region. Although E1a protein sequences encoded by conserved regions 1 (aa 40 to 70) and 2 (aa 121-139) were shown to interact with pRB, transfection of myogenic factors such as Myf-5 and MyoD into pRB-minus SAOS cells revealed that trans-activation of muscle specific reporter genes is independent of pRB. Therefore, pRB is not a likely candidate for the putative coactivator.

To elucidate the mechanisms by which Myf-5 is involved in muscle cell differentiation, we focused on L6 cell lines expressing estrogen receptor/E1a chimeras. Hormone dependent activation of E1a protein should allow us to identify putative cofactors that might be involved in Myf-5 mediated regulation of myogenesis. Here we present data on double-immuno-precipitations with antibodies directed against E1a and Myf-5 that may identify Myf-5 associated proteins involved either in cell cycle regulation and/or myogenic activities of Myf-5.

**W 164** **cDNA CLONING AND CHARACTERIZATION OF NOVEL TEF-1-RELATED FACTORS THAT BIND TO THE M-CAT MOTIF OF THE MYOSIN HEAVY CHAIN β GENE,**

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The M-CAT (GTIIC) motif in the rabbit myosin heavy chain (HC) β promoter is essential for muscle-specific transcription of the myosin HCβ gene. We have previously identified both muscle-specific and ubiquitous factors (A1 and A2, respectively) that bind to the M-CAT motif. Screening of a mouse cardiac cDNA library with a human TEF1 cDNA probe at low stringency had resulted in isolating three distinct clones: one encodes a mouse homologue of TEF1 (mTEF1) and the others encode mTEF1-related proteins (TEFR1 and 2). We have concentrated on examination of TEFR1. Three TEFR1 transcripts (7, 3.5, and 2 kb) are expressed in differentiated skeletal myotubes but not in myoblasts or in non-muscle cells. By screening a Sol8 myotube cDNA library, we isolated two distinct clones (TEFR1a and b) which seem to be alternatively spliced products. TEFR1a encodes a 427 a.a. protein containing a TEA motif (DNA binding domain) related to mTEF1. The amino acid sequence of TEFR1b is the same as that of TEFR1a except that 43 a.a. is deleted from next to the TEA DNA-binding domain. The *in vitro* transcription/translation products of mTEF1, TEFR1a and b specifically bound to the M-CAT motif in gel mobility shift assays. The mobility of DNA-protein complexes of TEFR1b was similar to that of the muscle-specific A1 factor. However, the mobility of DNA-protein complexes of mTEF1 and TEFR1a was similar to that of the ubiquitous A2 factor. Overexpression of TEFR1a and b repressed the expression of the TK-CAT reporter constructs containing M-CAT motifs. However, overexpression of the fusion proteins of TEFR1 and GAL4 DNA binding domain trans-activate the expression of the CAT reporter constructs containing GAL4 DNA binding sites. Thus, the TEFR1a and b contain transcriptional activation domains but may require co-factor(s) for full function.

**W 165** **REGULATION OF THE E2F TRANSCRIPTION FACTOR BY MUSCLE CELL DIFFERENTIATION**

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We have examined the regulation of the E2F transcription factor upon differentiation of muscle cells. E2F regulates many genes involved in growth control. E2F is also the target of regulation by diverse cellular signals and can form complexes with growth suppressor (retinoblastoma (RB), p107) and cell cycle proteins (cyclins A, E and cdk). Both the RB and p107 proteins can inhibit E2F function, which is consistent with their growth suppressor function.

We have examined the E2F DNA-protein complexes, protein levels and phosphorylation in both undifferentiated and differentiated muscle cells. Using the gel shift assay, we find that there are distinct DNA-protein complexes in undifferentiated and differentiated C2 muscle cells. In particular, a new E2F complex was present upon differentiation, but is not observed in undifferentiated cells. Furthermore, in muscle cells that are blocked in differentiation, the differentiation-specific complex was absent and E2F complexes characteristic of undifferentiated cells were observed. The identity of the proteins in the various E2F complexes is currently under investigation.

As determined by Western blots of extracts from undifferentiated and differentiated cells, the level of the E2F-1 protein was unchanged with differentiation. By in vivo labelling and immunoprecipitation, E2F-1 was phosphorylated in both undifferentiated and differentiated cells, and E2F-1 phosphorylation levels appear to be reduced in differentiated cells. Because specific E2F complexes were formed depending on differentiation state and E2F protein levels remained constant, these observations suggested that the regulation of E2F upon differentiation could be through protein-protein interactions and/or phosphorylation.

**W 166** DIFFERENTIAL EXPRESSION OF A/T-RICH DNA BINDING PROTEINS IN EARLY EMBRYOGENESIS,

M.A.Q. Siddiqui, S. Goswami, S. Ghatpande, E. Mascareno, M. Dhar, M.A. Baig, A. Deshpande, and S. Carleton Department of Anatomy & Cell Biology, State University of New York Health Science Center Brooklyn, Brooklyn, NY 11203

The complexities of morphogenetic changes that occur in the developing heart reflect changes in transcriptional activity of the muscle cell type specific genes. Using gene specific DNA primers and reverse transcriptase-polymerase chain reaction assay, we have analyzed the early chicken blastodermal development by monitoring the onset of the cardiac and skeletal muscle specific myosin light chain (MLC2) gene expression and the appearance of the cognate DNA binding protein factors. We have observed that the first discernable transcript of cardiac MLC2 gene appears in stage 5 and that of skeletal MLC2 in stage 8 embryos. We have previously shown that the cardiac muscle specific activation of MLC2 transcription is due to two A/T-rich sequence elements (element B and A) and their DNA binding protein factors. Element B is identical to the myocyte enhancer factor (MEF-2) target site and element A is similar to the CArG-box sequence. The repression of cardiac MLC2 transcription in skeletal muscle is mediated by a negative regulatory element (CSS) and its binding factors. Using gel mobility shift assay, we have observed that the DNA binding activity of cardiac specific element B binding protein, BBF-1, distinguishable from MEF-2, begins to appear at stage 5, consistent with the appearance of cardiac MLC2 mRNA, whereas the MEF-2 activity is detectable at stage 8 at which time the skeletal MLC2 mRNA appears. Differential appearance of DNA binding proteins is also observed with the CArG- box DNA as probe. The expression of the ubiquitous TATA binding factors (TBFs), on the other hand, remains more or less unchanged throughout the development. We conclude that the expression of the muscle specific A/T-rich DNA binding proteins undergo distinct changes, consistent with the onset of myogenic program for transcription of cardiac and skeletal MLC2 gene, and may, therefore, be involved in regulation of the differential expression of tissue specific MLC2 genes in early development.

**W 167** DISTINCT MECHANISMS OF MYOGENIC INIHIBITION BY THE HLH PROTEINS *Mtwist* AND ID,

Douglas B. Spicer and Andrew B. Lassar, Harvard Medical School, Department of Biological Chemistry and Molecular Pharmacology, 240 Longwood Ave., Boston, MA 02118.

The MyoD family of myogenic basic-helix-loop-helix (bHLH) proteins plays a pivotal role in the differentiation of skeletal muscle and are exclusively expressed in the myotome of developing somites and in the mature muscles that are derived from these cells. *twist* is a member of the bHLH family of proteins and was originally identified in *Drosophila* as a gene necessary for embryonic gastrulation and formation of mesoderm. During *Drosophila* larval development, *twist* is expressed in skeletal muscle precursor cells which subsequently turn off *twist* expression as these cells differentiate into muscle. *twist* homologues have been isolated from both *Xenopus laevis* and mouse and these are specifically expressed in virtually all of the mesoderm except the myotome, and are also present in the anterior neural crest cells. Since there is such a contrasting pattern of expression between *twist* and the myogenic bHLH genes, we have tested whether there may be a reciprocal inhibition between these two classes of bHLH regulator. We have found that *Mtwist* is able to inhibit the transactivation of a muscle creatine kinase promoter-CAT reporter construct (MCK-CAT) by MyoD in C3H10T1/2 embryonal fibroblasts. This inhibition was significantly more efficient than that of two other dominant negative HLH proteins, ID and a mutated MyoD construct (MyoD-Bpro) containing a proline in its basic domain which prevents it from binding DNA. In order for the myogenic bHLH proteins to activate muscle-specific transcription they must heterodimerize with the ubiquitously expressed bHLH proteins such as E12 or E47 ("E" proteins). ID and MyoD-Bpro are thought to inhibit wild type MyoD function by preferentially binding to the limiting E proteins and therefore prevent any active myogenic bHLH protein/E protein heterodimers from forming. In support of this we were able to "rescue" the inhibition of MyoD transactivation by ID and MyoD-Bpro by cotransfecting excess E protein. The inhibition of MyoD transactivation by *Mtwist*, however, could not be "rescued" even with 10-fold excess E protein. *Mtwist* also inhibited transactivation by a MyoD-VP16 transactivation domain fusion protein, however excess E protein was able to reverse this inhibition suggesting that the inhibition by *Mtwist* is brought about by "squelching" a coactivator needed by MyoD.

**W 168** STRUCTURE AND EVOLUTIONARY HISTORY OF THE HUMAN SARCOMERIC MYOSIN HEAVY CHAIN GENE FAMILY,

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Based on our previous comparisons of myosin heavy chain (MHC) coding sequences, we have generated data to support the hypothesis the sarcomeric MHC gene number in H. sapiens and most other mammals is eight. We have cloned and partially characterized all eight human MHC genes as DNA fragments in cosmid and/or YAC vectors, enabling the construction of a high resolution physical map. Sequence comparisons between MHC coding regions have been utilized to estimate evolutionary distances within the gene family. We propose an evolutionary history for the modern human MHC gene loci and discuss patterns of developmentally regulated gene expression in this regard. Finally, we discuss the clinical implications of these findings.

**W 169** **Specific Binding of an Actin-like Protein, MS2, to a TC-II Enhancer Element of the MCK Gene,**

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Region s4 of the MCK enhancer is able to bind AP2, but its control appears to be more complex. Different site-specific mutants reveal both enhancing and suppressing activity from this region. Pure AP2 can bind to region s4, but since AP2 appears to be absent from muscle cells, other factors in muscle must be responsible for s4's observed activities. Muscle nuclear extracts do contain factors different from AP2 that bind region s4; skeletal factor MS2 and cardiac factor PC1 bind region s4 with specificity. Competitive bandshifts with oligonucleotides related to region s4 reveal that factor MS2's binding specificity depends strongly on the TC-II motif that binds AP2, and only weakly on the p53 binding site just upstream or on the κB-related site just downstream. DEPC footprints confirm the binding of MS2 to the TC-II motif at region s4, with a different pattern of protection than that seen with AP2. Factor MS2 was affinity purified by an established method for magnetic method of DNA-binding factors; this method has been modified to directly confirm the identity of the purified material by coupled bandshift. Affinity-purified MS2 resembles actin by mobility on denaturing gels, by amino acid content and N-terminal blockage, and by immunoreactivity. The immunologic similarity of MS2 to actin, and its different binding properties from actin, have been confirmed by bandshift-western blot of purified MS2. The similarity of enhancer-binding factor MS2 to actin suggests a mechanism through which MS2 could coordinate transcription with nuclear structure.

**W 170** PHOSPHORYLATION STUDIES OF MYOD

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We are examining the question of which protein kinases may regulate MyoD. Using an assay developed by Michael Karin's group, we have identified a kinase activity in C2 myoblast and myotube extracts that binds with very high affinity to bacterially-produced GST-MyoD protein; even after washing the cellular proteins bound to GST-MyoD in buffer containing 2M NaCl, significant kinase activity remains. We have roughly mapped the phosphorylation sites to the N- and C-termini by using existing deletion mutants of GST-MyoD, which correlates with the presence of three consensus casein kinase II (CKII) sites.

Indeed, CKII from C2 cell extracts does bind GST-MyoD as determined by immunoblot analysis, and GST-MyoD is an in vitro substrate for purified CKII. We have demonstrated by tryptic peptide mapping that a subset of the sites phosphorylated by extracts is phosphorylated by CKII. Heparin, a well-known inhibitor of CKII, inhibits the phosphorylation of GST-MyoD by purified CKII. However, the same concentration of heparin is not capable of inhibiting the kinase activity present in extracts. This observation, in conjunction with the tryptic peptide mapping result, suggests that at least one other kinase in addition to CKII is capable of binding tightly to GST-MyoD. Studies are currently underway to identify this kinase(s) and to determine whether they play a role in controlling MyoD activity.

**W 171** A NOVEL DNA-BINDING PROTEIN CONTAINING A *FORKHEAD* DOMAIN IS INVOLVED IN MUSCLE-SPECIFIC TRANSCRIPTION, Rhonda S. Bassel-Duby, and R. Sanders Williams, Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, TX 75235
A transcriptional control element, CCAC box, has been defined within a muscle-specific promoter region of the myoglobin gene. We used λgt11 expression cloning with oligonucleotides corresponding to the CCAC box to isolate a clone encoding a DNA-binding protein, termed CBF1. Protein expression of CBF1 is concurrent with muscle development and is elevated in muscle subjected to chronic contractile activity. Determination of the predicted amino acid sequence of CBF1 revealed it to be a member of a new family of DNA-binding proteins, defined by a 110 amino acid domain first identified in the *forkhead* gene of *Drosophila melanogaster*. *Forkhead* functions in the *Drosophila* embryo as a region-specific homeotic gene that promotes terminal, in contrast to segmental, development. Other members of the *forkhead* family, HNF-3α, β, and γ, also have been identified in vertebrates, where they also function as developmental regulators. Our current results provide the first suggestion that members of this family may be involved in control of gene expression in cardiac and skeletal muscle.

**W 172** **CK II-dependent phosphorylation of the muscle-specific transcription factor Myf-5 is necessary for transactivation.** Barbara Winter[1], Olaf-Georg Issinger[2], and Hans-Henning Arnold[1], Department of Cell and Molecularbiology, TU of Braunschweig, Spielmann Str.7, 38106 Braunschweig, FRG, [2]University of Saarland, Medical School, Human Genetics, Blg.68, 66421 Homburg/Saar, FRG

Transcription of muscle-specific genes is regulated by a family of transcription factors that includes Myf-5, MyoD, MRF-4, and myogenin. These proteins share homology within a basic-helix-loop-helix domain (bHLH) that mediates dimerization and DNA-binding. Each protein activates myogenesis when expressed in nonmyogenic cells. An intriguing property of myogenic HLH proteins is their differential expression pattern during myogenesis. Myf-5 and MyoD are present but apparently functionally silent in undifferentiated myoblasts and become activated upon terminal differentiation. In contrast, myogenin expression and activity start at the onset of differentiation and becomes prevalent only in myotubes. The regulation of Myf-5 and MyoD activities in myoblasts suggest the involvement of posttranslational mechanisms. Specific phosphorylation events mediated by protein kinases have been shown to modulate the activity of a variety of transcription factors within the cell. Here, we present in vitro data on Myf-5 phosphorylation with casein kinase II (CK II) demonstrating that Myf-5 is a substrate for this protein kinase. In transient transfection experiments using mutant Myf-5 expression constructs, we identified phosphorylation sites in the amino-terminal activator domain and in helix II which are both essential for the biological activity of the Myf-5 protein. Mutations which prevent phosphorylation of these sites result in proteins that still dimerize and bind to DNA but fail to transactivate. These results demonstrate that the Myf-5 protein is a substrat for CK II phosphorylation in muscle cells and CK II modifies the transactivating capacity of this transcription factor. It therefore may play an important role for the regulation of myogenesis.

*Muscle Gene Regulation I*

**W 200** CONTROL OF ANF TRANSCRIPTION DURING CARDIAC DEVELOPMENT, Ali Ardati, Stéphane Tremblay and Mona Nemer, Laboratoire de développement et différenciation cardiaques, Institut de recherches cliniques de Montréal (IRCM), Montréal (Québec) CANADA H2W 1R7
Studies of tissue-specific transcription and isolation of muscle-specific transcription factors have greatly contributed to significant advances in understanding skeletal muscle differentiation. In contrast, the mechanisms underlying cardiac muscle differentiation and cardiac-specific gene expression remain largely unknown. We have used the atrial natriuretic factor (ANF) gene as marker to study the mechanisms of transcription during heart development. ANF is the major secretory product of the heart and the ANF promoter directs high level tissue-specific expression in cardiac cells in primary cultures and in transgenic mice. The ANF gene is expressed at a high level in both atria and ventricles at a very early stage of heart development and, while ANF transcription is constitutive in atria, ventricular ANF transcription is switched off within the first postnatal week. Primary cardiocytes derived from fetal or neonate rats of various ages were used to analyze the molecular mechanisms underlying spatial and developmental control of cardiac transcription. Using a combination of deletion analysis, we have identified an enhancer region sufficient for cardiac- and stage-specific expression of ANF. Deletion of this enhancer markedly reduced promoter activity in cardiac myocytes and derepressed ANF promoter activity in non-cardiac cells. Dissection of the enhancer revealed the presence of two subdomains which functioned in a tissue- and stage-specific manner. Mutagenesis experiments and promoter reconstruction assays revealed an important role for a CarG element specifically in differentiated cardiac cells and *in vitro* DNA binding studies showed that the ANF-CarG element is bound by a cardiac-specific complex distinct from the one recognizing the c-*fos* serum response element (SRE). Another tissue-specific element of the enhancer was active specifically in atria and ventricles at early differentiation stages. This element is bound by a novel cardiac factor unrelated to the Mef or to the helix-loop-helix family of nuclear *transcription* factors. Thus, the expression of the ANF gene appears to be under control of positive and negative elements which are active in a stage- and tissue-specific fashion.

**W 201** CHARACTERIZATION OF MULTIPLE CIS-ACTING ELEMENTS REGULATING SERCA2 GENE EXPRESSION IN CARDIAC AND SKELETAL MUSCLE CELLS, Debra Baker, Junaid Shabbeer, Jennifer Larsen, Richard A. Walsh, Muthu Periasamy, Division of Cardiology, University of Cincinnati College of Medicine, Cincinnati, OH 45267.
The major focus of our research is to understand the transcriptional regulation of the cardiac/slow twitch sarcoplasmic reticulum $Ca^{2+}$-ATPase (SERCA2) gene. Previously, we have shown that a 1.1 Kb upstream DNA sequence is able to direct expression of the SERCA2 gene both *in vivo* and *in vitro*. To further delineate additional upstream cis-acting elements, we have made a series of SERCA2 promoter-CAT constructs extending from -7 kb upstream to -72 bp and transfected them into C2C12 myoblasts, myotubes, and NIH 3T3 fibroblasts. The same promoter sequences were spliced with a luciferase reporter and are being tested *in vivo* by injection into rat hearts. Our preliminary analysis suggests that multiple cis-acting elements are involved in the control of SERCA2 promoter activity. We have found that a 17 bp element located at -275 appears to be important for maximal levels of SERCA2 expression in C2C12 cells. This element interacts with multiple proteins as identified from gel mobility shift assays. These factors display a distribution of binding activity that correlates with promoter strength. We will be presenting new data on additional promoter sequences involved in both tissue specific and developmental regulation of the SERCA2 gene.

**W 202 Rates of Somite Formation and Muscle-Specific Gene Expression in Growth-Selected Lines of Japanese Quail.** Jeremy L. Barth[1], Julie Morris[1], Henry Marks[2] and Robert Ivarie[1], Department of Genetics[1] and the ARS, SEPRL, USDA[2], University of Georgia, Athens, GA 30602.
Two Japanese quail lines have undergone long-term selection for low (line L) and high body weight (line P) at 4 weeks post-hatch. After 30 and 91 generations of selection, respectively, the L line weighed 2.3-fold less and the P line 3-fold more than the control line C. Similar differences were found in *pectoralis* muscle weights one day after hatch while the amount of DNA per gram of muscle tissue was nearly identical in all lines. Thus, differences in muscle mass and body weight appear to be due in part to stem cell hypoplasia (line L) and hyperplasia (line P) occurring during embryogenesis. We have shown that embryos of the high weight P line delay somite formation from stage 10 (10 somites) through stages 14-15 (22-25 somites) and proposed that delayed development increased myogenic stem cell divisions thereby changing the number of cells committed to muscle in later stage birds[1]. This suggested that for the low weight line L, an accelerated rate of somite formation might decrease stem cell growth and subsequently reduce the number of committed cells. To test this, somites were counted in L and C embryos at at 37.25 (n=13 for L, 16 for C), 39.5 (n=18 for L, 21 for C) and 42.5 hours (n=89 for L, 54 for C) of incubation. At 42.5 hours, L embryos had 16.1 and C, 13.2 somites. This increase of 2.9 somites was highly significant. The gain was only 0.7 somites at 39.5 hours, and L and C embryos each had 10.4 somites at 37.25 hours. Clearly, L embryos accelerate their rate of somite formation after stage 10, the same point at which P embryos begin to delay theirs. To show whether altered embryonic development reflects changes in expression of specific genes, embryos are being analyzed by whole-mount *in situ* hybridization and imunocytochemical detection[2] for expression domains of genes affecting muscle lineage (myogenic regulatory factors) and mesodermal pattern formation (*mox1, mox2, dsl1*). We are also determining whether altered somite formation is a property to P and L segmental plates or whether external factors may play a role. [1]Coutinho, L. L., J. Morris, H. L. Marks, R. J. Buhr and R. Ivarie. (1993) *Development* 117, 563-569; [2]Coutinho, L. L., J. Morris and R. Ivarie. (1992) *Biotech.* 13, 722-724.

**W 203** AN E-BOX MOTIF WITHIN THE MUSCLE-SPECIFIC INTRON 1 ENHANCER OF THE HUMAN DMD GENE DISPLAYS DISTINCTIVE FACTOR BINDING PROPERTIES. Lucine O. Bosnoyan[1], Ronald G. Worton[1], Peter N. Ray[1], and Henry J. Klamut[2], [1]Department of Genetics and Research Institute, The Hospital for Sick Children, 555 University Avenue, Toronto, Ontario, M5G 1X8, and [2]the Department of Medical Biophysics, University of Toronto and the Ontario Cancer Institute, Princess Margaret Hospital, 500 Sherbourne St., Toronto, Ontario, M4X 1K9.
The Duchenne muscular dystrophy (DMD) gene is expressed primarily in skeletal and cardiac muscle and is induced as human myoblasts differentiate into multinucleated myotubes *in vitro*. We have previously identified sequences upstream of muscle exon 1 which regulate transcriptional induction of DMD gene expression with myoblast differentiation. In this study we describe a muscle-specific enhancer element within intron 1 of the gene. These sequences significantly enhance reporter gene expression in an orientation- and position-independent manner upon transfection into rat H9C2(2-1) myoblasts and myotubes. No activity is observed in 3T3 fibroblasts. We have used deletion analysis to define a 195 bp core enhancer region containing three MyoD/E-box and two MEF2 binding site homologies. These sequences mediate the formation of five protein-DNA complexes in electrophoretic mobility shift assays (EMSA) using nuclear extracts from H9C2(2-1) myotubes. Three of these complexes are effectively competed by a 30 bp oligonucleotide spanning one (E-1) of the three E-box homology motifs. No competition was observed with oligonucleotides spanning the other two E-box concensus regions, or with either oligonucleotides or genomic clones containing muscle creatine kinase gene enhancer MyoD/E-box binding sites. This result suggested that MyoD is not involved in nuclear complex formation within the DMD gene enhancer core. DNase footprinting, however, shows clear protection of the E-1 E-box motif, and oligonucleotides having mutations in the E-1 E-box motif no longer compete for factor binding in EMSA. These results indicate that the factor binding properties of the E-1 E-box motif within the DMD gene muscle intron 1 enhancer are distinct from E-box motifs within the muscle creatine kinase gene enhancer.

**W 204** MYOCYTE-SPECIFIC ENHANCER FACTOR 2 (MEF2) PROTEINS ACTIVATE TRANSCRIPTION OF THE MUSCLE REGULATORY FACTOR MRF4, Brian L.Black, James F. Martin and Eric N. Olson, Department of Biochemistry and Molecular Biology, The University of Texas M. D. Anderson Cancer Center, Houston, TX 77030
Differentiation of myoblasts to mature skeletal muscle involves the induction of a wide variety of muscle-specific genes. The transcription of many of these genes is induced by a family of myogenic basic helix-loop-helix (bHLH) transcription factors which include MyoD, myogenin, MRF4, and Myf5. These myogenic bHLH proteins regulate transcription of many muscle-specific genes directly by binding to an E-box motif located in the control regions of muscle-specific genes. However, since many downstream muscle-specific genes do not contain an E-box in their control regions, other factors must be necessary for transcriptional control of the full myogenic program. Recent evidence has suggested that an additional family of transcription factors, the MADS box containing myocyte-specific enhancer factor 2 (MEF2) proteins, are required for the control of the muscle-specific transcriptional program. This family of transcription factors includes MEF2A, B, C, and D. Overexpression of myogenin results in induction of the MEF2C gene suggesting that the MEF2 genes may be downstream of the myogenin bHLH proteins. However, the myogenin gene contains a MEF2 recognition element which is required for full transcriptional activity. These data suggest that the myogenic bHLH proteins and the MEF2 family of proteins are part of a complex regulatory network. To begin to dissect this regulatory network we investigated whether the MEF2 proteins could activate the promoter of the MRF4 gene. We cotransfected expression vectors encoding either MEF2A, MEF2C, or MEF2D along with a reporter plasmid containing the MRF4 promoter linked to the CAT gene. Coexpression of any of the MEF2 factors caused a greater than tenfold increase in CAT activity as compared to the activity of the reporter construct alone. In addition, we examined the ability of each of the MEF2 factors to activate the transcription of various myogenic genes. These results indicate that transcriptional activation of the MRF4 promoter requires the expression of MEF2.

**W 205** A COOPERATIVE COMPLEX ON THE CARDIAC ACTIN PROMOTER REACTS WITH SP1, SRF, AND MYOGENIN ANTIBODIES, Elzbieta Biesiada, Vittorio Sartorelli, and Larry Kedes, Department of Biochemistry and Molecular Biology and Institute for Genetic Medicine, University of Southern California, School of Medicine, Los Angeles, CA, 90033.
The composite human cardiac α-actin (HCA) promoter is activated in skeletal myogenic cells by interaction with the transcriptional activators: serum response factor (SRF), Sp1, and a member of the myogenic determinant factor (MDF) family. We report the formation of a multiprotein complex on the HCA promoter whose assembly requires the presence of at least three factors interacting with CArG-, GC- and E-boxes which are the DNA binding sites recognized by SRF, Sp1, and MDF, respectively. This complex displays cell-type restricted features since it is formed only when the HCA promoter is simultaneously occupied by multiple activators derived from myogenic cells: the binding of one activator requires the presence of the other two. Proteins eluted from the HCA:multiprotein complex reacted with a monoclonal antibody directed against myogenin or with antisera raised against Sp1 and SRF. The presence of the complex on the HCA promoter is necessary but not sufficient to ensure transcription because permutation of the binding sites for SRF, Sp1 and MDFs resulted in preservation of the complex but abolishment of transcription. Taken together our results indicate that cooperative protein:protein interactions are critical for the formation of a transcriptionally productive multiprotein complex on a composite muscle specific promoter and that Sp1, SRF and myogenin are components of a protein complex formed on the HCA promoter.

**W 206** A DEVELOPMENTALLY-REGULATED EXON EXPRESSED IN FETAL TROPONIN Ts, Margaret M. Briggs, Melinda Maready, and Fred Schachat, Department of Cell Biology, Duke University Medical Center, Durham, NC 27710
Developmentally specific isoforms of troponin T (TnT) are expressed in fetal and neonatal skeletal muscle of rabbit and rat. Immunological cross reactivity suggests that these novel TnTs arise from the fast TnT gene, and cDNAs encoding these proteins contain fast TnT coding sequences. The cDNAs also contain 36 additional nucleotides inserted between exons 8 and 9 that define a new cassette exon in the fast TnT gene, whose alternative splicing is developmentally regulated.

In the rabbit, the fetal TnTs and their mRNAs comprise nearly all the TnT protein and mRNA at 6 days before birth. Beginning slightly before birth and continuing through the first week of neonatal development, they are gradually replaced by a well-characterized fast isoform TnT3f. The parallel changes in fetal TnT cDNAs and protein isoforms during development suggests that TnT expression is regulated primarily by changes in the alternative splicing process, rather than at subsequent steps, such as translation.

Expression of the fetal exon appears to be regulated in a manner that is quite different from that of the other cassette exons in the 5'-region of the fast TnT gene. The fetal exon is expressed specifically during fetal and neonatal development, whereas the other 5'-cassette exons are expressed in both developing and adult muscle. Examination of the rat (Breitbart and Nadal-Ginard) and rabbit genomic sequences reveals a feature that distinguishes it from the other 5' exons and which likely accounts for its developmentally regulated splicing. Unlike the other 5'-cassette exons, the fetal exon has a rare non-consensus acceptor splice site at its 5' end (aagA is present as opposed to the consensus, cagG) (Shapiro and Senapathy, 1987). The ability to recognize this non-consensus splice site appears to be the critical difference between fetal and late neonatal muscle splicing mechanisms as the mRNAs for the major fetal TnT, fetal TnT-3, differs from its replacement, TnT3f, solely by the inclusion of the fetal exon.

**W 207** DE-METHYLATION OF THE *myoD* DISTAL ENHANCER CORRELATES WITH *myoD* GENE EXPRESSION. Brian P. Brunk, David J. Goldhamer and Charles P. Emerson Jr, Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, PA 19111, Department of Cell and Developmental Biology, University of Pennsylvania Medical School, Philadelphia, PA 19104
The distal *myoD* enhancer located 20 kb upstream of the *myoD* gene is sufficient for correct developmental regulation of a lac-Z reporter gene in muscles of transgenic mice (Science, **256**, 538-542). Interestingly, when transfected into tissue culture cells, this enhancer drives expression of a reporter gene in both muscle and non-muscle cell lines. This suggests that the chromosomal *myoD* enhancer in non-muscle tissue culture cells is under cis-regulation that represses the activity of the enhancer, perhaps by inhibiting accessibility to DNA binding regulatory proteins. To investigate this possibility, we are conducting DNAseI hypersensitivity and DNA methylation analyses. The distal enhancer is hypersensitive and un-methylated in muscle cell lines which express the *myoD* gene while it is not hypersensitive and is heavily methylated in non-muscle cell lines. Consistent with these data, the enhancer is less methylated in DNA isolated from muscle tissue of newborn mice than in DNA isolated from a variety of non-muscle tissues. Our results also indicate that the enhancer is methylated by at least the 16 cell stage of development. Thus, methylation could account for the differences in *myoD* enhancer-reporter gene expression in transgenic mice versus transfected cells in culture and may play a role in *myoD* gene regulation in the developing embryo.

**W 208** STRIATED MUSCLE AND NON-MUSCLE FACTORS BINDING TO CArG, A/T-RICH, AND MEF2 ELEMENTS OF THE MUSCLE-SPECIFIC MUSCLE CREATINE KINASE ENHANCER, Jean N. Buskin, Sharon L. Amacher, and Stephen D. Hauschka, Department of Biochemistry SJ-70, University of Washington, Seattle, WA 98195
The mouse muscle creatine kinase (MCK) gene region from position -1256 to -1050 confers high transcriptional activity specifically to skeletal and cardiac muscle. A variety of elements within this region have been delineated using transient transfections[1] and transgenic mice[2]. We now report a survey of factors binding to three positive elements, CArG, A/T-rich, and MEF2, as detected by gel-shifts, combined preparative gel shift-carbethoxylation interference, and expression library screening. Nuclear extracts from the two striated muscle types as well as from heart-derived non-muscle cells were compared. Each extract contained at least two factors binding to the CArG site; these differed in electrophoretic mobility and carbethoxylation interference pattern, but appeared to be similar among cell types. One shifted complex contains serum response factor or an antigenically related factor as ascertained with antiserum. Expression library screening of a myocardiocyte cDNA library using the A/T-rich site as probe revealed that the site is recognized by the octamer binding protein Oct-1. Two distinctly migrating factors binding to the A/T-rich site were detected in nuclear extracts from all cell types examined, and additional bands were present in non-muscle cells. One common band gave a clear interference pattern which was similar between cell types. This complex is identical or antigenically related to Oct-1 as assessed using antiserum, and it has higher affinity for an authentic "octamer" element from the immunoglobulin heavy chain enhancer than for the MCK A/T-rich site. Analytical gel-shift gels yielded MEF2 binding factor bands with similar mobility in the three cell types, while preparative gels revealed factors with different mobilities and interference patterns.

1. Amacher *et al.*, Mol. Cell. Biol. 13:2753-2764 (1993)
2. Donoviel *et al.*, submitted

**W 209** REGULATION OF *myogenin* EXPRESSION DURING MOUSE EMBRYOGENESIS. Tse-Chang Cheng, Brian Tseng, Peter Cserjesi, William H. Klein, John P. Merlie*, and Eric N. Olson, Department of Biochemistry and Molecular Biology, The University of Texas M. D. Anderson Cancer Center, Houston, TX 77030 and *Department of Molecular Biology and Pharmacology, Washington University School of Medicine, St. Louis, MO 63110
Expression of the myogenic helix-loop-helix (HLH) protein myogenin in the muscle cell precursors within somites and limb buds is among the earliest events associated with myogenic lineage determination in vertebrates. A 1565 bp 5'-flanking sequence of the *myogenin* locus was sufficient to direct the expression of a *lacZ* gene during mouse embryogenesis in the same pattern as that of the endogenous gene. Mutations in the *myogenin* promoter that abolish binding sites for myogenic HLH proteins or myocyte enhancer factor-2 (MEF-2) suppressed transcription of a linked *lacZ* transgene in subsets of myogenic precursors in mouse embryos. (Cheng et al., *Science* **261:** 215-218.) By crossing *myogenin*-lacZ mice with mice carrying a myogenin-null allele, we further defined the contribution of myogenin to its own expression in embryogenesis. We also analyzed expression a *lacZ* gene driven by tandem copies of the MEF-2 binding site linked to a basal promoter. The *lacZ* transgene was expressed in developing somites, heart, limb buds, and nervous system, suggesting that functioning MEF-2 factors are present in these regions. These results suggest that myogenic HLH proteins and MEF-2 participate in activating myogenin transcription in the embryo.

**W 210** REGULATION OF CARDIAC TROPONIN T ALTERNATIVE SPLICING,
Thomas A. Cooper and Jackie Ramchatesingh, Department of Pathology, Baylor College of Medicine, Houston, TX 77030.

Alternative splicing is a determinative regulatory event for the expression of divergent protein isoforms in striated muscle. To investigate the mechanism of regulated splicing, we are using the avian cardiac troponin T (cTNT) gene which produces two mRNAs via inclusion or exclusion of exon 5. This splicing decision is developmentally regulated such that exon 5 inclusion predominates (>90%) in the early embryo and exon skipping predominates (>95%) in the adult. We are using transient transfection of cTNT minigenes into primary skeletal muscle cultures to localize cis elements required for regulated alternative splicing. To identify the relevant trans-acting factors, a cell-free splicing assay for in vitro synthesized cTNT RNAs has been established [Cooper, T.A. (1992) J. Biol. Chem. 267, 5330-5338]. Two lines of investigation will be presented: (i) The alternative exon contains a novel purine-rich exon splicing element (ESE) that is required for exon inclusion [Xu et al. (1993) Mol. Cell. Biol. 13, 3660-3674]. Purine-rich motifs that are required for splicing have recently been found in several exons from different genes. This is the first splicing element to be identified within a vertebrate exon. RNA binding assays are in progress to identify the exon binding factors involved in exon recognition. (ii) The cis elements required for regulated splicing are local to exon 5. Our results demonstrate that the ESE serves as a general splicing element and is not required for regulated alternative splicing in muscle cultures. The pre-mRNA cis elements that serve as "targets" for factors mediating regulated splicing are located outside of the exon. Experiments are in progress to localize regulatory elements that are necessary and sufficient to confer regulation to a heterologous alternative exon. A model for regulated alternative splicing will be presented.

**W 211** CONSERVED AND SUBOPTIMAL SEQUENCES REGULATE SKIP SPLICING OF TRANSCRIPTS OF THE *DROSOPHILA MELANOGASTER* MYOSIN HEAVY-CHAIN GENE,
Richard M. Cripps, Dianne Hodges, Martin E. O'Connor, Jennifer A. Suggs & Sanford I. Bernstein, Department of Biology and Molecular Biology Institute, San Diego State University, San Diego, CA 92182.

The *Drosophila melanogaster* myosin heavy-chain gene, *Mhc*, encodes all of the muscle myosin heavy-chain protein in the fly. Different protein isoforms are generated by alternative RNA splicing. The 3' penultimate exon, exon 18, is either excluded from mature mRNA in larval muscles and some adult muscles, or is included in mature mRNA in most adult muscles. We have shown previously that the 3' and 5' splice sites of exon 18 are suboptimal and not recognized in an *in vitro* splicing system made from a *Drosophila* non-muscle cell line; however, exon 18 is included in fully processed mRNA if both splice sites are altered to consensus sequence. Flies transformed with *Mhc* minigenes expressing mutant versions of exons 17 through 19 recognize exon 18 in larvae only when both splice sites are mutated to consensus.

To define additional sequences which regulate this splicing choice, we have sequenced the 3' end of the *D. virilis Mhc* gene, where the transcripts appear to be processed in the same manner as those of *D. melanogaster*. Conserved non-coding sequences were deleted from the *Mhc* minigene, and splicing was assayed *in vivo* to determine if these regions are important for regulated splicing. Deletion of a large part of exon 18 did not affect splicing; however, deletion of a conserved polypyrimidine tract upstream of exon 18 abolished adult splicing but did not affect larval splicing. We hypothesize that this polypyrimidine tract may be the site of action of a *trans*-acting splicing factor that is present in adult muscles and promotes inclusion of exon 18 in *Mhc* mRNA, but absent or inactive in larval muscles. To test this model, we have generated additional constructs which change the sequence of the tract.

To obtain muscle cell nuclear extracts from *D. melanogaster* to isolate the putative *trans*-acting factor, we are attempting to generate immortalized muscle cell lines. We are expressing *c-myc* or SV40 large T antigen under the control of muscle-specific promoters. Flies expressing *c-myc* in adult indirect flight muscles are unable to fly, but the muscle cells still differentiate to produce relatively normal myofibrils. The nuclear morphology of these cells suggests they may be undergoing apoptosis, a phenomenon observed in vertebrate cells when *c-myc* is expressed in the absence of serum. We are attempting to isolate continuously growing muscle cell lines from pupal thoraces of the *c-myc* transformed flies.

**W 212 Position Dependent Repression and Activation by cis-elements with identical core sequence (GAAGCTTC) regulate cardiac tissue specificity.**
Manya Dhar, Eduardo Mascareno and M.A.Q. Siddiqui.Department of Anatomy and Cell Biology, State University of New York Health Science Center at Brooklyn. Brooklyn, NY 11203.

Tissue specific regulation of Myosin Light Chain 2 (MLC2) gene in cardiac muscle is achieved through the involvement of two proximal activators elements, a MEF-2 binding site (element B) and a CArG like sequence (element A), and two distal elements, an upstream negative element (CSS), (-340 to -310), which is able to repress transcription of cardiac MLC2 in skeletal muscle cell, and an activator/repressor sequence IRE, in the first intron, can relieve the repression induced by CSS in skeletal muscle . CSS is also able to exert repression on heterologous promoters, such as MCK (muscle creatine kinase) and ANG (angiotensinogen) promoters in skeletal muscle cell. Introduction of IRE in the same recombinant plasmid can override the CSS mediated repression of heterologous promoters in a position-dependent fashion. The negative CSS and positive IRE elements contain a common core nucleotide sequence, GAAGCTTC, which is the target site for both positive and negative regulatory proteins. In gel mobility shift assays IRE promotes a complex formation similar to that produced by CSS. Taken together, these data suggest that cardiac tissue-specificic expression of MLC2 gene is accomplished through the precise modulation of both positive and negative regulatory transcription factors. During repression, the complex formation induced by CSS may alter the basal transcription apparatus in such a way as to cause a decrease in the level of transcription. Whereas in activation, IRE squelches the CSS binding proteins, obliterating the repression and the basal transcription apparatus becomes fully active.

**W 213** INTERACTIONS BETWEEN THE TATA BOX REGION AND UPSTREAM PROMOTER MOTIFS IN THE TRANSCRIPTIONAL REGULATION OF THE IIB MyHC GENE,
Thierry Diagana, Daniel North, Carole Jabet, and Robert G. Whalen, Department of Molecular Biology, Institut Pasteur, 25 rue du Dr. Roux, 75724 Paris cedex 15, FRANCE.

Skeletal muscle is submitted to various regulatory mechanisms that control its expression. We have cloned the gene that codes for the adult mouse Myosin Heavy Chain IIB (MyHC IIB), which is specifically expressed in fast glycolytic fibers. Our long term goal is to understand the molecular processes that regulate the expression of this gene by the analysis of its 5' flanking region.

A -192 bp construct is sufficient to obtain transcriptional activity in quail myotube that have been stably transformed by a temperature-sensitive strain of the Rous sarcoma virus. However, in the C2 mouse myogenic cell line, this construct is only active after the cotransfection of any one of the four bHLH myogenic factors. This phenomenon is independent of the presence or integrity of a proximal E box. Mutant bHLH proteins, which are unable to convert 10T1/2 fibroblasts into myogenic cells, are unable to activate the -192 bp construct.We have identified two regions that are important for the activity of this promoter in both quails cells and cotransfected C2 cells. One of these region located between -140 bp and -190 bp contain two adjacents AT-rich motifs, both of which have putative MEF2 binding sites. In order to analyse more precisely the roles of these two motifs we have cloned each of them upstream of a minimal promoter construct which extends to -40 bp. We showed that the AT2 motif can highly enhance transcriptional activity in both C2 myoblasts and myotubes without cotransfection of myogenic factors. The AT1 motif, however still requires cotransfection of myogenic factors to be able to activate this minimal promoter.

The other region which is critical for this activity is the TATA box. Both of the AT-rich motifs require the specific TATA sequence found in MyHC IIB to exercise their enhancer function. If this TATA box is changed to that of either SV40, IgH, or albumin, these motifs are no longer able to act as enhancers.

**W 214** REGULATION OF ACTIN EXPRESSION DURING MUSCLE GROWTH OVER THE MOULT CYCLE IN LOBSTERS, Alicia J. El Haj and Paul Harrison, School of Biological sciences, University of Birmingham, Edgbaston, Birmingham, B15 2TT
Muscle growth in crustacea is an intermittent process centred around the moult when the old exoskeleton is shed and the animal expands in size. This intermittent growth makes these animals good models for investigating regulation mechanisms for muscle protein expression. Longitudinal growth of muscle fibres occurs by addition of new sarcomeres over the moult (1) coinciding with elevated total protein synthesis rates in the muscle (2). Recent studies have shown that actin proteins are synthesized during pre and postmoult period in the leg muscles. In order to assess the transitions in mRNA levels for actin over the moult cycle, a cDNA for lobster actin has been isolated (3). Total RNA from leg, claw and abdominal muscles have been extracted and hybridized for actin mRNA levels from 60 animals taken at two daily intervals throughout a 45 day moult cycle. In contrast to actin protein levels , actin mRNA levels remain constant within a narrow range throughout the moult cycle in all three muscles examined. These results indicate that actin protein synthesis in the lobster during the moult is a result of translational or posttranslational control.
Levels of ecdysteroids, which are involved in the regulation of the moult cycle in these animals, are elevated during the pre and postmoult period(4) coinciding with periods of muscle growth. To determine the role of ecdysteroids in regulating actin synthesis, in vitro intermoult leg muscle preparations were treated with ecdysteroids. Actin mRNA levels and protein synthesis did not increase in response to hormone treatment alone. However, in vivo injections of ecdysteroids in intermoult animals resulted in elevated protein synthesis rates indicating a possible role for an additional factor or factors.
During lobster muscle growth, actin transcription may not be controlled by ecdysteroids directly. Instead, ecdysteroids may be acting at a protein level and/or may involve additional intermediaries to control muscle growth over the moult.

1)El Haj et al (1984) J. Crust. Biol,4,536-545
2)El Haj and Houlihan (1987) J.Exp. Biol.,127,413-426
3)P.Harrison and A.J.El Haj(in press) Molecular Marine Biol. and Biotech.
4)El Haj et al (1992) In Molecular Biol of Muscle (Ed. A.J.El Haj), C.O.B.pp 151-165.

**W 215** IDENTIFICATION OF A NEW CIS-ACTING REGULATORY ELEMENT IN THE MCK ENHANCER. Christine Fabre-Suver and Stephen D. Hauschka, Department of Biochemistry, University of Washington, Seattle WA 98195.
Regulatory regions of the mouse muscle creatine kinase (MCK) gene have been well characterized. To date six cis-acting regulatory elements have been identified in the 5' enhancer region. They are, from 5' to 3', the CArG, AP2, A/T rich, Left, MEF1 and MEF2 sites. Analysis of sequence alignments of the human, mouse, rat and rabbit MCK enhancer regions, indicates that these sites are well-conserved portions of the DNA. However several other regions within the enhancer exhibit high similarity between species. These regions may represent additional control elements required for muscle specific expression of the MCK gene. To test this hypothesis, mutants of one conserved as well as several non-conserved regions, were examined in the context of (enh 206)80MCKCAT in which the enhancer is placed upstream of the (-80 to +7 bp) MCK basal promoter ligated to the CAT reporter gene. Each construct, has been tested by transient transfection assays in MM14 skeletal muscle cells. A 10 nucleotide mutation of the region between the AP2 and the A/T rich site, reduces the enhancer activity 10 fold. Part of this sequence is similar (7/8 nt) to the MCBF/TEF1 consensus DNA-binding site N($^{A/}{}_{G}$)CATNC($^{C/}{}_{T}$)($^{T/}{}_{A}$)NN identified in troponin T (Farrance et al. 1992, JBC 267: 17234). Further mutational analysis of this region as well as nuclear extract binding assays are in progress to better delineate this novel potential regulatory element.

**W 216** TRANSCRIPTIONAL REGULATION OF THE CREATINE KINASE GENE IN EMBRYONIC AND FETAL MUSCLE CELLS. Stefano Ferrari, Renata Battini, Susanna Molinari, *Giulio Cossu and §Robert Kelly. Istituto di Chimica Biologica, Università di Modena, 41100 Modena, Italy, *Istituto di Istologia ed Embriologia Generale, Università di Roma "La Sapienza", 00161 Roma, Italy, and § Département de Biologie Moleculaire, Institut Pasteur, 75724 Paris Cedex, France.
Embryonic myoblasts generate myotubes which express both in vivo and in vitro slow and fast myosin heavy chains (MyHC), but no muscle-specific isoforms of metabolic enzymes, such as muscle creatine kinase (MCK) or β-enolase. Conversely, fetal myoblasts generate myotubes which express fast, but not slow, MyHC and high levels of MCK and β-enolase. Primary cultures of mouse embryonic and fetal myoblasts were transfected with a series of plasmid constructs containing the promoter and variable lengths of the 5'-flanking region of the MCK gene (up to -3300) cloned 5' of the CAT reporter gene. While fetal muscle cells showed CAT activity with all the constructs that contained the MCK enhancer, embryonic cells did not show reporter activity with any of the transfected constructs. Co-transfection with a β-gal construct driven by the α-skeletal actin gene promoter demonstrated that both embryonic and fetal cells had undergone terminal muscle differentiation. These data suggest that a different set or ratio of transcription factors might be responsible for the differential expression of the MCK gene in embryonic and fetal muscle cells. Preliminary experiments suggest that protein/DNA interactions at the MCK enhancer region are not involved, since nuclear extracts prepared from embryonic, fetal and C2C12 cells show the same pattern of retarded bands in a band-shift assay.

**W 217** CHARACTERIZATION OF CARDIAC GENE cis-REGULATORY ELEMENTS IN THE VERY EARLY STAGES OF CHICK HEART MORPHOGENESIS *in vivo*, Steven A. Fisher, Kenneth Walsh and Cindy J. Forehand, Department of Physiology, Case Western Reserve University, Cleveland, OH 44106

We have developed a transfection method to study transriptional regulation during early stages of chick embryo cardiogenesis. Stage 10-17 embryo hearts are efficiently transfected by plasmid DNA-liposome complexes with the intact embryo in culture or within the entire egg. We show that promoter/luciferase reporter constructs containing -610, -658 and -2000 bp (relative to the transcription start site) of ANF, SRCaATPase and α-skeletal actin DNA promote transcription *in vivo* at these early stages. In addition, using a combination of deletion constructs,site-directed mutation and heterologous promoter we demonstrate the importance of the proximal CArG consensus sequence in enhancing transcription of the skeletal α-actin gene in the primitive heart tube *in vivo*. This technique should be valuable in dissecting the elements responsible for the expression of many cardiac genes during early cardiac morphogenesis *in vivo* as well as for regional specification in the developing heart.

**W 218** CIS ACTING ELEMENTS INVOLVED IN THE REGULATION OF THE CHOICE OF EXONS 6A AND 6B FOR THE THREE GENES $\alpha_F$ *TM*, $\alpha_S$ *TM* AND $\beta$ *TM* IN AVIAN MUSCLE AND NON MUSCLE CELLS M.Y. Fiszman, L. Balvay and A.-M. Pret, Department of Molecular Biology, Pasteur Institute, Paris, France.

The tropomyosin family consists of approximately 15-20 different isoforms which, in the avians, are encoded by 4 genes. Each of these genes encodes, at least, one isoform which is specific for one type of striated muscle, hence their nomenclature: $\alpha_F$ *TM*, $\alpha_S$ *TM*, $\alpha_C$ *TM* and $\beta$ *TM*.

The three genes, $\alpha_F$ *TM*, $\alpha_S$ *TM* and $\beta$ *TM*, contain a pair of internal exons 6 which are differentially used for the synthesis of the transcripts coding for the non muscle specific transcripts. Thus $\alpha_F$ TM non muscle specific transcripts contain either one of the two exons 6 while $\alpha_S$ TM non muscle specific transcripts contain essentially exon 6B and $\beta$ TM non muscle specific transcripts contain mostly exon 6A.

We have made minigene constructs containing chicken genomic DNA fragment spanning from exon 5 to exon 7 for the three genes and used them to transfect muscle and non muscle cells in order to define the cis acting elements which are involved in this differential expression. The results of these experiments will be presented and a general model for the use of these internal exons will be proposed.

**W 219** ALTERNATIVELY PROCESSED ISOFORMS OF CELLULAR NUCLEIC ACID BINDING PROTEIN DIFFERENTIALLY REGULATE THE HUMAN $\beta$-MYOSIN HEAVY CHAIN GENE, Irwin L. Flink, Joseph J. Bahl, and Eugene Morkin, Departments of Medicine, Pharmacology, and Physiology, and the University Heart Center, University of Arizona College of Medicine, Tucson, AZ 85724

Expression of the $\beta$-myosin heavy chain ($\beta$MHC) gene is an important phenotypic marker of differentiation in cardiac muscle. $\beta$MHC is the major myosin isoform expressed during early development and in the adult human ventricular myocardium. Changes in $\beta$MHC expression occur in rat models of cardiac hypertrophy and a missense mutation in this gene underlies some cases of familial hypertrophic cardiomyopathy. Analysis of a series of human $\beta$MHC gene constructs containing progressive deletions in the 5' flanking region has localized a repressor element (RE) at positions -332/-300, which is immediately upstream from a strong positive element (-298/-277). To identify binding factors for RE, a 49-bp restriction fragment containing RE was used to screen a cardiac expression library. A 0.6 kb cDNA was isolated which encodes a $Zn^{2+}$-finger polypeptide, Cellular Nucleic Acid Binding Protein (CNBP$\alpha$). This protein was initially identified in liver as a putative transcriptional regulator of the rate-limiting enzyme in cholesterol biosynthesis. An additional clone was identified (CNBP$\beta$) with an identical sequence except for a deletion of 21 nucleotides encoding 7 amino acids in the linker region between the first two fingers. When cotransfected with a $\beta$MHC reporter construct, CNBP$\alpha$ suppressed activity in a dose-dependent manner, whereas cotransfection of CNBP$\beta$ appeared to relieve repression. Thus, the $\beta$MHC gene may be differentially regulated by alternatively spliced products of CNBP.

**W 220** MYOD AND MYOGENIN ACT ON THE CHICKEN MYOSIN LIGHT CHAIN 1 GENE AS DISTINCT TRANSCRIPTIONAL FACTORS. Atsuko Fujisawa-Sehara, Atsushi Asakura, Tohru Komiya, Tomomi Sato, Takako Yagami-Hiromasa, Yoko Nabeshima, and Yo-ichi Nabeshima, Division of Molecular Genetics, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1 Ogawahigashi, Kodaira, Tokyo 187, Japan

Expression of MyoD, myogenin, MRF4 and Myf-5 convert non-muscle cells to muscle cells. In an attempt to analyze the roles of these factors, we have investigated their effects on transcription driven by the promoter of the chicken myosin alkaline light chain (MLC1) gene. The activation by CMD1 or c-myogenin (chicken MyoD or myogenin) was dependent on the existence of a muscle-specific regulatory region (MSR) located from -2096 to -1743. Its distal half containing a pair of E boxes (CANNTG) had been previously characterized as an enhancer responsive to CMD1 but not to c-myogenin. We report here the identification of another enhancer in MSR which is preferentially responsive to c-myogenin. Deletion and mutation analyses indicated that this enhancer requires a single E box and its flanking sequences. Furthermore, analysis of chimeric proteins of CMD1 and c-myogenin indicated that regions outside the basic-helix-loop-helix (bHLH) domain of c-myogenin are involved in the specificity of the enhancer. These results show that CMD1 and c-myogenin act on the MLC1 gene by recognizing different upstream DNA sequences and that direct or indirect interactions between the regions outside the bHLH domain and flanking sequences of E boxes are involved in the target sequence-specificity.

**W 221** COMPARISON OF MUSCLE CREATINE KINASE GENE REGULATION IN STABLE TRANSFECTIONS, TRANSIENT TRANSFECTIONS AND TRANSGENIC MICE, DeeAnn L. Gregory, Jean N. Buskin, Dorit B. Donoviel, and Stephen D. Hauschka, Department of Biochemistry SJ-70, University of Washington, Seattle, WA 98195

Muscle creatine kinase (MCK) gene regulation has previously been studied using regulatory regions of the gene attached to the CAT reporter gene in transient transfections of the mouse skeletal muscle MM14 cell line[1] and in transgenic mice[2]. We now report results of stable transfections in MM14 cells and compare the results with the other methods. In both stable and transient transfections, the upstream 206-bp enhancer (-1256 through -1050) fused to the -80 through +7 basal promoter confers expression similar to the entire -3300 to +7 region. In contrast, transgenic mouse skeletal muscle expression is several orders of magnitude lower in the minimal enhancer-promoter than in the larger construct. In all three assay systems, the -1256 to +7 region has similar activity to the -3300 to +7 region. A mutation in the MEF1 (right E-box) element in the -1256 to +7 MCK construct decreases expression 30-fold in transient transfections, but less than 2-fold in stable transfections. In transgenic mice there is no statistically significant difference between the mutant and wild type constructs, showing a greater resemblance to the stable transfectants than to the transient transfectants. In the minimal enhancer-promoter construct, the same MEF1 mutation reduces expression 100 or 20-fold, respectively, in transient and stable transfectants, yet has no statistically significant effect in transgenic mice. A mutation of the A/T-rich site within the enhancer in the -1256 to +7 MCK context led to 10-fold lower expression in both transient and stable transfections, and several orders of magnitude lower expression in transgenic animals. In conclusion, no two of these methods yield similar results for all constructs, and the differences observed cannot be explained entirely by either integration of the DNA or whole mouse physiological effects.

1. Amacher *et al.*, Mol. Cell. Biol. 13:2753-2764 (1993)
2. Donoviel *et al.*, submitted

**W 222** IDENTIFICATION OF MUSCLE SPECIFIC CIS-REGULATORY ELEMENTS FOR THE AdSS1 GENE ENCODING ADENYLOSUCCINATE SYNTHETASE, Oivin M. Guicherit, Amy L. Lewis and Rodney E. Kellems, Department of Biochemistry, Baylor College of Medicine, Houston, TX 77030
Adenylosuccinate synthetase (AdSS) is one of three enzymes that constitute a muscle specific metabolic pathway termed the purine nucleotide cycle (PNC). These three enzymes regulate the adenine nucleotide pool in muscle and therefore play a central role in regulating muscle energy metabolism. Genetic deficiencies associated with this pathway show phenotypes consistent with its postulated role in muscle energy metabolism. To assess the role of AdSS, and thus the PNC, during muscle development we have made use of transgenic mice to identify cis-sequences that control muscle specific gene expression during embryogenesis and in the adult. Using the full length muscle AdSS1 cDNA as a probe we identified a set of genomic clones spanning the AdSS1 gene. The transcription start site was determined with the aid of RNase protection and primer extension assays. Sequence analysis of the 5' flanking region revealed the presence of putative muscle specific cis-regulatory elements. To test the promoter and 5' flanking region we used either 1.5 kb or 6.5 kb of DNA from the 5' flanking region of the AdSS1 gene to drive the expression of a lacZ reporter gene. No ß-galactosidase expression was observed from three independent transgenic mouse lines carrying the 1.5AdSS1/lacZ fusion gene. In contrast, embryos carrying the 6.5AdSS1/lacZ transgene showed strong segmented expression in the somites across the entire flank from as early as day 8.5 of gestation. By 11.5 days of gestation patches of expression were also observed in the forelimb, the neck area and the facial area. All regions showing ß-galactosidase activity reflected structures and/or organs known to contain mesodermally derived tissue which can differentiate into muscle progenitors. This pattern of expression suggests that the 6.5 kb AdSS1 5' flanking region is sufficient to promote gene activation in muscle during embryogenesis. Current efforts are aimed at localizing the muscle specific elements as well as other regulatory elements associated with the AdSS1 gene.

**W 223** AN E-BOX/MCAT HYBRID MOTIF CONFERS MUSCLE-SPECIFIC AND cAMP-INDUCIBLE EXPRESSION OF THE RAT CARDIAC αMYOSIN HEAVY CHAIN (MHC) GENE, Mahesh P. Gupta, Madhu Gupta, and Radovan Zak, Departments of Medicine and Biochemistry & Molecular Biology, University of Chicago, Chicago, IL. 60637
Although several neuroendocrine factors are known to modulate the expression of MHC isoforms, very little is known about signaling mechanisms involved in determining cardiac muscle phenotype. Using primary cultures of fetal rat cardiac myocytes we have found that elevation of intracellular levels of cAMP induced αMHC mRNA expression. This effect of cAMP was dependent upon the presence of serum in the culture medium and it was found sensitive to cycloheximide. In transient transfection analysis of the αMHC gene promoter fused to CAT reporter gene we have determined that muscle specific transcriptional activation of the αMHC/CAT expression was mediated by an immediate upstream (-54 to -42 bps) cis-regulatory element of the αMHC gene which also conferred cAMP responsiveness of the promoter. This element (GGCACGTGGAATG) is related neither to CRE nor to AP-2 consensus sequences, but it appears to be an E box/MCAT hybrid motif. In mobility gel shift assay, protein DNA interaction could be effectively competed by oligonucleotides containing MCAT of chicken cardiac troponin T or GTII-c binding motif of $SV_{40}$ enhancer sequences but not by myoD binding element of the human cardiac actin gene. When oligonucleotides containing mutations in the MCAT region of troponin T MCAT binding site were used no competition was observed. We have also found by methylation interference and transfection analyses that besides MCAT sequences, center CG dinucleotides of the E box motif CACGTG were essential for protein binding to this element and for its functional activity. Furthermore, factor binding to these consensus sequences was found to be substrate for *in vitro* phosphorylation by cAMP-dependent protein kinase-A which favours its DNA-binding ability. These results indicate that transactivation of the αMHC gene expression by cAMP is mediated by an unique E box/MCAT hybrid motif which is recognized by one or more phosphoprotein(s).

**W 224** THE HUMAN TROPONIN I SLOW PROMOTER DIRECTS MUSCLE FIBER SPECIFIC EXPRESSION IN TRANSGENIC MICE Edna Hardeman[1], Linda Levitt[1], Lei Zhu[2], Josephine Joya[1], Robert Wade[2], Gary Lyons[3], [1]Children's Medical Research Foundation, Locked Bag 23, Wentworthville NSW 2145, Australia; [2]Department of Biological Chemistry and Program in Cell and Molecular Biology, University of Maryland School of Medicine, Baltimore, MD 21201; [3]Department of Anatomy, University of Wisconsin, Madison WI 53706.
Troponin I is a muscle-specific protein involved in the calcium mediated contraction of striated muscle. Three troponin I isoforms have been identified, each encoded by a separate gene and expressed in a fiber-type specific manner. Expression of the slow isoform is restricted to slow twitch skeletal muscle fibers and the conductive tissue in the heart of the adult. In order to delineate regions of the gene which are necessary for this restricted expression in the adult, we generated transgenic mice carrying a 4.2kb segment of the 5' flanking sequence plus 11bp of the 5'UTR from the human troponin I slow gene linked to the bacterial chloramphenicol acetlytransferase (CAT) gene. Four lines expressing the transgene were analysed by Northern blots, enzymatic, histochemical, and *in situ* hybridisation techniques. In all lines, CAT expression was restricted to those muscles known to contain slow twitch fibers. The relative levels of CAT transcripts in soleus, diaphragm, and gastrocnemius muscles approximated that of the endogenous mouse TnI slow transcript levels. In order to determine whether transgene expression was restricted to slow twitch fibers, we identified slow fibers in the soleus, diaphragm, and gastrocnemius muscles using an anitbody specific for β MHC. CAT activity in frozen sections was detected by the method of Donoghue et al.[1] CAT protein was detected only in those fibers containing β MHC protein. CAT transcripts and endogenous mouse TnI slow transcripts were examined in hearts from one line of mice by *in situ* hybridisation. CAT and TnI slow transcripts were detected in the atrioventricular node. Therefore, in this study we show that the 4.2kb segment of 5' flanking DNA plus 11 bp of the 5'UTR of the human TnI slow gene is sufficient to direct fiber-type and tissue-specific expression in the adult.

1. Donoghue, M.J. et al (1991) J. Cell Biol. 115:423-434.

**W 225** **CELL TYPE-SPECIFIC EXPRESSION FROM THE BOVINE *myf5* PROMOTER,** Robert Ivarie, Richard Shimkets and Robert A. Worrell, Genetics Department, The University of Georgia, Athens, GA 30602.
*Myf5* is a member of the *myoD* family of myogenic factors which also includes *mrf4/herculin* and *myogenin.* It is the first myogenic factor to appear during mammalian embryogenesis and analysis of the promoter may lead to the isolation of upstream factors. We have isolated a bovine genomic clone of *myf5* which contains 4.2 kb of 5' and 5 kb of 3' flanking sequences. Reporter gene fusions with various regions of the *bmyf* clone have been analyzed for promoter activity in primary embryonic myoblasts and fibroblasts from Japanese Quail. The 4.2 kb *bmyf* 5' flanking region, extending to a *PstI* site at +151 in the 5' untranslated leader, drove basal expression in proliferating myoblasts but not in fibroblasts. Unlike *myogenin,* it was not induced in differentiating myotubes. Deletion of the distal 5' regions up to position -131 increased promoter activity, indicating the presence of negative regulatory elements. These elements also suppressed expression from the *HSV tk* promoter as well as the *bmyf* proximal promoter (-131 to +151) but they were not cell type-specific. A 0.4 kb *XbaI* fragment, which did not have significant enhancer activity when fused to a promoter by itself, was required for activity of the proximal *bmyf* promoter in myoblasts when the negative elements were also present. Deletion the 5' untranslated leader and transcription start from the proximal promoter did not alter its activity in myoblasts or its cell type-secificity. This minimal promoter (-131 to -13) contains the TATA box and a canonical Sp1 site. Myoblast-specific expression from this promoter did not depend on either the negative elements or the 0.4 kb *XbaI* fragment described above. Gel-shift and footprinting experiments are in progress to determine the sites through which myoblast-specific expression is controlled.

**W 226** MYOGENIC FUNCTION OF MEF2 AND CROSS REGULATORY NETWORK WITH THE MYOGENIC bHLH FACTORS

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Both the myogenic bHLH factors and the MEF2 factors are essential activators of skeletal muscle gene regulation. Since many muscle specific enhancers and promoters contain and require adjacent MEF2 and Ebox binding sites for their gene expression (eg. MCK, MLC1/3, and myogenin), we asked whether the corresponding factors physically interact and functionally cooperate in transactivating muscle genes. Cotransfection experiments of different reporters along with the expression plasmids of MEF2 and Myogenin into CV1 cells demonstrated transcriptional cooperativity between these factors, which was both enhancer and spacing independent. We next demonstrated that these factors physically interacted in two different assays. First, using gel retardation assays, we showed that these proteins cooperate and form a tertiary complex when they bind DNA. Secondly, using co-immunoprecipitation assays, we showed that MEF2 and myogenin physically interacted without the presence of DNA. We next mapped the domains of each protein necessary for the interaction in vitro: the highly conserved MADS domain of MEF2 and the three amino acids in the basic and the junction domains of MyoD which have been implicated in the myogenic function of MyoD.

Since the myogenin promoter contains a MEF2 essential for its muscle specific expression and since MEF2 is induced at the critical stage of muscle differentiation, we asked whether MEF2 is sufficient to induce the myogenic program in non muscle cells. Stable cell lines expressing MEF2A in 10T1/2 cells induced the critical markers of myogenesis both at the RNA and protein levels: MyoD, Myogenin, MHC, and the alpha isoform of actin. Similar results were obtained in 3T3L1, 3T3F442A, and Swiss 3T3 cell lines. Using 10T1/2 cell lines permanently expressing MyoD, the converse experiment showed the induction of all four MEF2 proteins. These results demonstrate for the first time a non bHLH factor induces the skeletal muscle phenotype and suggests that at least two different but interactive pathways exist in generating and maintaining the muscle program.

**W 227** TRANSCRIPTIONAL REGULATION OF THE MOUSE MYOSIN LIGHT CHAIN 1F/3F LOCUS, Robert Kelly, Serge Alonso, Achim Schneider, Shahragim Tajbakhsh, and Margaret Buckingham, Institut Pasteur, 28 rue du Dr Roux, 75724 Paris Cedex 15, France.

Myogenesis involves complex spatial and temporal control of muscle-specific gene expression; in order to dissect regulatory mechanisms involved in this control we have analysed the sequence elements involved in transcription of the mouse MLC1F/3F gene. This gene encodes the two myosin alkali light chain isoforms of fast skeletal muscle fibres, MLC1F and MLC3F, transcribed from two promoters which are differentially activated during mouse embryogenesis - the MLC1F at day 9.5 and the MLC3F at day 13.5. In the rat gene an enhancer element downstream of the locus has been shown to confer muscle-specific expression on the MLC1F promoter in cultured cells and transgenic mice (Rosenthal *et al.* 1989, PNAS **86**:7780). The mouse enhancer is conserved with that of the rat both at structural and functional levels, and furthermore increases expression 30-fold from a MLC3F-reporter gene construct in C2/7 muscle cells. In transgenic mice a -2kb MLC3F promoter plus 3' enhancer drives reporter gene (lacZ) expression in the majority of skeletal muscle masses, although the transgene is activated at 9.5 days of development. We have identified a second regulatory element in the first intron of this gene which also confers a 30-fold increase on MLC3F expression; MLC1F is activated, but this effect is only 20% of that seen with the downstream enhancer. The combined action of both elements on the MLC3F promoter is 3-5 times stronger than that of either element alone; the intronic element has been delimited to an 800bp region by transient transfection studies. We are examining the relative roles of the intronic and 3' regulatory sequences *in vivo* by comparing the expression patterns of mice transgenic for reporter constructs with and without the intronic element.

**W 228** TRANSCRIPTIONAL REGULATION OF TWO MOUSE MUSCLE GENES

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Tissue and cell type specific transcription is determined by the interaction of trans-acting factors with several regulatory modules of a gene: (i) the TATA box and/or the initiator region, (ii) the proximal promoter region and (iii) enhancer(s). In order to dissect different levels of transcriptional regulation in vitro, we studied the promoter of the myosin light chain 1A (MLC1A) gene and the distal enhancer of the cardiac α-actin gene. Both examples involve new specific protein/protein interactions.

We demonstrate that the general transcription factor TFII-I, which facilitates the assembly of the basic transcription machinery, binds to the pyrimidin-rich initiator region of the MLC1A gene. This protein/DNA interaction is impeded by addition of myogenin or E12, respectively. Heterodimerization of myogenin and E12, however, restores TFII-I binding to the initiator region. Immunoprecipitation experiments suggest physical interactions between TFII-I (a 120 kDa protein containing several HLH-like motifs) and myogenin or E12, respectively.

The complex regulation of temporal and spatial gene expression often seems to involve one or several enhancers in addition to the proximal promoter. We have characterized an enhancer for the α-cardiac actin gene whose function depends on the presence of both, a specific E-box and a second element (1P) which lacks any obvious consensus sequence for known DNA binding proteins. Since cotransfection with myogenic factors from the bHLH family is not sufficient for transactivation, an additional factor binding to 1P might be crucial for enhancer function. We have evidence for cell type specific protein/DNA complexes on 1P involving an ubiquitous transcription factor.

**W 229** REPRESSOR-MEDIATED TRANSCRIPTIONAL INDUCTION OF THE HUMAN DMD GENE MUSCLE PROMOTER.

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The Duchenne muscular dystrophy (DMD) gene is expressed in skeletal and cardiac muscles and is induced as upon myoblast differentiation *in vitro*. We have previously shown that 150 bp upstream of muscle exon 1 of the human DMD gene regulates the expression of reporter gene constructs in a muscle-specific manner when transfected into primary human and mouse myoblasts and the rat H9C2(2-1) myogenic cell line (Klamut, H. et al. (1990) Mol. Cell Biol. 10:193). A CArG box motif within this region has been reported to be necessary for transcriptional activation (Gilgenkrantz, H. et al. (1992) J. Biol. Chem. 267: 10823), and our studies show that deletion of the single E-box motif results in a 50% decline in promoter activity. We have also identified two additional regulatory elements within this region. Deletion of a "negative regulatory domain" between -82 and -150 bp results in a 5-fold increase in reporter gene expression in H9C2(2-1) myotubes. Sequences responsible for high levels of reporter gene expression in the absence of the negative regulator are located within 17 bp of the ATA box. This "activation domain" is equally active in both pre- and post-differentiated H9C2(2-1) myoblasts, but is inactive in 3T3 fibroblasts. These results suggest that the repressor domain acts to down-regulate DMD gene expression prior to differentiation, and are substantiated by electrophoretic mobility shift assays in which changes in nuclear complex formation with myoblast differentiation are evident using both a full-length probe and oligonucleotide probes corresponding to the negative regulatory domain. No significant changes are observed using oligonucleotide probes spanning the E-box and proximal activation domains. These results are consistent with a repressor-mediated mechanism for transcriptional induction of DMD gene expression with myogenic differentiation.

**W 230** THE ROLE OF *CIS*-ACTING ELEMENTS IN THE DEVELOPMENTAL EXPRESSION OF THE β MYOSIN HEAVY CHAIN GENE *IN VIVO*,
Stephanie Knotts, Alejandro Sánchez, Hansjörg Rindt, and Jeffrey Robbins, Division of Molecular Cardiovascular Biology, TCHRF, Cincinnati, OH 45229.
The β myosin heavy chain (MHC) gene is regulated at the transcriptional level and is expressed both in skeletal and cardiac muscle. In mice, β MHC is expressed in the ventricle and skeletal muscles prior to birth, with expression becoming restricted to slow skeletal muscles after birth. Three *cis*-acting elements in the 5' upstream region are important for muscle specific expression of β MHC *in vitro*: an MCAT or TEF-1 binding site, a C-rich region, and a βe3 region, which binds an unidentified factor. To elucidate the role of these *cis*-acting sites and their corresponding *trans*-acting factors in directing the developmental expression of the β MHC gene in cardiac and skeletal muscle, an *in vivo* approach is required. We have developed a method for the immunocytochemical detection of the chloramphenicol acetlytransferase (cat) protein which allows visualization of reporter gene expression in transgenic mice during development. This method allows us to assess the importance of various *cis*-acting regions of the β MHC promoter at key developmental time points. We have previously characterized the β MHC promoter in transgenic mice using 5.6 kb of the 5' upstream region linked to the *cat* gene. Using PCR, we have mutated the MCAT binding site, the C-rich region, and the βe3 region in the context of this 5.6 kb-cat construct and generated transgenic mice. The expression pattern of the reporter gene in these transgenic animals is being analyzed before and after myoblast differentiation and compared to mice generated with the non-mutated 5.6 kb-cat construct.

**W 231** **REGULATORY REGIONS OF THE MUSCLE CREATINE KINASE GENE ASSOCIATED WITH COPY NUMBER-DEPENDENT EXPRESSION,**
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Exogenous genes stably transfected into cultured cells and transgenic animals typically yield expression levels unrelated to transgene copy number. Analysis of DNA flanking the MCK gene locus was undertaken to test for the presence of locus control regions which, in a limited number of transgenes, have been shown to confer copy number-dependent, integration site-independent expression.

MM14 myoblast subclones were selected for stable integration of various MCK gene constructs containing a unique 21 bp insert. Thirty subclones of -5500MCK, -3300MCK and fifteen of -800MCK were analyzed for transgene expression and copy number. All constructs contained the indicated amount of 5' flanking DNA, the entire transcribed region, and 2 kb of 3' flanking DNA. Transcript levels from both introduced and endogenous MCK were assayed via RNAse protection with a labelled RNA probe spanning the unique insert in the 7th exon and/or by RT-PCR analysis with primers spanning this insert. Introduced transcripts in -800MCK subclones were typically <0.01 of the endogenous level (10 of 15 subclones) with highest expression level at 0.03 of endogenous MCK. Subclones containing -3300MCK fell between 0.1 and 1X endogenous levels. Subclones of -5500MCK demonstrated highest expression levels (0.1X to 20X). Southern analysis performed to date among these various stable lines indicates the highest correlation of transgene copy number to transgene expression in the -5500MCK subclones ($r = .99$, $n = 8$) with expression per copy at 0.1X of the endogenous gene.

Two mouse lines carrying the -5500MCK transgene have been established. Both exhibit muscle-specific regulation of the transgene and expression above endogenous levels. These data are consistent with the hypothesis that MCK upstream regions confer copy number-dependent, integration site-independent expression. DNAse hypersensitivity analysis is underway to determine regions serving as potential locus regulatory elements.

**W 232** ROLE OF THE PROMOTER IN THE TRANSCRIPTIONAL REGULATION OF THE MURINE IIB MYOSIN HEAVY CHAIN GENE,
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The murine adult fast IIB myosin heavy chain (MyHC) gene is expressed in fast-contracting, glycolytic skeletal muscle fibers, and only appears at a late stage in development. Its expression can be precociously induced by administration of thyroid hormone. In order to understand the role of the promoter in this restricted expression, we have studied the binding of proteins to this region.

There are two E boxes in the first kb (at -940 bp and -65 bp), whose sequences conform well to the consensus sequences of MyoD and myogenin homodimers. Nuclear proteins from C2 cells produce a footprint around the proximal E box; overlapping and adjacent to it are potential binding sites for AP1, CTF, and thryoid hormone receptors. However, neither E box appears to be functionally important for the activity of the promoter, as measured by transient transfection assays, even though the E box at -940 bp binds myogenic bHLH factors well. This is striking because for the promoter constructions to be active in C2 myogenic cells, they must be co-transfected with an expression vector for one of the myogenic bHLH factors. The myogenic factors which activate the best are MRF4, and MyoD, both of whose expression is correlated with the expression of the IIB MyHC gene.

There are two A/T-rich sequences between -140 bp ("mAT1") and -190 bp ("mAT2"), which are conserved in several other MyHC genes. Both of these sites can bind MEF2 and an unidentified protein we call 2BB2. It appears that the binding of 2BB2 to mAT2 is stabilized by MEF2 bound to mAT1. Transfection studies show that MEF2 must be bound to mAT1, and that either MEF2 or 2BB2 must be bound to mAT2, for the promoter to be active. 2BB2, which appears to be a homeo- domain protein, is present at all stages of myogenic differentiation. There are several isoforms of MEF2, one of which (MEF2C) appears only late in myotube development, coincident with the expression of the endogenous IIB MyHC gene. In addition, between the two mAT sites there is a palindromic sequence which binds a protein found only in myo- tubes. At -100 bp there is a CArG box; this region binds two proteins, SRF and CTF/NF1, both of which are considered ubiquitous. The bind- ing of these proteins induces a bend in the DNA, which could be required for bringing together the mAT sites with the region around the TATA box, whose precise sequence is important for promoter activity.

**W 233** MYOCYTE-SPECIFIC ENHANCER-BINDING FACTOR 2 (MEF2) AND THYROID HORMONE RECEPTOR (TR) ASSOCIATE AND SYNERGISTICALLY ACTIVATE THE α-CARDIAC MYOSIN HEAVY CHAIN GENE.
Youngsook Lee, Bernardo Nadal-Ginard, and Vijak Mahdavi, Department of Cellular and Molecular Cardiology, Children's Hospital, Harvard Medical School, Boston, MA 02115

Expression of the cardiac α-myosin heavy chain (MHC) gene responds to developmental, hormonal, physiological, and pathological stimuli. Its tight tissue-specificity is controlled mainly by its 5' regulatory sequences, which include a $T_3$ receptor (TR) binding site (TRE) and two A/T-rich sequence motifs, which constitute putative target binding sites for the recently cloned MEF2 transactivators. MEF2 proteins are detected only in striated and smooth muscle and brain. Transient expression assays using reporter constructs bearing the 5' regulatory region with the basal cardiac α-MHC promoter linked to chloramphenicol acetyl transferase (CAT) were carried out in several muscle and non-muscle cells. The constructs containing the -368 regulatory region, or the proximal A/T-rich and the TRE motifs were expressed in primary cardiocytes and in Sol8 myotubes in the presence of $T_3$ but not in non-muscle cells. These constructs showed a 15-fold induction in non-muscle cells upon the cotransfection of MEF2 and TR in the presence of $T_3$, whereas neither factor by itself activated the α-MHC reporters. The reporter constructs containing the proximal A/T-rich and the TRE motifs linked to the heterologous herpes simplex virus (HSV) thymidine kinase (TK) promoter also showed synergistic transactivation when both MEF2 and TR were cotransfected in non muscle cells. Electrophoretic mobility shift assays confirmed the binding of MEF2 and TR to the proximal A/T-rich and TRE motifs, respectively.

The possibility of direct physical interaction between MEF2 and TR was tested using GST-fusion MEF2 and in vitro translated TR, or vice versa. Binding assays demonstrated that MEF2 and TR specifically binds to one another in vitro. The MADS domain of MEF2 and DNA-binding domain of TR are necessary and sufficient to confer the interaction with one another. These data suggest that synergistic activation of α-cardiac MHC gene expression requires the binding of MEF2 and TR to their respective cis-acting elements as well as direct interactions between these two factors.

**W 234** CONSTRUCTION OF AN INDUCIBLE SELECTION SYSTEM DEPENDING ON MUSCLE SPECIFIC SPLICING, Domenico Libri, Marc Y.Fiszman* and Michael Rosbash, Dep. of Biology, Brandeis University, Waltham, MA, 02254 *Institut Pasteur, Paris, France.

The gene coding for β tropomyosin in chicken contains two mutually exclusive exons, 6A and 6B, one of which (6B) is spliced only in skeletal muscle tissue (or differentiated myotubes in culture), while the other is spliced in all the other tissues. Skipping of exon 6B in most cells depends on the existence of a negative control: a secondary structure in the exon and sequence elements in the upstream intron are part of this control. In the aim of setting up a genetic screen to identify putative trans factors which activate splicing of the exon in muscle cells, we have been working to the construction of a selection system inducible by exon 6B splicing.

A genomic fragment containing exon 6B and the flanking introns was inserted in a selectable marker (β geo) carried by a vertebrate expression vector (kindly provided by P. Soriano). β geo codes for a fusion protein having both the functions of β galactosidase and $Neo^r$. Only the spliced product containing exon 6B will keep β geo in phase, while skipping of the exon will yield a non functional protein. As a positive control, two cis mutations activating splicing of the exon have been introduced in separate constructions. The functionality of the system was first assayed by monitoring β gal activity in transiently transfected cells, and secondly by the capability of the three constructions to yield stable transfectants being G418 resistant.

**W 235** AN RT-PCR ANALYSIS OF MYOGENIC DETERMINATION FACTOR EXPRESSION DURING CHICK SOMITE AND LIMB BUD DEVELOPMENT, Jennifer H. Lin-Jones, Howard M. Stern, and Stephen D. Hauschka, Departments of Biochemistry and Zoology, University of Washington, Seattle, WA 98195

Myogenic determination factor genes (MyoD, myogenin, myf 5, MRF 4) have the ability to activate the muscle program in non-muscle cells. However, the role of these genes during embryogenesis remains unclear. Previous studies in our lab have shown that muscle cell precursors are present in the embryonic chick limb bud at stages when in situ hybridization analysis indicates that no myogenic determination factor transcripts are present. We have used PCR primers designed to amplify the chicken homologs of the myogenic determination factor cDNAs to study the activation of myogenic determination factor expression in the forelimb buds, hindlimb buds and isolated preps of somites from chick embryos at various stages during development. The myogenic determination factor genes appear to be activated at different times in the limb buds. MyoD, myogenin, and MRF 4 are transcribed after stages in which myogenic precursors can already be found in the limb. The myf 5 expression pattern in the limb bud is currently being characterized. Data on skeletal and cardiac α-actin expression in the limbs as a marker for skeletal muscle differentiation will also be presented. Preliminary experiments with RT-PCR of isolated embryonic somites have been done to determine the somitic pattern of myogenic determination factor expression. MyoD transcripts have been detected as early as H and H stage 10, several stages earlier than in situ hybridization data has detected MyoD. Expression patterns of the other three myogenic determination factors in the developing somites are currently being examined.

**W 236** INTRAGENIC REGULATORY ELEMENTS MEDIATE THE LATE INDUCTION OF THE GLYCEROPHOSPHATE DEHYDROGENASE GENE DURING SKELETAL MYOGENESIS, Helena M. Madden, Steven N. Perrin, Claire M. Steppan, and Deborah E. Dobson, Department of Biochemistry, Boston University School of Medicine, Boston, MA 02118

Transcriptional activation of the adult glycerophosphate dehydrogenase (GPD) gene occurs quite late in the skeletal myogenesis program when compared to contractile genes such as the myosin light chain 1/3 (MLC1/3) locus. *In situ* hybridization of mouse embryo tissue sections indicates that GPD transcripts accumulate in a restricted set of limb muscle fibers beginning 17.5 days post coitum, which is 4-6 days later than the initial appearance of transcripts encoding the bHLH myogenic transcription factors and many muscle contractile proteins. This late induction of muscle GPD *in vivo* is recapitulated during the differentiation of mouse C2C12 cells *in vitro*. Transcripts for MLC1/3 and myosin heavy chain appear within 16 hours of the differentiation program while GPD mRNA is not detected until 36 hours after induction of differentiation. We therefore expect that factors controlling GPD expression are likely to include some important differences from other skeletal muscle genes such as MLC1/3.

Using a DNA transfection approach, we identified a GPD intragenic region that, in concert with the GPD promoter, controls the late differentiation-dependent activation of muscle GPD in C2C12 cells. This activation is independent of the activity of the MyoD1 family of muscle transcription factors. To localize GPD temporal regulatory elements, we constructed a series of chimeric CAT expression vectors with various combinations of the MLC1 promoter, MLC1/3 enhancer, GPD promoter, and GPD enhancer, introduced them into C2C12 cells, and monitored CAT activity as a function of time in the myogenic program. We find that GPD temporal regulatory elements reside in the intragenic enhancer, not the GPD promoter. Further delimitation of these temporal regulatory sequences is ongoing.

**W 237 REGULATION OF THE HUMAN SMOOTH MUSCLE α-ACTIN GENE EXPRESSION**

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The *in vitro* transient expression in several muscle cell lines showed that the human smooth muscle α-actin gene has at least two positive transcriptional regulation regions; one is an upstream region from the transcription initiation site and the other is the first intron region. These regions include three transcriptional DNA motifs; CArG box, A/TBF site and E box.

In *in vitro* studies, however, tissue-specific expression of the human smooth muscle α-actin gene was not clear, because these expression also observed in fibroblast cells. Next, we made several transgenic mice having these transcriptional regulatory regions following a CAT gene. In the transgenic mice having the upstream region, the CAT expressions were weak in all tissues we examined. Whereas, the transgenic mice having the both regions showed very strong CAT expressions in aorta, intestine, uterus and heart, but weak in liver, muscle etc. Therefore, not only the upstream region but also the first intron region was necessary for the smooth *muscle α-actin gene* expression in mouse tissues. So far, the most of the expression tissues were smooth muscles, but it is not clear that the expression is aorta-specific. Now we are investigating the CAT expressions in cellular level.

**W 238 A MODEL FOR ALTERNATIVE SPLICING: INTERPLAY BETWEEN NEGATIVE AND POSITIVE CONTROL ELEMENTS.** Takayuki Morisaki, Hiroko Morisaki and Edward W. Holmes. Department of Medicine and Genetics, University of Pennsylvania, Philadelphia, PA 19104.

Alternative splicing is a fundamental mechanism for expanding gene diversity - commonly used in myocytes. We have used AMPD1 as a model gene to identify cis-acting sequences that control alternative splicing. Results may be clinically relevant because of the potential rescue of AMPD deficiency through alternative splicing. Prior studies have established that Exon 2 is intrinsically defective and difficult to recognize. Transfection studies with mutant minigene constructs establish that the predominant factor leading to masking of Exon 2 is its length: if the exon is "expanded" from 12 to 24 nt, it is no longer subject to masking, suggesting steric hindrance between the 3' and 5' splice sites surrounding this exon. Prior studies have also suggested that recognition of Exon 2 is facilitated by sequences in the downstream intron. Transfection studies with mutant minigene constructs have identified a region in the middle of Intron 2 that markedly enhances recognition of Exon 2. Taken together the results suggest a mechanism that may be common for alternative splicing : a defective unit, in this case a very short exon, is paired with a facilitating unit, in this case an element in the downstream intron. This interplay between positive and negative elements, modulated by cellular trans-acting factors, may determine the ultimate splicing pattern that lead to alternative mRNA isoforms.

**W 239 Activation of mouse *myogenin* and MyoD gene transcription by 3,5,3'-Triiodothyronine: A direct role for the thyroid hormone and retinoid X receptors** *George Muscat, Lesley Mynett Johnson, Russell Griggs, Dennis Dowhan and Michael Downes.* University of Queensland, Centre for Molecular Biology, St Lucia , 4072, QLD., AUSTRALIA.

Thyroid hormones are major determinants of skeletal muscle differentiation and play a role in regulating contractile protein isoform expression. Triiodo-L-thyronine treatment of myogenic cell lines and adult rodents, promotes terminal muscle differentiation, results in increased *MyoD* gene transcription, and precocious fast twitch fiber development. Recently, it has been demonstrated that MyoD selectively accumulates in fast twitch fiber types and that thyroid hormone treatment results in the significant induction of MyoD and fast myosin heavy chain mRNAs in slow twitch fibers. We have identified $T_3$ response elements (TRE) in the mouse myogenin (1) and MyoD promoters. These sequences conferred appropriate $T_3$ responses to an enhancerless SV40 promoter. In vitro binding studies showed that the thyroid hormone receptor $\alpha$ (TR$\alpha$) formed a heterodimeric complex with either the retinoid X receptor $\alpha$ or $\gamma$ (RXR$\alpha$, RXR $\gamma$), on the myogenin and MyoD TREs that was specifically competed by other well characterised TREs and not by other response elements. Analyses of this heterodimer indicated that the complex was efficiently competed by direct repeats of the AGGTCA motif separated by 4 nucleotides as predicted by the 3-4-5 rule. Mutagenesis of the TREs indicated that the sequence of the direct repeats (AGGTCA) and the 4 nucleotide gap were necessary for efficient binding to the heterodimeric complex. In conclusion our data suggest that the TREs in the *myogenin* and *MyoD* genes are a target for the direct binding of TR$\alpha$ and RXR$\alpha/\gamma$. The present studies in the context of our past work, which demonstrated that the skeletal $\alpha$-actin promoter (2) was directly regulated by TR and RXR, firmly suggest that $T_3$ simultaneously targets the hierarchical *MyoD* gene family of regulators and the contractile protein genes directly in skeletal muscle. Fine tuned expression is probably achieved by the MyoD family and other intermediate regulators such as MEF-2, that are part of a positive autoregulatory loop that maintain the required threshold levels of these trans-regulators. In conclusion these results provide a molecular basis for the effects of $T_3$ on myogenesis and the activation of MyoD gene expression in slow twitch muscle fibres.

1. Downes et al.(1993), Cell Growth and Differentiation 4, *in press*
2. Muscat et al.(1993), Cell Growth and Differentiation 4, 269-279

**W 240 EXPRESSION OF THE QUAIL SLOW MYOSIN HEAVY CHAIN 3 GENE AND ESTABLISHMENT OF THE SLOW SKELETAL MUSCLE CELL LINEAGE**
William Nikovits, Jr., Gangfeng Wang and Frank E. Stockdale Stanford University, Dept. of Medicine, Stanford CA. 94305-5306

Using a monoclonal antibody specific for slow myosin heavy chain (MyHC) isoforms, we have isolated and sequenced in their entirety overlapping cDNA and genomic DNA clones encoding the complete quail slow MyHC 3 isoform, the first among the three known avian slow MyHC isoforms to be sequenced. Slow MyHC 3 shows greatest sequence homology with mammalian $\alpha$-cardiac and $\beta$-cardiac/slow MyHC isoforms. Muscle-specific sequence motifs, M-CAT elements and an A/T-rich region similar to a MEF-2 binding site, are being analyzed in transient transfection studies. The highest level of slow MyHC 3 expression is observed in the embryo in the atria. In addition, slow MyHC 3 is transiently expressed in developing limb muscle at the earliest stages of development, during the time when distinctions between the fast and fast/slow muscle fibers first become apparent. Its expression during embryonic development makes it a useful reagent to study the mechanism whereby the slow muscle cell lineage is established. Slow avian skeletal muscle fibers arise in vivo from a discreet myoblast population. Because the slow MyHC 3 gene is transiently expressed in the developing limb in fibers formed from embryonic myoblasts its expression may be a marker of commitment to the slow muscle cell lineage. Factors which regulate the expression of slow MyHC 3 must be present prior to transcription of the gene, and by identifying the factors which restrict expression of the slow MyHC 3 gene to fibers of the fast/slow lineage, one may identify differences in the myoblast precursors. We have begun analyzing the cis-elements required for expression of the slow MyHC 3 gene in slow skeletal and atrial muscle. By studying the regulation of the slow MyHC 3 gene, we may develop an understanding of the mechanism by which different muscle cell lineages are established.

**W 241 *SNO* HAS TRANSCRIPTION FACTOR ACTIVITY, TRANSACTIVATING THE MYOSIN LIGHT CHAIN1 MUSCLE-SPECIFIC ENHANCER** Sonia H. Pearson-White and Kristen Jessen, Departments of Pediatrics and Microbiology, MR4 Bldg Box 3002, University of Virginia Medical Center, Charlottesville, VA 22908.

The *ski* family genes, *ski* and *sno*, encode nuclear proteins that are not members of any of the major transcription factor families. *Ski* and *sno* appear to function in muscle development: (1) Overexpression of either chicken *ski* or *sno* can convert quail embryo cells to the myogenic (muscle) pathway (Stavnezer et. al.), and (2) Hughes and colleagues have shown that transgenic mice over-expressing *ski* in their skeletal muscles exhibit hypertrophy of certain types of fast myofibers. Mutant *ski* fails to convert quail embryo cells to muscle but retains its ability to activate MyoD and myogenin, suggesting that overexpression of mutant *ski* can interfere with myogenesis.

In contrast to the MyoD family of genes, which are expressed muscle-specifically, *ski* and *sno* genes are expressed in all human and murine tissues examined, including muscle. Recent transfection experiments in our laboratory suggest that *snoN* can transactivate the muscle-specific MLC1CAT plasmid containing the MLC1 promoter and MLC downstream enhancer (N. Rosenthal), about five-fold in C2C12 myofibers. *SnoN* can also dramatically increase transactivation by MyoD; co-transfection of *snoN* with a ß-actin-MyoD expression plasmid increased levels of CAT activity 24-fold over that obtained with MyoD alone.

We have isolated a novel alternatively spliced cDNA for *sno*, *snoI* (for insertion), that accumulates specifically in skeletal muscle in humans. *SnoI* is an insertion of 1330 nucleotides in place of 7 nucleotides of *snoN*, but would predict the translation of a truncated protein, 399 amino acid residues instead of the 684 residues in *snoN*. It is thus effectively a deletion mutant of *snoN*. This alternative exon is absent from rodents. We have not so far detected any transactivation activity when *snoI* is co-transfected with the MLC1CAT reporter, suggesting that this activity resides in the C-terminal 285 residue portion of *snoN* that is missing from *snoI*. However, when *snoI* is co-transfected with MyoD, a 5-10-fold increase in CAT activity is seen. Thus, the N-terminal 399 amino acid residues of *snoN* that are also contained in *snoI* and highly conserved with *ski* contain the MyoD-interacting activity. These studies suggest that *sno* interacts with MyoD to form a transcription factor complex involved in muscle regulation.

**W 242** A COMPLEX DISTAL ENHANCER ACTIVATES TRANSCRIPTION OF QmyoD, THE EARLIEST MyoD FAMILY MEMBER EXPRESSED IN QUAIL SOMITES.

Deborah F. Pinney, Fabienne Charles de la Brousse, Alexander Faerman, Moshe Shani, Kimiaki Maruyama, Charles P. Emerson, Jr., Fox Chase Cancer Center, Philadelphia, PA and Volcani Center, Bet Dagan, Israel.

Recent studies have begun to elucidate the molecular basis of skeletal myogenic determination and differentiation. A tissue-specific set of regulatory genes, the myoD family, has been identified and their function established as transcriptional activators of differentiation-specific contractile protein genes. *In situ* hybridizations of early quail embryos have shown that quail myoD (QmyoD) is expressed first in the somites, followed by Qmyf5 and the Qmyogenin. It is particularly important to understand the developmentally regulated expression of QmyoD because of its premier expression and apparent auto-activation and cross-activation functions.Transient DNA transfections of primary quail myoblasts and fibroblasts with CAT or Lac Z reporter constructs have identified a multi-element enhancer complex 12 kb 5′ to the transcription initiation site. Part of the enhancer complex has both tissue specificity and response to autoactivation by constitutively expressed QmyoD. Sequence and Southern blot analyses show a high degree of DNA sequence conservation among avians but not with mammals. We have shown that the enhancer complex controls expression appropriately in transgenic mice suggesting conservation even with mammals of the regulatory factors that interact with the enhancer elements. Thus, our analysis has identified regulatory elements important for the appropriate developmental expression of QmyoD and will allow further investigation of upstream genes in the myogenic regulatory pathway.

**W 243** **TISSUE SPECIFIC EXPRESSION OF MURINE SMOOTH MUSCLE γ-ACTIN FUSION GENES IN TRANSGENIC MICE AND IN CULTURED CELLS,**

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Smooth muscle gamma-actin (SMGA) is the principal element of thin filaments found within the gastrointestinal system and urogenital tract. The expression of this gene is restricted to smooth muscle cells making it a hallmark of smooth muscle differentiation. We are carrying out an analysis to define the *cis*-acting elements that are involved in the regulation of the SMGA gene. This is being done using hybrid genes that contain various segments of the murine SMGA gene linked to the *CAT* gene as reporter. A hybrid gene containing about 14 kilobases (kb) of the enteric actin gene has been used to generate six transgenic lines containing up to 30 copies. This construct contains 4.9 kb of upstream sequence, exon 1, intron 1, and a portion of exon 2 up to the start codon for translation. Both *CAT* assays of tissue homogenates and immunohistochemical staining for this enzyme indicate that expression is restricted to smooth muscle cells. Moreover, preliminary results suggest that the expression of the reporter in the transgenic mice shows the same developmental timing and cell-specific pattern as the endogenous SMGA. We have also assessed the expression of this hybrid gene in three continuous cell lines: DDT1 MF2, a smooth muscle tumor cell line derived from hamster; $C_2C_{12}$, a mouse skeletal muscle myoblast line; and RPMI1846, a malignant melanoma cell line from hamster, as well as primary rat intestinal smooth muscle cell cultures. Preliminary deletion analyses suggest that the regulatory elements capable of directing expression of the SMGA gene in a tissue specific manner are primarily located in the region -571 to +30. However, there is evidence for additional elements both upstream and in intron 1 that contribute to the temporal pattern of expression during development and affect tissue specificity.

**W 244** ACTIVATION OF THE MYOGENIN GENE: A DIRECT RESPONSE TO MYF-5 ACTIVITY IN PERMISSIVE NON-MUSCLE CELLS,

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Myf-5, MyoD, Myogenin, and MRF-4 are muscle specific transcription factors. Overexpression of a single factor converts various non-muscle cells to myoblasts capable of differentiating into myotubes. Under these conditions auto- and crossactivation of members of the myogenic factor gene family can be observed which leads to difficulties in the assessment of the primary gene targets for each individual factor and its contribution to myogenesis. The temporal pattern of Myf gene expression has revealed a sequential order suggesting a possible functional hierarchy during mouse development.

In order to identify direct targets for Myf-5 activity, we expressed hybrid proteins consisting of Myf-5 and the hormone binding domain of the estradiol receptor in mouse fibroblasts. This approach permits to induce Myf-5 activity by the addition of ß-estradiol to the culture medium. The specific function and regulation of these Myf-5 fusion proteins was demonstrated by transactivation of muscle-specific reporters.

By using RT-PCR, myogenin was found to be one of the early genes expressed upon Myf-5 activation in C3H 10T1/2 fibroblasts. Detectable levels of myogenin mRNA accumulated within 3 hours following the addition of hormone. This activation was not inhibited by cycloheximide or emetine suggesting that Myf-5 leads to myogenin expression without de novo protein synthesis. These results indicate that other transcription factors such as Mef-2 which is required for myogenic gene activation are already present in C3H 10T1/2 cells.

In contrast, no myogenin activation by Myf-5 was observed in 3T3 fibroblasts. The nature of the difference between various cell lines is currently under investigation.

**W 245** MUTATIONAL ANALYSIS OF THE MURINE α-MYOSIN HEAVY CHAIN GENE *IN VIVO*,

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The expression of the myosin heavy chain (MHC) genes is regulated primarily on the transcriptional level. The modulation of MHC gene expression depends on the interaction of *cis*-acting elements with *trans*-acting transcription factors. We have investigated the role of three of these elements, two thyroid hormone response elements (TREs) and a CArG element, in the promoter of the α-MHC gene using transgenic mice. The TREs have been implicated in mediating the upregulation of the α-MHC gene in the ventricles, which coincides with a surge of circulating thyroid hormone levels at the time of birth. In the atria, α-MHC is expressed constitutively throughout development. Another element, the so-called CArG box, has been shown to play an essential role in the expression of actin genes. This element is also present in the α-MHC gene's upstream region. We have analyzed the effects of directed mutations of these elements *in vivo* using chloramphenicol acetyltransferase reporter gene (*cat*) constructs in transgenic mice. The mutations have been made in the context of the α-5.5 promoter construct, which has previously been shown to confer high levels of tissue-specific expression. The mutation of TRE1 at position -149 with respect to transcriptional start site had little or no effect on transgene expression, while the mutation of TRE2 at position -120 reduced *cat*-activity to about 10% of the wild-type levels. However, the simultaneous mutation of both TREs led to a significantly greater reduction of transgene expression than the mutation of each of the TREs individually. These results indicate that interactions mediated through TRE1 can sustain a lower level of expression in the absence of TRE2. The replacement of the CArG element with a non-related sequence did not result in a detectable change of the transgene's expression level or tissue profile as compared to the wild-type construct, indicating that this element does not play a crucial role in the transcriptional regulation of the α-MHC gene.

**W 246** RECONSTITUTION OF THE BIOLOGICAL SIGNAL OF BASIC-FGF IN L6E9 MYOBLASTS. Dina Ron, Ronith Reich-Slotky and Ester Shaoul, Department of Biology, Technion-Israel Institute of Technology, Haifa 32000, Israel.

Basic-FGF (bFGF) is a potent mitogen of skeletal muscle derived cell lines and a potent inhibitor of their differentiation. Basic-FGF delivers its biological signal via cell surface tyrosine-kinase receptors. We cloned two closely related but distinct receptors for bFGF, designated FGFR1 and FGFR4, and studied their ligand binding properties. One of the problems in studying signal transduction by FGF is the lack of appropriate cell lines because most known cell lines express more than one type of FGFR. To overcome this problem we used the L6E9 rat myoblast cell line to overexpress FGFR1 and FGFR4. L6E9 cells are devoid of any high affinity receptors for FGFs and do not respond mitogenically to bFGF and other members of the FGF family. Following transfection, L6E9 cells acquired the ability to bind bFGF and aFGF as shown by binding saturation assay and cross-linking experiments. Moreover, both FGFR1 and FGFR4 expressing cell lines could respond mitogenically to bFGF and aFGF. Cells expressing FGFR1 could grow in serum free media only in the presence of bFGF or aFGF. Thus, we were able to reconstitute the signal transduction pathway for FGFs in myoblasts. The effect of the expressed receptors on the differentiation of the transfected myoblast cells will be discussed in more details.

**W 247** **FAST MUSCLE-SPECIFIC AND POSITION INDEPENDENT EXPRESSION OF HUMAN ALDOLASE A TRANSGENES,** Salminen, M., Maire, P., Concordet, J.P., Kahn, A. and Daegelen, D., ICGM, U 129 INSERM, 24, rue du Fg St Jacques, 75014 PARIS, FRANCE.

Regulation of the human aldolase A gene is especially complex with three alternative promoters localized in a small 1.6 kb region, giving rise to three types of mRNAs, which differ in their 5' non-coding ends, while the coding region is the same. In transgenic mice, the two ubiquitously active promoters, pN and pH, are both already transcribed in fetal tissues and, in adult, the highest levels of N- and H-type mRNAs are observed in heart and skeletal muscle. A third promoter, pM, which lies in between the two ubiquitous promoters, is turned on at birth and becomes strongly active in mouse muscles from the 15th day, reaching a maximal level of expression in mature adult muscle. This M-type mRNA accumulates in muscles composed mainly of fast-twitch glycolytic fibers.

We have used aldolase A M-promoter as a model to enable the identification of factors responsible for a fast muscle-specific expression. With a deletion analysis we have delimited the pM control region to a 200 bp DNA-sequence characterized by a muscle-specific DNAse I hypersensitivity.

For most genes, expression in transgenic mice is influenced by surrounding chromatin at the site of integration. As a result, transgenes are not always expressed in a copy-related manner and it is usual to find ectopic activity of promoters. We have studied over 30 lines of transgenic mice, and in all of them pM was functional in a roughly copy-dependent way. Elements that confer a position-independent expression on the aldolase A transgenes seem to colocalize with the same 200 bp region which is needed for tissue-specific expression of pM.

**W 248** THE E BOX IS ESSENTIAL FOR EXPRESSION OF THE CARDIAC ACTIN PROMOTER IN SKELETAL BUT NOT IN CARDIAC MUSCLE, Ilona S. Skerjanc and Michael W. McBurney, Cancer Research Group, Department of Medicine, University of Ottawa, Ottawa, Ontario, Canada, K1H-8M5

The P19 embryonal carcinoma cell line is capable of differentiating into both cardiac and skeletal muscle. Skeletal muscle differentiation is known to involve the myogenic family of basic helix-loop-helix (bHLH) proteins, such as myoD. MyoD binds to the DNA sequence CANNTG, called the E box, and can transform various cell lines into skeletal muscle. Many muscle proteins, including cardiac $\alpha$-actin, are expressed in both embryonic cardiac and skeletal muscle. However, no members of the myogenic family of bHLH proteins have been found in cardiac muscle. If a "cardiac myoD" exists, the E box should be essential for cardiac expression of the cardiac $\alpha$-actin promoter. We found that mutating the E box of this promoter had no effect on promoter activity in cardiac muscle but ablated promoter activity in skeletal muscle. Thus the E box is not necessary for the cardiac $\alpha$-actin promoter activity in P19 derived cardiac muscle but is essential for activity in skeletal muscle. Furthermore, myoD expressed in P19 cells induced skeletal muscle at the expense of cardiac muscle during P19 cell differentiation induced with dimethysulfoxide. These results suggest that the two muscle pathways are independent and mutually exclusive.

**W 249** REGULATION OF *MYOD* PROMOTER/ENHANCER BY HOMEOBOX-CONTAINING GENE *MSX1* (*HOX-7.1*)

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The pattern of *Msx1* (formerly *Hox-7.1*) expression during embryonic development suggests that it may play a crucial role during cellular proliferation and differentiation. Previous work has demonstrated that forced-expression of *Msx1* in F3 myoblasts can either block or delay terminal differentiation [1]. The steady-state levels of *MyoD* transcripts are also down-regulated in *Msx1* expressing cells that fail to differentiate. We have now addressed whether or not the regulatory effects of *Msx1* upon *MyoD* are at the transcriptional level. Forced-expression of *MyoD* in *Msx1* expressing cell lines results in robust myotube formation, therefore restoring the differentiation potentials of these previously non-differentiating cells. These results imply that down regulation of *MyoD* is necessary for the inhibition of terminal differentiation by *Msx1*. We further analyzed the effects of *Msx1* upon the cis regulatory elements of the *MyoD* gene [2]. The activity of the *MyoD* enhancer/promoter is significantly lower in *Msx1* expressing cells as compared to that in controls. A stronger inhibitory effect is observed when *Msx1* is co-transfected with the *MyoD* enhancer/promoter construct. These observations indicate that *Msx1* can suppress *MyoD* expression and inhibit terminal differentiation by acting on *MyoD* cis regulatory elements. We are currently addressing whether the effects of *Msx1* upon *MyoD* regulatory elements are direct or indirect by mobility shift assays.

1 Song, K., Wang, Y. and Sassoon, D.A. (1992) Nature 360:477-481
2 David J. Goldhamer, Alexander Faerman, Moshe Shani, and Charles P. Emerson, Jr. (1992) Science 256: 538-542

**W 250 INTERPLAY BETWEEN A NEGATIVE ELEMENT AND A CArG BOX IN THE HUMAN DYSTROPHIN MUSCULAR PROMOTER,**

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The muscle-specific promoter of the dystrophin gene is active in skeletal, cardiac, and smooth muscles and is specifically stimulated during ex vivo myogenic differentiation.
The study of this promoter revealed a striking conservation between rodents and human of the 140 bp upstream from the cap site (93% of homology). This proximal region confers a muscle preferential expression. It contains both an inhibiting region, spanning from nucleotide position -140 to -96 which is active in all cell types, and an activating region containing a CArG box which accounts for the muscle preferential expression.
By transient transfection in several cell lines, we have defined a 25 bp element responsible for the inhibitory effect. Gel retardation experiments show that this short sequence specifically binds different nuclear factors depending on whether the nuclear extracts used are from myogenic or non-myogenic cell lines.
The functional interactions between the negative element and the contiguous CArG box, as well as the cognate binding proteins, are currently under investigation.

**W 251 Id mRNA EXPRESSION DURING XENOPUS EMBRYONIC DEVELOPMENT,** Georges Spohr, Hong Zhang and Sorogini Reynaud, Cell Biology Department, Sciences III 30, Quai E. Ansermet, 1211 Genève 4, Switzerland

Transcriptional activation by bHLH proteins can be negatively modulated by HLH proteins of the Id family. These lack the basic DNA binding element. To characterize Id mRNAs expressed in *Xenopus* embryos, a cDNA library prepared from neurulas was screend using a probe derived from human Id-I. Two types of cDNAs were isolated. They are identical in the 5'-untranslated and in the coding region but differ in the 3' untranslated part. Comparison with the corresponding genomic sequence suggests the existence of an optional splicing site in the 3'-untranslated part. Northern blot analysis shows that Id mRNA starts to be transcribed during gastrulation. Transcription decreases after tailbud stage but remains detectable in adult tissues such as liver and heart. Microinjection experiments carried out in oocytes reveal that the protein encoded by the isolated *Xenopus* Id mRNA inhibits transcriptional activation by MyoD of a CAT reporter gene presenting four MCK E-boxes. Whether this acticity plays also a role *in vivo* during *Xenopus* myogenesis by inhibiting transcriptional activation by bHLH myogenic factors is presently investigated.

**W 252 ANALYSIS OF MUSCLE-TYPE SPECIFIC SPLICING OF THE Mhc PRE-mRNA IN DROSOPHILA**

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All muscle myosin heavy chain isoforms are derived from the alternative processing of the single Mhc pre-mRNA transcript in Drosophila. In the thorax, the utilization of the different alternative exons has been shown to be tightly regulated (Hastings and Emerson, JCB 114:263). One such alternative exon, exon 11e, is found utilized only in the indirect flight muscle (IFM), which is the major flight muscle in the fly. We have begun an analysis of sequence elements that appear likely to function *in cis* to regulate this splice choice specificity. Two elements, based on their high degree of conservation between D. melanogaster and D. virilis are of initial interest. One element is the 5'splice donor of exon 11e, which is conserved, non-consensus and unique among other alternative exon 11s, and the second is a 100 nt conserved domain located within the intron 5' to constitutive exon 12. We have assembled a panel of Mhc mini-gene constructs to explore the function of these elements in maintaining splice choice specificity in transgenic flies. A minigene that contains the wild-type exon 11 domain in conjunction with the β-galactosidase reporter gene has been shown to be regulated appropriately in transgenic flies, thus indicating that all splice regulatory elements are within this construct. Preliminary data from transgenic flies carrying mutant mini-gene constructs suggest that both the 5'donor and the conserved domain (putative intronic control region) are important for maintaining splice choice specificity.

**W 253 TRACKING *MYF*-5 EXPRESSION AFTER HOMOLOGOUS RECOMBINATION WITH LACZ,** Shahragim Tajbakhsh, Didier Rocancourt, Giulio Cossu and Margaret Buckingham. Department of Molecular Biology, Pasteur Institute, 25 rue du Dr. Roux, 75724 Paris Cedex 15, France.

*Myf*-5 is one of the four myogenic factor genes present in mice and is the earliest to be expressed before skeletal muscle formation in the embryo. We have mutated the endogenous mouse *myf*-5 gene in embryonic stem cells (ES) and introduced the LacZ reporter gene in phase with *myf*-5 coding sequences. ES cells carrying the myf-5 mutation were injected into mouse blastocysts and precompaction morula to obtain chimeric mice. By using ES cells in chimaeric embryos containing the targeted or randomly integrated *myf*-5/nlsLacZ construct, we demonstrate that 5.5 kbp of *myf*-5 upstream flanking sequence directs some skeletal muscle expression, but this is neither qualitatively nor quantitatively equivalent to that of the endogenous gene. Using the targeted cells, we show that all skeletal muscles express ß-galactosidase, even after birth, indicating that *myf*-5 expression, although downregulated, continues to be detected. This expression is not confined to primary myotubes which are derived from embryonic myoblasts, but is also detected in muscles containing different adult fibre types. *Myf*-5 is initially expressed in the medial half of the somite, before muscle formation. Its subsequent localization in the somite as the myotome develops is consistent with observations on myotome formation in birds. However, the details of this process are less well understood. We discuss two models for myotome formation in the context of our findings. In other studies, we have used a cultured limb bud assay to show that myogenic precursors that have migrated from the somite arrive in the early limb bud and are *myf*-5 negative. This result suggests that *myf*-5, and the remaining myogenic factors, do not play a role in muscle cell determination. Although *myf*-5 expression is confined to skeletal muscle, we report that this myogenic factor is also expressed in the CNS, both in regions of the mesencephalon and diencephalon, and the neural tube.

(These studies were carried out in collaboration with Charles Babinet for the initial blastocyst injections and E. Bober and H. Arnold who provided the mouse genomic fragments)

**W 254** TISSUE-SPECIFIC EXPRESSION OF THE MYOSIN HEAVY CHAIN GENE IN *DROSOPHILA MELANOGASTER*.

Kien Trinh, Phillip A. Singer, Norbert Hess and Sanford I. Bernstein, San Diego State University, Department of Biology and Molecular Biology Institute, San Diego, CA 92182.

In *Drosophila melanogaster*, a single muscle myosin heavy chain (*Mhc*) gene is expressed in all larval and adult muscles. We have used P element-mediated germ line transformation of *lacZ* fusion reporter constructs to determine sequences required for tissue-specific transcription of this gene. The region of the *Mhc* gene from -107 to +2095, including the transcription start site to exon 2, when fused to *lacZ* exhibits muscle specific ß-galactosidase expression. Deletion of a 1.5 kb region within intron 1 from this construct, shows reduced levels of ß-galactosidase expression in the indirect flight muscle, in the small cells of the jump muscle and in larval muscle. Overlapping regions within the -107 to +2095 fragment were analyzed for enhancer activities under a heterologous *hsp70-lacZ* promoter construct. Larval and adult indirect flight muscle-specific expression of ß-galactosidase is observed with a 1.5 kb fragment from +196 to +1667 of intron 1. We also performed DNase I footprinting experiments using nuclear extracts from *Drosophila* embryos to identify trans-acting factor targets on the *Mhc* DNA sequence. Portion of an AT rich sequence from +1350 to +1500 of intron 1 are specifically protected by nuclear factors from embryos after the initiation of muscle differentiation. This sequence has some homology to a vertebrate muscle-specific transcription factor (MEF-2) binding site. *In vivo* experiments are in progress to determine the mininum sequence within intron 1 which is required for muscle specific expression of the *Mhc* gene.

**W 255 CHARACTERISATION OF A SOX GENE EXPRESSED IN MUSCLE DEVELOPMENT.**

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The Sox (SRY-box) motif is a class of DNA binding domain related to the high mobility group (HMG) found in the testis determining factor (SRY) and a growing number of other genes with a variety of functions. We have isolated a number of novel genes containing this HMG-box DNA binding motif from embryonic chick cDNA libraries. One of these genes (cSox9) is expressed in the somites of embryos and adult skeletal muscle. It is also expressed at high levels in the notochord. Preliminary analysis indicates that cSox9 is differentially spliced and is present as a 3.4kb transcript in embryonic muscle and a 1.3kb transcript in adult muscle. We are currently in the process of characterising these transcripts further. Whole mount *in situ* hybridisation analysis of stage 20 chick embryos revealed that cSox9 is predominantly expressed in the notochord and somites. The embryonic and adult expression patterns of cSox9 suggest a role in muscle development.

**W 256 MULTIPLE REGULATORY ELEMENTS MEDIATE SLOW SKELETAL MUSCLE TROPONIN I GENE EXPRESSION DURING *IN VIVO* AND *IN VITRO* MUSCLE DEVELOPMENT**

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Three isoforms of troponin I (TnI) have been identified: two skeletal muscle isoforms, $TnI_f$ and $TnI_S$, which are expressed exclusively in fast-twitch and slow-twitch skeletal muscle myofibers, respectively; and a single cardiac isoform,$TnI_C$, which is expressed in both the atria and ventricles of the heart. Each of the TnI isoforms is encoded by a separate gene. $TnI_S$ is the predominant TnI isoform gene expressed early in cardiac muscle development and whenever skeletal muscle fibers form[1]. Thus, the study of $TnI_S$ gene regulation provides valuable information about the early steps of striated muscle differentiation and about later periods during which muscle fiber type is established. We have used the *in situ* hybridization technique to examine the expression of TnI isoform genes during mouse embryonic development, and have examined the spatial and temporal expression of a -4200TnIsCAT gene introduced into transgenic mice (See abstract of Hardeman et al). While the -4200TnIsCAT transgene exhibits appropriate fiber-specific expression in adult skeletal muscles, the transgene is aberrantly expressed during embryonic devlopment. Not all embryonic striated muscle tissues expressing the endogenous mouse $TnI_S$ gene express the -4200TnIsCAT transgene. This suggests that multiple regulatory components mediate the spatial and temporal control of $TnI_S$ gene expression, and that important regulatory sequences may be absent in this promoter construct. We have conducted transfection studies with primary neonatal heart cells and skeletal muscle cell lines which have identified additional cell type-specific regulatory sequences downstream from the transcriptional start site of the $TnI_S$ gene. A second muscle-specific enhancer element has been identified within the first intron of the human $TnI_S$ gene. The role of internal regulatory elements during *in vivo* muscle gene expression is being investigated.

1. Sutherland et al (1993) Dev. Dynamics 196:25-36.

**W 257** Transcriptional Regulation of the *Drosophila* Tropomyosin I Gene.

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Myogenesis in Drosophila occurs in two phases. The first is an embryonic phase beginning with myoblast fusion at 9-11 hours of development (stages 13/14) to form multinucleated myotubes by 13 hrs (stage 15) and producing visceral and somatic muscles of the embryo and larva. A second phase of myogenesis occurs in the pupa and produces the visceral and somatic muscle of the adult and the indirect flight muscles (IFM) and tergal depressor of the trochanter (TDT) or jump muscles in the thorax of the adult. We are characterizing the cis-acting sequences involved in regulating both the temporal and muscle-specific pattern of tropomyosin I (TmI) expression during both phases of myogenesis.

We are using *lacZ* fusions to identify cis acting regulatory elements in the intron of the *TmI* gene. Our results show that a 350 bp fragment is sufficient for expression in all muscles in embryos, larvae, and adults. A 70 bp subfragment drives muscle specific expression in all muscles except for IFM and TDT muscles. We are using site directed mutagenesis to identify specific sequences involved in muscle activation. In addition, we are further characterizing the elements necessary for expression in IFM and TDT muscle.

Finally, we have found that the 350 bp enhancer appears to contain negative regulatory elements. A mutation in an E-box (HLH protein binding site) results in expression in nervous system without affecting expression in muscle, suggesting that HLH proteins may play a role in repressing muscle gene expression in nervous tissue. We are currently using genetic methods to identify possible candidates for these regulators.

**W 258** REGULATION OF RABBIT SERCA2 GENE EXPRESSION BY THYROID HORMONE RECEPTOR. Zarain-Herzberg, A.[@], Marques, J.,[@] and Sukovich, D.[§] [@]Division of Cardiovascular Sciences, Department of Physiology, University of Manitoba, St. Boniface General Hospital Research Centre, 351 Taché Avenue, Winnipeg, Manitoba, R2H 2A6, Canada, and [§]Cardiovascular Sciences, DuPont Merck Pharmaceutical Co., Experimental Station, E400/5458 Wilmington, DE, 19880-0400

The sarcoplasmic reticulum (SR) is an internal membrane system that plays a essential role in the regulation of cytoplasmic $Ca^{2+}$ concentration during the contraction/relaxation cycle of skeletal, cardiac, and smooth muscle. Active $Ca^{2+}$ transport from the cytoplasm into the SR of the muscle cell is mediated by a cardiac sarco(endo)plasmic reticulum $Ca^{2+}$-ATPase (SERCA). It has been previously demonstrated in rats and rabbits that thyroid hormone (T3) increases the relative level of cardiac sarco(endo)plasmic reticulum $Ca^{2+}$-ATPase (SERCA2) mRNA in cardiac muscle cells. In this study we have analyzed the effect of T3 on the SERCA2 gene expression using fetal chicken primary cardiac myocytes and C2C12 skeletal muscle cells in culture. Northern blot analysis of both cell types demonstrates that thyroid hormone (T3) induced three fold accumulation of SERCA2 mRNA compared to cells grown in media lacking of T3. We have engineered deletion constructs containing various lengths of the 5' flanking region from the rabbit SERCA2 gene that we have previously cloned. A stable transfectant in C2C12 containing a chimeric SERCA2/CAT gene construct including -254 bp of SERCA2 5'-flanking region showed increased transcription activity upon the addition of 50 nM T3. We have analyzed the expression of several deletion constructs spanning 1102 bp of 5'-upstream sequence of the SERCA2 gene by functional expression assays. Transient co-expression of the T3 receptor $\alpha 1$ with various SERCA2/CAT deletion constructs showed *trans*-activation of chimeric constructs containing more than -267 bp, indicating that a thyroid hormone responsive element was localized, at least in part, to the region -267 to -72 bp. T3-receptor/DNA binding assays demonstrated binding of the rat T3 receptor $\alpha 1$ to a fragment containing a proposed T3 response element located between position -254 and -72 in the 5'-flanking region of the SERCA2 gene.

**W 259** A YB-1/p30 COMPLEX BINDS TO THE HF-1a CARDIAC REGULATORY ELEMENT AND MEDIATES VENTRICULAR MUSCLE SPECIFIC EXPRESSION OF THE MYOSIN LIGHT CHAIN-2v GENE, Yimin Zou, Hong Zhu, Wanda F. Reynolds, and Kenneth R. Chien, Department of Medicine, University of California San Diego, La Jolla, CA 92093 and La Jolla Cancer Reserach Foundation, La Jolla, CA 92037

The myosin light chain-2v (MLC-2v) gene displays positional specification to the ventricular segment of the linear heart tube at day 8.0 of murine embryogenesis. In transient assays in cultured ventricular muscle cells and in transgenic mice, two adjacent regulatory elements (HF-1a and HF-1b/MEF-2) are required to maintain ventricular chamber specific expression of the MLC-2v gene. To characterize the HF-1a binding factor(s), a rat neonatal heart cDNA library was screened with an HF-1a binding site, resulting in the isolation of nine overlapping cDNA clones. Each of these independent clones encoded $EFI_A$, the rat homolog of the human Y-box binding protein first found to bind to the class II histocompatibility gene promoter. Bacterially-expressed and affinity-purified $EFI_A$ protein binds to the HF-1a site in a sequence specific manner, and antisera against the rat $EFI_A$ and the Xenopus YB-3 can specifically supershift the endogenous HF-1a complex in the rat cardiac myocyte nuclear extracts. The HF-1a sequence contains AGTGG, which is highly homologous to ATTGG, the "inverted CCAAT" core of the $EFI_A$ binding site in other genes. In transient assays in cardiac and Cos cells, co-transfection of an $EFI_A$ expression vector with a 250 bp MLC-2 luciferase fusion gene increased the reporter activity by 4.3 fold specifically in the cardiac cell context, and in a HF-1a site dependent manner. The $EFI_A$ complexes with an unknown protein in cardiac myocyte nuclear extracts to form the endogenous HF-1a binding activity. Immunocoprecipitation using an epitope tag approach reveals that $EFI_A$ associates with a single major protein of ~ 30kd (p30) in cardiac cells, which is distinct from the 28kd co-factor found in Cos cells. This study suggests that YB-1, in association with a 30kd protein partner, binds to the HF-1a site and, in conjunction with HF-1b/MEF-2, mediates ventricular chamber specific expression of the MLC-2v gene. Exploring the role of the p30 co-factor in the ventricular chamber restricted expression of the MLC-2v gene will be of interest.

## *Muscle Proteins*

**W 300** A NOVEL LIM-DOUBLE FINGER PROTEIN PROMOTES MUSCLE CELL DIFFERENTIATION

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To identify genes which are induced in skeletal muscle by denervation, we constructed a subtractive cDNA library from adult rat diaphragm enriched in cDNAs expressed seven days after denervation.
One strongly induced clone showed high homology to a LIM-double-finger protein. The LIM-double-finger motif binds zinc and is present on a small group of highly regulated genes, including some homeobox proteins. The expression of the new protein is essentially restricted to skeletal and cardiac muscle. Low level expression is found in adult skeletal muscle; interestingly, abundance is comparable in adult cardiac muscle and in skeletal muscle after denervation.
Induction of the gene during in vitro myogenic differentiation (C2 cell line) occurs comparatively late. Early overexpression of the protein in stably transfected C2 cells leads to marked acceleration of differentiation and overexpression of muscle-specific proteins.
Localization of the protein changes from nuclear in myoblasts to subsarcolemmal in myotubes. Upon transient expression in nonmyogenic cells, nuclear and cytoplasmic localization along subsarcolemmal actin fibers can be observed. Finally, apoptosis occurs with high frequency when the protein is overexpressed in some nonmyogenic cell lines.
The protein is highly conserved during evolution (rat, chick, Drosophila), suggesting that it may play an important role in muscle cell differentiation. It can be hypothesized that the correct timing and cell type of expression of the novel LIM-double-finger protein is critical for functional development of striated muscle.

**W 301** MINIPARAMYOSIN, A MUSCLE INSECT SPECIFIC PROTEIN : MOLECULAR CHARACTERIZATION OF THE GENE, DEVELOPMENT AND ISOFORM DIVERSITY. J. Arredondo, M. Maroto, M. San Roman, R. Marco & M. Cervera. Departamento Bioquimica(UAM) & Instituto Investigaciones Biomedicas(CSIC). Facultad Medicina. Arzobispo Morcillo 4. Madrid 28029. SPAIN.

The *Drosophila* paramyosin/miniparamyosin complex gene spands 12.3 Kb and is located at the 66D14. To characterize the complex, partial nucleotide sequence has been carried out and the position of the intron-exon boundaries has been deteminated. Primer extension analysis and S1 nuclease digestion has also been done to confirm that paramyosin and miniparamyosin mRNAs arise from two overlapping transcription units with distinct promoters. In fact, the miniparamyosin transcription start has been found within a paramyosin intron.
We have cloned the paramyosin/miniparamyosin gene from *D. virilis*. These genomic clones include the promotor regions. The sequencing of the promotor regions of *D.melanogaster* and *D. virilis* clones is now in progress. Comparison between the genes will help to elucidate specific regulatory sequences.
The expression of miniparamyosin during development has been studied. The protein is present in adult fibrillar and tubular muscles(in relative different quantities) but not in embryonic/larval muscles. Interestingly, the expression of the protein and the mRNA is coordinated until recient emerged imagos, but not in the adult stage. Double immunofluorescence, using a specific polyclonal antibody generated in the lab, suggests a different localization of the protein depending the type-fiber (tubular or fibrillar). Two-dimesional analysis have shown several isoforms, all *in vivo* phosphorylated.

**W 302** Analysis of Single Missense Mutations in the β Myosin Heavy Chain Protein in Cultured Cardiomyocytes and Transgenic Mice, K. David Becker*, Kim Gottshall*, Jean-Claude Perriard† and Kenneth R. Chien*, *Univ. of Calif., San Diego, La Jolla, CA., †Inst. for Cell Biology, Swiss Federal Inst. of Tech., Zurich, Switzerland.

Familial hypertrophic cardiomyopathy is an autosomal dominant disease that is genetically heterogeneous. Some portion of families with this disease have point mutations in their β myosin heavy chain (β MHC) gene, producing single amino acid changes, that cosegregate with the disease phenotype. To approach a molecular understanding of this disease we have cloned human β MHC cDNA's downstream of the myosin light chain 2 ventricular specific promoter. In vitro mutagenesis has been utilized to generate several different β MHC clones each having one of the point mutations previously reported. The human cDNA's have been epitope tagged in order to facilitate detection. Two different epitopes have been inserted in two different N-terminal positions to control for the sequence and site of epitope addition. The expression of these clones in primary cultures of neonatal rat ventricular cardiomyocytes is being analyzed. Transfected cardiomyocytes appear to incorporate either tagged wild type or tagged mutant β MHC proteins into sarcomeres as detected by the epitope specific antibody. The utilization of two distinct epitopes will facilitate the analysis of direct competition between mutant and wild type β MHC proteins in singly co-transfected cardiomyocytes. Confocal microscopy is currently being used to determine the sarcomeric structure and assembly of myofibrils in these cells. Transgenic mice have also been generated using either wild type or mutant β MHC cDNA's. These transgenic lines are being characterized for transgene expression and manifestation of cardiomyopathy.

**W 303** THE ROLE OF INTEGRIN $\alpha5\beta1$ IN THE MYOBLAST TO MYOCYTE CONTROL POINT, David Boettiger, Hee Yong Yoon, Mindy George-Weinstein, A. Sue Menko, and Motomi Iwamoto, Department of Microbiology, University of Pennsylvania, Philadelphia, PA 19104

The transition from replicating myoblast to post mitotic myocyte can be controlled by presence of growth factors and by interactions with the extracellular matrix and thus represents a control or checkpoint for myogenic differentiation. Although at least 4 members of the integrin family are present on the myoblast, 2 appear to be inactive for cell binding and could not be detected associated with adhesion structures. The specific interaction required for the differentiation signal was identified as $\alpha5\beta1$ integrin. This interacts with fibronectin attached to the culture substrate. The use of antibodies to perturb this process allowed us to demonstrate that a successful signal required that the fibronectin be attached to a rigid substrate suggesting that the cell is using this system to probe mechanical aspects of its microenvironment. The establishment of the fibronectin-$\alpha5\beta1$ binding lead to a rise in the level of MyoD and a drop in Myf5 mRNA levels detectable within 6-12 hours, followed by an increase in myogenin mRNA and other specific differentiation marker mRNA's. This signal was relatively transitory because it also led to a down regulation of the binding activity of $\alpha5\beta1$ integrin without change in its protein level. This appears to be accomplished by a conformational change in the receptor mediated by the developmental process. This down regulation was important for the morphological changes in producing first the bipolar myocyte and the subsequent myotube.

**W 304** **Expression and Functional Analysis of *Drosophila* Muscle Myosin Isoforms.**
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The single 19 exon muscle myosin heavy chain (MYHC) gene of *Drosophila* has the potential to generate 480 distinct protein isoforms via regulated alternative RNA splicing. Previous work has demonstrated that several of these alternative exons are expressed in developmentally and muscle type specific patterns. The recent solution of the S1 subfragment crystal structure allows the placement of these alternative exon choices into the high resolution structural domains of MYHC.

In order to determine how alternative exon useage influences MYHC function, we have begun to express MYHC isoforms using molecular cloning techniques and a baculovirus expression system. These constructs are also being used to develop transgenic *Drosophila* in which the functional properties of these alternative isoforms can be examined in intact muscle. This approach is facilitated by the availability of viable null MYHC mutants which provide a unique background for the expression and analysis of alternative isoforms. We will present preliminary data on the biochemical properties of some alternative isoforms and the initial results of transgenic expression.

**W 305** EFFECT OF DENERVATION ON TERMINAL DIFFERENTIATION OF RABBIT SLOW- AND FAST-TYPE MUSCLES. CONTRACTION VELOCITY AND MYOSIN ISOFORM CHANGES ARE OPPOSITE TO THOSE FOUND IN THE RAT, Anne d' Albis, Francis Goubel, René Couteaux, Chantal Janmot and Jean-Claude Mira, Laboratoire de Biologie Physicochimique, URA CNRS 1191, Bâtiment 433, Université Paris-Sud, 91405 - Orsay, France.

Denervation of the soleus and gastrocnemius medialis was performed on eight-day-old rabbits and its effects were examined fifty two days later by mechanical, cytochemical, and biochemical methods.

The contralateral soleus exhibited the properties of a slow-type muscle: slow contraction and relaxation velocities, a predominance of slow-type oxidative fibers as defined by histochemical and immunocytochemical determinations, and of slow-type myosin isoforms. The contralateral gastrocnemius exhibited the properties of a fast-twitch muscle: fast contraction and relaxation velocities, a predominance of fast-type non-oxidative fibers and fast-type myosin isoforms.

Denervation of rabbit muscles caused the differentiation of the two muscles towards slow-type muscles. Both denervated soleus and gastrocnemius exhibited slow contraction and relaxation velocities, a predominance of slow-type oxidative fibers and slow-type myosins. Some of these slow-type myosins were normal type, made up of slow-type heavy and light chains, and the others hybrid type, made up of slow-type heavy and regulatory light chains and of fast-type essential light chains.

Thus, innervation in the rabbit appears to be a determining factor for muscle fiber differentiation into a fast-type phenotype, but it is not necessary for muscle fiber differentiation into a slow-type phenotype, whereas the opposite is the case in the rat.

**W 306 SMOOTH MUSCLE MYOSIN LIGHT CHAIN KINASE EXPRESSION IN SKELETAL AND CARDIAC MUSCLE: FUNCTIONAL IMPLICATIONS**, Paul Herring, Department of Medicine, Indiana University School of Medicine, Krannert Institute of Cardiology, 1111 W. 10 th St. Indianapolis, IN 46202

Myosin light chain kinases (MLCK) are important enzymes involved in the regulation of muscle contraction, cell movement, secretion and cell division. There are two major forms of MLCK which can be distinguished biochemically and structurally, a skeletal muscle specific form and a smooth/non-muscle form. The restricted expression of the skeletal muscle kinase suggests that it is probably the smooth/nonmuscle MLCK which is involved in regulation of more general processes such as cell division. The main kinetic difference between the two forms of MLCK is that the smooth muscle MLCK has a marked preference for smooth or nonmuscle light chains as a substrate, whereas the skeletal muscle enzyme can phosphorylate either skeletal or smooth muscle light chain equally well. Immunoblot, immunofluorescence and RNase protection analysis of skeletal muscle cells have revealed that during development of skeletal muscle *in vitro* a paradigm exists in which the smooth muscle MLCK is expressed in the committed, undifferentiated myoblasts and upon differentiation and induction of skeletal muscle myosin, this form is largely replaced by the specific skeletal muscle MLCK. Similar studies of adult tissues have demonstrated that the smooth muscle MLCK is expressed in adult skeletal and cardiac muscle and represents the primary MLCK expressed in cardiac muscle. This latter finding has important implications for cardiac myosin light chain phosphorylation as the smooth muscle MLCK phosphorylates cardiac myosin light chains relatively poorly as compared to smooth muscle myosin light chains. This could account for the very low rates of cardiac myosin light chain phosphorylation observed *in vivo*.

**W 307 DEFECTS IN THE MYOSIN ROD PERMIT NORMAL THICK FILAMENT ASSEMBLY BUT CAUSE MUSCLE DEGENERATION**, William A. Kronert, Annabeth Fieck, Patrick T. O'Donnell, Jim O. Vigoreaux*, John C. Sparrow**, Sanford I. Bernstein, Biology Department, San Diego State University, San Diego, CA 92182, *Department of Zoology, University of Vermont, Burlington, VT 05405, **Department of Biology, University of York, U.K.

The molecular defects of three myosin mutants in *Drosophila melanogaster* have been determined to be in the α-helical rod. All three mutants, $Mhc^{6}$, $Mhc^{13}$ and $Mhc^{19}$, are homozygous-viable and dominant flightless. All have normal size classes of *Mhc* mRNA and near normal levels of MHC protein in the thorax and leg muscles.

In $Mhc^{13}$ and $Mhc^{19}$ the indirect flight muscles (IFM) of newly enclosed adults appear normal but as development proceeds the IFM fibers super-contract pulling the muscles apart; electron microscopy shows a random array of thick and thin filaments; there are Z-bands but the M-lines are absent.

We have found that all three mutant MHC proteins display a proteolytically sensitive cleavage site both *in vivo* and *in vitro*. N-terminal sequencing of the ~70 kD proteolytic fragment maps it to the rod region of the protein.

Two-dimensional gel studies of $Mhc^{6}$, $Mhc^{13}$ and $Mhc^{19}$ has revealed that an IFM-specific thick filament protein known as flightin (Vigoreaux et al., 1993, *J. Cell Bio.* 121:587-598) is reduced in these mutants. Further studies on very young $Mhc^{13}$ adults show that a single form of flightin can be detected early on but that additional phosphorylated forms that normally accumulate later in development are absent. It is possible that the mutation affects the flightin-binding site on MHC, preventing flightin's post-translational modification on the thick filament

Single mutations were found in the coding region for each of the mutants. In $Mhc^{13}$ and $Mhc^{19}$ the mutation changes a glutamic acid in the rod to a lysine. The mutation is 237 amino acids downstream from the proteolytic site. This particular amino acid is conserved in all muscle myosins that have been studied. In the case of $Mhc^{6}$ the mutation affects an amino acid located five residues downstream of the change in $Mhc^{13}$ and $Mhc^{19}$, converting an invariant arginine residue to a histidine. This region of the myosin rod is encoded by a constitutive exon, suggesting that there are conserved domains in the MHC protein that are critical for specific functions in certain muscle types. We are currently examining models of myosin coiled-coil assembly to shed light on the defects in these mutants and determining the ultrastructural defects in the $Mhc^{6}$ mutant.

**W 308 RECOMBINANT CARDIAC ISOMYOSINS : STRUCTURE-FUNCTION STUDIES. APPLICATIONS TO MUTANT MYOSINS IN FAMILIAL HYPERTROPHIC CARDIOMYOPATHY**, J.J. LEGER with the collaboration of : AM.Cathiard, B.Cornillon, P.Eldin, M.Le Cunff & D.Mornet, INSERM U300, Fac.Pharmacie, 34060 MONTPELLIER cedex, FRANCE

Our aim was to investigate the molecular basis of functional differences between the two main cardiac α and β myosin heavy chains, and evaluate possible functional changes resulting from different point mutations in ß myosin heavy chain genes from different hypertrophic cardiomyopathic families.

Recombinant DNA methods were used to obtain soluble, undenatured fragments of the ß-type heavy chain of human cardiac myosin subfragment-1 (S-1). These fragments were preselected lengths, and could include protease-sensitive segments that are destroyed when other preparation, methods are used. Actin binding by each of the three contiguous segments (residues 1-248, 249-524, 518-722, essentially spanning the entire S-1 heavy chain) was obtained. ATP binding, comparable to that of native S-1, was demonstrated only with a segment consisting of residues 1-524. Competition among the various fragments for actin was also studied. Preliminary results also suggest that a functional full-length recombinant S1 has ATP hydrolysis capacity and is relevant for further *in vitro* motility assays of mutated or chimaeric myosins.

**W 309 ANALYSIS OF A MUSCLE VARIANT OF MICROTUBULE-ASSOCIATED PROTEIN 4**, M.E. Mangan and J.B. Olmsted, Department of Biology, University of Rochester, Rochester, NY 14627

MAP 4 is a ubiquitous structural microtubule-associated protein. Previous work from our lab has shown that MAP 4 is encoded by a single gene from which multiple transcripts are generated (West et al., 1991. J. Biol. Chem. 266:21886-21896). In most tissue types, dominant classes of 5.5 and 6.5 kb transcripts are observed; the major size differences arise from alternative polyadenylation (Code and Olmsted. 1992. Gene 122:367-370). However, more dominant transcripts of ~8 and 9 kb are present in both skeletal and cardiac muscle. Mouse $C_2C_{12}$ cells that undergo differentiation from myoblasts to myotubes in culture have been used to examine MAP 4 during myogenesis. Indirect immunofluorescence shows that MAP 4 is present in both myoblasts and myotubes, and co-localizes with microtubules. Northern analyses indicate a switch from the 5.5/6.5 to the 8/9 kb transcripts by day 4 of culture in differentiation medium, when fusion of the myoblasts into myotubes is occuring. To investigate the origins of transcript heterogeneity in muscle, a cDNA library made from differentiated $C_2C_{12}$ cells (gift of E. Olson) was screened with probes derived from the ubiquitous MAP 4 transcripts. Positive clones were subcloned and examined for sequences that differed from those obtained previously. The major transcript variation identified to date arises from a single 3.2 kb insertion within a region (b) of the projection domain immediately adjacent to the basic microtubule binding domain of the protein. A potential ORF derived from a partial sequence contiguous with the existing ORF predicts an elongate structure with partial homology (40%) to repeated domains of neurofilament protein H. Using probes unique to the new sequence, preliminary in situ hybridization analyses show that the muscle MAP 4 transcript is present in somites and pre-thoracic muscle of embryonic day 11 mice, and in head and neck muscles, tongue, intercostal muscles, and diaphragm of embryonic day 13 mice. These data suggest that a unique form of MAP 4 with a distinctive projection domain structure is significant for function in muscle tissues. (Supported by NIH GM22214 to JBO)

**W 310 OVER-EXPRESSION OF STRIATED β-TROPOMYOSIN DURING MURINE CARDIAC DEVELOPMENT.**

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Tropomyosins (TMs) comprise a family of actin-binding proteins which play an important role in the regulation of contractility in muscle (cardiac, skeletal, smooth) and nonmuscle cells. In the myocardium of adult small mammals, the principal striated muscle isoform of TM is α, while fetal and hypertrophic myocardium express both the α- and β-TM isoforms. Our previous studies have shown that the ratio of striated α- to β-TM mRNA in murine cardiac tissue dramatically changes during the embryonic to adult transition. To further elucidate the role of the striated muscle-specific α- and β-TM isoforms during cardiac development, we generated transgenic mice over-expressing the striated β-TM. A β-TMstr isoform cDNA driven by an α-myosin heavy chain promoter which had been shown to be cardiac specific was used to generate transgenic mice. Nine individual transgenic lines were generated and the transgene's expression was analyzed by quantitating the amount of β-TM transcripts. There is a significant (>20-fold) increase in levels of β-TMstr mRNA, with little variation among these transgenic lines. To determine whether over-expression of β-TMstr results in compensatory expression by other contractile protein genes (myosin heavy chain, actin and troponin T), RNAs from the transgenic and nontransgenic hearts were analyzed. Surprisingly, results demonstrate that there are no drastic changes in the expression of these genes. Currently, we are assessing the biochemical and physiological effects of the over-expression of β-TMstr on cardiac musculature in these mice.

**W 311** PHOSPHORYLATION OF MYOGENIN AND REGULATION OF THE ACETYLCHOLINE RECEPTOR GENES EXPRESSION BY ELECTRICAL ACTIVITY AND BY PROTEIN KINASE C

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Muscle differentiation is accompanied by a drastic reduction of acetylcholine receptor (AchR) molecules outside the NMJ (extrajunctional AchR). This reduction results from a down regulation of AchR gene expression. Compared to cultured electrically inactive myotubes, spontaneous electrically active ones display about 1/3 AchR molecules on their surface. Cultured myotubes thus provide a model system for muscle regulation of AchR gene expression during cell differentiation.

We have analysed the potential role of myogenin in the regulation by electrical activity of the expression of the acetylcholine receptor (AChR) α-subunit gene in cultured chick embryonic myotubes. The state of phosphorylation of myogenin was followed by [$^{32}$P]-labeling and immunoprecipitation with an anti-myogenin antibody. In electrically active myotubes myogenin is phosphorylated while it is dephosphorylated in electrically silent myotubes following tetrodotoxin (TTX) treatment. Myogenin dephosphorylation is also observed upon incubation of myotubes with GF109203X, a pharmalogical agent which specifically inhibits protein kinase C (PKC) activity. Both treatments cause similar increases in the expression of the AChR protein. The effects are not additive. Thus TTX and GF109203X most probably affect a common process. Recombinant chick myogenin binds to myogenic sites (E box) present in the AChR α-subunit promoter but loses this binding capacity after phosphorylation. As a working hypothesis we propose that repression of AChR biosynthesis by electrical activity results, at least partly, from phosphorylation of myogenin via the PKC pathway.

**W 312 THE MOLECULAR STRUCTURE OF MUSCLE-TYPE COFILIN AND ITS TISSUE EXPRESSION.**

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Cofilin is an actin-modulating protein of about 20 kDa which was first discovered in mammalian brain, but now it is known that this protein is widely distributed in muscle and non-muscle cells. We observed that cofilin is enriched in denervated or dystrophic degenerating skeletal muscle as well as in the developing muscle. It is likely that this protein is involved in redistribution of actin during assembly and/or disassembly of myofibrils. By means of immunoblotting combined with two dimensional electrophoresis, we found that two cofilin variants, muscle-type (M-type) and non-muscle-type (NM-type), exist in mammals. During in vitro myogenesis of mouse C2 cells, expression of the M-type cofilin was up-regulated. Therefore, characterization of the M-cofilin would be indispensable to understand the role of cofilin in muscle cells. We cloned cDNAs encoding M-cofilin from cDNA library of C2 myotubes and determined the sequence. The deduced peptide sequence contained a nuclear transport signal and putative actin-binding sequence as reported in NM-cofilin. The sequence showed about 80 % identity with mouse NM-cofilin sequence and, interestingly, higher homology (95 % identity) with that of chicken cofilin which is common to both muscle and non-muscle cells in the case of chicken. The message for mouse M-cofilin was detected preferentially in striated muscles, C2 myotubes and testis by Northern blotting, while the message for NM-cofilin was in variety of non-muscle tissues.

**W 313** COMPLEXITY OF AVIAN SARCOMERIC MYOSIN HEAVY CHAINS IN DEVELOPING AND ADULT MUSCLES: CORRELATION AT PROTEIN AND GENOMIC LEVELS.

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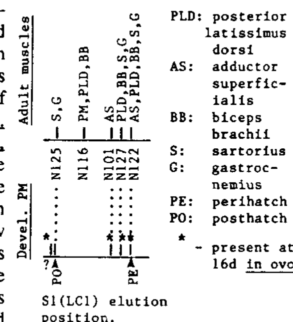
We have studied myosin isoform complexity in avian muscles by high resolution anion-exchange chromatography of purified S1 species (J. Muscle Res. & Cell Motility 9, 552 (1988);ibid, 12, (1991); Mol. Biol. Cell 3, 912a (1992); Rushbrook et al, in preparation). Each unique myosin heavy chain head elutes as 2 peaks in this system, S1(LC1) and S1(LC3), S1(LC1) eluting first due to the additional positive charge of the LC1 N-terminal region.

Five unique heavy chains were found to be expressed differentially in six adult fast muscles and a progression of six heavy chains in the developing pectoralis major muscle (PM). Co-chromatography suggested identity of many developmental and adult forms.

Sequencing of a unique CNBr peptide near the N-terminus confirmed identities and permitted correlation of protein forms with genomic species identified in the laboratory of Jeff Robbins (J. Biol. Chem. 262, 16536 (1987); ibid 261, 6606 (1986). The results are summarized to the right. The complexity of expression emphasizes the question of how presumed functional differences in the proteins mesh with the differing requirements of fibers during development and differentiation.




S1(LC1) elution position.

**W 314** CARDIOTIN, A STRUCTURAL AND AGE-RELATED COMPONENT IN THE MYOCARDIUM

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Cardiotin, a recently described component in the cardiovascular system (Schaart *et al.*, Eur. J. Cell Biol. 61, 1993), reacts with the monoclonal antibody R2G. Application of immunofluores-cence assays, revealed that cardiotin is expressed in the myocardium of several species and also, but weakly, in skeletal muscle. Cardiotin is not found in smooth muscle tissue, or any other type of mesenchymal, epithelial or neural tissue. The cardiotin distribution pattern was different from that of other sarcomeric proteins, such as desmin, myosin, actin, titin, nebulin, and desmoplakins, and showed a longitudinal filamentous localization between the myofibrils. The cardiotin filamentous staining reaction is oriented perpendicularly to the typical cross-striations observed with antibodies to desmin, and was normally found spanning several sarcomeres. Immunoblotting and immunoprecipitation experiments have shown that cardiotin is a high molecular weight protein restricted to cross-striated muscle. Cardiotin subunits have a molecular weight over 300 kDa. Cardiotin cannot be solubilized from cardiac muscle tissue by nonionic detergents or high concentrations of KCl and KI, suggesting a structural role in the myocardium.

Immunohistochemical studies on heart tissue of different species and of different age (i.e., human, monkey, and rabbit) showed that the expression of cardiotin in the myocardium is age-related. During embryonic development no immuno-reactive cardiotin could be detected, while directly after birth a weak reaction pattern was observed. In adult myocardium an abundant expression of cardiotin was found. The subcellular localization and the age-related expression of cardiotin suggest a possible link with the sarcoplasmic reticulum.

Immunoelectron microscopy and studies at the molecular level are in progress to detetermine its distribution, and the structural and cellular function of cardiotin.

**W 315** INTERMEDIATE FILAMENT NESTIN EXPRESSION DURING MYOGENESIS AND IN MUSCLE DISORDERS.

Thomas Sejersen[1], Urban Lendahl[1], Gunnar Sjöberg[1], and Lars Edström[2], Department of Cell and Molecular Biology[1], and Department of Neurology[2], Karolinska Institute, Stockholm, S 171 77

The intermediate filament protein nestin was originally discovered in neuroepithelial stem cells, and subsequently found also in somites of developing embryos. It constitutes a sixth class of intermediate filaments, distantly related to vimentin and desmin, the intermediate filaments previously found in skeletal muscle cells. We present results showing that the intermediate filament transitions during muscle development involve a transient expression of nestin mRNA and protein. Nestin protein was found up to the postnatal myofiber stage, where it colocalized with desmin in Z bands. Despite its more distant structural relationship with desmin and vimentin, nestin formed a cytoplasmic filamentous network shown by computorized image analysis to co-localize with the other two intermediate filamaments in myoblasts and in multinuclear myotubes of the human fetal myogenic cell line G6. In light of the general assumption that regenerating muscle cells pass through the same sequential events as developing muscle, we analyzed the expression pattern of the three intermediate filaments in biopsies from patients with Duchenne/Becker muscular dystrophy and polymyositis. In both diseases, areas of muscle regenaration stained positive for nestin and vimentin, and also stained more intensely for desmin than surrounding fibers. Nestin was, however, the only intermediate filament to be found specifically in regenerating muscle cells, vimentin being present in fibroblasts and other mesenchymal cells, and desmin in all myofibers.

Results will also be presented on the expression of nestin in rhabdomyosarcomas.

**W 316** THYROID HORMONE REGULATED, DIFFERENTIAL EXPRESSION OF Ca-ATPase ISOFORMS (SERCA1 AND SERCA2)IN SKELETAL MUSCLE FIBERS.

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Increasing levels of thyroid hormone ($T_3$) induce a shift from slow to fast contractile properties of skeletal muscle. The slow isoform of myosin heavy chain (MHC) is replaced with the fast isoform. The simultaneous increase in relaxation rate is caused by an increased expression of sarcoplasmic reticulum (SR) Ca-ATPase (SERCA). However, $T_3$ increased the mRNA levels of both the fast muscle isoform (SERCA1) and the slow muscle isoform (SERCA2). Here we investigated the apparent absence of an isoform shift of SERCA1 and -2 at the single fiber level. Rat soleus muscles from hypothyroid (4 weeks $KCLO_4$ treatment), euthyroid and hyperthyroid rats (euthyroids receiving 1 to 14, daily $T_3$ injections) were analyzed. Antibodies and RNA probes specific for SERCA1 and -2 and slow and fast MHC were used in immunohistochemical and *in situ* hybridization analysis of serial muscle sections. In the transition from hypo- to hyperthyroidism the total level of SERCA1 protein increased over ten fold, whereas that of SERCA2 remained unchanged. At the single fiber level, all fibers expressed SERCA2 in hypothyroidism with 10% also containing SERCA1. In hyperthyroidism the total SR activity was dramatically increased with SERCA1 expressed in all fibers. The expression of SERCA1 was tightly linked to that of the fast MHC isoform. The number of SERCA2 positive fibers was down to 70%, but expression levels in most of these fibers were higher than in hypothyroidism. Fibers expressing SERCA2 also expressed slow MHC, but SERCA2 levels could be increased while those of slow MHC were already going down.

Under the present experimental conditions two populations of fibers can be distinguished in soleus muscle. One in which high levels of $T_3$ stimulate expression of both SERCA1 and -2 with partial replacement of slow MHC by fast MHC, and one in which a complete slow to fast switch for the SERCA and MHC isoforms is induced.

**W 317** FUNCTIONAL ANALYSIS OF M-CADHERIN IN CELL CULTURE AND TRANSGENIC MICE.

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A number of cell adhesion molecules (CAMs) including members of the cadherin and immunoglobulin superfamilies are expressed in skeletal muscle and are important im myoblast fusion. A member of the cadherin superfamily called M-cadherin has recently been described and has a highly specific pattern of expression. (Moore and Walsh. Development (1993) *117*, 1409). A full length rat cDNA encoding muscle cadherin has been isolated and the predicted protein it encodes is 96% conserved with the mouse homologue, with the signal and prepeptide amino acid sequences being less conserved than the other domains. When introduced into mouse 3T3 cells under the control of a constitutive viral promoter, the cDNA expresses a 125kD protein which comigrates with mouse M-cadherin from C2 cell cultures, in immunoblots with affinity purified antibodies directed against an extracellular sequence of M-cadherin. Transfected cell lines expressing M-cadherin have increased $Ca^{++}$ dependent adhesion compared to control cell lines of transfected cells expressing muscle cadherin with a deletion in the cytoplasmic domain. When introduced into C2 cells, M-cadherin has a marked effect on fusion of myoblasts in culture; relatively low level ectopic expression of rat M-cadherin enhances the rate of C2 myoblast fusion and differentiation. As M-cadherin is expressed in developing somites during commitment of myoblasts to terminal differentiation, the effect of M-cadherin transfection may be to potentiate the cellular recognition and adhesion occuring in myogenesis. Guided by these results, domain-deleted forms of muscle cadherin have been placed under the control of muscle specific promoters for expression of potentially dominant negative alleles in transgenic mice, to analyse the consequences of altered myoblast fusion during embryonic development.

**W 318** M-CADHERIN AND ITS ROLE DURING MORPHOGENESIS OF SKELETAL MUSCLE OF SOMITIC ORIGIN. Anna Starzinski-Powitz, Human Genetics for Biologists, J.W. Goethe University, Siesmayerstr. 70, D-60054 Frankfurt a. M.;FRG.
M(muscle) cadherin, isolated from myogenic cells, is a member of the multigene family of calcium-dependent cell adhesion molecules, the cadherins. These are involved in diverse processes such as cell recognition and cell sorting, cell polarisation and tumor metastasis. In a series of studies, we investigated the putative role of M-cadherin during morphogenesis of skeletal muscle. We found that blocking the function of M-cadherin on myogenic cells in culture inhibits fusion of myoblasts as well as accumulation of muscle specific Troponin T protein. Furthermore, M-cadherin-blocked cells show an increased BrdU incorporation when compared to non-blocked cells indicating that M-cadherin provides a signal for myoblasts to become postmitotic. During prenatal mouse development, M-cadherin protein is specifically expressed on myogenic cells of somitic origin and is first detectable at E10$^{1/3}$. M-cadherin changes its subcellular localisation from evenly distributed to being clustered later in development. Postnatally, M-cadherin is expressed on all satellite cells and most likely also in the regions of the myofiber restricted to the domains beneath the satellite cells and is transiently upregulated in regenerating muscle. In conclusion, our analysis of the expression pattern and function of M-cadherin implies that it is a central molecule in the regulation of myogenic morphogenesis in prenatal and postnatal mouse development.

**W 319** STRUCTURE-FUNCTION ANALYSES OF STRIATED MUSCLE MYOSIN, H. Lee Sweeney, Zhaohui Yang, James T. Stull, Leslie Leinwand, Huisheng Feng and Lynn Faust, Department of Physiology, University of Pennsylvania School of Medicine, Department of Physiology, Philadelphia, PA 19104-6085.

In order to study structure/function relationships of myosin, we have developed several parallel approaches. To address the function of the myosin light chains, we are expressing wild type and mutant essential (alkali) and regulatory light chains in *E. coli*. We then replace the endogenous light chains in permeabilized skeletal muscle fibers with the bacterial synthesized light chains and perform functional measurements, including force development and shortening velocity. This approach has been utilized to demonstrate modulatory roles for the light chains of skeletal muscle myosin. In rabbit striated muscle, phosphorylation of myosin regulatory light chains has been observed to increase both force and the rate of force redevelopment. Our mutagenesis studies suggest that the mechanism of this effect is that phosphorylation changes the net charge in a critical region of the N-terminus, which in turn alters interactions between the regulatory light chain and myosin heavy chain. We have also shown that the essential (alkali) light chain of myosin can modulate shortening velocity. The region involved is the extended N-terminus of the light chain, which we speculate forms interactions with the C-terminus of actin. To begin structure/function analyses on the myosin heavy chain, we have developed baculovirus-driven expression of myosin as well as expression of exogenous myosin heavy chains in quail myogenic cultures. In the case of baculovirus expression, the myosin can be assessed in solution, while the myogenic culture system generates myotubes for muscle mechanical studies. To date, we have examined mutations in the myosin heavy chain that cause hypertrophic cardiomyopathy.

**W 320** SMOOTHELIN, A STRUCTURAL PROTEIN IN SMOOTH MUSCLE CELLS.
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Smoothelin, a structural component in smooth muscle cells, reacted with a mouse monoclonal antibody, R4A (Schaart et al., J. Muscle Res. Cell Motil. 14(2): 236, 1993). Tissue specificity and interspecies cross reactivity of the MoAb were tested by immunofluorescense staining on frozen material and by 1D- and 2D-SDS-PAGE/ Western blotting analysis. Smoothelin was demonstrated to react with all smooth muscle cells, but was not found in cardiac or skeletal muscle cells. The smoothelin cellular organization resembles the filamentous patterns of desmin and α-smooth muscle actin. However, immunoblotting showed smoothelin to be a 59 kDa protein with a electrophoretic mobility different from desmin and α-smooth muscle actin.
From human colon smooth muscle cell, poly-A$^+$ RNA was isolated and a cDNA library (λ-ZAP, Stratagene) was constructed. From this library R4A immunopositive clones were selected. The selected clones have been characterized by Southern and Northern blotting analysis and by sequencing. So far, no homologies with other genes have been found.

**W 321** DEVELOPMENTAL REGULATION OF THE STRUCTURE AND FUNCTION OF α7β1 INTEGRIN IN SKELETAL MUSCLE, Weigwang Wang and Stephen J. Kaufman, Department of Cell and Structural Biology, University of Illinois, Urbana, IL 61801
The α7β1 integrin heterodimer is a developmentally regulated extracellular matrix binding protein on the surface of differentiating and adult skeletal muscle. As the sole functional laminin-binding integrin on skeletal myoblasts α7β1 mediates the response of these cells to a laminin-rich environment and this includes increased cell mobility, maintenance of proliferation and myoblast localization at the sites of secondary fiber formation. Once fibers form, the α7β1 integrin localizes along their periphery where it likely serves to help associate these cells with the extracellular matrix. It is also localized at myotendinous junctions where it tethers fibers and thereby plays an important role in force generation. We report here that the diversity of functions of the α7β1 integrin at different stages of myogenesis and in the adult is mediated by its capacity to bind multiple ligands and by the developmentally regulated expression of three alternate cytoplasmic domains of the α7 chain. The α7A, B and C isoforms are generated by alternate RNA splicing and the three amino acid sequences thus derived have distinct motifs and membrane signal transducing capacities. The α7B form is expressed on precursor cells and in replicating myoblasts. Two regions in the α7B cytoplasmic domain have homology to those in the catalytic phosphotransfer domain of serine/threonine protein kinases, a sequence of 15 amino acids is homologous to that in many protein tyrosine phosphatases, and there is a potential actin binding sequence and a unique 3-fold DXHP repeat. Binding ligand causes a change in conformation in the α7B cytoplasmic domain and promotes its association with the cell cytoskeleton. Upon terminal differentiation, there is a decline in expression of α7B and commensurate expression of α7A and then α7C.

In addition to binding laminin, the α7β1 integrin also binds to fibronectin and to a lectin, L-14. L-14 is synthesized in replicating myoblasts but it is not secreted until these cells commence fusion. Our experiments demonstrate that L-14 binds to both laminin and the α7β1 integrin and it can effectively inhibit the association of these molecules. Modulation of α7β1 interactions with its ligands by L-14 is selective: L-14 does not bind to fibronectin, nor does it interfere with the binding of α7β1 with fibronectin. A consequence of the onset of L-14 secretion with terminal differentiation is a shift in myoblast interactions away from laminin to fibronectin, and we believe this is significant to further myogenic development.

**W 322** *IN VIVO* EFFECTS OF MIS-EXPRESSION OF AN EMBRYONIC ISOFORM OF THE MYOSIN HEAVY CHAIN GENE IN *DROSOPHILA MELANOGASTER*, Linda Wells, Norbert Hess, Venetia L. Collier, Kevin A. Edwards, and Sanford I. Bernstein, Biology Department and Molecular Biology Institute, San Diego State University, San Diego, CA 92182

In *Drosophila*, transcripts of the single MHC (myosin heavy chain) gene are alternatively spliced to generate the necessary isoforms for various muscle functions. To determine whether there are functional differences between these isoforms, flies were transformed with a P element containing an embryonic-specific cDNA under the control of the MHC promoter region. Expression of this MHC isoform produced a flightless phenotype in wild-type flies. However, flies transformed with the genomic MHC gene are capable of flight (unpublished data, Cripps, et. al.). Since actin to myosin ratios are critical for flight, various lines were constructed with different actin to embryonic myosin gene ratios. The embryonic myosin was not capable of restoring flight ability in any of these backgrounds; thus it appears to be the isoform itself, and not the expression levels that produce the phenotype. Electron microscopy reveals structural abnormalities in the transformed lines in both wild-type and null backgrounds, including fraying and fusing of the myofibrils. This effect is due to use and/or aging in the wild-type background, as the pupal myofibrils appear normal. The indirect flight muscle and embryonic MHC isoforms clearly cannot substitute for each other, indicating that the alternative exons confer unique functional properties.

**W 323** THE ROLE OF TITIN IN MUSCLE DEVELOPMENT AND SARCOMERE FORMATION, D. M. Wright*, M. Gautel@, S. Labeit@ & M. Peckham*. * Molecular Biology and Biophysics Group, King's College London, 26-29 Drury Lane, London, WC2B 5RL, UK and @ EMBL, D-69012 Heidelberg, Germany.

Titin is a large (mol. wt. 3,000kD) myosin-binding protein found in the thick filaments of cardiac and skeletal muscle. One molecule is about 1μ m long and spans the length of the half sarcomere; the N terminus of the protein is anchored in the Z disk and the globular C terminus binds to proteins within the M line. Sequence analysis has shown that most of the protein contains repeats of two classes of globular domains; an Immunoglobulin G II like domain (CI) and a fibronectin type III like domain (CII). There are at least four different functional regions in titin. In the I band titin is elastic, and may centre the thick filament in the sarcomere. In the A-band, super repeats of eleven of CI and CII domains in titin match the helical repeat of the thick filament, and may enable titin to regulate the length of the thick filament. In the A band/M-line region there is a functional serine/threonine kinase in titin, the substrate of which is unknown. In the M-line region of titin, there is a multiple phosphorylation site, the phosphorylation of which is developmentally regulated suggesting that it is important for the ordered incorporation of titin, from Z-disk to M-line, into the nascent sarcomere. To test some of these hypotheses, we are using gene targeting to either knockout the titin gene, or to truncate the titin gene at the 3' end in a novel conditionally immortal mouse myogenic cell line; *H2k^b*-tsA58 (see Morgan *et al, Dev. Biol.* in press). So far, we have obtained isogenic clones of titin for the 3' and 5' ends of the gene by screening a mouse genomic library in phage λ. These clones have been restriction mapped and fragments have been subcloned in preparation for making replacement vectors for gene targeting. These replacement vectors will be electroporated into *H2k^b*-tsA58 myoblasts. Cells in which the gene has been correctly targeted (as determined by PCR and southern analysis) will be allowed to form myotubes to analyse the effects of the mutation on sarcomere development, by light and electron microscopy.

**W 324** Myocyte enhancer factor 2 A (MEF2A) is associated with two proteins in cultured mouse skeletal muscle cells. Yie-Teh Yu, Division of Cardiology, Department of Medicine and Biochemistry. School of Medicine, Vanderbilt University, Nashville, TN 37232

The tissue-restricted transcription factor myocyte enhancer factor 2 (MEF2) is essential for the full activity of many muscle-specific enhancers and promoters in heart, skeletal muscle, and smooth muscle cells. Recent studies of transgenic mice have also confirmed that MEF2 activity is required for the development of skeletal muscle *in vivo*. Subsequent cloning of cDNAs encoding human and mouse MEF2 activity indicates that MEF2 activity comprises a multigene family of four members, MEF2 A, B, C, and D. The expression of MEF2 A, C, and D has been shown to be developmentally regulated in differentiated C2 cells, a mouse skeletal muscle cell line. In order to elucidate the molecular mechanisms by which the MEF2 activity is regulated during skeletal myogenesis, the expression of MEF2A, C, and D in three mouse skeletal muscle cell lines as well as the identification of MEF2A associated factors have been investigated.

Rabbit polyclonal antibodies against individual human MEF2A, MEF2C, and MEF2D polypeptides have been generated. The specificity of each MEF2 member-specific antiserum has been demonstrated by electrophoretic mobility shift assay and immunoprecipitation assay. These MEF2 member specific antisera have been used to study the expression of MEF2A, C, and D during skeletal myogenesis *in vitro*. In C2 myotubes, which had been maintained in the differentiation medium for three days, the expression of MEF2A was most abundant, followed by MEF2D, as shown in the immunoprecipitation assays. The expression of MEF2C was not detectable. Similar expression patterns were also observed in mouse skeletal muscle cell lines, BC3H1 and Sol8 cells, at similar stages of differentiation *in vitro*.

Interestingly, two proteins, P1 and P2, were co-immunoprecipitated with MEF2A in all three skeletal muscle cell lines. However, only P1 was co-immunoprecipitated with MEF2D. Thus, P1 and P2 are candidate MEF2A cofactors. The expression and identification of P1 and P2 are currently being investigated.

*Muscle Gene Regulation II*

**W 325** EXPRESSION OF ACETYLCHOLINESTERASE AND MYOSIN HEAVY CHAIN GENES DURING *IN VITRO* DIFFERENTIATION OF SATELLITE CELLS ISOLATED FROM FAST AND SLOW RABBIT MUSCLES, Francis Bacou, Catherine Barjot, Christiane Goblet*, Robert G. Whalen*, Omar Jbilo and Arnaud Chatonnet, INRA, Différenciation cellulaire et Croissance, 34060 Montpellier Cedex 01, and *Institut Pasteur, 75724 Paris Cedex 15, France
Among the factors responsible for initiating and sustaining differences between muscle fiber types, it has been proposed that intrinsic factors establish a state of myoblast commitment that defines the developmental fates of myoblasts. Recent studies have been focused on the existence of similar cellular processes during the differentiation of satellite cells (SC). Here we show that cultured SC isolated from fast (100% type II) and slow (100% type I) rabbit muscles do present differences in relation with muscle fiber type. The number of SC isolated per gram of muscle is lower in fast ($1.1x10^6$) than in slow ($2.7x10^6$) muscle. Under the same conditions of culture, "fast" SC proliferate at a lower rate and differentiate latter than "slow" SC. Thus, myogenin and MyoD mRNAs are expressed in cultured "slow" SC first, and appear before fusion in both types of cultures. Moreover, no adult myosin heavy chain (MHC) isoform is detected in "fast" SC cultured for 13 days, whereas cultures from "slow" SC express neonatal, and adult slow and fast isoforms at that stage. From the 16th day of culture onwards, neonatal and adult MHC are expressed in both types of cultures. Those made from fast muscle express neonatal and adult fast isoforms; those made from slow muscle express neonatal, and adult slow and fast MHC isoforms. Thus, only SC isolated from slow muscle express *in vitro* the adult slow MHC isoform. These results are shown on both proteins and their mRNAs. Acetylcholinesterase (AChE) activity and their mRNAs are expressed after fusion only, and their levels are higher in SC cultured from slow than fast muscles, as observed in the muscles themselves. However, both types of cultures present a similar AChE molecular form pattern.

**W 326 EXPRESSION AND REGLATION OF THE HUMAN CARDIAC TROPONIN I GENE.** Pankaj Bhavsar, Nigel Brand, Magdi Yacoub and Paul Barton, Department of Cardiothoracic Surgery, National Heart & Lung Institute, London SW3 6LY, UK.
The troponin complex plays a key role in the regulation of calcium-mediated contraction in striated muscle. In several species, including man, the cardiac isoform of Troponin I (TnIc) is expressed solely in cardiac muscle. We have examined therefore the expression of TnIc in the human heart as a model of cardiac-specific gene expression. We have identified a transition in Troponin I gene expression in the human heart such that during fetal development there is a switch from co-expression of both the slow skeletal and cardiac isoforms to exclusively the cardiac isoform soon after birth. Parallel changes in mRNA and protein abundance occur during this period suggesting that this transition may be regulated at the level of transcription. We have demonstrated that TnIc is encoded by a single copy gene located on chromosome 19p13.2-q13.2. The TnIc gene, which was isolated in a series of overlapping genomic phage, consists of 8 exons covering 6.2kb. The sequence of the promoter of this gene reveals several conserved sequence elements of potential interest including a putative MEF-2 binding site. Current experiments are aimed at determining the functional role of these elements through transient transfection studies into primary cardiocytes and a variety of skeletal muscle and non-muscle cell lines.

**W 327** HORMONAL AND NEURONAL CONTROL OF MHC EXPRESSION IN REGENERATING MUSCLE, K. J. Bockhold and R.G. Whalen, Department of Molecular Biology, Pasteur Institute, Paris, France.
The expression of different MHC isoforms is under the control of several factors, including the state of innervation and thyroid hormone levels. Muscle regeneration following injection of venom from the snake *Notechis scutatus scutatus* into rat soleus was used to study the role of innervation and excess thyroid hormone in influencing MHC gene expression. MHC protein and mRNA content were determined in muscles regenerating in the presence/absence of innervation or in the hyperthyroid state in order to further understand a) how these factors affect MHC expression in regenerating muscle and b) at what level e.g. transcription or translation. The mRNA analysis involved a qualitative (PCR) and a quantitative (slot blot hybridization) assessment of various isoforms of MHC. Protein analysis, using immunohistochemistry, was conducted on the same samples. This allowed for the identification of different MHC isoforms at different stages during regeneration.

Our results indicate that the initial expression of the β MHC gene is nerve independent; however, innervation is required for β MHC mRNA and protein accumulation. Innervation also acts to suppress the accumulation of the immature and fast isoforms of MHC. In the absence of innervation IIB and/or IIX MHC protein accumulate and large amounts of IIA MHC mRNA, but little of its protein. This suggests that (i) innervation suppresses IIA MHC mRNA accumulation and (ii) a regulatory mechanism exists which modulates II MHC protein accumulation. Thyroid hormone causes a transition to the fast isoforms of MHC, from β to IIA MHC in hyperthyroid regenerates and from IIX to IIB MHC in hyperthyroid/denervated regenerates. The results suggest that innervation plays a greater role than thyroid hormone in determining MHC expression in regenerating rat soleus muscle.

In summary, MHC production appears to be primarily controlled at the level of transcription or mRNA stability; however, translational or post-translational control may play a role for IIA MHC protein production. The results also suggest that satellite cells are not preprogrammed to express a particular MHC isoform, but rather are capable of expressing either fast or slow MHC. Subsequent expression of MHC isoforms is modified by factors such as innervation and thyroid hormone.

**W 328** ACTIVATION OF THE TYPE -A,B NATRIURETIC FACTOR GENES IN MOUSE P19 AND D3 STEM CELL CULTURES INDUCED FOR CARDIAC MYOGENESIS.
Poppo H. Boer*, William Lear* and Jenny Phipps. University of Ottawa Heart Institute and Department of Biochemistry*, 40 Ruskin Street, Ottawa, Ontario, Canada, K1Y 4E9, and National Research Council of Canada, M54, Montreal Road, Ottawa, Ontario, Canada K1A 0S2.

We examined the temporal transcriptional activity profiles of the gene for type-A and B natriuretic factor, ANF and BNF, and other cardiac muscle specific genes in cultured multipotential mouse cell lines. There exists no stable cardiac myocyte cell lines, and primary adult cells may be subject to de-differentiation and this complicates studies on control of cardiac muscle gene expression. We used P19 embryonal carcinoma cells and D3 embryonic stem cells which were induced for in vitro cardiac myogenesis and displayed spontaneous beating activity. RNA was isolated at regular intervals throughout the differentiation programs and on Northern blots it revealed abundant cardiac α-actin transcripts beginning at Day 6, reaching maximum levels during Days 7 to 8 and declining to low levels by Days 11 - 15. The transcriptional activation profile of the BNF gene, like that established for the type-A natriuretic factor gene, was similar to that of the induced α-actin gene, but there were quantitative differences that were best assayed by reverse transcriptase-mediated polymerase chain reactions. Myosin heavy chain α- and β-isoform genes were also transcriptionally activated in P19 and D3 developmental models. The induced BNF transcript levels in the P19 model system greatly exceed those induced in D3 cells and they may reach up to 15 - 20% that of adult mouse ventricular muscle tissue, whereas the P19 ANF gene was induced to a level about 10% that of adult atrial muscle. This opens up the possibility of defining regulatory control elements for ANF/BNF gene expression in stably transfected cell populations. Supported by the Medical Research Council of Canada and the Heart and Stroke Foundation of Ontario.

**W 329** SELECTIVE EXPRESSION OF VENTRICULAR (V) MYOSIN HEAVY CHAIN (MHC) IN DIFFERENT AVIAN SKELETAL MYOGENIC LINEAGES, Blanca Camoretti-Mercado, Smilja Jakovcic, and Radovan Zak, Department of Medicine, University of Chicago, Chicago, IL. 60637


We have shown that a MHC immunologically indistinguishable from the adult chicken ventricular isoform is present during early avian development of skeletal muscles and in regenerating adult anterior latissimus dorsi (ALD) muscle after cold injury. We have cloned the V-like MHC cDNA expressed in ALD muscle at 3 d. of regeneration which by nucleotide sequence comparison with V-MHC revealed 98% homology in the 3'-untranslated region (UT). Expression of V-MHC in primary and secondary myotubes as well as in quiescent satellite cells was analyzed by RNase protection assay. Total RNA was isolated from myotomes, forelimb (FL) and hindlimb (HL) at embryonic days (E) 4 to 10, from pectoralis muscle (PM), whole wing (W) and leg (L) muscles (devoid of skin) at E12 as well as from PM, ALD and L at E16, E17 and E19. Specific protected fragments were compared to those of the control adult V with a probe specifying the coding or the 3'-UT region of V-like MHC. Expression in the most rostral myotomes could be detected already at E4 which continued throughout the embryonic period and disappeared completely by E19. Presence of V-MHC mRNA could be first seen in primary myotubes of FL and HL at E5-6, and increased steadily up to E9-10. When secondary and quiescent satellite cells predominate, expression decreased and was no longer detected in L or W at E19. The same kinetics with high level of mRNA expression was also found in developing PM and leg muscles which is in contrast to regenerating adult muscles where activated satellite cells expressed minute amounts of V-MHC. These results show, that V-MHC transcripts are present in developing muscles at comparable levels, irrespective of their location and future load demands, however, there is a selective expression in embryonic myotubes but not in fetal myotubes or quiescent satellite cells.


**W 330** TRANSCRIPTION FROM THE CHICKEN $\alpha$ ACTIN PROMOTOR INCREASES IN RESPONSE TO STRETCH-OVERLOAD. James A. Carson, Zhen Yan, Robert J. Schwartz[1], Frank W. Booth & Craig S. Stump. Dept. Physiology & Cell Biology, The University of Texas Medical School, Houston Tx 77030. [1]Dept. of Cell Biology, Baylor College of Medicine, Houston Tx 77030.


The objective of this study was to determine the regulation of the chicken skeletal $\alpha$-actin gene in the intact chicken during hypertrophy of the anterior latissimus dorsi (ALD) muscle by determining; i) if transcription from the $\alpha$-actin promoter increased during hypertrophy induced by stretch-overload, ii) the region of the $\alpha$-actin promoter responsive to stretch-overload. Four deletions of the 2090 base pair $\alpha$-actin promoter (Mol. Cell. Biol. 6:2462, 1986)(-74, -98,-202,-424) were made. The right and left ALD muscles of three week old male chickens were injected with 50$\mu$g of an actin promoter-luciferase construct and 50 $\mu$g of RSVCAT in a volume of 20$\mu$l. Two days post injection the chickens had a weight (10% of their body weight) attached to the left wing, producing hypertrophy of the left ALD muscle, while the contralateral wing served as the control. Six days of wing weighting increased the mass of the stretch-overloaded ALD 90% above the contralateral control. The -2090 actin-luciferase chimeric gene significantly increased luciferase activity 209%, in the stretched (1.29$\pm$.26 TRLU/CAT) versus control (.61$\pm$.10 TRLU/CAT) ALD. The -424, -202 and -98 deletion constructs all had significantly higher luciferase activity in the hypertrophied versus the control ALD (229%,125%,225%, respectively). The -74 actin-luciferase construct did not show a significant difference in luciferase activity (-10%) between stretch and control wings. These data show that skeletal $\alpha$-actin gene transcription in the ALD muscle of the chicken is up-regulated during stretch-overload induced hypertrophy. The -74 to -98 base pair region of chicken skeletal $\alpha$-actin promoter, containing *serum* response element 1 (-83 to -92), could be responsible for the increase in $\alpha$-actin gene transcription during hypertrophy induced by stretch-overload in the chicken ALD muscle. Supported by NIH AR 19393.


**W 331** GENETIC MAPPING AND PATHOLOGY OF THE NEUROMUSCULAR DISEASE IN KYPHOSCOLIOTIC (KY) MICE. Coulton, G.R.[1], Skynner, M.J.[1], Barrett, M.[2], Moss, J.[2] Gangadharan, U.[3], Entwhistle, A.[4] Beckers-Bleukx, G.[5] Maréchal, G.[5] Brown, S.D.M.[3], van Mier, P.[6] & Mason R.M.[1] Dept. Biochemistry[1] & Dept. Histopathology[2], Charing Cross and Westminster Medical School, London, UK. Dept. Biochemistry, St. Mary's Hospital Medical School, London, UK[3]. Ludwig Institute of Cancer Research, London, UK[4]. Dept. des Physiologie, Université Catholique de Louvain, Brussels, Belgium[5]. Dept. Anatomy and Neurobiology, Washington University, St. Louis, USA[6].


Ky mutants exhibit a bilateral neuromuscular disease where predominantly slow twitch muscles undergo extensive post-natal necrosis and regeneration but regenerated fibres are smaller and weaker than normal. The most striking feature of ky is the extensive motornerve sprouting and complete loss to normal endplate structure. Pre- and post-synaptic abnormalities have been analysed by *in vitro* and *in vivo* (See van Mier et al., This meeting) visualisation of junction-associated proteins (JAPs: eg. AChR and 43kD) by immunohistochemistry or $\alpha$-bungarotoxin-FITC. Expression of a several JAPs was analysed by northern blot. There appears to be continual expression to the $\gamma$-AChR subunit in adult ky muscles. Both AChR and 43kD protein were abnormally distributed but were always in close association, suggesting that loss of AChR clustering *per se* is not the primary ky lesion. However, our data suggests that the ky mutation is in a protein which maintains junction integrity and function. Chronic remodelling of ky muscles is associated with sole expression of the slow myosin heavy chain in muscles such as soleus. The ky lesion appears to precipitate succession of changes in cell function resulting in progressive pathology culminating in spinal deformity.
An interspecific backcross segregating the ky mutation maps the ky locus to a small region of chromosome 9, ky is non-recombinant with the microsatellite marker D9Mit24 and lies in a conserved linkage group that encompasses human chromosome 3. It is striking that ky maps close to, but is recombinant with, a group of genes coding for junction-associated proteins including, s-laminin, collagen 7 and dystroglycan. Having identified the map position of ky we have eliminated all the obvious candidates for the ky gene. The ky mutation appears to lie in a gene coding for an as yet unidentified nmj-associated protein which causes the death of muscle fibres and massive neural sprouting.


**W 332** EXPRESSION OF THE MEF2 GENE FAMILY DURING MOUSE DEVELOPMENT, P. Cserjesi, D.G. Edmondson, G.E. Lyons*, J.F. Martin and E.N. Olson, Department of Biochemistry and Molecular Biology, M.D. Anderson Cancer Center, Houston, TX 77030, *Department of Anatomy, The University of Wisconsin Medical School, Madison, WI 53706


Activation of the myogenic program is regulated by members of the myogenic basic-helix-loop-helix (bHLH) family of muscle specific transcription factors. These factors act through a common DNA consensus sequence known as an E-box which is found in most, though not all, skeletal muscle specific genes. The recent finding that activity of the myogenin promoter requires an A/T rich DNA element which binds MEF2 suggests that a complex interaction between members of MEF2 and myogenic bHLH family of factors is involved in myogenic regulation. The regulation of genes lacking E-boxes and the activity of the skeletal muscle genes in cardiocytes, which apparently lack myogenic bHLH factors, is consistent with E-box independent regulatory pathways in both skeletal and cardiac muscle differentiation. A feature common to many genes regulated in an E-box independent fashion is the presence of a MEF2 regulatory site. It appears that the MEF2 family of transcription factors plays a role in both bHLH dependent and independent regulatory pathways. Recently, the four members of the mouse MEF2 family have been cloned. In order to better understand the role of MEF2 in muscle development, we undertook an *in situ* analysis of the developmental expression of all four members of the MEF2 family. Our analysis shows that the MEF2 family of genes is expressed in overlapping but distinct patterns of expression. Early expression in heart and somites suggest that the MEF2 family of transcription factors plays an important role in determination and tissue specific gene expression in these two muscle types.

**W 333 A CARP MYOSIN HEAVY CHAIN GENE THAT IS EXPRESSED IN SMALL MUSCLE FIBRES**

**Steven Ennion and Geoffrey Goldspink, Royal Free Hospital School of Medicine University of London, London NW3 2PF, U.K**

Whilst the expression of different isoforms of both the heavy and light chains of myosin are well characterised in mammals, little is known about the myosin isoforms present in fish. Work in our laboratory has focused on the genes coding for the myosin heavy chain protein in carp (Cyprinus carpio). We have constructed a carp genomic library and isolated 28 different lambda clones which contain myosin heavy chain (MyoHC) gene sequences. We present here the partial characterisation of one of the carp MyoHc isoforms we have isolated. The genomic clone FG2 was restriction mapped and partially sequenced to reveal the location of various exons. The clone contains a complete MyoHc gene which is approximately 12.0 k.b long and transcribes to a mRNA of approximately 6000 nucleotides. Analysis of sequence data generated from the exons of this clone revealed a high degree of homology with published mammalian skeletal muscle MyoHc genes. Intron sequences however are approximately 50% shorter than in mammalian MyoHc genes, explaining why at the genomic level the gene is about half the size of the mammalian MyoHc genes. Northern blot analysis using the 3' untranslated region of the gene showed that expression of the FG2 isoform is only observed in the white muscle of adult carp which have been subjected to an increase in environmental temperature. No expression was observed in carp acclimatised to 10°C. In Situ hybridisation studies using a digoxigenin labelled 3' untranslated region probe showed that expression of the FG2 isoform was limited to small budding like processes from the large white muscle fibres in adult carp acclimatised to 28°C. Muscle growth in fish occurs by both fibre hypertrophy and fibre hyperplasia whereas mammals are restricted only to fibre hypertrophy with no increase in fibre number once embryonic development is complete. We hypothesize that this isoform of the myosin heavy chain is expressed in the cells responsible for muscle fibre hyperplasia in fish. Supported by the NERC and SERC (UK).

**W 334 REGULATION OF CONTRACTILE PROTEIN mRNA LEVELS IS ALTERED IN RESPONSE TO SPACEFLIGHT**

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Ten rats were exposed to 9 days of zero-gravity aboard the NASA SLS-1 space mission (June 1991). Fast (EDL) and slow (soleus) muscles were removed upon return to earth. From these tissue samples, levels of the fast and slow isoform mRNAs from 6 gene families that encode contractile proteins of the thick and thin filaments were quantified. In the EDL muscle there was an increase in fast mRNA levels from 23 - 232% in all families except TnC, with no change in slow isoform mRNA levels. Changes were seen in most slow mRNA levels in the flight soleus muscle; TnCslow levels increased, and MLC1slowa, TnIslow, αTmslow, and MLC 2slow levels decreased. All fast mRNA levels increased in the flight soleus muscle from 217 - 1100% above control levels. These changes in isoform mRNA levels resulted in a shift to a faster mRNA profile for each gene family in the flight soleus muscle with no change in the flight EDL muscle. We can conclude that exposure to zero-gravity results in 1) an increase in fast contractile protein mRNAs in both fast and slow muscles 2) differing regulation of slow mRNA accumulation in the slow soleus muscle and 3) a transition to a faster mRNA profile for each contractile protein gene family in the soleus muscle. This suggests that exposure to non-gravity increases either the transcription or stability of fast contractile protein mRNAs in all muscles independent of phenotypic or morphological changes. In contrast, the response of slow mRNA levels was more complex among the gene families indicating unique regulatory mechanisms in response to zero-gravity exposure.

**W 335 MULTIPLE MCBF/TEF-1 mRNAs AND PROTEIN ISOFORMS ARE PRESENT IN CARDIAC AND SKELETAL MUSCLE,** Iain K.G. Farrance, Simonetta Ausoni* and Charles P. Ordahl. Department of Anatomy and Cardiovascular Research Institute, University of California, San Francisco, CA 94143.

M-CAT elements are required for the expression of many cardiac and skeletal muscle-specific promoters. The factor that binds M-CAT sites, MCBF, has been shown to be biochemically and immunologically related to TEF-1, a human transcription factor required for SV40 promoter activity. Multiple TEF-1 isoforms (TEF-1A, TEF-1B, TEF-1C, and TEF-1D) have been cloned from chicken cardiac muscle (see abstract by Larkin, S. B. et al.). Here we report on the expression of TEF-1 isoforms in muscle and non-muscle tissues by RNase protection, protein blotting and mobility shift PAGE. We find that TEF-1 transcripts are expressed at higher levels in cardiac and skeletal muscle relative to other tissues with the chicken homologue of human TEF-1 (TEF-1A) predominant. Mobility shift PAGE and protein blotting with TEF-1 antibodies show that multiple TEF-1 isoforms are expressed in all tissues examined with highest level in cardiac and skeletal muscle. Preliminary data show that one isoform and protein:DNA complex may be muscle specific. Experiments are in progress to determine which cDNAs correspond to each protein isoform and also to determine if TEF-1 is post translationally modified. (This work was supported by a NIH postdoctoral fellowship to I.K.G.F., by a Telethon (Italy) postdoctoral fellowship to S.A., and grants HL35561, HL43821 and GM32018 from NIH to C.P.O.)

*The first two authors contributed equally to the work in this abstract

**W 336 CHARACTERIZATION OF TWO MYOSIN HEAVY CHAIN GENES WHICH ARE TRANSIENTLY EXPRESSED DURING DEVELOPMENT AND PHYSIOLOGICAL ADAPTION,** **Geoffrey Goldspink, Thomas Jaenicke and Peter Butterworth, Royal Free Hospital School of Medicine, University of London, NW3 2PF, UK.**

Skeletal muscle is able to adapt to different activity pattern during growth and in response to altered physiological conditions. Adaptation can be achieved by quantitative as well as qualitative changes in gene expression. One of the most remarkable processes of adaptation takes place during development around birth, when the skeletal musculature becomes life supporting to the animal. On physiological/histochemical level, one observes a differentiation into fiber subtypes which are characterized by their ATPase rate and their oxidative capacity. We studied the regulation involved in this process and report here the isolation of two new myosin heavy chain isoforms, one expressed before this developmental change, the other transiently during adaptational processes. We screened a rabbit genomic library with myosin heavy chain (MyHC) unspecific oligonucleotide probes and isolated 54 independent clones. Two clones were randomly chosen for further studies. They both cover the N-terminus of the S1-head and code for so far MyHC isoforms genes. The first clone was identified as a neonatal isoform by both sequence comparison and in situ hybridization. For in situ hybridization experiments, a subclone (OC41S), covering the first 15 amino acids of the myosin genes - a highly variable region between isoforms - was prepared. This probe hybridized with tissue from soleus, tibialis anterior (TA) of two and four weeks old animals. The second clone was also characterized as coding for a skeletal muscle MyHC isoform by sequence comparison. Again, we subcloned the region coding for the first few amino acids (clone D7H9n) and studied its expression by Northern hybridization. This probe hybridized only to RNA from tongue, but not to RNA from the other studied tissues (embryonic leg, adult soleus and TA). Interestingly, in the rabbit TA muscle subjected to stretch immobilisation for two days, we detected a hybridization\signal with this probe. The pattern of the tissue distribution and its expression in stretch-stimulated muscle equals the pattern described on the protein level by Schiaffino and by Pette for the type IIc/d myosin. To study this further we subcloned the promoter region for this gene. Studies relating to its regulation of expression are in progress. Supported by the Wellcome Trust, UK.

**W 337** EXPRESSION OF SMOOTH MUSCLE MYOSIN HEAVY CHAIN IN A NOVEL VASCULAR SMOOTH MUSCLE CELL LINE FROM TRANSGENIC MICE HARBORING SV40 LARGE T ANTIGEN GENE, Kazuhide Hasegawa, Shoji Oda, Nobuaki Yanai*, Masuo Obinata*, Yuzuru Matsuda, Vessel Research Laboratory Co., Ltd., Tokyo, Japan, * Touhoku University, Sendai, Japan.

During development and arteriosclerosis, vascular smooth muscle cells (VSMCs) express three distinct phenotypes: embryonic and adult ones in normal vessel, and malignant one in arteriosclerosis. Smooth muscle myosin heavy chain (SM1) is known to be a reliable marker for the normal phenotype and is greatly reduced in arteriosclerotic lesion. However, the mechanism of the transformation in arteriosclerosis remains obscure mainly due to the lack of cultured VSMC of normal phenotype which retains SM1 expression.

In this study, we established a SM1-expressing VSMC line (SVS 30-2-6 cell) from transgenic mice harboring temperature-sensitive SV40 large T antigen gene. The transgenic mice method is new and powerful for establishing cell lines which retain differentiated phenotypes from various tissues. SVS 30-2-6 cells showed the hill-and-valley morphology and the temperature-sensitive growth. Moreover, the expression of not only SM1 but also α-actin and h-caldesmon was detected by immunofluorescent method and/or by western blot analysis. Thus this clone is the first one which retains differentiated characteristics of VSMC, especially the expression of SM1 and h-caldesmon.

In SVS 30-2-6 cells, we found that SM1 and non-muscle myosin heavy chain were expressed in a growth phase-dependent manner: SM1 was expressed scarecely in the growing phase but expressed markedly either in the stationary phase or at non-permissive temperature. On the other hand, the expression of non-muscle myosin heavy chain was abundant in the growing phase, and reduced either in the stationary one or in non-permissive condition. These results indicate the correlation of VSMC proliferation and the expression of myosin heavy chain isoforms, and that SVS 30-2-6 cells show two phenotypes (normal and malignant) in a growth phase-dependent manner.

In conclusion, SVS 30-2-6 cells are quite valuable for the study on the transformation of VSMC, and on the regulation of SM1 expression which is revealed to be closely related with VSMC proliferation.

**W 338** MUSCLE-SPECIFIC EXPRESSION OF THE RAT *MRF4* GENE IN *XENOPUS*, Timothy J. Hinterberger, Quentin Reuer, and Jesse L. Owens, Biomedical Program and Department of Biological Sciences, University of Alaska Anchorage, Anchorage, AK 99508

The tissue-specific transcription factor MRF4 is present in mammals and *Xenopus* exclusively in differentiated skeletal muscle cells, including both multinucleate myofibers and embryonic myotomal myocytes. In an earlier study to identify *cis*-acting regions that regulate tissue- and stage-specific *MRF4* gene expression, rat myogenic cells were transfected with rat *MRF4* gene clones which contained up to 8.5 kb of 5'-flanking sequence (Hinterberger et al., *Gene* 117:201, 1992). Transcription of the introduced *MRF4* genes increased substantially as the transfected myoblasts differentiated into myofibers, and the maximal increases required the presence of more than 1.5 kb of 5'-flanking sequence. However, unlike endogenous *MRF4*, low levels of mRNA from all of the introduced genes were detected in undifferentiated myoblasts and in non-muscle cell lines. To test whether the cloned genes would display similarly relaxed regulation when placed in cells in vivo, *LacZ* reporter constructs under the control of either 8.5 kb or 430 bp of *MRF4* 5'-flanking sequence were injected into 1- or 2-cell *Xenopus* embryos. The animals were examined for ß-galactosidase activity from 1 day to 2 months later. In this situation, activation of the cloned rat *MRF4* gene appeared to be regulated more stringently. Expression from the 8.5-kb construct was confined to skeletal muscle in both embryos and tadpoles and was developmentally regulated in parallel with endogenous *MRF4*. No expression was observed from the 430-bp *MRF4* construct, suggesting that *MRF4* activation in normally developing embryonic muscle cells requires upstream sequence elements not essential for expression in established cell lines in vitro.

**W 339** DIFFERENTIAL EXPRESSION OF $\alpha$1, $\alpha$2, AND $\beta$1 THYROID HORMONE RECPTOR GENES IN DEVELOPING RAT SKELETAL MUSCLE, Rebecca K. Hoffman, Mitchell A. Lazar*, Neal A. Rubinstein†, and Alan M. Kelly, Departments of Pathobiology, *Medicine, and †Cell & Developmental Biology, University of Pennsylvania, Philadelphia, PA 19104.

Skeletal muscle development follows an orchestrated pattern of expression of many muscle-specific genes. Many of these genes, including the myosin heavy chain (MHC) genes, occur as developmentally regulated and tissue-specific isoforms that have been shown to be responsive to thyroid hormone (triiodothyronine, $T_3$) in a highly muscle-specific manner. We have asked if the $T_3$-responsiveness of individual muscles may be due, in part, to the distribution of $T_3$ receptors in those muscles. Using northern analysis and ribonuclease protection assays (RPAs) we have examined the levels of expression of the $\alpha$1, $\alpha$2, and $\beta$1 $T_3$ receptor genes in developing soleus, extensor digitorum longus (EDL), and diaphragm muscles in the rat. We have found that all three receptors are expressed before birth; $\alpha$1 and $\alpha$2 message levels reach a maximum at postnatal days 15-20, then decrease gradually to adult levels. At all ages examined, $\alpha$1 and $\alpha$2 message levels are higher in EDL muscles compared to soleus or diaphragm. $\beta$1 message levels increase gradually from before birth to a maximum at postnatal day 20, then decrease to adult levels. $\beta$1 message levels are higher in soleus and diaphragm compared to EDL. In hypothyroid and hyperthyroid developing muscles $\beta$1 message levels are increased compared to control muscles by postnatal day 30. These changes in message levels are greater in soleus than in EDL; in the diaphragm, $T_3$ receptors show less change in response to $T_3$ levels. $T_3$ receptor expression is under neuronal control; postnatal denervation in the soleus and EDL results in a marked decrease in $\alpha$1 message levels, and a slight decrease in $\alpha$2 levels.

**W 340** SUPERFAST MYOSIN HEAVY CHAIN AND LIGHT CHAIN-2 GENES ARE SPECIFICALLY EXPRESSED IN CAT JAW MUSCLES, Joseph F.Y. Hoh and Han Qin, Department of Physiology, University of Sydney, NSW, 2006, Australia.

Jaw and limb muscles are derived from different lineages of myoblasts. In carnivores, primates and some bats, a unique superfast muscle fibre type is found in jaw muscles. Superfast fibres express a unique subset of myofibrillar proteins, namely, myosin, tropomyosin and C-protein. Transplantation experiments reveal that satellite cells of jaw and limb muscles are different lineages of myogenic cells capable of expressing different subsets of myofibrillar proteins, raising the question of how this is brought about.

As a first step in approaching this problem, we have cloned the superfast myosin heavy chain (MyHC) and light chain-2 (MyLC2) genes, using polyclonal antibodies against these proteins to isolate cDNA clones from an expression library in the λ-ZAP vector constructed from mRNA from cat jaw muscles. A 4 kb superfast MyHC clone was isolated and sequenced. A 609 bp MyLC clone encompassing the entire coding sequence was isolated. These sequences show only about 60% homology with published MyHC or MyLC2 sequences derived from limb or cardiac muscle, suggesting that superfast myosin is of very ancient origin. However our superfast MyLC2 showed 89% homology with a human MyLC2 sequence (hummyl5) recently submitted to the Genbank by Collins and Hayden. A comparison of the deduced amino acid sequences showed that our clone and hummyl5 comprise 173 residues, compared with 169/170 residues for mammalian limb skeletal MyLC2, the difference representing mainly an insertion of 4 residues in the N-terminal region and a deletion at the C-terminus. We conclude that our clone represents superfast MyLC2 and that hummyl5 is a superfast human homologue.

The expression of superfast MyHC and MyLC2 was studied by Northern blotting, using molecular probes which correspond principally to the 3'-non coding sequences of the clones. These genes were found to be expressed in cat jaw muscle, but not in cat limb or cardiac muscle.

**W 341** DNA TRANSFECTION ANALYSIS OF THE RABBIT SMOOTH MUSCLE MYOSIN HEAVY CHAIN (MHC) GENE PROMOTER AND UPSTREAM SEQUENCES. Robert C Kallmeier, Chandra Somasundaram and Philip Babij. Dept. Physiology, University College, London WC1E 6BT, England.

To study the regulation of myogenesis in vascular smooth muscle cells (VSMC) we have used the smooth muscle MHC gene as a model since its expression is known to be tissue-specific and developmentally regulated. A fragment of the rabbit smooth muscle MHC gene extending to -2.3kb upstream of the transcription start-site was isolated and sequenced in both directions. Several consensus DNA binding motifs were identified including six E-boxes and two CArG boxes. A set of 5' end deletion mutants was created all of which included the TATA box and ended at position -4bp from the start-site. The mutants were sequenced and ligated into the reporter gene pCAT Basic for transient transfection experiments in primary cultures of rabbit aorta VSMC. The highest relative CAT activity was obtained from mutant pRSMHC-1,328 which was 24-58 fold above control. Longer mutants extending upstream to pRSMHC-2,301 showed a decreased response but had similar activities that were still 17-29 fold greater than control. The relative CAT activity from mutant pRSMHC-1,221 which lies immediately downstream of pRSMHC-1,328 was very low, being only 3-9 fold above control. This relatively low level of CAT activity was also obtained from the three shortest mutants from -112 to -420. Mutants between -573 and -754 showed slightly higher activities above this basal level. To determine whether the measured CAT activities might be tissue-restricted, mutants were transfected into several other cell lines. In rabbit RK13 (kidney epithelial) and rat L6 (skeletal muscle) no CAT activity was detectable from mutants pRSMHC-2,301 and pRSMHC-1,328 (the most active in VSMC). Furthermore, in the rabbit aorta VSMC cell line Rb-1 which does not express smooth muscle MHC (unpublished), the level of CAT activity from the above two mutants was only 5-7 fold above control. These results suggest that smooth muscle MHC may be regulated by VSMC specific factors.

**W 342** DIFFERENTIAL EXPRESSION OF THE DIHYDROPYRIDINE RECEPTOR AND THE RYANODINE RECEPTOR DURING SKELETAL MUSCLE DEVELOPMENT. Jan Kyselovic, Abhijit Ray, John J.Leddy, Jeffrey Wigle and Balwant S.Tuana, Department of Pharmacology, University of Ottawa, Ontario, Canada K1H 8M5.
Excitation-contraction (E-C) coupling in skeletal muscle is critically dependent on the dihydropyridine (DHP) receptor and the ryanodine (RY) receptor. The DHP receptor operates as $Ca^{2+}$ channel and a voltage sensor in transverse tubules while the RY receptor is the $Ca^{2+}$ release channel of sarcoplasmic reticulum. It is believed that in adult skeletal muscle, a stoichiometric relationship exists between the DHP and the RY receptors such that the electrical signal may be physically transmitted from the DHP receptor to the RY receptor to induce $Ca^{2+}$ release. Little is known about the molecular basis of E-C coupling during early phases of muscle development. The developmental expression of the DHP and RY receptors in skeletal muscle was investigated during development of rat myotubes in culture as well as during embryonic and postnatal development in the rat. Through the use of specific gene probes and radioligand binding ([$^3$H]-PN 200-110 (DHP) and [$^3$H]-Ryanodine) assays, we examined the ontogenesis of the DHP and RY receptors. At early stages of development, the number of DHP receptors dramatically increased. The density was increased up to day 7 in tissue culture as compared with day 20 postnatally. After this time period, the number of DHP receptors remained at a constant level. This process was accompanied by a parallel increase of the 6.5 kb mRNA transcript encoding the $\alpha 1$ subunit of the DHP receptor. No change in affinity of the receptor for the DHP was detected over the development period studied. In contrast, appearance of the RY receptor plateaued at day 10 in culture compared with day 25 postnatally. The increased expression of the RY receptor coincided with increase number of RY binding sites. This development of the RY receptor was associated with a change in Kd value for RY in tissue culture and in postnatal development. The discrepancy between the time course of appearance of the DHP receptor and the RY receptor suggests that the mechanism of E-C coupling during early development is distinct from that in the adult. (supported by MDAC)

**W 343** TRANSCRIPTIONAL REGULATION OF THE MOUSE GENE ENCODING MUSCLE-SPECIFIC ENOLASE
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In birds and mammals, the glycolytic enzyme enolase (2-phospho-D-glycerate-hydrolyase) exists as dimers formed from three structurally related subunits, $\alpha$, $\beta$ and $\gamma$, encoded by separate genes. The gene for $\beta$-enolase subunit ("$\beta$-gene") is expressed only in striated muscles. We have previously shown that $\beta$-gene transcripts are first detected in the embryo, in the cardiac tube and in newly formed myotomes, and further accumulate at selected stages of foetal and post-natal muscle development in the mouse (A. Keller *et al* M.O.D. 38: 41-54, 1992 ; M. Lucas *et al* Differentiation 51: 1-7, 1992). In addition, $\beta$-gene belongs to an interesting subset of muscle-specific genes showing transcriptional activity in cultured replicating myoblasts, prior to the onset of terminal differentiation. The $\beta$-gene therefore provides a useful model sytem for investigating the molecular mechanisms involved in the multistep regulation of myogenesis. The studies reported here concern the mechanisms by which $\beta$-gene expression is regulated in cultured myogenic cells. We have simultaneously measured the rates of synthesis and degradation of $\beta$-mRNA in proliferating C2 myoblasts as well as in differentiating myotubes, using a procedure based on the isolation of newly-synthesized RNA following pulse-labeling in the presence of thiouridine (Johnson *et al*. Proc. Natl. Acad Sci USA 88: 5287-5291, 1991). The results show that the accumulation of $\beta$-mRNA in the course of terminal differentiation is primarily transcriptionally regulated. We have therefore initiated studies aimed at characterizing the cis-acting DNA sequences and cognate trans-acting factors responsible for the low transcriptional activity of $\beta$-gene in myoblasts as well as its increased activity in myotubes. We have cloned and sequenced the mouse $\beta$-gene and flanking regions and identified a single transcription start site. The upstream as well as intragenic regions show putative binding sites for trans-acting regulatory factors and their functional significance has been assessed by transient transfection analysis in C2 and in non-muscle cells. A 230 bp region present into the first intron was found to be indispensable for $\beta$-gene activity in myotubes and presents the properties of a muscle-specific enhancer. It includes consensus sequences for MEF2 and MyoD1 which form specific complexes with nuclear proteins, as shown by gel shift assays. A *detailed functional analysis of this enhancer is in progress.*

**W 344** TRANSCRIPTIONAL REGULATION OF THE *COX8H* GENE FOR CYTOCHROME c OXIDASE SUBUNIT VIII-HEART.
Margaret I. Lomax and Jeremy S. Dasen, Department of Anatomy and Cell Biology, University of Michigan Medical School, Ann Arbor, MI 48109.
Cytochrome *c* oxidase (COX), the terminal complex of mitochondrial electron transport, is critically important for oxidative metabolism in highly aerobic tissues such as heart and slow-twitch skeletal muscle. Mammalian COX is composed of 13 polypeptide subunits. Subunits I-III are encoded in mitochondrial DNA and carry out the catalytic functions; the 10 smaller subunits are encoded in nuclear DNA. Although the exact function of these nucleus-encoded subunits is unknown,. they may modulate cytochrome oxidase activity in response to different physiological signals or metabolic environments Bovine COX has tissue-specific isozymes: a heart (H) or contractile muscle isozyme, and a liver (L) or non-muscle isozyme. These isozymes contain different isoforms of three nucleus-encoded COX subunits: VIa, VIIa, and VIII. The H isoforms are found only in striated muscle such as heart and skeletal muscle and are thus heart/muscle isoforms. We are analyzing the transcriptional regulation of the bovine gene for COX subunit VIII-heart (*COX8H*) to indentify muscle-specific enhancer elements that regulate respiratory genes. The *COX8H* gene contains two small exons separated by a single 1.2-kb intron. The basal promoter of the *COX8H* gene contains a single TATA element and several putative muscle-specific enhancer sequences: tandemly duplicated E-boxes and several AT-rich sequences flanked by CC and GG dinucleotides that could be either CarG or MEF-2 binding sites. In transient transfection experiments, a 400 bp region of 5'-flanking sequence containing these elements and the transcription initiation site drives efficient expression of the CAT reporter gene in differentiated C2C12 myotubes. The COX8H and SV40 promoters are equally active in HeLa cells, but the COX8H promoter is approximately 5-fold more active than SV40 in differentiated C2C12 myotubes. Currently, deletion constructs are being tested to define the minimal promoter region and the transcriptional control elements in this region. Additional upstream and intron sequences from this gene are being tested functionally for muscle-specific enhancers.

**W 345** IN SITU mRNA DISTRIBUTION OF SARCO (ENDO) PLASMIC RETICULUM $Ca^{2+}$-ATPase ISOFORMS DURING ONTOGENY IN THE RAT,
Anne-Marie Lompré, Marielle Anger, Françoise Marotte, Lydie Rappaport and Jane-Lyse Samuel, INSERM U275-LOA, ENSTA-Ecole Polytechnique -91120 Palaiseau, URA CNRS 1131, Faculté des Sciences, 91405 Orsay and INSERM U127-Hopital lariboisière - 75010 Paris, France.
The existence of several isoforms of the sarco(endo)plasmic reticulum $Ca^{2+}$-ATPase (SERCA), encoded by three different genes and produced by alternative splicing of pre-mRNA transcripts, has been established by cDNA cloning. The two SERCA1 gene products were shown to be expressed in a developmentally regulated manner in skeletal muscle. One of the SERCA 2 mRNA isoform (2a) is expressed in adult cardiac, slow skeletal and some smooth muscles. The other SERCA 2 mRNA isotype (2b), as well as SERCA 3 mRNA were both observed in a broad variety of tissues. However, the cellular localization of these isoforms in adult tissues as well as the temporo-spatial evolution of their expression during ontogeny was unknown.

We have used *in situ* hybridization to determine the cellular distribution of three of these mRNA isoforms, SERCA 2a, SERCA 2b and SERCA 3 during rat ontogeny. We demonstrate that early in embryogenesis, SERCA 3 mRNA is highly expressed in the heart tube and is also present in the yolk sac. In 14-16 days embryos, SERCA 3 mRNA has disappeared from the heart but is expressed in the aorta and in discrete foci of the liver. Later on, its expression is restricted to the arterial endothelium and to some epithelial cells. SERCA 2a mRNA is coexpressed with SERCA 3 mRNA in the heart tube and remains expressed in the cardiomyocytes throughout life. It is transiently expressed in skeletal muscle at the onset of differentiation. In early fetal life, SERCA 2b is expressed in the mesenteric area and thereafter in all cell types at various levels. Our data indicate that, 1) expression of SERCA 2b is neither tissue-specific nor developmentally regulated 2) expression of SERCA 2a and SERCA 3 isoforms is regulated in a cell specific manner during development and suggest that the SERCA 3 gene plays a role in controlling the function of endothelial cells during vasculogenesis.

**W 346** CLONING MUSCLE-SPECIFIC GENES FROM ENHANCER TRAP LINES IN *DROSOPHILA MELANOGASTER* ,
Michelle Mardahl, Nancy Drain, Sanford I. Bernstein, Greg L. Harris, Molecular Biology Institute, San Diego State University, San Diego, CA 92182
We are interested in characterizing proteins that are important in muscle structure or function and to learn how these proteins interact and are used differentially in individual muscle types. P-element-mediated enhancer detection in *Drosophila melanogaster* was used to identify genes that are expressed specifically in muscle. This technique utilizes a reporter gene contained within a transposable element that has inserted at random in the genome. Transcriptional regulatory elements located near the site of insertion will influence the expression of the reporter gene in a tissue specific or developmental manner. An advantage of this detection system is its design to facilitate rapid cloning of the flanking genomic region in the vicinity of insertion by plasmid rescue. Two enhancer trap lines that exhibited muscle-specific staining of the *lac-Z* reporter gene product in cryo-sectioned adult flies were used in this study. Both lines express the reporter gene strongly in the indirect flight muscle but not in other muscles. The position of the P-element insertions have been mapped on polytene chromosomes by *in situ* hybridization. One line mapped to 22AB, which is an area not previously noted to contain a muscle-specific gene, and the other maps to 66D, close to the paramyosin gene location. Several mutants for each enhancer line that have been generated by P-element excision are homozygous lethal indicating that these genes may be required for viability. The plasmid rescue procedure was used to isolate genomic DNA from each of the gene regions. These clones are being used to isolate cDNA clones which may correspond to the muscle-specific genes. cDNA clones will be analyzed by DNA sequencing.

**W 347** DELINEATION OF THE CARDIAC TROPONIN T EXPRESSION PATTERN DURING MURINE DEVELOPMENT,
John J McAuliffe, Erica Roulier, Bruce Aronow, and David Witte. Department of Anesthesia, University of Cincinnati and Childrens' Hospital Research Foundation, Cincinnati, OH 45229
Cardiac troponin T is expressed during fetal development in both heart and cardiac muscle. In heart the slow migrating, "embryonic" ,isoform has been previously found to be expressed first and the faster "adult" form subsequently (in rat and chick). The pattern of isoform expression was determined using PCR analysis of RNA from whole embryos and embryo tissues and *in situ* hybridization of embryo sections using cTNT isoform specific probes . Additionally the murine cTNT gene was cloned and the 5' flanking sequence and first intron sequenced. A parental transgene construct consisting of 4.6 kb 5' flanking sequence and the entire first intron with luciferase as a reporter gene was injected into FVB/N pronuclei and the resulting lines were analyzed for tissue specific and developmental expression patterns. The results indicated that the "adult" isoform of cTNT is the first expressed in the embryo at 7.5 - 8 day pc. The embryonic isoform appears at about 9-9.5 day pc at which time both forms are coexpressed in heart. The "adult" form ceases to be expressed at 10 days and then reappears at 11.5 days in heart but not in somites. This pattern of expression has not been previously described. Early somites and limb buds contain exclusively the embryonic isoform but at later stages both isoforms are coexpressed in skeletal muscle. Skeletal muscles expression begins to wane just before birth. The transgene was expressed at high levels (5 orders of magnitude above baseline) in adult heart with little expression elsewhere. Expression was copy numbers dependent in three of four lines; the non dependent line exhibited some ectopic expression. In the copy dependent lines, mid gestation embryo expression in heart was less than in adult heart and expression in limbs and axial skeleton was 3 orders of magnitude above baseline compared to 2 orders at 3days post birth. The parental construct appears to direct high level and specific expression of the reporter gene.

**W 348** ANALYSIS OF THE MYOSIN HEAVY CHAIN GENE FAMILY IN DIFFERENT CANINE BREEDS
**Vidyanand Mohan-Ram and Geoffrey Goldspink, Royal Free Hospital School of Medicine, University of London, NW3 2PF, UK.**
Comparative molecular biology studies in our laboratory have shown how muscle genes can result in different contractile characteristics in different muscles across species. This work is now being applied to different breeds of dog, a species within which there are wide variations in speed, strength and stamina. The work in our laboratory has focused on the 5'-end of the canine muscle myosin heavy chain (MyHC) gene which encodes the myosin crossbridge to determine how form and function have been selected for at the gene level. This approach has begun to yield results that can be used in breed and dog type identification. Due to extensive sequence homology across species we are using human MyHC PCR primers to amplify a region which includes the ATP-binding site and hypervariable loop of MyHC isoforms from canine genomic DNA. We have found that two PCR products of approx. 0.9kb and 0.8kb are amplified from all canine samples. However, we have also identified an additional 0.6kb product that is consistently amplified from unrelated pitbull terrier samples. Southern blot analysis using an internal probe has confirmed that the products are myosin ATP-binding sites. These products are now being subcloned and sequenced and compared with other known S1 sequences. Restriction analysis of the PCR products offers refinement of the technique. By using MspI we generate extra fragments from only those amplifications containing the 0.6kb product. So far this approach has provided a potential means by which the pitbull fighting dog can be distinguished from other canine breeds. This work is supported by th UK Kennel Club.

**W 349** ISOLATION OF cDNAs ENCODING FAST AND SLOW ISOFORMS OF LOBSTER MYOSIN HEAVY CHAIN,
Donald L. Mykles and Julie L.S. Cotton, Department of Biology, Colorado State University, Fort Collins, CO 80523
Slow- and fast-twitch fibers from the striated muscles of the American lobster, *Homarus americanus*, contain different isoforms of myosin heavy chain (HC), paramyosin, and troponin. In addition, fast fibers possess a 75-kDa regulatory protein that is not expressed in slow fibers. We have isolated cDNAs encoding fast and slow myosin HCs, 75-kDa protein, and tropomyosin. The deduced amino acid sequences of the myosin HCs and tropomyosin clones show high similarities to those genes isolated from other species. The fast myosin HC cDNA (1.5 kb) codes for the last 413 residues of the protein and is 73% identical to myosin HC from *Drosophila* striated muscle. The slow myosin HC cDNA (4 kb) was partially sequenced; the deduced amino acid sequence of the terminal 92 residues is 84% identical to that of the lobster fast myosin HC. The 3' untranslated regions (UTRs) of the two clones share little identity, thus making it possible to synthesize isoform-specific probes for northern and southern blot analyses. The tropomyosin cDNA (1.4 kb) contains the coding region for the C-terminal half (143 amino acids) and is 81% identical to *Drosophila* tropomyosin. The 75-kDa cDNA (2.4 kb) showed no strong sequence identity to any protein in the database. Northern blot analysis showed that fast myosin HC and 75-kDa protein are only expressed in fast fibers, while tropomyosin, which shows no isoformic diversity, is expressed in both fiber types. Southern blot analysis using a probe synthesized from the 3' UTR of the slow myosin HC cDNA showed exclusive hybridization with slow myosin HC clones. *In situ* hybridization showed that the myosin HC message is largely confined to the intermyofibrillar space and the subsarcolemmal cytoplasm of the fiber periphery and sarcolemmal infoldings. With probes specific for each of the slow or fast myosin HC mRNAs, we will examine the fiber-type-specific expression of myosin HC genes during fiber transformation. The predictability of transformation in this species may allow the eventual isolation of genes that initiate and coordinate myofibrillar protein isoform expression. Supported by NSF (DCB-8958270) and NIH (RR-08041).

**W 350 THE EFFECTS OF DENERVATION ON THE ACTIVATION STATE, SUBUNIT PROTEIN AND mRNA LEVELS OF PHOSPHORYLASE b KINASE.**
Dean C. Ng and Donal A. Walsh. Department of Biological Chemistry, School of Medicine, University of California, Davis, CA 95616.

Phosphorylase kinase contains of four each of four different subunits, α, β, γ, and δ. The α, β, and γ subunits are unique to this enzyme and the δ subunit is calmodulin. Denervation decreases enzyme level in muscle. The mechanism of this down regulation is unknown. In this report, we have examined the effects of denervation on the **total activity** and **activation state** of the enzyme and the **protein** and **mRNA** levels of each subunit.

The total enzyme activity is reduced to 30% of control value 8 days after denervation. Denervation did not cause any significant increase of 6.8/8.2 activation ratio suggesting that the holoenzyme is not activated prior to degradation. The amount of each of the three unique subunits is reduced to a new plateau of 10 to 40% of control value by day 8.

Is the reduction of each subunit due to the reduction of its mRNA? The γ subunit mRNA decreases to a steady state level of 20% of control value 3 days following denervation. The reduction of the γ subunit mRNA precedes the reduction of activity and subunit protein levels by about 5 days. Whereas, in contrast, the mRNA levels for the α and β subunits did not diminish significantly for at least 13 days.

Denervation thus reduces the amount of each subunit protein but only the mRNA level of the γ subunit. While the reduction of the catalytic subunit is sufficient to account for the reduction of the total enzyme activity in muscle, the data presented here suggest that the stability of the α and β subunits may depend on the presence of the γ subunit. The activation ratio, which would be high if free gamma subunit were present, is low throughout the time course of this experiment suggesting that the gamma subunit is not release as an active protein during denervation induced degradation of phosphorylase kinase.

**W 351** SEARCH FOR MYOGENIC FACTORS IN A CARDIAC TUMOR CELL LINE, Shi Niu, Parker B. Antin, Irwin L. Flink and Eugene Morkin, Departments of Medicine, Pharmacology, Physiology, and the University Heart Center, University of Arizona College of Medicine, Tucson, AZ 85724
Myc-embryonic quail cardiomyocytes (MEQC) is a cell line derived from 3-day quail embryo hearts transformed with v-myc. MEQC can be induced to express muscle-specific proteins by co-culture with NIH 3T3 cells, suggesting that this cell line contains determinants of cardiac myogenesis. The expression of muscle-specific proteins is inhibited, however, when MEQC is treated with bromodeoxyuridine (BUdR) before co-culture. Transcription of skeletal myogenic factors, such as MyoD, is inhibited by treatment with BUdR, blocking differentiation in skeletal myoblasts. We hypothesize that the failure of MEQC differentiation in the presence of BUdR may be caused by the inhibition of transcription of cardiac myogenic determinants. Three cDNA clones, pX10, pX27, and pX19 have been isolated from a stage 17 chick heart cDNA library using a probe prepared by subtractive hybridization of mRNAs from untreated and BUdR-treated MEQC cells. Northern blotting demonstrates that mRNAs encoded by pX10, pX27, and pX19 are down-regulated by treatment with BUdR. By sequence analysis pX10 is a calcium-dependent neutral protease. The COOH-terminal portion of pX27 is homologous to human lung fibroblast and rat brain heparin sulfate proteoglycan core protein. No homology has been found between pX19 and any gene in the current database. During chick embryogensis, the mRNAs encoded by pX10, pX27 and pX19 are expressed in multiple tissues. Possibly, one or more of these proteins may play a role in cardiac myogenesis.

**W 352** ALTERATIONS IN CONTRACTILE PROTEIN GENE EXPRESSION IN RESPONSE TO TARGETED ABLATION OF ALPHA TM STR EXONS IN EMBRYONIC STEM CELLS. Pajak L., Howles P., Doetschman T., and D.F. Wieczorek. Department of Molecular Genetics, Biochemistry, and Microbiology, University of Cincinnati Medical School, Cincinnati, Ohio 45267.
Alpha Tropomyosin (alpha TM) is a member of a multigene TM family generating many tissue and developmentally regulated isoforms. The striated muscle isoform of alpha TM (alpha TM str) is expressed in both skeletal muscle and cardiac muscle, where it is the predominant TM isoform expressed in the adult myocardium. Previous analyses of TM gene expression during in vitro differentiation of embryonic stem (ES) cells demonstrate that nonmuscle, smooth muscle, striated muscle and neural isoforms of TM can be detected; the temporal expression of these isoforms coordinates well with developing structures including neural cells and a primitive myocardium. Specifically, the alpha TM str isoform is initially detected on day 5 in differentiated ES cells which is prior to the onset of muscle contraction, a phenomena also observed during murine cardiac development. To further elucidate the role of alpha TM str in development and function of the cardiomyocyte, the alpha TM str exons were ablated via homologous recombination in ES cells. Analysis of targeted alpha TM str ES cells demonstrates that these cells differentiate to the same extent in vitro as wild type ES cells as indicated by similar percentages of contracting EBs observed during differentiation of targeted and wild type ES cells. A molecular analysis of TM expression is being performed on targeted and wild type ES cells to determine alterations and compensatory events in TM gene expression. Additionally, quantitative RT-PCR experiments demonstrate that atrial myosin light chain and cardiac troponin C transcript levels are reduced in the alpha TM str targeted ES cells suggesting coordinate regulation of contractile protein gene expression occurs in ES cells.

**W 353** EXPRESSION OF MYOSIN HEAVY CHAIN GENES IN NOVEL CONDITIONALLY IMMORTAL MOUSE MYOGENIC CELLS *H2K*$^{b}$-tsA58.
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A novel cell line has been isolated (Morgan *et al*, Dev. Biol. in press) from the transgenic mouse *H2K*$^{b}$-tsA58 (Jat *et al*, 1990 P.N.A.S. **88**, 5096-5100).The cells have a single copy of the temperature sensitive SV40 virus T-antigen gene under transcriptional control of the γ-interferon (γ-IF) promoter (*H2k*$^{b}$). Cells isolated from neonatal mouse muscle grow at 33$^{o}$C + γ-IF (T-antigen produced and stable) and differentiate into myotubes at 39$^{o}$C - γ-IF (T-antigen not produced and unstable). Cell division stops within 24 hours at 39$^{o}$C. Sarcomeres form at about 3 days, and mature striated myotubes form by 7-8 days. About 80 clones have so far been isolated. 4 of these have been analysed for expression of the myosin heavy chain (MHC) genes by RNAse protection assays (probes to MHC kindly donated by M. Buckingham), by western analysis and by immunostaining (antibodies kindly donated by H. Blau). We found that the myoblasts do not express MHC. Embryonic myosin is expressed in the first 24 hours after the switch to 39$^{o}$C. Neonatal myosin was found in 7 day myotubes. However, there were some clonal differences. We intend to use this information to target the expressed genes (by homologous recombination) to look at the effects of mutations in these genes on muscle development and contraction in these cells.

**W 354** REGULATION OF ADULT MYOBLAST-SPECIFIC GENE EXPRESSION, Charlotte A. Peterson and Jane M. Taylor, Departments of Medicine and Biochemistry and Molecular Biology, University of Arkansas for Medical Sciences, McClellan Veterans Hospital, Little Rock, AR 72205
In adult muscle, satellite myoblasts are responsible for the maintenance of skeletal muscle mass. These cells proliferate and differentiate in response to muscle damage or degeneration, thereby regenerating muscle fibers. Factors that actively regulate gene expression in myoblasts have not been described, but such regulators may play a role in specifying the satellite myoblast lineage. Muscle regulatory molecules identified to date (MyoD, Myogenin, Myf-5, MRF4) only activate muscle-specific gene expression upon differentiation. We have identified a muscle-specific gene product, β-enolase, that is expressed not only in differentiated myotubes, but also in proliferating adult myoblasts, thus providing the marker necessary to identify the molecular mechanisms that control gene expression in adult myoblasts. The *cis*-regulatory DNA sequences responsible for controlling the human β-enolase gene are currently being defined. Through analysis of the expression of reporter gene constructs containing different amounts of DNA flanking the β-enolase gene following transfection into cells, we have identified a 230 base pair region of DNA which promotes high level expression in myoblasts but not in fibroblasts. This region is not active following myoblast differentiation suggesting that different regions of DNA control β-enolase gene expression prior to and following differentiation in skeletal muscle. Furthermore, this region promotes myoblast-specific expression in association with a heterologous promoter. The 230 bp β-enolase *cis*-regulatory DNA sequence is now being analyzed for the presence of binding sites for regulatory factors. Proteins that bind to the novel regulatory DNA sequences will be isolated from an adult human myoblast cDNA expression library and their role in skeletal muscle will be characterized.

**W 355** THE ANTISENSE FUNCTION OF A CLONED SUBSPECIES OF THE CYTOPLASMIC TRANSLATION INHIBITORY RNA OF CHICK EMBRYONIC MUSCLE, Satyapriya Sarkar, Arpad Molnar, Yan Du and Z-C Zheng, Department of Anatomy and Cellular Biology, Tufts University Health Science Campus, Boston, MA 02111
Previous work by us has shown that a heterogeneous cytoplasmic translation inhibitory RNA (iRNA; 60-150 nucleotides size range) is present as RNP particle (iRNP) in chick embryonic muscle. The iRNAs act as a potent inhibitor of the initiation phase of mRNA translation in vitro. One resolved iRNA subspecies strongly inhibits the translation of muscle mRNAs in a discriminatory manner in contrast to non-muscle mRNAs. A cDNA library for iRNA was prepared in pUC18 vector. The 114 nucleotide insert of one clone was subcloned in the pBluescript vector. The in vitro transcript of this insert (only the 5'-3' and not the reverse orientation) showed inhibition of muscle poly A$^{+}$ mRNA translation in vitro. The derived sequence of the iRNA indicates that it is a new sequence in which 27 nucleotides at the 3' and 5' ends can form base paired stem. This iRNA has complementary (antisense) sequences involving about 100 nucleotides to a domain of 28S RNA which is highly conserved in eukaryotes e.g. yeast, slime mold, plant, vertebrate and mammals. The 5'-3' in vitro transcript hybridizes to a 120 nucleotide iRNA component of iRNP. The iRNA also shows antisense complementarily of about 15 nucleotides to a 5' leader segment of myosin light chain 2 mRNA. Since 28S rRNA and 60S ribosomal subunit is involved in initiation complex formation, these results suggest that translational inhibition mediated by the antisense iRNA may act as a novel mechanism of controlling cellular mRNA levels during the transition of embryonic to adult muscle. The iRNA action may alter the cellular translation patterns in conjunction with the transcriptional control of myogenesis.

**W 356** EXPRESSION PATTERNS OF MUSCLE TRANSCRIPTION FACTORS DURING REGENERATION. Edward Schultz, Karen M. Krabbenhoft and Gary E. Lyons Department of Anatomy, University of Wisconsin, Madison, WI 53706
Intact (IS) or denervated (DS) rat soleus muscles were induced to regenerate by injection of the myotoxin, Notexin (10μg/ml). Muscles were collected at various time points between 0-28 days and examined for the expression of embryonic MHC, myogenin, MyoD, myf5, MEF2C and MEF2A by in situ hybridization. In both IS and DS muscles each of the genes exhibited a significant increase in expression during the first 0-4 days post injection. Expression of embryonic MHC, myogenin, MEF2C remained high through at least 10 days then gradually decreased between 10-28 days. At 28 days expression was still present in isolated fascicles of the muscle. In general, expression tended to appear first at the peripheral margins of the muscle where it was very punctate and over individual cells at the periphery of myofibers, presumably first over satellite cells and then over satellite cell-derived myoblasts. At later intervals expression was distributed throughout the muscle belly in both a punctate and diffuse pattern over regenerated myofibers. MyoD, myf5 and MEF2A were not detected or were present at very low levels in IS muscles beyond 10 days. Denervation altered the expression pattern of some but not all of the factors examined. Denervation had the most pronounced effect on myogenin and MEF2C each of which remained at their highest levels of expression throughout the 28 day regeneration period studied. MyoD was also elevated in DS muscles when compared to IS but not to the extent of myogenin and MEF2C Denervation had the least effect on MEF2A and myf5 which remained at low levels beyond 10 days in both IS and DS. These studies suggest that MyoD in regenerating rat soleus muscles shows a similar expression pattern to that reported in mixed mouse muscles but myogenin and MEF2C are expressed at high levels for a longer duration than in mixed muscles. The co-expression of MEF2C and myogenin is consistent with the possible regulatory role of MEF2 in myogenin expression. Finally, these studies also suggest that nerve plays a role in the down-regulation of all factors examined, with the possible exception of MEF2A and myf5.
Supported by NASA NAG 2-671, NSF DCB9017085 (ES) and the Muscular Dystrophy Association (GEL).

**W 357** ANALYSIS OF DIPTERAN ACTIN GENES SUGGESTS CONSERVATION OF REGULATORY ELEMENTS BETWEEN VERTEBRATES AND INVERTEBRATES,

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We have sequenced the coding as well as 5' and 3' flanking regions of a housefly, *Musca domestica (Md)* actin gene, *Mdact1*. Comparison of the coding sequence reveals a 17.2 and 18.1% difference between it and the *Drosophila act79B* and *act88F* genes, respectively. At the level of deduced amino acid sequence, *Mdact1* differs from *act79B* by 1.6% and from *act88F* by 4.8%. In the former comparison all six substitutions are conservative, while in the latter case 5 of 18 amino acid differences are not. The intron position at codon 307 is identical to that observed in *act79B* and *act88F*; there is no length or sequence conservation between these introns. There is no sequence conservation in the 3' transcribed untranslated regions. The 5' flanking region of *Mdact1* harbors sequences that resemble the TATA box and transcription initiation site of *act79B* in sequence and position relative to the translation initiation codon. There are also several CArG box-like elements, found in both *act79B* and *88F*. These elements are present in all vertebrate actin gene promoters studied to date, as well as in several contractile and non-contractile protein genes where their regulatory function was demonstrated. Furthermore, the E boxes (binding sites for myogenic bHLH proteins) found in promoters of vertebrate muscle specific genes, are present in the 5' flanking region of *Mdact1* gene and *act79B* and *88F* genes. The conservation of these elements between vertebrates and invertebrates suggests they may perform similar roles in the housefly. The marked similarities in the deduced amino acid sequences as well as the putative TATA box, transcription initiation site and polyadenylation signal, which are absent from the corresponding regions of *act88F*, suggest that *Mdact1* is a functional ortholog of the *Drosophila act79B* gene.
(Supported by NIH Grant GM 08167-14)

**W 358** ISOLATION AND CHARACTERIZATION OF PORCINE MYF GENES

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The different members of the MyoD (myf) gene family fulfil key functions in the determination and differentiation of myocytes towards myofibres, the number of which has been related to growth capacity in meat producing animals. We are interested in the occurence of allelic variation within the porcine myf genes and their association with differences in muscle fibre numbers and growth capacity within pig breeds, which might be useful in breeding programs.

We started the isolation and characterization of the porcine myf genes. A myf-4 specific PCR reaction on porcine genomic DNA enabled the cloning and sequence determination of the coding and intron regions of the porcine myf-4 gene. Comparison with the human and mouse myf-4 sequences revealed great homology within the coding regions (more than 93%). Screening of a porcine genomic library with the human myf-cDNA fragments (ATCC) resulted in the isolation and purification of 37 myf-positive clones. Rapid identification of the myf-4 gene containing clones was performed by myf-4 specific PCR amplification. Presently these clones are further characterized (sequence determination of the 5' and 3' non-coding regions of the myf-4 gene) and used for (1) chromosome localization of the myf-4 gene by *in situ* hybridization and (2) generation of specific probes for RFLP-analysis.

**W 359 Transcriptional Repression of the MCK Gene in Mechanically Overloaded Skeletal Muscle of Transgenic Mice.**

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Adult striated muscle demonstrates remarkable adaptability in response to a broad range of physiological and pathophysiological stimuli. However, the molecular pathways and regulatory molecules which underlie skeletal muscle plasticity remain obscure. To better understand this process we have examined the molecular mechanism(s) which govern the expression of the mouse muscle creatine kinase (MCK) gene in mechanically overloaded plantaris muscle of transgenic and nontransgenic mice. Northern blot analysis revealed that the level of endogenous MCK specific mRNA transcripts were repressed 3.5 fold in the overloaded plantaris muscles of nontransgenic and transgenic mice after 2 days of overload. To identify the cis-acting DNA sequence(s) involved in the repression of MCK gene expression by mechanical overload, multiple independent lines of three different mouse MCK-CAT transgenes were studied. Analysis of transgene expression levels as measured by chloramphenicol acetyltransferase activity (CAT assays) identified an upstream DNA fragment located between nucleotides -3300 and -1256 (transgenes E21 and E10) which strongly represses (5.6 fold) MCK gene transcription. Expression of transgenes DL16 (-1256 to +7) and E7A (E-117 to +7) revealed a modest trend (2 fold) toward repression. These findings where further supported when CAT mRNA transcripts representing each transgene were measured by Northern blot. To determine if endogenous MCK protein was repressed we analyzed protein extracts from control and overloaded muscles. Results demonstrate that the MM-CK isozyme (100% in skeletal muscle) activity or quantity was repressed (2.6 fold) in mechanically overloaded plantaris muscle while other MCK isoforms (MB or BB) were not induced to compensate. These data provide evidence that the mouse MCK gene is transcriptionally repressed in response to work overload, and that this event is governed by upstream regulatory sequences which are distinct from those which direct muscle-specific and differentiation-specific expression of the MCK gene.

**W 360** MUSCLE CELLS DERIVED FROM DIFFERENTIATION OF EMBRYONAL CARCINOMA CELLS EXPRESS THE CARDIAC ISOFORM OF THE $\alpha_1$ SUBUNIT OF THE DIHYDROPYRIDINE RECEPTOR.

Balwant S. Tuana, Elaine Petrof, Jan Kyselović and Christine Pratt, Department of Pharmacology, University of Ottawa, Ottawa, Canada K1H 8M5.

Pluripotent embryonal carcinoma cells (P19 cells) can differentiate into muscle cells in the presence of DMSO. During differentiation into muscle, the cells acquire characteristics of embryonic cardiac myocytes with the onset of spontaneous contractile activity at days 6 to 7 in culture. In order to understand the developmental aspects of the mechanism of excitation-contraction (E-C) coupling in these cells we have investigated the presence and expression of the dihydropyridine (DHP) and ryanodine (RY) receptors during differentiation. Ligand binding studies with PN200-110 indicated a time dependent increase in DHP binding sites upon DMSO treatment, which plateaued between days 7 & 9 and saturation isotherms indicated a single high affinity binding site with a $K_D$ of 0.1 nM. DMSO differentiated P19 cells were found to express the cardiac specific $\alpha_1$ subunit of the DHP receptor using RT-PCR and Northern blot analysis. The appearance of the 9 kb mRNA transcript encoding the $\alpha_1$ subunit increased during differentiation and coincided with the increase in the number of DHP binding sites and the onset of spontaneous contractile activity. The skeletal muscle isoform of the $\alpha_1$, $\alpha_2$ and $\beta$ subunits of the DHP receptor were not expressed in these cells. Ligand binding studies with RY and Northern blot analysis with RY receptor cDNA suggested that neither the cardiac or skeletal muscle isoforms of the RY receptor was expressed in these muscle cells. The spontaneous contractile activity in these cells was abolished by the application of the DHP's suggesting that the cardiac isoform of the $\alpha_1$ subunit of the DHP receptor was operating in the apparent absence of the $\alpha_2$ subunit to deliver $Ca^{2+}$ sufficient to trigger contraction in these cardiocytes.

**W 361** SYNAPTIC REMODELING STUDIED OVER TIME IN LIVING KYPHOSCOLIOTIC (KY) MICE, Peter van Mier.
Department of Anatomy and Neurobiology, Washington University School of Medicine, St. Louis.
Kyphoscoliotic mice display a neuromuscular disorder in which the body musculature undergoes extensive atrophy in the first six postnatal months. Muscle atrophy starts in a sub-group of paraspinal and limb muscles in the first weeks after birth (Bridges et al., 1992, Muscle & Nerve 14:172-179). About four months later all muscles including muscles in the neck and head region are affected to varying degrees. Fixed muscles from these animals showed extensive sprouting and muscle atrophy.
To study the dynamic events taking place at the neuromuscular junctions early on during muscle atrophy we followed living neuromuscular junctions in the KY mouse sternomastoid muscle beginning six weeks after birth when this muscle shows no sign of muscle atrophy. Using vital dyes that stain living nerve terminals and rhodaminated α-bungarotoxin (Rh-αBTx) to label postsynaptic acetylcholine receptor (AChR) sites, neuromuscular junctions were visualized with conventional epifluorescence and low-light level video microscopy. Within several weeks after their initial viewing, endplates began to show abnormalities in that parts of the initial postsynaptic AChR areas had shrunk or disappeared. When restained with Rh-αBTx, frequently new AChR sites were found nearby the original junction. Examined at high magnification, secondary folds of affected endplates appeared not continuous (as in normal mice) but rather broken up, missing small patches of labeled AChR's along their lengths. Once begun, remodeling occurred in many of the neuromuscular junctions over a period of 4-8 weeks during which muscle fibers appeared to atrophy and some degenerated completely. Postsynaptic remodeling of junctions was always accompanied by sprouting of the overlying nerve terminals. To see whether this remodeling is caused by the postsynaptic muscle fiber per se or whether other factors are at play, we have begun to follow individual muscle fibers in denervated muscles to see if the same remodeling occurs.

**W 362** TRANSCRIPTIONAL ACTIVITY OF CAT FUSION CONSTRUCTS OF ACHR β-, γ-, AND ε-SUBUNIT GENE PROMOTERS, V. Witzemann, I. Dürr, C. Berberich, W. Kues, M. Numberger, and M. Koenen, Abteilung Zellphysiologie, Max-Planck-Institut für medizinische Forschung, Jahnstr. 29, D-69120 Heidelberg, FRG
We have isolated promoter regions of the β-, γ-, and ε-subunit genes of the nicotinic acetylcholine receptor from rat muscle in order to analyse their transcriptional regulation when fused to the chloramphenicoltransferase reporter gene. The ε-subunit gene carries a single E-box element within a palindromic sequence region in close proximity to the promoter. Point mutions suggest that this region, although not essential for transcription in muscle and not under the control of known myogenic factors, could modulate the promoter activity in muscle and non-muscle cells. The β- and γ-subunit genes are activated by muscle-intrinsic differentiation programs before their expression becomes modulated by the formation of neuromuscular junctions. Both rat genes contain functionally important tandem E-box elements which act as enhancers and can be separated from the basal promoter region carrying a M-CAT box in case of the β-subunit-specific promoter. These genes are probably transactivated by myogenic factors during myogenesis as well as in denervated or pharmacologically disused muscle and show a clear preference for myogenin in cotransfection experiments. The regulation of transcriptional activity of the γ-subunit promoter by Id, a negatively acting myogenic factor, or ProteinkinaseC inhibitors has been analysed.

**W 363** CYTOCHROME C CHIMERIC GENE EXPRESSION IN RAT SKELETAL MUSCLE, Zhen Yan and Frank W. Booth, Department of Physiology and Cell Biology, University of Texas Houston Health Science Center, Houston, TX 77030

Cytochrome c (cyt c) plays an essential role in the electron transport in the mitochondria of skeletal muscle. Increased contractile activity mediates an increase of cyt c protein concentration in skeletal muscle, which is partly due to an increase in cyt c mRNA. For example, slow-twitch (ST) muscle undergoing tonic activity has higher cyt c protein and mRNA concentration than fast-twitch (FT) muscle undergoing phasic work. The purpose of this study was to determine if the expression of a cyt c chimeric gene might be higher in ST muscles than in FT muscles, using direct plasmid injection. Various lengths of rat somatic cyt c promoter regions (supplied by Richard Scarpulla) were fused to 5' end of firefly luciferase cDNA. These chimeric genes were co-injected with pRSVCAT plasmid intramuscularly into adult rat skeletal muscles. Luciferase activities were measured and corrected for pRSVCAT activity. Promoter activity of a construct (pRC4L/-631) containing 631 nucleotides upstream from the transcription initiation site was 40-fold higher than that containing only 66 nucleotides (pRC4L/-66). This data indicates that the region between -631 and -66 contains sequence element(s) necessary for high transcriptional activity of the cyt c promoter in adult rat skeletal muscle. The full length promoter construct (pRC4L/-726) was injected into soleus muscle (predominantly ST fibers) and white portion of vastus lateralis (predominantly FT fibers). Luciferase activity from pRC4L/-726 was 4-fold higher in soleus muscle than in white portion of vastus lateralis muscle. This supports that rat somatic cyt c gene transcription is higher in ST muscle than in FT muscle. It is suggested that cyt c gene transcription may be regulated in response to increases in contractile activity and a contractile activity response element(s) may be in the cyt c promoter. Supported by NIH AR19393.

**W 364** EXPRESSION OF THE ATRIAL-SPECIFIC MYOSIN HEAVY CHAIN AMHC1 AND THE ESTABLISHMENT OF ANTERO-POSTERIOR POLARITY IN THE DEVELOPING CHICKEN HEART, Katherine E. Yutzey, Jeanne T. Rhee, and David Bader, Department of Cell Biology and Anatomy, Cornell University Medical College, New York, NY 10021.
A unique myosin heavy chain cDNA(AMHC1) which is expressed exclusively in the atria of the developing chicken heart was isolated and used to study the generation of diversified cardiac myocyte cell lineages. The pattern of AMHC1 gene expression during heart formation was determined by whole mount in situ hybridization. AMHC1 is first activated in the posterior segment of the heart when these myocytes initially differentiate (Hamburger and Hamilton stage 9+). The anterior segment of the heart at this stage does not express AMHC1 although the ventricular myosin heavy chain isoform is strongly expressed. Throughout chicken development, AMHC1 continues to be expressed in the posterior heart tube as it develops into the diversified atria. The early activation of AMHC1 expression in the posterior cardiac myocytes suggests that the heart cells are diversified when they differentiate initially and that the anterior heart progenitors differ from the posterior heart progenitors in their myosin isoform gene expression. The expression domain of AMHC1 can be expanded anteriorly within the heart tube by treating embryos with retinoic acid as the heart primordia are fusing. Embryos treated with retinoic acid prior to the initiation of fusion of the heart primordia express AMHC1 throughout the entire heart forming region and fusion of the heart primordia is inhibited. These data indicate that retinoic acid treatment produces an expansion of the posterior (atrial) domain of the heart and suggests that diversified fates of cardiomyogenic progenitors can be altered. This posteriorizing influence of retinoic acid could be the result of inappropriate regulation of morphogens or homeobox regulatory proteins. Current investigations are focused on the mechanisms by which the atrial and ventricular lineages are established during heart formation (st. 8-12) and on the molecular basis of anteroposterior polarity in the developing heart.

*Growth Factors; Oncogenes*

**W 400** ACCUMULATION OF MUSCLE THROMBOSPONDIN AT THE NEUROMUSCULAR JUNCTION

Pico Caroni, Virginie Terrasson, Corinna Schneider and Silvia Arber, Friedrich Miescher Institute, 4002 Basel, Switzerland

We identified a novel member of the thrombospondin family by a subtractive hybridization approach with the aim to isolate genes induced seven days after denervation in adult skeletal muscle.
The expression of the novel thrombospondin in muscle is restricted to interstitial cells (in situ hybridization data); it is not expressed by muscle cells.
In adult skeletal muscle the protein accumulates at the neuromuscular junction (visualization with an antibody against the unique C-terminal end of the protein), although no obvious prefered expression by synaptic interstitial cells is observed.
Full induction of the gene after denervation slightly lags behind the peak of proliferation of muscle interstitial cells. After denervation or paralysis muscle thrombospondin initially (3 days) accumulates at the neuromuscular junction. 8 days after muscle inactivation junctional accumulation is not detectable anymore, and the protein displays a widespread fibrillar distribution in interstitial spaces.
During embryonic development, high levels of muscle thrombospondin accumulate at boundaries between muscle and the surrounding tissue.
Due to its modular structure, muscle thrombospondin may affect multiple aspects of extracellular signalling in the the developing and regenerating neuromusclular system. These may include modulation of growth factor and proteolytic activity, and regulation of cell and neurite growth.

**W 401** THE EPSILON SUBUNIT GENE OF THE NICOTINIC ACETYLCHOLINE RECEPTOR IS ACTIVATED BY ARIA/rHRG, Gerald C. Chu and John P. Merlie, Department of Molecular Biology and Pharmacology, Washington University School of Medicine, St. Louis, MO 63110

The modulation of acetylcholine (ACh) receptors located at the neuromuscular junction takes place at multiple levels. One such level is the regulation of expression of the individual ACh receptor subunit genes. The motor neuron seems to play a dual role regulating the transcriptional activity of these genes: First, motor neuron-induced electrical activity influences ACh receptor gene expression. Second, chemical factors may be present at the nerve terminal which can locally induce receptor expression, rendering the underlying synaptic muscle nuclei transcriptionally distinct. We are interested in the regulation of the epsilon subunit of the ACh receptor. Expressed postnatally, this subunit is only present in the adult isoform of mammalian ACh receptor. In addition, of all the subunit genes, the epsilon subunit has the most spatially restricted expression.

Previous studies have shown that ARIA, a factor which increases cell surface expression of acetylcholine receptors in chicken muscle cultures, also greatly increases epsilon subunit mRNA in mouse muscle cells. Thus, ARIA has been proposed to regulate transcriptional events at the neuromuscular synapse. We have begun to examine more closely the effects of ARIA on the transcriptional activity of the AChR epsilon subunit gene.

We demonstrate that an 8kD peptide corresponding to the EGF-like domain of the mammalian homologue of ARIA, heregulin ß1 (rHRG), retains properties characteristic of native ARIA protein. Primary muscle cultures from transgenic mice carrying the epsilon promoter fused to a reporter gene exhibit a dramatic increase in reporter activity upon addition of rHRG, demonstrating that rHRG increases AChR epsilon subunit mRNA by activating transcription. Similar effects of rHRG on the epsilon promoter are also observed in a transient transfection system. Using this transfection system, we have been able to examine the regulatory sequences within the epsilon promoter that are responsible for conferring ARIA/rHRG inducibility.

**W 402** ANALYSIS OF THE FUNCTIONAL ROLE OF NCAM IN NEUROMUSCULAR DEVELOPMENT USING TRANSGENIC MICE.M.S. Fazeli, D.J. Wells, and F.S. Walsh
Dept. Experimental Pathology, UMDS, Guy's Hospital, London Bridge, London SE1 9RT. U.K. and Royal Vet. College, Campden Town, London.
A number of studies have indicated a fundamental role for NCAM in recognition events associated with neurite outgrowth and myoblast fusion. Thus the outgrowth of neurites from a variety of neurons is enhanced when cultured on top of a monolayer of cells expressing a number of NCAM isoforms. Furthermore, overexpression of NCAM in C2 myoblasts results in a significant enhancement of fusion. To extend these findings and further examine the role of NCAM in such phenomena *in vivo*, we have generated a number of transgenic mice expressing human NCAM cDNA, such as the 125 kD muscle specific GPI-anchored isoform, under the control of the skeletal muscle alpha-actin and creatine kinase (MCK) promoters. Both promoters drive high level expression of the transgene in a tissue specific manner, with the heart being the only other tissue expressing detectable levels of the transgene. Skeletal muscle expression under the control of the alpha-actin promoter appears to be 2 - 3 fold higher in a fast (extensor digitorum longus) compared to a predominantly slow muscle (soleus), in contrast to the uniform transgene expression driven by the MCK-promoter. A number of transgenic mice have been also generated where the above promoters drive the expression of the 125 kD GPI anchored form containing the VASE exon. *In vitro* studies have indicated that the expression of VASE containing NCAM isoforms down regulate its ability to promote neurite outgrowth and also inhibit C2 myoblast fusion. Therefore the expression of this isoform of NCAM might be expected to act in a dominant negative fashion. Transgenic animals harbouring the same cDNAs under the control of the myogenin promoter, which has a much earlier developmental pattern of expression, are being currently generated. Further analysis of muscle development and regeneration in these transgenic mice is underway.

**W 403** SUBTRACTIVE CLONING OF A NOVEL SYNAPSE-SPECIFIC cDNA FROM RAT DIAPHRAGM, Mark A. Velleca, Mia C. Wallace and John P. Merlie, Department of Molecular Biology and Pharmacology, Washington University School of Medicine, St. Louis, MO 63110
Synaptic nuclei of innervated muscle transcribe acetylcholine receptor genes at a much higher level than extrasynaptic nuclei. In order to isolate putative synaptic regulatory molecules responsible for the unique transcriptional potential of synaptic nuclei, we have taken a subtractive hybridization approach. cDNAs prepared from the endplate zone of rat diaphragms were enriched for synaptic clones by subtractive hybridization to cDNAs prepared from extrasynaptic regions of the same muscle; this procedure resulted in an enrichment of approximately 1,000 fold. Clones from the subtractive library were then screened for synapse-specific expression by Northern blot analysis.
We are currently characterizing clone # 7H4 which hybridizes to two RNAs (1.6 kb and 6 kb) that are 10 fold more abundant in endplate(+) than endplate(-) regions of adult rat diaphragm. Both RNAs are skeletal muscle-specific, as no expression is detected in various non-skeletal muscle tissue and cell line RNA preparations. Several overlapping cDNAs and 5' RACE products have been cloned and sequenced; we believe a composite sequence of 1577 bp represents the full-length clone of the 1.6 kb RNA. Recently, we have begun cloning and sequencing the 6 kb RNA. 7H4 appears to be a novel sequence with no homologies to any current entry in available databases. A Southern blot of rat genomic DNA digested with several restriction enzymes and probed with different 7H4 probes demonstrates a simple pattern of bands indicative of a single-copy gene.

**W 404 IN VITRO HEART AND MUSCLE FORMATION: THE EFFECT OF THE INACTIVATION OF THE DESMIN LOCUS IN DIFFERENTIATING EMBRYONIC STEM CELLS,** Georg Weitzer, Jai Up Kim and Yassemi Capetanaki, Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77030, USA.
One of the first steps during embryonic cardiac and skeletal muscle development is the expression of the muscle specific intermediate filament protein desmin in replicating presumptive myoblasts. Desmin is the only muscle-specific protein detected so far in the neuroectoderm where it is transiently expressed at 8.25 dpc. At 8.5 dpc it appears in the heart rudiment and then at 9 dpc in the myotome. The biological role of desmin, however, during these early steps of myogenesis as well as in adult muscle is unknown. Our recent data using anti-sense RNA to inhibit desmin expression in vitro suggests that this IF protein may play a fundamental role in early stages of myogenesis. In order to investigate the functional role of desmin during muscle development in vivo we attempted to disrupt the desmin gene by homologous recombination. Therefore, we constructed a replacment targeting vector by disrupting the first exon through insertion of a neo-casette. 28% of the G418- and FIAU-resistant ES cell colonies carried an inactivated desmin allele. These cells were further selected for a second homologous recombination event to obtain ES cell with both of the desmin alleles disrupted. ES cells can be made to form embryoid bodies, which differentiate into all types of muscle cells, allowing the study of differentiation of these cells in vitro. The effects of single and double knockouts of the desmin gene on heart and skeletal muscle formation in differentiating ES cells were very dramatic and will be discussed. ES cells with single desmin knock outs were also injected into mouse embryos to produce germ-line chimeras, which are currently bred to obtain germline transmission. These mice with heterozygous and homozygous loss of desmin will be used to investigate the role of desmin on myogenic differentiation, development and normal muscle function.

## *Muscle Disease and Gene Therapy*

**W 405** EFFECT OF AGING ON HISTOLOGICAL, BIOCHEMICAL AND CONTRACTILE PROPERTIES OF SKELETAL MUSCLES FROM DYSTROPHIN DEFICIENT (*mdx*) MICE. Richard T. Abresch, Sandra A. Walsh, Mark A. Wineinger, Gregory T. Carter, Sylvia J. Horasek, and William M. Fowler, Jr., Department of Physical Medicine and Rehabilitation, University of California, Davis,CA.
Although *mdx* mice have the same genetic defect as patients with Duchenne muscular dystrophy (DMD), they do not show similar extreme functional deficits. It has been reported that the lack of symptoms in the *mdx* mouse is due to the ability of the dystrophin deficient limb muscles of the *mdx* mouse to regenerate and recover after an initial intense period of degeneration. Our studies demonstrate distinct abnormalities in limb and diaphragm muscles of the *mdx* mouse at all ages. Although the regenerated extensor digitorum longus (EDL) muscles appear stabilized with little degeneration after 10 weeks of age, the resulting fibers range from small immature fibers to hypertrophied, centrally nucleated fibers that do not fall into distinct fiber type groups (type I, IIa and IIb) as do the control EDL. The adult *mdx* diaphragm exhibits marked permeability to fluorescein isothiocyanate indicating an abnormal membrane permeability and it displays an infiltration of connective tissue similar to the pathology seen in DMD. The slow soleus, fast EDL muscles and the diaphragm from mature *mdx* mice all display a non-functional hypertrophy, increased size with no change or a decrease in strength, and all have reduced capillarity. The *mdx* EDL, after the initial necrotic period, is significantly weaker and more fatiguable than the control EDL. The fatigue and histologic data are evidence that the muscles of the *mdx* mice never mature completely. Plasma creatine kinase, commonly used to indicate muscle injury, is elevated in young *mdx* mice and our data show even further elevation in the mature *mdx* mice. This study confirms the pathology of the diaphragm of the adult *mdx* mouse and clearly demonstrates that in the dystrophin deficient limb muscles of the *mdx* mouse there are ongoing abnormalities which do not correlate directly with the initial period of degeneration and continue in the adult mouse beyond the age of maturity.

**W 406** AN EM STUDY OF IMMUNOGOLD-LABELLED BFGF IN DYSTROPHIC mdx AND NORMAL MOUSE DIAPHRAGM, Judy E. Anderson and Karen A.H. Penner, Department of Anatomy, University of Manitoba, Canada R3E 0W3

A proposed relation between muscle bFGF, a mitogenic growth factor, and the success of muscle regeneration (Growth Factors 9:107; 1993 and Dev Biol 147:96; 1991) was examined in a double-blind experiment, by comparing soleus and diaphragm muscles in mdx and control mice. Immunogold-labelled bFGF was quantified on fixed, resin-embedded sections. A labelling index (LI) was derived from the gold particle density in known areas of cytoplasm and nuclei of certain cells. Myoblasts, myotubes, regenerated and intact fibers, and satellite, endothelial, and fibroblast cells were identified by EM. Very low labelling was seen in sections exposed to immune serum presorbed with bFGF, or without primary antibody. Dystrophic damage to regenerated fibers was much more common in mdx diaphragm than soleus, while control diaphragm had more injured and centrally-nucleated fibers than control soleus. Statistically lower LIs were found in mdx than control diaphragm. By comparison, mdx soleus cells had higher LIs than cells in control soleus or mdx diaphragm muscle. FGF-LI significantly decreased as myogenic cells differentiate in both strains. Among myogenic cells, myoblasts with early myofilaments had the highest LI, while control endothelial cells had larger LI than myoblasts. Endomysial collagen in mdx diaphragm also showed heavy gold labelling. These findings suggest that the amount of bFGF sequestered in muscle tissue (both myogenic and non-myogenic cells) may correlate with the response of different muscles to compensate for dystrophic insult or normal muscle strain. This may have implications for promoting the success of cell and gene therapies for DMD. Supported by a grant from MDAC and studentship from MHRC (KP).

**W 407** POSSIBLE INVOLVEMENT OF MITOCHONDRIAL DNA ON RAT AGEING, Kimiko Asano*, Masahiko Nakamura+ & Akira Asano+, *College of Medical Technology, Kyoto University, Sakyo-ku, Kyoto 606, JAPAN and +Institute for Protein Research, Osaka University, Suita, Osaka 565, JAPAN
Recently, some investigators suggested the participation of mitochondrial DNA on the animal aging through the accumulation of the damages on the DNA which is followed by the decay of mitochondrial activity. However, the amounts of such DNA damages were too little for the support of the direct relationship between these phenomena. We have been studying the age-dependent qualitative and quantitative changes of rat mitochondrial DNA for several years, and reported that the contents of mtDNA in rat liver and heart muscles decreased strikingly upon aging. For accurate measurement of mtDNA content, we measured the ratio of color intensity of Southern blots using LINE probe for genomic DNA and probes for mtDNA sequences. As the results, mtDNA of hepatic cell and cardiac muscle cell extensively decreased during aging of rat fed on 60% restriction diet, but did not decrease in thigh muscles of the same rats. We would like to clarify the role of the mitochondrial DNA on the cell aging and differentiation.

**W 408** EXPRESSION OF Zbu1, A HUMAN BRAHMA-RELATED GENE, IN NORMAL AND MALIGNANT CELLS. Natalia Bogdanova, Xiaohua Gong, Nadia Rosenthal, Cardiovascular Research Center, Massachussets General Hospital, Charlestown, MA 02129.

The Zbu1 gene (a *brahma*-related gene) is distinct from genes encoding Drosophila brahma, yeast SNF2/SW2, and RAD5, a super family which appears to be involved in co-regulation of transcription by chromatin reconfiguration. Preliminary evidence suggests that the Zbu1 gene product may be a DNA sequence-specific binding protein with a target in the myosin light chain 1/3 enhancer.

In normal human tissues, Zbu1 is highly expressed in skeletal muscle, heart, and brain. Lower expression has been observed in lung, liver, kidney, pancreas and placenta. There are three Zbu1 transcripts which are encoded by a single locus. The Zbu1 expression patterns in a variety of malignant cell lines and in primary tumors of different origins are currently being analysed by RPA and in situ hybridization.

The structure of the Zbu1 protein, which includes helicase domains similar to the related Drosophila and yeast proteins, suggests that it may be involved in co-activation of gene expression. Expression of the Zbu1 in stably transfected C2C12 myoblasts changes their phenotype (cells growth dramatically slows down) and potential effect of the gene on cell growth and differentiation is currently being analysed.

**W 409** EFFECT OF VOLUNTARY WHEEL-RUNNING EXERCISE ON MUSCLES OF THE *mdx* MOUSE, Gregory T. Carter, Mark A. Wineinger, Sandra A. Walsh, Sylvia J. Horasek, Richard T. Abresch, and William M. Fowler, Jr., Department of Physical Medicine and Rehabilitation, University of California, Davis, CA.
The purpose of this study is to determine whether dystrophin-deficient *mdx* mice (a model of Duchenne muscular dystrophy) are functionally impaired and more susceptible to muscle injury than normal C57 mice. Studies of muscle injury in the *mdx* mouse using external stimulation either *in vitro* or *in vivo* have produced varying results and it is not clear how *mdx* muscles adapt to exercise. We studied *mdx* and control C57 mice that ran on metered mouse wheels for four weeks to determine if voluntary exercise, that allows normal muscle recruitment patterns, differentially affects the functional, contractile, and morphometric response of these mice. Our results show *mdx* mice are significantly impaired when compared to controls. The distance young *mdx* mice ran was 67-78% of the C57 mice, while the adult *mdx* mice ran only 31-48% of the adult controls. Plasma creatine kinase levels of the adult *mdx* mice (initially higher than the young *mdx*) are reduced to the level of the young after the exercise period, even with their limited running. No further histologic changes were evident in hematoxylin and eosin stained sections of the exercised soleus and extensor digitorum longus (EDL) muscles beyond the pathology normally present in the muscles of the *mdx* mouse. *In vitro* contractile studies of the EDL and soleus muscles that were conducted after the exercise period showed that the slow soleus muscle of young and adult *mdx* mice exhibited hypertrophy with no changes in strength or fatiguability. In contrast, the young C57 mice increased strength and the adult C57 mice became less fatiguable. In the adult *mdx* mice, the fast EDL exhibited slight hypertrophy with a loss of strength, as did the adult C57 EDL. However, the adult *mdx* EDL had only 78% of the tetanic strength of the C57 EDL. The EDLs from the young animals of both lines exhibit no changes after the exercise. These results indicate that the soleus and EDL muscles of the *mdx* mouse adapt differently than those of the C57 mouse to even moderate exercise.

**W 410 FIBER TYPE-SPECIFIC ACTIVITY OF A MUSCLE GENE PROMOTER DIRECTLY INJECTED INTO RAT SKELETAL MUSCLE,** Shari J. Corin and Robert Wade*, Department of Biological Chemistry and *Program in Cell and Molecular Biology, University of Maryland School of Medicine, Baltimore, MD 21201
Fast twitch and slow twitch skeletal myofibers express distinctive patterns of contractile protein isoforms. These fiber-type specific isoforms often are encoded by multigene families, the members of which are differentially transcribed in different types of muscle. The mechanisms responsible for this differential, i.e. fiber type-specific, gene regulation remain unknown. In order to study this problem, we cloned and characterized the human slow twitch skeletal muscle troponin I gene ($TnI_S$) and its associated 5' flanking region, which contains a muscle-specific promoter and enhancer (Corin et al., submitted). In adult mammals, the $TnI_S$ gene is preferentially expressed in slow twitch skeletal muscle. Moreover, the 5' flanking region exhibits fiber type-specific promoter activity in transgenic mice (see abstract by Hardeman et al.). As a first step toward developing a transient assay by which to identify fiber type-specific regulatory elements, we injected slow (soleus) and fast (extensor digitorum longus, EDL) twitch muscles of live rats with an expression plasmid containing the $TnI_S$ 5' flanking region fused to a luciferase reporter gene (-4200$TnI_S$luc), along with an internal control plasmid containing the SV40 promoter fused to a CAT reporter gene. We observed that the $TnI_S$ promoter was preferentially active in slow twitch muscle. To control for the possibility that the differential activity in soleus vs. EDL might be due to factors other than relative $TnI_S$ promoter activity, we performed a similar experiment with a plasmid containing the human skeletal $\alpha$-actin promoter linked to the luciferase gene. Skeletal $\alpha$-actin is the major actin isoform in both slow and fast twitch skeletal muscle; therefore the skeletal $\alpha$-actin promoter would not be expected to exhibit differential activity between fiber types. As expected, the skeletal $\alpha$-actin promoter was equally active in slow and fast twitch muscle. Thus, appropriate fiber type-specific regulation of a muscle gene promoter can be observed by means of an *in vivo* transient assay. *Cis*-acting elements that direct fiber type-specific $TnI_S$ promoter activity now are being identified.

**W 411** DIRECT GENE TRANSFER IN MUSCLE FOR GENETIC VACCINATION AGAINST HEPATITIS B VIRUS (HBV)

Heather L. DAVIS§†, Marie-Louise MICHEL#, Maryline MANCINI#, Simon C. WATKINS*, Martin SCHLEEF† and Robert G. WHALEN† §Physiotherapy Program and Dept. of Physiology, University of Ottawa, CANADA; *Dept. of Cell Biology and Physiology, University of Pittsburgh; #Unité de Recombinaison et Expression Génétique, INSERM U163, and †Département de Biologie Moléculaire, Institut Pasteur, Paris, FRANCE.

The possibility of inducing an immune response to a protein expressed directly from an introduced gene represents an attractive alternative to classic vaccination. There are several ways to carry out *in vivo* gene transfer into skeletal muscle and we have shown that pure plasmid DNA is superior to either adenoviral or retroviral vectors in mature muscle, and equivalent to adenovirus in regenerating muscle. The use of plasmid DNA for genetic vaccination offers many advantages including ease of design and preparation, chemical stability, lack of immunogenicity, and no risk of inadvertent viral infection. We injected plasmid DNA encoding the Hepatitis B surface antigen (HBsAg) driven by a viral promoter (pCMV-HB.S) into hindlimb muscles of mice, which were found to subsequently contain HBsAg-expressing myofibres, as demonstrated by indirect immunofluorescence and confocal microscopy. The sera obtained from these animals 10 days after gene transfer contained significant levels of HBsAg and low levels of antibodies to it (anti-HBsAg). Over the following 2 mos, serum levels of anti-HBsAg steadily increased whereas those for HBsAg fell, most likely due to the neutralizing effect of the antibodies. Within 2 wk of DNA injection, 91% of the mice were seropositive [i.e., > 1 milli-International Unit/ml (mIU/ml) of anti-HBsAg] and this increased to 100% by 8 wk, at which time all mice had titres greater than 100 mIU/ml (10 mIU/ml is recognized as being sufficient in humans to confer protection against natural HBV infection). Similar results were obtained by injecting plasmid DNA into hindlimb muscles of rats and rabbits using the Biojector® needle-free jet injection system. We have thus demonstrated that intramuscular injection of plasmid DNA can give rise to synthesis, processing and secretion of a foreign protein into the circulation which leads to an appropriate antibody response. These findings are important for application to therapeutic uses other than vaccination, and provide a model with which to study immunological responses to foreign proteins expressed directly from a gene introduced into skeletal muscle.

**W 412 RETROVIRAL-MEDIATED HUMAN DYSTROPHIN GENE TRANSFER,**

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We have produced and expressed a variety of cDNA-based genes encoding full-length normal human dystrophin and smaller BMD-based mutant forms which have been tested in tissue culture and in transgenic *mdx* mice. In the case of the BMD-based minigenes recombinant retroviral particles have been produced and used to directly infect and transduce *mdx* muscle cells both *in vitro* and following intramuscular injection *in vivo* (3-13% fibre conversion for over 9 months; Dunckley *et al*, Hum Mol Genet, 2, 717). While higher gene transfer efficiencies have been reported in muscle using adenoviral vectors, retroviral vectors still represent the only gene transfer system which offers the potential of stably-integrated permanent gene transfer into skeletal satellite cells. In order to increase transfer efficiency, several novel packaging cell systems and proviral structures have been examined with the aim of increase viral titres, and the use of producer cell implants *in vivo* to enhance transfection efficiency have been investigated. In the case of full-length cDNAs encoding normal dystrophin (> 11Kb) these are likely to be more efficacious and acceptable candidates for gene therapeutic applications, but are thought to probably exceed the packaging capacity of currently available MLV-based retroviral vectors. However this possibility remains to be experimentally determined. We have therefore also constructed various retroviral plasmids expressing full-length human dystrophin cDNAs which are now being examined for packaging efficiency.

**W 413** M-LAMININ DEFECT IN THE DYSTROPHIC *dy/dy* MOUSE,

Hong Xu[1], Peter Christmas[2], Xiao-Rong Wu[1], Kiichi Arahata[3], Ulla Wewer[4], and Eva Engvall[1,2], [1]La Jolla Cancer Research Foundation, La Jolla, CA, [2]Department of Developmental Biology, Wenner-Gren Institute, University of Stockholm, Sweden, [3]Department of Neuromuscular Research, National Institute of Neuroscience, Tokyo, Japan, and Institute of Anatomical Pathology, University of Copenhagen, Denmark.

Laminin is a large 800 kD protein that belongs to a family of basement membrane (BM) proteins. The laminins are composed of three subunits, a heavy chain and two light chains, which are assembled into a crosslike structure. The first laminin to be expressed during development is composed of chains designated A, B1, and B2; A being the heavy chain. M-laminin is a laminin which has the homologous heavy chain M in place of the A chain. M-laminin is expressed prominently in mature muscle and peripheral nerve. We have found that M-laminin is defective in the dystrophic *dy* mouse. The phenotype of this mouse is due to a spontaneous mutation that was discovered in the Jackson Laboratory in 1951. The disease is inherited as an autosomal recessive and the mutation is known to be located on chromosome 10, but the nature of the mutation is not known. Indirect immunofluorescence on tissue sections of homozygous and wild type mice with M chain specific antibodies showed the lack of M chain in BMs of dystrophic mice but normal M chain in heterozygous and wild type littermates. Western blotting with several antisera to different portions of the M chain confirmed the complete absence of M chain in the *dy/dy* mice. Transmission electron microscopy of muscle showed that the *dy/dy* mice have very thin and fragmented BMs as expected from the lack of a major BM component. M chain mRNA could not be detected by Northern blotting, although low levels of mRNA were detected by PCR. Southern blotting of fragments of genomic DNA failed to detect any defect in the M chain gene in the *dy/dy* mice. The lack of M chain in the *dy* mice may be due to a point mutation resulting in low levels of mRNA being expressed or the mRNA being unstable.

**W 414** THE USE OF Y-CHROMOSOME SPECIFIC PROBES TO MONITOR THE MOVEMENT AND SURVIVAL OF CELLS IN MYOBLAST TRANSPLANTATION STUDIES.

Ying Fan*, Manfred W. Beilharz+, Moira Maley* Miranda Grounds*, Departments of Pathology* and Microbiology+, The University of Western Australia, WA, 6009

Experimental myoblast transplantation as a therapy for muscular dystrophy requires a nuclear marker to follow the fate of (donor) skeletal muscle precursor cells (myoblasts) transferred into host muscles. Specific DNA sequences on the Y-chromosome represent a powerful tool for identifying male cells introduced into female hosts and are applicable to all cell types. Y-chromosome DNA is specific for the nucleus, does not require transcription, is not diluted by a division or reutilised upon cell death, and generally is exempt for problems of immunological rejection. In our laboratories, *in situ* hybridisation with a digoxigenin labelled mouse specific Y-chromosome probe has been used to monitor donor male cells in transplantation studies of myoblasts (1) and glial cells (2). In addition, a dog specific Y-chromosome probe has been developed for studies of myoblast transplantation in the canine model of X-linked muscular dystrophy (3).

Here we report the results of studies using the mouse Y-chromosome probe on: (i) the movement of cells between whole muscle isografts and host muscles; and (ii) the long term survival - up to 6 months - of male C57B/10sn myoblasts transplanted into female mdx mice.

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**W 415** EFFECT OF TRIPLET REPEAT NUMBER ON MYOTONIC DYSTROPHY PROTEIN KINASE (*DMPK*) mRNA LEVELS IN ADULT AND CONGENITAL MYOTONIC DYSTROPHY,

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Myotonic dystrophy (DM) is an autosomally dominant inherited disease in which system-wide abnormalities are caused by a triplet repeat (CTG) expansion within the 3' untranslated region of the DM protein kinase (*DMPK*) gene. To determine the effect an expanded repeat region has on *DMPK* gene expression, we have separated the chromosome 19 homologues from an adult DM patient into different cell lines by way of somatic cell hybridization. Reverse transcription/polymerase chain reaction (RT/PCR) amplification of coding regions from the *DMPK* gene has shown an absence of *DMPK* mRNA from the mutant allele (~ 133 repeats), whereas mRNA production from the normal allele (13 repeats) remains unaffected. In contrast to adult DM, no *DMPK* mRNA from either the normal or mutant alleles was detected in muscle tissue from a congenital DM patient. Since congenital DM patients generally have larger repeat sizes than those found in adult DM, our data suggests that the larger CTG expansion suppresses *DMPK* gene expression from the normal allele as well, further reducing *DMPK* mRNA levels and heightening the penetration of the disease. To determine if this hypothesis is correct, somatic cell hybrids are being constructed from both congenital DM myoblasts and fibroblasts to separate the normal and mutant *DMPK* alleles. We predict that the normal allele from congenital DM patients will be re-expressed once it is removed from the influence of the mutant allele.

**W 416** MYOSIN HEAVY CHAIN ISOFORMS AND MYOGENIC TRANSCRIPTIONAL FACTORS IN MYOTONIC MICE,

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The recessive autosomal myotonia of the mouse "arrested development of righting response" (ADR) is characterized by an electrical after-activity associated to after-contractions which are independent of neuromuscular transmission. This is due to a decreased chloride muscle conductance, the consequence of a transposon inserted into the gene coding for the chloride channel protein, destroying its coding potential for several membrane-spanning domains. $ADR^{CRP}$ or cramp mouse is an ethylnitrosourea-induced neuromuscular mutant which has been shown to be allelic to the original spontaneous mutant, ADR. Among numerous biochemical modifications such as a decrease in parvalbumin, transitions from Myosin Heavy Chain IIB (MyHCIIB) to MyHCIIA have been observed (Reininghaus et al., 1988). We thus studied the expression of various MyHC mRNA transcripts either by the slot blot method or by a more sensitive method, the polymerase chain reaction, to assay low level transcripts. mRNA expression of MyHCneo, MyHCIIA, MyHCIIB, MyHCIIX and β/slow MyHC has been determined.

Myogenic factors of the MyoD family play a role in the induction of muscle-specific gene transcription and are regulated by electrical activity and differently expressed on different electrical activity pattern. We studied therefore the expression of mRNA transcripts coding for MyoD, myogenin and herculin in the ADR mutants. Expression of MyHCIIB mRNA decreases drastically. MyHCIIA, MyHCIIX and β/slow MyHC mRNAs increases whereas MyHCneo expression is not observed. MyHC transitions in the myotonic muscles thus progress towards a more oxydative phenotype. Concerning the transcription factors, MyoD and herculin mRNAs decrease and myogenin mRNA increases. Results obtained on the cramp mutant are qualitatively identical but the modifications are of various intensity depending on the transcript observed. In conclusion, the expression of MyHCIIB and herculin decreases substantially in the muscles of ADR mice. Herculin expression is thus associated with the expression of other markers of glycolytic fibers (MyHCIIB, parvalbumin), either as a pathological feature of myotony or as an intrinsically fiber-type specific protein.

**W 417** MOLECULAR PHYSIOLOGY OF MYOTONIA: DEFECTS OF THE MUSCULAR CHLORIDE CHANNEL GENE *Clc-1* AND ACTIVITY DEPENDENT REGULATION OF ITS EXPRESSION

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We have previously shown that hereditary myotonias (ADR in the mouse, Thomsen and Becker type in man) are due to mutations in the gene *adr/Clc-1* (Chr 6, mouse), *CLC-1* (Chr 7q, man) that codes for the muscular chloride channel ClC-1. Three non-complementing (and therefore probably allelic) murine myotonia mutations have been analyzed at the nucleic acid sequence level. The standard allele *adr* is due to an insertion of a retroposon, $adr^{mto}$ is a nonsense *mutation at codon* 47, and $adr^{K}$ is a missense mutation, Ile → Thr at the conserved position 553. - The level of ClC-1 mRNA is extremely low in myotubes and is steeply upregulated during rodent postnatal development. In adult muscles, ClC-1 mRNA levels are lower in slow than in fast muscles in the wildtype (WT) mouse, but high in both muscle types in the myotonic ADR mouse. Denervation leads to rapid down-regulation in WT but not in ADR muscle, indicating that spontaneous myotonic activity may substitute for neural stimulation. This difference in the response to denervation of WT and ADR muscles was also observed for acetylcholine receptor α-subunit and for myogenin and MyoD1 mRNAs. Myogenic factor (myf) binding sequence motifs were found in the 5' non-coding sequences of the *CLC-1* gene, indicating myf mediated transcriptional regulation.

We thank Dr. E.N. Olson, Houston, for the myogenin probe.
Supported by the Deutsche Forschungsgemeinschaft (SFB 223 and Schwerpunkt "Muskelforschung") and MDA.

**W 418 INVESTIGATION OF GENE REGULATORY MECHANISMS IN CARDIAC HYPERTROPHY**,

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The changes in expression of the genes for contractile proteins which accompany myocardial hypertrophy serve as a paradigm for the investigation of transcriptional regulation mechanisms in cardiac adaptation. This is exemplified by the selective increase in the phosphorylatable isoform of myosin light chain (MLC2) reported for both rat and human hypertrophied hearts. Since the regulated expression of myosins in cardiac hypertrophy reflects changes in the genomic activity, it is of interest to investigate the mechanisms which control the transcription of these genes in response to signals associated with hypertrophy. To this end, our laboratory has previously isolated the genes for chicken and rat ventricular MLC2 and identified several cis-acting regulatory sequence elements in the promoters. With synthetic DNAs encompassing the regulatory promoter elements, mobility shift assays were performed with nuclear proteins isolated from spontaneously hypertensive rats (SHR) and age-matched normotensive rats, WKY. SHR are genetically predisposed to hypertension induced myocardial hypertrophy and serve as a model for evaluating the molecular basis of transcriptional changes during hypertrophy. Specific qualitative and quantitative changes were observed in nuclear proteins from SHR which recognize A/T-rich activator sequences in the MLC2 promoter. These sequences, elements A and B, are homologous to the CArG box and the MEF-2 binding site, respectively. A developmental analysis of the complex formation suggests that the changes observed can be correlated with the onset of hypertension-induced hypertrophy in the SHR heart. Thus, it appears that distinct modulations in transcription factors can account for changes in MLC2 gene expression in the diseased heart. Several putative clones of B element binding factors have been obtained by screening a cardiac expression library: characterisation and sequence analysis are in progress.

**W 419** TARGETED DISRUPTION OF THE CARDIAC α–ACTIN GENE IN MOUSE EMBRYONIC STEM CELLS.

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In the adult rodent, cardiac α–actin generally is the predominant actin in the heart although a low level of this actin is present in skeletal muscle [McHugh, K.M., Crawford, K., and Lessard, J.L. Dev. Biol. 148:442:458 (1991)]. In addition, cardiac α–actin comprises a significant fraction of the total actin found in early myoblasts within the myotome and fetal skeletal muscle. However, neither the physiological significance or developmental relevance of cardiac α–actin is known. In order to examine these issues, we have made use of gene targeting strategies to disrupt the single cardiac α–actin gene in embryonic stem cells from the mouse. Since isogenic DNA appears to give higher targeting efficiencies, we have isolated and completely sequenced the cardiac α–actin gene in 129/SvJ mice. We have employed a targeting strategy based on the use of the HPRT minigene [Reid, L.H., Gregg, R.G., Smithies, O., and Koller, B.H. Proc. Natl. Acad. Sci. USA 87:4299-4303 (1990)] to disrupt this actin gene at the DNA sequence corresponding to the 21st amino acid residue. After electroporation of ES cells (E14) with the targeting fragment, potential clones were obtained and, following serial propagation in HAT medium, they were analyzed by nested PCR. Proper targeting in these clones was confirmed by southern hybridization. Chimeric mice are being generated by microinjection of cells from one of the targeted ES-cell clones into C57Bl/6 blastocysts and implantation in pseudopregnant females. Once germ-line chimeras are obtained, we plan to make use of both heterozygous and homozygous null animals to assess the effects, if any, on embryogenesis, organization, and function of the heart and skeletal muscles. Although the restricted expression of this muscle actin should result in a readily defined phenotype, it is difficult to predict the outcome. If this mutation is not lethal, the basis for compensation will be determined and the structural and functional properties of isolated hearts will be assessed.

**W 420** EFFECTS OF AGE ON ACTIN, MYOSIN HEAVY CHAIN AND THYROID HORMONE RECEPTOR GENE EXPRESSION IN MUSCLES OF CONTROL AND MDX MICE,

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Murine X-linked muscular dystrophy (mdx) is a genetic desease characterized by an absence of dystrophin and a progressive myofiber degeneration in diaphragm, whereas limb muscles are minimally affected. The present experiment was carried out to analyse the effects of the mdx status during aging on gene expression in different muscles. We asked if certain fiber types or muscles are more capable of surviving progressive damage of disease or aging, and what characteristics of the muscle confer this survivability. Total RNAs were extracted from the heart, diaphragm, and gastrocnemius muscles at 2, 12, 17 and 28 months of age and subjected to northern blot analysis and ribonuclease protection assays. Our results show that the proportion of slow MHC mRNA and non-skeletal actin (β-actin) increased in mdx diaphragm by 17 months of age, suggesting a shift towards a slower contractile fiber type and an increase in the proportion of non-skeletal muscle tissues including fibroblasts. In diaphragm of 28 month old control mice, the levels of mRNA coding for all specific muscle proteins studied dramatically decreased while that of β-actin mRNA increased. Fast IIB MHC mRNA was only detected in gastrocnemius, in which levels were lower in mdx compared to control mice. In both control and mdx animals IIb MHC mRNA decreased from 2 to 17 months. Preliminary results suggest a higher level of β1 thyroid hormone receptor ($T_3R$) mRNA in mdx than control diaphragm and heart muscles at 2 months of age. No differences were observed for α1 and α2 $T_3Rs$.

**W 421** MHC CLASS II EXPRESSION IN HUMAN MUSCLE CELLS AT DIFFERENT MATURATIVE STAGES

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It has been recently reported that cells, devoid of immune functions, may acquire APC activity after stimulation with inflammatory cytokines such as IFN-γ. Muscle cells have been demonstrated to express MHC molecules after IFN-γ treatment as well as to be able to effectively present antigen to autologous T cell lines (1). To evaluate the possible involvement of muscular cells in amplifying and sustaining local immune reactions, we studied MHC class II molecule expression on human muscle cells at different maturative steps: proliferating myoblasts, differentiated myotubes and innervated mature myofibres. By RT-PCR experiments and immunocytochemistry, both MHC class II transcripts and molecules expressed on the plasmalemma were found on proliferating myoblasts and aneurally multinucleated myotubes after IFN-γ treatment. Innervated myotubes, expressing a mature differentiated phenotype, seem to loose their responsiveness to IFN-γ as shown by the negative immunostaining for MHC class II surface molecules. Our results suggest that human muscle cells posses the potential of behaving as non-professional APC mainly at the early stage of maturation. This acquired function may account for their direct involvement in local immunobiological events and emphasize the differences between proliferative and maturative stages in muscular cells.

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**W 422** **SINGNALING PATHWAYS IN SATELLITE CELL MYOGENESIS : DIFFERENTIAL EFFECTS OF PKA AND PKC TARGETING DRUGS.**

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Signaling pathways involved in myogenesis are not yet fully understood. The effects of several ser/thr kinase inhibitors and of TPA, drugs which alter PKA and/or PKC intracellular activities were tested on myogenesis. We have shown that substances which inhibit PKA activity highly enhance myogenesis of satellite cells grown in primary cultures. Myogenin but not MyoD mRNA level was increased by these drugs. *In vivo*, PKA inhibiting drugs improve the regeneration of crushed muscle when injected in the muscle at the time of injury.

Further results strongly suggested that PKC exerts a negative control on PKA activity in differentiating satellite cells. The possibily that different PKC isoforms are involved in satellite cell myogenesis *in vitro* and *in vivo* is now being evaluated.

**W 423** CARDIAC GENE EXPRESSION FROM THE MLC3 PROMOTER,

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We are studying the transcriptional activation of the myosin light chain (MLC) 1/3 locus in mouse. This locus consists of two promoters separated by 10kb and a downstream enhancer. The upstream MLC1 promoter is activated at 9.5 days p.c. while the MLC3 promoter is activated a full four to five days later. To investigate the mechanisms of activation of this promoter, we generated a transgenic mouse containing a minimal MLC3 promoter region and a MLC enhancer fragment which was sufficient to drive expression in muscle cell lines. Characterization of a mouse containing these elements revealed this construct was expressed exclusively in skeletal muscle except for an apparently aberrant expression in cardiac muscle.

Examination of endogenous MLC3 gene expression in adult mouse hearts detected a high atrial and low ventricular expression pattern by *in situ* hybridizations and ribonuclease protection assays. Direct injections of the transgenic construct into the ventricular walls of rat hearts demonstrates that this construct is transcriptionally active *in vivo*. To further delineate the mechanisms for transcriptional activation of the MLC3 promoter in cardiac muscle, we are conducting a deletional analysis of this transgenic construct using direct cardiac injections accompanied by the generation of new transgenic animals.

**W 424** HYPOTHYROIDISM DECREASES REGENERATION BY *MDX* LIMB MUSCLE,

Laura M. McIntosh & Judy E. Anderson, Dept of Anatomy, University of Manitoba, Winnipeg, Canada, R3E 0W3.

It was previously shown that a simple metabolic disturbance (hypothyroidism) changed the phenotype of *mdx* dystrophy to resemble that of Duchenne dystrophy (DMD) and decreased the formation of myotubes and removal of debris from crush-injured tibialis anterior muscle (TA). In order to confirm these findings and to study their etiology, hypothyroidism (0.05% PTU in drinking water for 8 wks) was induced in *mdx* and control mice and the cell types and their activities were studied. Muscle precursor cells (mpcs) and inflammatory cells were located in pre-set zones of crush injured TA 4 days post-injury by double immunofluorescence staining for basic fibroblast growth factor (bFGF) and neural cell adhesion molecule (NCAM). Tissue amounts of these proteins were determined by Western blotting. Autoradiography was also performed in order to label DNA synthesis. We found that cells stained with α-NCAM and tissue amounts of NCAM were decreased in treated *mdx* TA. Although this NCAM is not specific to mpcs, greater numbers of cells were labelled in *mdx* than control TA. At 2 days post-injury, the proportion of radiolabelled PMNs, myotubes and other cell types was increased in treated compared to untreated *mdx* mice. No differences were found between control untreated and treated TAs. These results confirm that muscle regeneration is decreased in treated *mdx* mice, but show that control mice are not as affected by hypothyroidism. The regenerative response of mpcs in hypothyroid mice may involve a combination of delayed inflammatory infiltration and a smaller population of mpc/satellite cells in the hypothyroid dystrophic muscle at the time of crush. Funded by the Medical Research Council of Canada (LM) and a grant from the Muscular Dystrophy Association of Canada (JEA).

**W 425** CHARACTERIZATION OF A GENETIC LOCUS EXPRESSED IN 10T1/2 FIBROBLASTS THAT INHIBITS MYOGENESIS IN L6 MYOBLASTS.

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Despite the fact that 10T1/2 fibroblasts are myogenic when transfected with MyoD or treated with azacytidine, we have found that the myogenic phenotype of L6 myoblasts, which express Myf-5 and not MyoD, is extinguished in whole cell hybrids between L6 cells and 10T1/2 fibroblasts. In contrast to the parent L6 cells these hybrids do not form myotubes and do not express myosin heavy chain. Analysis of myogenic regulatory gene expression reveals that Myf-5 mRNA is present in L6 cells as well as in the hybrids, whereas MyoD is absent in both cell types. Myogenin mRNA is present in L6 cells but is extinguished in the hybrids. Therefore, myogenin extinction occurs despite the continued expression of Myf-5 mRNA. In order to determine the genetic basis of extinction, 10T1/2 chromosomes were transferred into L6 cells by microcell fusion. Transfer of a fragment of chromosome 16 is sufficient to extinguish myogenin and myosin heavy chain expression, indicating the presence of a muscle-specific extinguisher on mouse chromosome 16.

**W 426** FIBER TYPE DIFFERENTIATION IN PATHOLOGICAL HUMAN FETAL MUSCLES,

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Further insight into the factors involved in muscle development might be achieved from the study of material from naturally occurring human genetic defects affecting the neuromuscular system. We studied four cases of the lethal congenital contractures syndrome (LCCS), a form of arthrogryposis multiplex congenita, involving severe reduction in the number of α-motoneurons and extremely hypoplastic muscles. Limb muscles (20 to 33 weeks of gestation) consisted of very few groups of small fibers which were strongly stained with mAb against embryonic and fetal myosin heavy chains (MHC). Approximately half of the fibers were also strongly stained with an mAb against slow twitch MHC. Most of these fibers were unstained with the mAb against fast twitch MHC, whereas all other fibers were moderately to strongly stained. No muscle spindle fibers could be identified. Thus, differentiation into distinct fiber types expressing slow or fast MHC isoforms occurred in spite of the absence of motoneurons. These findings are in agreement with results obtained in rat and mouse following early denervation in utero.

Limb muscles of a case of familiar Fetal Akinesia Deformation Sequence (FADS) (35 weeks of gestation + 9 weeks postnatal) were immature, contained numerous profiles of nerve trunks and prominent muscle spindles with very high numbers of intrafusal fibers. The increased number of intrafusal fibers was partly due to splitting of the original fibers, a finding previously observed in the rat after long-term motor denervation. Deposits of AChE were detected in many intrafusal fibers but neurofilament protein was surprisingly absent in many, suggesting that their innervation was deficient in spite of the abundance of nerves in the muscle. Systematic studies of fetal material in which a neuromuscular defect is suspected might yield important clues to the understanding of human muscle differentiation.

**W 427** THE EFFECTS OF HYPERTHYROIDISM ON MDX AND CONTROL MUSCLE REGENERATION, A.N. Pernitsky and Judy E. Anderson, Department of Anatomy, University of Manitoba, Canada, R3E 0W3.

Thyroid hormone plays a key role in normal muscle cell proliferation and differentiation, and in the maturing expression of myosin heavy chain genes. It has also recently been shown to down-regulate basic fibroblast growth factor (bFGF). To determine a possible role for thyroid hormone in muscle repair, 5.5-week-old mdx dystrophic and control mice were made hyperthyroid with triiodothyronine (T3) while littermates served as euthyroid controls. After 3.5 weeks, the tibialis anterior (TA) muscles were crush-injured and allowed to recover for 4 days. $^3$H-thymidine (ip) 24 hours before sacrifice allowed a study of muscle cell turnover. Morphometry (H&E) and immunohistochemistry (bFGF, NCAM, embryonic myosin) were performed on various muscles. All T3-treated mice showed decreased muscle weight ($p<.001$) in uncrushed muscle, and decreased myotube density ($p<.05$) in crushed TA. The area of active dystrophy in uncrushed mdx TA was also decreased by T3 ($p<.05$). Myofiber size ($p<0.05$) in uncrushed TA and myotube diameters ($p<0.01$) in zones adjacent to the crush were smaller in T3-treated controls only compared to untreated control TA, and fewer bFGF-positive mononuclear cells ($p<0.001$) were observed in this group. However, mdx myotubes were always larger than control myotubes ($p<0.01$). Results suggest that T3 may impair muscle repair more in controls than mdx, possibly by altering the balance between myoblast proliferation, myotube maturation and inflammatory processes.
Supported by an MHRC studentship (AP) and a grant from the MDAC (JEA).

**W 428** PHENOTYPIC EFFECTS OF UNIFORM AND MOSAIC EXPRESSION OF A TRUNCATED DYSTROPHIN TRANSGENE IN SKELETAL MUSCLE OF *mdx* MICE, Jill A. Rafael and Jeffrey S. Chamberlain, Department of Human Genetics, University of Michigan Medical School, Ann Arbor, MI 48109
Duchenne muscular dystrophy results from mutations in the dystrophin gene. Although the symptoms of this disease are primarily muscle-associated, isoforms of dystrophin are produced in many non-muscle tissues. Alternative splicing of 5 exons within two regions of the C-terminal domain of the protein generates tissue- and developmental stage-specific isoforms of dystrophin. To study the function of these alternatively spliced exons, we generated transgenic mice expressing a construct (MDA-Δ330) missing four exons deleted in an isoform of dystrophin normally present in the brain. Mice expressing this isoform in all skeletal muscle fibers, on a mutant *mdx* background, appear to have normal muscle morphology through 3.5 months of age. Therefore, this region of the protein does not seem to be critical for normal dystrophin function in limb skeletal muscle and diaphragm. Analysis of two additional lines of transgenic *mdx* mice that display a mosaic expression pattern from the MDA-Δ330 transgene reveals a dramatic difference in the pathophysiology of the two mouse lines that is dependent on the degree of mosaicism. Mice expressing this dystrophin isoform in less than 50% of their muscle fibers display necrosis and fibrosis of skeletal muscle typical of the *mdx* phenotype. However, mice with greater than 70% dystrophin-positive fibers display a normal muscle morphology. Together, these results suggest that the entire dystrophin C-terminus is not required for the prevention of muscular dystrophy, and that high level expression of dystrophin in a large percentage of fibers may prevent degeneration of neighboring dystrophin minus fibers.

**W 429** ARTERIAL DELIVERY OF SKELETAL MYOBLASTS TO THE HEART, Shawn W. Robinson, Peter C. Cho, Michael A. Acker[1], and Paul D. Kessler, Departments of Medicine and Surgery, Johns Hopkins University School of Medicine, Baltimore, MD 21205 and [1] University of Pennsylvania School of Medicine, Philadelphia, PA 19104
Skeletal muscle is unique in its capacity for repair and regeneration. This property has been attributed to the presense of adult myoblasts in mature skeletal muscle. In contrast stem cells can not be identified in the heart of the adult, which consists of terminally differentiated cardiomyocytes. From a clinical perspective it might be beneficial to generate new striated muscle into the heart to replace or augment damaged cardiomyocytes. We report the incorporation, long-term survival, and differentiation of skeletal myoblasts following their introduction into the arterial supply to the heart. One million C2C12 myoblasts expressing cytoplasmic (C2-MFGlacZ) or nuclear-localized (C2-Zn, a gift of E. Ralston) β-galactosidase were injected into the ventricular cavity of anesthetized C3H mice. Cardiac tissue was obtained at intervals up to 6 months after injection and processed for β-galactosidase histochemistry, immunofluorescence microscopy, and electron microscopy. LacZ positive myoblasts were indentified in 39 of 60 (65%) of experimental animals up to 6 months after injection. Immediately after the injection, myoblasts were arrested within the myocardial capillaries. One week after injection lacZ positive mono- and multi-nucleate cells that co-expressed a fast-twitch isoform of the sarcoplasmic reticulum calcium ATPase (SERCA1) were present in the cardiac interstitium. Injected cells aligned with the fiber axis and formed recognizable sarcomeres. Electron-dense "gap-like" junctions formed betweeen myoblasts-derived cells and cardiomyocytes. Starting four months after the injection, some of the implanted cells showed aspects of the slow-twitch phenotype, including induction of phospholamban expression. We conclude that arterial delivery of myoblasts is a novel method for the introduction of new striated muscle or recombinant proteins into the heart.

**W 430** INVESTIGATION OF THE ROLE OF THE MYOTONIC DYSTROPHY KINASE (DMK) IN THE DIFFERENTIATION OF CULTURED MYOBLASTS.

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Recently, the mutation underlying DM has been identified as trinucleotide repeat amplification in the 3' untranslated region of a gene encoding a protein kinase (DMK). We have demonstrated that expansion of this repeat causes a marked increase in the steady state mRNA levels of DMK in tissues from severely affected individuals (Sabourin *et al.*, Nature Genetics, 1993, in press). Primary myoblast cultures from normal and congenital patients have been established and antibodies to the kinase have also been produced. Immunocytochemical analysis of myoblast cultures shows that a major isoform of DMK localizes to the nuclei of myoblasts and myotubes.

Several authors have reported a marked delay of muscle maturation and an abundance of satellite myoblasts in tissue sections of congenital DM patients (Reviewed by Harper, 1989 "Myotonic Dystrophy" ). Supporting these observations, our data show that DM myoblasts induced to differentiate *in vitro* show a delay of terminal differentiation as evidenced by a lack of myosin heavy chain-positive cells. Induced DM myoblasts weakly express muscle lineage-specific genes, such as MCK and V-MLC1. Furthermore, in contrast to normal myoblasts, induced DM myoblasts show low levels of myogenin expression following induction of differentiation. Interestingly, MyoD is expressed at similar levels in both normal and DM lines, suggesting that the inhibition caused by the myotonic phenotype blocks one or several very early steps during differentiation of skeletal muscle. Supporting the hypothesis that DM is due to overexpression of DMK, induction of terminal differentiation in the presence of DMK antisense oligonucleotides rescues the myotonic phenotype in DM myoblasts. The effects of under- and over-expression of DMK on C2 differentiation have been investigated and will be presented. Analysis of DMK expression in myogenic systems will provide insights into the effects of trinucleotide repeat expansion in the 3'-UTR of DMK, the dominant inheritance pattern of DM and the pathogenesis of the disease.

**W 431** MUSCLE FIBER TYPE-SPECIFIC GENE REGULATORY ELEMENTS IDENTIFIED BY *IN VIVO* TRANSFECTION,

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Four major fiber types (type 1, 2A, 2X and 2B) can be identified in rat skeletal muscle by the distribution of distinct myosin heavy chain transcripts (1). The regulatory sequences and the transcription factors responsible for fiber type-specific gene expression are not known. Intramuscular injection of plasmid DNA into mature muscles could be used to study the expression of foreign genes, however the efficiency of gene transfer is extremely low. We have recently found that the efficiency of gene transfer *in vivo* is markedly improved by pretreatment with the myotoxic drug, bupivacaine, that induces muscle degeneration followed by regeneration (2). We are now using this experimental system to study muscle fiber type-specific gene regulation. Initial experiments were performed with myosin light chain 1 slow (MLC1s) gene. Regenerating fast (extensor digitorum longus) and slow (soleus) muscles were transfected 3 days after bupivacaine treatment with plasmids containing fragments of the 5'-flanking region of the mouse MLC1s gene linked to bacterial CAT or β-gal genes. Reporter gene product was determined 10 days after bupivacaine treatment, when the endogenous MLC1s gene is expressed at high levels in the soleus but not in the extensor digitorum longus muscle. Several constructs also showed a higher level of expression in the regenerating soleus and deletion analysis has revealed a slow-specific element in the MLC1s gene promoter.

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**W 432 ADENOVIRAL VECTORS FOR GENE TRANSFER OF FULL LENGTH HUMAN DYSTROPHIN cDNAs,**

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Conventional adenoviral vectors are able to accommodate foreign DNA of up to 6.5kb which is insufficient for the incorporation of full length human dystrophin cDNA (12-14kb). However novel vectors have been developed with an increased capacity for exogenous DNA. These so-called pseudo-adenoviral (PAV) vectors contain the minimal sequences required for packaging of recombinant DNA into an adenoviral particle in the presence of a trans-complementing viral genome. We have shown that recombinant virions produced from a PAV reporter gene construct pPAVLacZ(n) are able to infect human muscle cells in culture. We have constructed full length dystrophin cDNA expression cassettes which utilise the muscle specific MCK and α-skeletal actin promoters; these constructs have been tested in tissue culture and in transgenic *mdx* mice and have been shown to express a recombinant protein of the appropriate size. We are generating adenoviral reagents which harbour the full length dystrophin and reporter-gene cDNAs for comparative somatic gene transfer studies in skeletal muscle. We will assess the ability of these constructs to transduce muscle cells *in vitro* and in the *mdx* mouse system with a view to using the constructs for gene therapy. The size limitations associated with current retroviral and adenoviral vectors means that only mutant BMD-based dystrophin minigenes have been advocated as attractive candidates for gene therapy of muscular dystrophy. These mutant dystrophins while remaining partly functional are however associated with significant (albeit relatively mild) disease pathology in patients; it thus remains likely that more acceptable improvements in the pathology will be obtained by expressing full length cDNAs encoding normal wild-type dystrophin.

**W 433** USE OF RETROVIRAL GENE INSERTION TO SELECTIVELY ABLATE SPECIFIC FIBERS IN EMBRYONIC MUSCLES

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Little is understood about the mechanisms of muscle fiber pattern formation in the early embryonic limb. We designed experiments in which avian retroviral vectors were used to deliver a diphtheria toxin-A (DT-A) gene to fibers of the early limb. The DT-A expression in this construct is under the control of the slow myosin heavy chain 3 (SM3) promoter so that all fibers that express SM3 will be induced to suicide. Previous work from our laboratory showed that the dorsal and ventral muscle masses for the muscles of the hind limb with a precise pattern of based on slow myosin heavy chain expression. Only a few primary fibers stain with monoclonal antibody S58 which recognizes the SM3. The intention is to eliminate prior to innervation those fibers responsible for the pattern and then to assess the fiber pattern that emerges as the muscles of the limb develop at early fetal stages. The following questions will be answered 1. How does elimination of only the slow primary fibers influence the early muscle pattern? 2. Will this effect the development of neighboring fibers, i.e. the fast primary fibers? 3. Will formation of entire muscles, such as medial adductor, be eliminated? 4. Will secondary muscle fiber formation be altered during fetal development? As a first step, we cloned a 1.1 kb BamHI-BglII SM3 regulatory region to the RCAN(B)-CAT vector, and a 0.8 kb SstI SM3 regulatory region into the RCAN(F)-CAT *vector. These two vectors were transfected into early passages of* embryonic day 10 chicken fibroblast by $CaPO_4$ transfection. Staining of these transfected cells with an antibody against the retrovirus capsid protein P 27 showed that the retrovirus particles spread quickly in the cultural cells. Retrovirus particles were harvested from the culture media with high titer and were used to infect ED6 quail atrial cells in culture (atrial cells that express SM3 at high levels).

**W 434 FATIGUE RESISTANCE OF RESPIRATORY MUSCLE FROM TRANSGENIC MICE EXPRESSING INCREASED CREATINE KINASE ACTIVITY,**

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Creatine kinase (CK) has been implicated in the maintenance of skeletal muscle intracellular energy supplies via its 1) ATP buffering capacity and 2) forming a shuttling system for ATP and ADP between mitochondrial and myofibrillar compartments. We explored the physiologic significance of CK activity by utilizing a transgenic mouse line that expressed the B subunit of CK leading to increased amounts of CK-MB, CK-BB, and total CK activity in a respiratory muscle, the external abdominal oblique (EO). We determined the 1) in vitro isometric contractile and fatigue properties and 2) myosin heavy chain (MHC) phenotypes of EO from transgenic (↑CK) [n=23] and control (CTL) [n=22] mice. Isometric twitch contraction and half relaxation times, force-frequency relationships, specific force outputs, and MHC phenotypes were not different between ↑CK and CTL animals. Fatigue resistance, however, was 22% higher in ↑CK as compared to CTL mice ($p<0.02$). The MHC phenotype of both ↑CK and CTL mice were characterized by abundant expression of MHC 2B. We conclude that increased CK activity and an altered CK isoform phenotype to the degree expressed in this transgenic line, are associated with a significant increase in EO fatigue resistance. Moreover, the comparable specific force outputs, and MHC phenotypes between the ↑CK and CTL lines suggest that force generating capacity, and the myosin heavy chains themselves, do not contribute to the difference in fatigue resistance between study groups. We speculate that the reaction catalyzed by CK activity contributes directly to increased fatigue resistance. (Supported by NIH HL 02491)

**W 435** GENE EXPRESSION FOLLOWING DIRECT INJECTION OF PLASMID DNA INTO SKELETAL MUSCLE.

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Myofibres take up and express plasmid DNA directly injected into skeletal or cardiac muscle of vertebrates. This technique has potential applications in local and systemic gene therapy, and as a means of vaccination. Quantity of DNA, the promoter used and the solute all affect the level of gene expression. We have shown expression of a reporter gene is significantly higher in young compared to old mice and males exhibit higher expression than females (Wells & Goldspink, 1992). Additionally, we have found that DNA uptake and expression is increased in regenerating muscles after pre-treatment with bupivacaine, with 2-3 times as many fibres expressing the reporter gene compared to the contralateral control. The same phenomenon is observed in the naturally regenerating dystrophic muscle of the mdx compared to control C57Bl10 mice. (Wells, 1993, in press).

Direct injection can also be used as a method for in-vivo localisation of recombinant muscle proteins. Dystrophin has been proposed to associate with the skeletal muscle membrane by way of a glycoprotein complex which interacts with its C-terminal domains. Direct injection of mdx muscles with recombinant genes encoding human dystrophin deletion mutants shows, however, that not only the C-terminus of dystrophin but also its N-terminal actin-binding domain can locate independently to the muscle sarcolemma. This observation suggests that the lack of sarcolemmal-associated dystrophin in Duchenne muscular dystrophy muscle may result from enhanced degradation of truncated mutation products rather than their inability per se to associate with the sarcolemma.

**W 436** GENETIC CORRECTION OF DYSTROPHIN DEFICIENCY IN TRANSGENIC MDX MICE.

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We have produced mdx mice transgenic for the full length and the recombinant (minigene) human dystrophin cDNAs driven by the α-skeletal actin and MCK promoters. A number of lines have been produced for each construct with transgene expression ranging from 1/10 to 10 times the wild type dystrophin expression in C57Bl10 control mice. We are examining the ability of the dystrophin 'minigene', a cDNA based on a very mild Becker muscular dystrophy patient, to alleviate the juvenile myopathy and prevent the fibrosis of the aging diaphragm that is typical of the dystrophic mdx mouse. We will present data on muscle morphology, immunolocalisation of transgene products and serum creatine kinase levels. Reductions in pathology associated with 'minigene' expression will be correlated to the level of expression and compared to the effects of full length cDNA expression at similar levels. We confirm our previous work in that there is significant reduction in the juvenile myopathy associated with 'minigene' expression [Wells et al., 1992 Hum. Mol. Genet. 1:35-40]. More detailed quantification of the performance of this 229 kD recombinant protein will be of great importance is assessing the likely role of this construct in gene therapy for Duchenne muscular dystrophy. Current retroviral and adenoviral vectors can accommodate the 'minigene' but not the full length dystrophin cDNA.

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**W 437 TRANSGENIC MICE WITH DEFICIENT CREATINE KINASE GENES.**

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The cytosolic Muscle-type Creatine kinase (M-CK) and the Sarcomeric Creatine kinase from mitochondria (Sc-CKmit) catalyse the reversible exchange of high energetic phosphate between creatine-P (PCr) and ATP, and are considered important enzymes in the energy metabolism of skeletal and cardiac muscle. Via homologous recombination in embryonal stem (ES) cells we have generated heterozygous/ homozygous mutant mice with (i) null-mutant M-CK genes (see van Deursen et al. (1993) Cell 74, 621-631), (ii) leaky-mutant M-CK genes or (ii) inactivated Sc-CKmit genes. These mice, as well as the original mutant ES cells in culture, were used to study how muscle adapts in respons to genetic changes in the CK-PCr system. Of the M-CK mutant mice, only the animals which lost M-CK activity completely showed adaptation in cellular architecture (reduced fibre size, reorganisation in number and size of the intermyofibrillar mitochondria) and increased storage of glycogen. Upon treatment of the null-mutant mice with the Cr analogue ß-guanidinopropionic acid these effects were even more pronounced, and resulted in a further reduction of the cross-diameter of type 2B fibres. Suprisingly, muscle burst activity and the flux of energy through the CK reaction (as determined by 31P NMR in vivo) were already affected at intermediate M-CK concentrations. Overall, for these latter parameters a close correlation with the level of M-CK expression was noted. Similar studies were performed with the Sc-CKmit deficient mice. Findings from biochemical, (immuno)histological and physiological typing on these mutants will be reported.

*Neuromuscular Junction*

**W 438** THE v-*src* ONCOGENE AFFECTS MYOGENIC DIFFERENTIATION AT DIFFERENT LEVELS: IMPLICATION OF TRANSCRIPTIONAL AND POST-TRANSCRIPTIONAL MECHANISMS. Stefano Alemà, Maria-Cristina Gauzzi, Sabrina Strano, Loriana Castellani* and Germana Falcone. Istituto di Biologia Cellulare and *Istituto di Neurobiologia, C.N.R., 00137 Rome, Italy.

*In vitro* transformation of replicating myogenic cells by a variety of oncogenes is always accompanied by inhibition of the transition to the terminally differentiated state. In primary quail myoblasts the v-*src* oncogene blocks myogenesis by controlling directly the transcription of muscle-specific genes, without affecting transcription of the myogenic regulatory factor MyoD. The MyoD protein accumulates in the nuclei of quail myoblasts transformed by a temperature sensitive v-*src* gene at both restrictive and permissive temperatures, as well as after reactivation of the oncoprotein in fully differentiated cells, and retains the ability to bind to the E-Box of a muscle specific enhancer. However, MyoD and myogenin proteins are functionally competent only in the presence of the inactive oncoprotein, as assayed by transient expression of CAT constructs containing muscle specific promoters. Finally, when $pp60^{v\text{-}src}$ is reactivated in postmitotic myotubes upon shift-down to the permissive temperature, a number of dramatic changes occur at the myofibrillar level, probably due to post-transcriptional mechanisms. First, within 1 hour, specific antibodies or rho-phalloidin stain the apperance in the myotubes of numerous "actin bodies", containing muscle-specific actin, a-actinin and vinculin. Then, within 6-12 hours, starts a sequential disassembly of sarcomeric proteins such as skeletal myosin, titin and nebulin. Experiments on the dissection of the pathways downstream to $pp60^{v\text{-}src}$, by the use of dominant negative mutants of *ras* and *raf* as well as specific inhibitors of PKC, will be discussed.

*Supported by AIRC, Telethon, and CNR-PF-Biotecnologie and ACRO.*

**W 439** A CELL CULTURE MODEL FOR THE STUDY OF SATELLITE CELL BEHAVIOR. John C. Angello and Stephen D. Hauschka, Department of Biochemistry, University of Washington, Seattle, WA 98195

In vivo skeletal muscle satellite cells are stimulated by trauma to replicate, producing a cohort of cells with myogenic potential. While the majority of these progeny commit to terminal differentiation, a few serve to re-establish the satellite cell population. The processes underlying the maintenance of this critical phenotype are not understood. We have used the MM14 satellite cell-derived mouse line to study this question via time-lapse videomicroscopy. Within single mouse myoblast clones there is a "stochastically-derived" subpopulation (<10% of the total cells) which exhibits satellite cell-like characteristics: upon mitogen withdrawal, these cells do not exit the cell cycle and undergo terminal differentiation as do their clonally-related siblings. Rather, during this period of apparent mitogen restriction, they continue to divide, albiet much more slowly [the mean cycling time increases from 9.7 hrs (± 7%) to 18.0 hrs (± 17%)]. Continued cell division does not depend on one daughter becoming committed. In fact, none of the cells which completed 4 doublings following mitogen withdrawal were committed. While these cells could still be stimulated by bFGF to resume rapid division (10.6 hrs ± 14%), this response was preceded by a 4-5 hr lag. Most importantly, the subsequent progeny recapitulated the earlier myogenic response to mitogen withdrawal. These results suggest that a minor subpopulation of MM14 cells can undergo a metabolic adjustment to mitogen-poor conditions which inhibits commitment to terminal differentiation and yet preserves myogenic potential.

**W 440** FIBROBLAST GROWTH FACTOR 1 AND MYOGENESIS D. BARRITAULT, L. SOULET, G. LEMAITRE, C. REY, M. JAYE*, J. GAUTRON AND M. MISKULIN.

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Satellite cells play an important role during muscle regeneration. Fibroblast Growth Factor 1 (FGF1) is a potent mitogen for these cells and is expressed within the myoblasts during the proliferation phase. However, FGF1 is undetectable when the cells differentiate, suggesting that this factor may negatively control this process. In order to test more accurately the role of FGF1, satellite cells were transfected with an FGF1 expression vector. One particular clone was studied which expressed constitutively FGF1 at 5 ng per $10^6$ cells. The biologically active monomer FGF1 was only detected in cell lysate but not in the cell culture medium. Although the FGF1-transfected satellite cells grew at the same rate as control cells during the proliferation phase they lost their capacity to differentiate. They were able to be subcultured at least over 200 divisions. Partial phenotypic reversion was observed after different treatments of transfected cells. Fusions can also be induced with more efficiency by transfection with FGF1 antisense.

Our results suggest a key role for FGF1 and its receptors in the control of myogenesis. The differentiation of muscle cells can be blocked if a high level of endogenous FGF1 is maintained.

**W 441** EXPRESSION OF MUTANT MyoD WITH DELETION OF PROTEIN KINASE C/FGF PHOSPHORYLATION SITE INHIBITS GROWTH OF RHABDOMYOSARCOMA CELLS, Peter Dias, Barbara Bugg, Michael Dilling, and Peter Houghton, Department of Molecular Pharmacology, St. Jude Children's Research Hospital, Memphis, TN 38101

Rhabdomyosarcoma (RMS), a soft tissue tumor, is thought to arise as a consequence of the loss of growth control of myogenic cells. Members of the MyoD family have been shown to be pivotal factors in orchestrating the expression of numerous genes associated with myogenic differentiation and inhibition of growth of normal, transformed and neoplastic cells. Growth and differentiation of myogenic cells is determined by a balance between opposing signals elicited by the myogenic bHLH regulatory factors and growth promoting cues. In Rh30 cells, autocrine growth is dependent upon IGF-II binding to the IGF-1 receptor. We postulate that this loop promotes proliferation and prevents MyoD function. Recently, Eric Olson's lab (Li *et al.*, Cell 71:1181, 1992) showed that a conserved site in the basic, DNA binding domain of myogenin (threonine 87) is a protein kinase C and FGF phosphorylation site. Deletion of this site in myogenin resulted in a mutant protein whose transcriptional activating potential was not repressed by FGF.

As PKC induction downstream of IGF-1R signalling has been proposed, we evaluated the role of phosphorylation of this conserved site in the activity of MyoD. We constructed an inducible expression vector that encodes a MyoD protein in which the conserved threonine residue (threonine 115) is mutated to alanine resulting in disruption of the phosphorylation site. This construct was transfected into Rh30 rhabdomyosarcoma cells and inhibits growth of the cells in low serum medium upon induction, whereas growth of cells transfected with the control vector or wild-type MyoD is not inhibited under similar conditions. These data suggest that PKC activity, possibly secondary to IGF-1R signalling, may relate to the undifferentiated/proliferative state of RMS. (Supported by NIH grants CA23099 and CA21765 and by American Lebanese Syrian Associated Charities.)

**W 442** INHIBITION OF MYOGENIC DIFFERENTIATION IN MYOBLASTS EXPRESSING A TRUNCATED TYPE II TGF-β RECEPTOR, Ellen H. Filvaroff, Reinhard Ebner, and Rik Derynck, Departments of Growth and Development, and Anatomy, Programs in Cell Biology and Developmental Biology, University of California at San Francisco, San Francisco, CA 94143-0640
Tranforming growth factor-β (TGF-β) is thought to play a role in mesenchymal cell development, and specifically in muscle differentiation, yet its precise role in the latter process remains unclear. TGF-β has been shown to both inhibit and induce myoblast maturation in vitro, depending on the culture conditions. To determine whether the type I or type II TGF-β receptor mediates the various TGF-β effects on myogenesis, C2C12 myoblasts were transfected with an expression vector for a truncated type II TGF-β receptor which has been shown to act as a dominant negative inhibitor of type II receptor signalling. In contrast to the parental cells, the transfected clones did not efficiently form myotubes nor induce expression of MyoD, myogenin, and several differentiation markers following incubation in low serum media. However, some differentiation markers continued to be expressed in the transfected cells suggesting that at least two pathways are involved in muscle cell differentiation. These cells could still growth arrest in low serum media, showing that decreased proliferation can be dissociated from differentiation. Unlike the several oncogenes known to block myogenic differentiation, the truncated TGF-β receptor did not induce myoblast transformation. Injection of the parental or the transfected C2C12 cells into the limb muscle of nude mice revealed quantitative and qualitative differences in their behavior, and suggested that myoblasts expressing the truncated TGF-β receptor could not fuse in vivo. Finally, retrovirus-mediated expression of MyoD in the transfected cells restored their ability to form myotubes in vitro, indicating that inhibition of myoblast differentiation by the truncated TGF-β receptor may depend on decreased MyoD expression. We propose that TGF-β signalling through the type II receptor is required for several distinct aspects of myogenic differentiation, and that TGF-β may act as a competence factor in this multistep process.

**W 443** IGF-I INHIBITS MYOGENESIS UNDER CONDITIONS IN WHICH ITS MITOGENIC ACTIONS CAN OCCUR, J. R. Florini, D. Z. Ewton, S. L. Roof, K. A. Magri, and F. J.McWade, Biology Dept., Syracuse Univ., Syracuse, NY 13244
Over the years, we have presented numerous reports on the myogenic actions of the IGFs, culminating in the observations that IGFs induce expression of the myogenin gene and that IGF-II is actively secreted by myoblasts in low-serum "differentiation" medium. To many observers, these reports seemed to conflict with the generalization that mitogens block myogenesis, presumably by preventing muscle cells from entering the postmitotic $G_0$ state from which myogenesis is initiated. It has been suggested that the apparently stimulatory effects of the IGFs result from their maintenance of the cells in a more viable state — i.e., the "happy cell" theory. Although these kinds of explanations have been eliminated, we have remained puzzled by our consistent observation that IGF-II (and insulin at very high levels) produces more extensive fusion and higher creatine kinase (CK) levels than does IGF-I, in spite of the fact that the IGF-I effects are observed at lower hormone concentrations, and much evidence indicates that the effects are mediated by the receptor that preferentially binds IGF-I. We have now found that the apparent differences among the IGFs are a matter of timing; i.e., the lower stimulation of myogenesis by IGF-I can be attributed to its greater stimulation of cell proliferation upon addition of hormone to L6A1 myoblasts in serum-free medium. Experiments comparing concentration dependencies at different times show that CK levels in IGF-I treated cultures lag approximately 24 hours behind those treated with IGF-II. When cells were treated under conditions that suppress cell proliferation (higher initial density, treatment with cytosine arabinoside), stimulation of myogenesis by IGF-I was as great as that by IGF-II. In fluorescence cell-sorted human myoblasts (a generous gift from Helen Blau), which exhibit a lower mitogenic response to IGF-I, this hormone stimulated CK elevation as much as did IGF-II. Thus the apparent controversy between those studying *inhibition* of myogenesis by FGF and other growth factors and those studying its *stimulation* by IGFs is resolved. There is, in fact, no exception to the generalization that mitogens inhibit myogenesis; the IGFs differ only in that they also induce expression of myogenin and thus stimulate myogenesis after cell division ceases. (Supported by NIH and the USDA).

**W 444** MYOGENIC HLH GENES AND bFGF INTERACTION IN MUSCLE CELLS OF THE mdx MOUSE. Kerryn L. Garrett and Judy E.Anderson, Department of Anatomy, University of Manitoba, Canada. R3E 0W3.
In addition to their mitogenic effects, fibroblast growth factors (FGF) have been well recognised as inhibitors of skeletal muscle differentiation in vitro. Members of the muscle specific helix-loop-helix (HLH) gene family, including myf5, MyoD, myogenin and MRF4, regulate myogenesis. Recently, FGF has been shown in cultured cells to trans-activate its own expression and suppress the expression of myogenin and MyoD, and thus prevent differentiation, suggesting a possible interaction between these genes. This was examined in vivo by in situ hybridisation to determine whether bFGF and the muscle HLH genes are co-expressed in myogenic cells of regenerating muscle. Muscle from the mdx mouse, which is characterised by repetitive cycles of degeneration and repair, demonstrated mononuclear cells containing MyoD, myogenin and bFGF transcripts in similar regions of the muscle: in areas of focal degeneration and repair, and particularly around the ends of damaged fibres. Using adjacent sections it was not possible to conclude co-expression. In myotubes sectioned transversely, these genes were expressed in the same newly regenerated fibres. A double probe hybridisation technique was developed to further investigate the apparent co-expression in mononuclear cells (specifically in muscle precursor cells). In addition, tritiated thymidine incorporation (as a measure of mitogenic activity) was examined to determine the proliferative status of cells expressing the myogenic regulatory genes and/or bFGF. The extent of co-expression of MyoD, myogenin and bFGF genes may imply their interaction in the genetic control of myogenesis in vivo. Work is supported by a grant from MHRC and a fellowship from MRC/MDAC (KLG).

**W 445** EFFECT OF EXPRESSION OF RAS EXCHANGE FACTOR ON SKELETAL MUSCLE DIFFERENTIATION
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Ras proteins are implicated both in the proliferative and differentiative pathways. But, while activation of ras proteins is essential for differentiation of PC12 cell lines, the activated ras oncogene has an inhibitory effect on differentiation of muscular cells. The percentage of ras proteins in the active state is set by the balance between GTP hydrolysis, induced by GTPase activating proteins, and GDP/GTP exchange stimulated by Guanine Nucleotide Releasing Factors, whose prototype is the product of the S.cerevisiae CDC25Mm gene. Exchange factors homologues to CDC25Mm have been recently isolated from mammalian cells. In this abstract we present data on the effect of the expression of two different forms of mouse CDC25Mm on myoblasts C2C12 differentiation. Cellular fusion index (FI) were measured after transfection with the CDC25Mm full length cDNA and compared with the index obtained from myoblasts transfected with a partial CDC25Mm cDNA correspondig to the catalitic domain, with the v-ras cDNA and a control plasmid. Our results showed a significant increasing of maturation processes, as demonstrated by the FI, in CDC25Mm full length transfected myoblasts, while CDC25Mm catalic domain transfection resulted in an increased proliferative capacity. The expression of the short CDC25Mm, lacking Db1 and PH domains, inhibits the differentiation probabmly through an activation of endogenous ras, while the differentiating effect of the whole CDC25Mm (form IV) suggests that this protein could activate other regulatory pathways that control the development of muscle cells.

**W 446 REGULATION OF CARDIAC MYOSIN LIGHT CHAIN-2 (MLC-2) GENE EXPRESSION BY PROTO-ONCOGENE C-JUN: INACTIVATION OF THE LEUCINE ZIPPER DOMAIN OF C-JUN CONVERTS IT FROM AN INHIBITOR TO AN ACTIVATOR,** **Shyamal K. Goswami, Saiyid Shafiq and M.A.Q.Siddiqui, Department of Anatomy and Cell Biology, SUNY Health Science Center at Brooklyn, Brooklyn, NY 11203**

**In skeletal muscle cells proliferation and myogenic differentiation are mutually exclusive events whereas embryonic and early neonatal cardiac cells in the differentiated state can undergo proliferation. Also, in response to various trophic signals including certain pharmacological agents such as angiotensin II, adrenergic agonists, phorbol esters etc. cardiac muscle cells develop a hypertrophic response which is characterized by a reprogramming of the transcriptional activity. During this process the expression of certain sarcomeric genes such as cardiac myosin light chain-2 (MLC-2) is upregulated and other genes such as atrial natriuretic factor and skeletal a-actin, which are expressed in myocardial cells only during early embryonic development, are reexpressed. The precise mechanism of the reprogramming of transcription during myocardial hypertrophy is not known, however, certain early response genes such as *c-fos*, *c-jun*, *egr-1* have been implicated in this process. Our laboratory has isolated and characterized the cardiac MLC-2 promoter to study its expression during myocardial development and hypertrophy. We have reported earlier that cotransfection of a plasmid harboring *c-fos* gene can inhibit the MLC-2 promoter/CAT fusion gene expression in cardiac muscle cells in culture. In this report we have examined the effect of c-Jun on the MLC-2 promoter activity. We demonstrate that c-jun inhibits the expression of the MLC-2 promoter/CAT fusion gene and this inhibition does not involve any specific cis-acting DNA elements in the MLC-2 promoter. Using various mutants of the *c-jun* expression plasmid we demonstrate that c-Jun can act as an inhibitor of MLC-2 gene expression via its interaction through the leucine zipper domain with an unidentified transcription factor in the cardiac muscle cells. Interestingly, inactivation of the leucine zipper domain of the c-Jun protein converts it into an activator of the MLC-2 promoter. Therefore, it appears that Jun may play an important role in transcriptional regulation in myocardial cells via its interaction with some other important transcription factors. The mechanism of this regulation is under study.**

**W 447** EVIDENCE FOR A CENTRAL ROLE OF THE EARLY GROWTH RESPONSE GENE-1 (Egr-1) IN MYOGENIC PROLIFERATION AND DIFFERENTIATION

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***Objective:*** Egr-1 is a prototypical member of a class of transcription factors expressed in response to exogenous growth factors. We have recently shown that antisense oligonucleotides complementary to the Egr-1 mRNA inhibit angiotensin-II induced protein synthesis in isolated rat cardiomyocytes. As a step towards defining regulatory target genes for the Egr-1 protein, we here investigated its role in myogenic cell lines.
***Methods and results:*** Numerous growth factors induced Egr-1 mRNA 5-25-fold (angiotensin II, endothelin, phorbol-myristate-acetate, fetal calf serum -FCS-, basic fibroblast growth factor -bFGF-, platelet-derived growth factor -PDGF-BB-) in Sol8 myoblasts. Growth factors that induced mitosis (FCS, bFGF, and PDGF-BB), shown by a parallel increase in DNA- and protein synthesis, lead to translation of the mRNA. In contrast, the differentiative stimulus insulin leads to a translational block of the Egr-1 mRNA. Furthermore, after stimulation with insulin, L6 cells which stably overexpressed Egr-1 (L6+) showed an impaired differentiated morphology as compared with normal L6 myoblasts and a clonal population stably transfected with an Egr-1 construct which contained a truncated DNA-binding domain (L6 trunc.). The DNA-synthesis of L6+ was increased twofold, as measured by $^3$H-thymidine incorporation. The creatine kinase level was severely inhibited (< 50% of native L6) in L6+. ***Conclusions:*** These results indicate a pivotal role of Egr-1 in the control of proliferation and differentiation of myogenic cells and will ultimately allow characterization of interaction partners of Egr-1, which suppress proliferation in the myocardium thus leading to hypertrophic (as opposed to proliferative) growth.

**W 448** THE INTERACTION OF EXTRACELLULAR MATRIX AND GROWTH FACTORS ON PRIMARY CULTURES OF ADULT SKELETAL MOUSE MUSCLE, Miranda D Grounds and Moira A Maley, Department of Pathology, University of Western Australia, WA, 6009

Primary cultures of skeletal muscle were used to investigate whether the superior regeneration seen in SJL/J compared with BALB/c muscle *in vivo* was reflected in a varied response of myoblasts from the two strains to growth factor (GF) *in vitro*. The effects on cell proliferation of bFGF, AA-AB- and BB-PDGF, TGF$\beta_1$ and LIF were assessed using $^3$H-thymidine incorporation on 24 hour primary muscle cultures and also on the mouse myogenic C2C12 cell line. With primary cultures grown on gelatin, proliferation increased with bFGF and PDGF, but was not affected by TGF$\beta_1$ or LIF: no difference was seen between the strains in the response to these exogenous factors. When cultures were grown on matrigel, the baseline proliferation in the absence of GF was higher and proliferation was not elevated by low doses of GF. The influence on proliferation of higher doses of GF and of matrigel components was assessed further. Significantly more myotubes were present in SJL/J than BALB/c cultures at 4 days on gelatin, but there was no difference in myotube formation on matrigel between the strains. LIF had no effect on myotube formation in any SJL/J cultures, but decreased the number of myotubes in BALB/c cultures on gelatin and increased the number on matrigel. There were generally more nuclei/myotube for SJL/J compared with BALB/c cultures. That the composition of the extracellular matrix can influence both cell proliferation and myotube formation emphasises the complexity of assessing the role of various factors in the *in vivo* environment.

**W 449 DIFFERENTIAL DISTRIBUTION OF β AND γ ACTIN mRNAs AND ITS REGULATION BY GROWTH FACTORS,** Peter Gunning and Mark Hill, Cell Biology Unit, Children's Medical Research Foundation, Locked Bag 23, Wentworthville NSW 2145, Australia.

The biological significance of actin isoforms is of interest in terms of both the regulation of cell cytoarchitecture and the evolution of structural protein multigene families. Previous gene transfection studies have indicated that the two non-muscle actin isoforms have different effects on myoblasts: elevated expression of β-actin promotes cell spreading whereas increased γ-actin expression is an antagonist. The localisation of these two mRNAs has been examined in order to evaluate whether changes in the size of cellular mRNA compartments may occur with alterations in isoform expression. We have found that β and γ actin mRNAs are differentially located in myoblasts. This was observed using non isotopic doube *in situ* hybridisation and was visualised with a confocal laser scanning microscope. The distribution of β actin mRNA was both perinuclear and concentrated at peripheral motile regions whereas γ actin mRNA was restricted to perinuclear regions. An identical result has been obtained with fibroblasts and macrophage. In contrast, double immunofluorescent visualisation of β and γ actin protein failed to reveal any significant difference in their disribution, suggesting that the mRNAs are used to localise synthesis rather than steady state distribution of the isoforms. We propose that functional differences between isoforms, such as cell spreading, could reflect the size of the peripheral actin mRNA pool and the resulting supply of free actin monomer for new microfilament formation. This diffference between the location of β and γ actin is under regulation by growth factors. Serum starvation of cells eliminates the peripheral pool of β actin mRNA. Readdition of serum restores peripheral β, but not γ actin mRNA within 2 minutes and correlates with increased membrane ruffling. We conclude that the differential distribution of actin isoform mRNAs is regulated by growth factors and involves active transport mechanisms.

W 450 INHIBITION OF MYOGENESIS BY bFGF: DOES POSTTRANSLATIONAL MODIFICATION OF THE MRF4 ACTIVATION DOMAIN PLAY A ROLE?, Sally E. Johnson, Yanfeng Kong, Serge Hardy and Stephen F. Konieczny, Department of Biological Sciences, Purdue University, West Lafayette, IN 47907

Transcriptional regulation of muscle specific genes is, in part, controlled by the basic helix-loop-helix (bHLH) myogenic regulatory factors. MRF4, as with other members of this transcription factor family, contains a DNA binding domain, a HLH domain and a transcriptional activation domain (TAD). The MRF4 TAD, located within the first 60 amino acids, functions as a heterologous activation domain in both mammalian and yeast model systems, provides partial specificity with regard to the activation of individual target genes and is required to fully induce the myogenic program. The MRF4 TAD also contains four candidate phosphorylation consensus sequences that potentially could be used as focal points for regulating MRF4 activity. Of these potential sites, serine residues 56/57 are part of a casein kinase II (CKII) consensus sequence. Initial *in vitro* experiments have shown that serines 56/57 are phosphorylated by CKII and that CKII-directed phosphorylation is abolished by mutation of these residues. Subsequent overexpression of MRF4 alanine 56/57 (MRF4 A56/57) in C3H10T1/2 fibroblasts results in an increase in transcriptional activity. However, as is the case for wildtype MRF4, MRF4 A56/57 activity is abolished by the addition of basic fibroblast growth factor (bFGF) to the culture medium. Similarly, transfection of C3H10T1/2 cells with a MRF4 expression plasmid in which MRF4 serines 31, 37 or 42 have been mutated yields identical results as those obtained for the mutated CKII site. That is, MRF4 A31, A37 and A42 activities are repressed in cells exposed to bFGF. Compilation of this data suggests that bFGF repression of myogenesis occurs independently of the phosphorylation status of the MRF4 TAD.

W 451 SPATIAL AND TEMPORAL EXPRESSION OF THE *sno* PROTOONCOGENE DURING EMBRYONIC MOUSE DEVELOPMENT. Stuart Kim*, Randall Moreadith#, and Gary E. Lyons*. *Department of Anatomy, University of Wisconsin Medical School, Madison, WI 53706. #Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, TX 75235.

The expression patterns of the *sno* gene, a novel protooncogene related to *ski* (see abstract by Lyons et al.) were examined by in situ hybridization in normal mouse embryos between 7.5 and 16.5 days post coitum (p.c.). Many embryonic cells expressed *sno* mRNAs at some level, but higher levels were detected in certain cells and tissues during development. Expression was prominent in the neuro-epithelium at 9.5 days p.c., and persisted in the cells of the neural tube and spinal cord through 16.5 days. Within the brain, *sno* expression was restricted to certain layers of neurons. Expression of *sno* during cardiac development was limited to two areas - the outflow tract at 10.5 days and endocardial cushions at 11.5-12.5 days p.c. *Sno* gene transcripts were detected in skeletal muscle cells between 12.5 and 14.5 days p.c. High levels of message in the mandibular arch and maxillary process were detected at 9.5 and 10.5 days, respectively. The observed hybridization patterns in the heart, branchial arches, and whisker follicles may represent *sno* expression in certain populations of neural crest cells. *Sno* mRNAs were detected in the developing retina between 11.5 and 13.5 days p.c. Cartilage in various areas, such as the ribs, and the limb buds contained elevated levels of *sno* message at 11.5 to 12.5 days. Transcripts were detected in cells around the developing olfactory cavity at 11.5 days and in cells surrounding the auditory vesicle at 14.5 days.

This research was supported by a grant from the Muscular Dystrophy Association to G.E.L.

W 452 ATRIAL NATRIURETIC PEPTIDE (ANP) AS A GROWTH FACTOR FOR CHICK EMBRYONIC CARDIOMYOCYTES, Masafumi Koide, Robert E. Akins, Hiroaki Harayama, Kenji Yasui, *Mitsuhiro Yokota, **Rocky S. Tuan. National Chubu Hospital, Obu, * Nagoya Univ., Nagoya, Japan, **Thomas Jefferson Univ. Philadelphia, PA 19107

Significant levels of ANP have been noted in the developing heart. In this study, the role of ANP in cardiac development was evaluated using cultured cardiomyocytes isolated from day 15 chick embryos. We analyzed ANP effects on: cell number, DNA synthesis, total RNA level, expression of cell cycle specific and sarcomeric proteins, and levels of LDH and CPK. ANP increased overall DNA synthesis (by BrdU incorporation, $P < 0.01$) and enhanced cell proliferation. Microscopically, the development of the cardiomyocyte network was distinctly enhanced by ANP. The level of LDH was lowered ($p<0.01$), but CPK was not altered by ANP. Expression of the G1-specific protein, PSTAIRE and PCNA, was raised, and the G2/M specific protein, cyclin B1, was not changed by ANP treatment. Cellular RNA content (by methyl green/pyronin staining) was elevated in ANP treated cells. Likewise, myosin and tropomyosin biosynthesis were significantly greater in ANP treated cells than in controls. An antibody against ANP and a specific ANP receptor antagonist, HS 142-1, antagonized and/or modulated the action of ANP on both cell proliferation and protein biosynthesis. These results indicate that ANP accelerates myocardial cell proliferation by enhancing entry into S phase and by increasing DNA synthesis during S phase specifically through the receptor mediated pathway. The *in vitro* effects of ANP on myocardial cell proliferation, together with the elevated levels of ANP seen *in vivo* during normal heart formation, suggests a possible autocrine function of ANP in embryonic cardiac development.

W 453 MYOGENIC DIFFERENTIATION AND CELL TRANSFORMATION: ARE THEY MUTUALLY EXCLUSIVE PHENOMENA?
**S.A. La Rocca**, D.H. Crouch*, D.A.F. Gillespie* and F. Tatò
Dip. Biologia Cellulare e dello Sviluppo, Università di Roma "La Sapienza", Rome-Italy, and *Beatson Institute for Cancer Research, Glasgow-UK.

Myogenesis represents a particularly attractive *in vitro* system for studying the genetic basis of differentiation and the relationship between proliferation and terminal differentiation. *In vitro* transformation of primary myoblasts by a variety of oncogenes is accompained by inhibition of terminal differentiation. We show in this study that the forced expression of the Myc protein transforms the growth phenotype of primary quail myoblasts and prevents their terminal differentiation in myotubes. The Myc-induced block of myogenic differentiation is associated with the absence of muscle specific gene products, such as myosin, and reduced expression of the positive myogenic regulatory factors of the MyoD family.
Mutant alleles in different Myc domains were used to clarify the mechanism through which the oncogene inhibits myogenic differentiation. Particularly Myc mutants in the Leucine Zipper domain (LZ), which impair or abolish Max dependent transcriptional activation, were tested for their ability to transform and inhibit the terminal differentiation.
The results will be discussed in the light of current models of Myc function.

Supported by Fondazione Cenci-Bolognetti, CRC, AIRC (Progetto Oncosoppressori) and CNR-PF ACRO.

**W 454** DEVELOPMENTAL EXPRESSION OF THE PROTO-ONCOGENE, *ski*, IN MOUSE EMBRYOS.

Gary E. Lyons*, Bruce Micales*, Mark Herr*, Hong Chen#, Stephanie Namciu# and Edward Stavnezer#+. *University of Wisconsin Medical School, Madison, WI 53706, #University of Cincinnati Medical Center, Cincinnati, OH 45267, +Present address: Case Western Reserve University, Cleveland, OH 44106

The protooncogene, *ski*, is thought to play a role in skeletal muscle development since it can induce muscle differentiation in quail nonmuscle cells[1] in vitro and can cause fiber type-specific hypertrophy in vivo in skeletal muscles of transgenic mice[2]. We examined the patterns of cellular *ski* gene expression by in situ hybridization and by Northern blot in normal mouse embryos between 7.5 days post coitum (p.c.) and birth. All embryonic and extra-embryonic cells express *ski* mRNAs at some level, but higher levels are detected in some cells and tissues during embryogenesis. Cells surrounding the somites that appear to be migrating neural crest, and the placenta have high levels of ski mRNAs at 8-9 days p.c. Neural crest derivatives such as whisker follicles and dorsal root ganglia contain higher levels of ski message at later stages. Myotomes do not express *ski* at high levels. These gene transcripts are upregulated in skeletal muscle at 12 days p.c., after MyoD and other basic, HLH myogenic factor genes have already been activated. *Ski* mRNAs remain elevated in skeletal muscle for several days. Cardiac muscle does not contain elevated levels of this gene transcript at any point examined. In developing limbs, these mRNAs are expressed at moderate levels in the interdigital region at 12.5 days p.c. and in limb muscle at 13 days p.c. At 15-16 days p.c., *ski* mRNAs are detected at high levels in the frontal cortex of the brain. At birth these gene transcripts increase approximately two-fold in lung tissue.

1. Colmenares, C. and Stavnezer, E. (1989) Cell 59, 293-303.
2. Sutrave, P., Kelly, A.M., and Hughes, S. (1990) Genes Dev. 4, 1462-1473.

This research was supported by grants from the Muscular Dystrophy Association to G.L. and from the NIH to E.S.

**W 455** A NEW MEMBER OF THE FIBROBLAST GROWTH FACTOR RECEPTOR FAMILY CLONED IN BIRDS: ITS EXPRESSION DURING EARLY EMBRYONIC DEVELOPMENT.

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We have cloned a new member of the fibroblast growth factor receptor family, FGFR5, from avian embryonic RNA. During elongating primitive streak stages, FGFR-5 is expressed in the rostral and lateral epiblast and in the Hensen's node. From 2.5 days of development (E 2.5) on, it is expressed in various ectoderm- and mesoderm-derived structures. Most striking is FGFR-5 expression in the skeletal muscle lineage. It is highly expressed in the early myotome and, at later stages, in all skeletal muscles of the embryo. From E9 to hatching, FGFR-5 expression in the muscles decreases dramatically but is maintained in satellite cells of adult muscles. We conclude that FGFR5 may play multiple roles in early avian development, including a specialized role in the early differentiation of skeletal muscle.

**W 456 TRANSFECTION OF HUMAN SATELLITE CELLS WITH AN IMMORTALISING VECTOR.**

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Human satellite cells have been transfected with a vector containing T antigen from SV40 under the control of the vimentin promoter. This vector was constructed by the group of Dr Paulin, and has been used successfully to transfect other mammalian cells. These cells were co-transfected with RSV-Neo and, after selection, growth properties of the selected cells were analysed. Myogenic differentiation was observed on two of these population, and the myogenic markers expressed were characterised. All these results were compared to the ones observed on the non transfected population, presented on another poster. Doubling time of the transfected cells was shorter than those observed in the original population, and the number of possible divisions was compared that observed in control. The *differentiation* was characterised by the expression of embryonic, fetal and slow myosin heavy chains, as well as fast type myosin light chains. The evolution of the length of the telomeres of the transfected cells was analysed, and conclusions concerning aging of the satellite cells and possibilities of obtaining an immortalised human myogenic cell line are discussed.

**W 457** pRB IS REQUIRED TO INHIBIT DNA SYNTHESIS BUT NOT DIFFERENTIATION OF SKELETAL MUSCLE CELLS

Bennett Novitch, Bart Williams[1], Tyler Jacks[1], and Andrew Lassar, Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115 and [1]Center for Cancer Research, Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139

In the process of skeletal muscle differentiation, proliferating myoblasts exit the cell cycle and enter a permanently non-dividing state. Early observations that myogenic differentiation can be blocked by infection of muscle cells with viruses such as SV40, which are now known to express oncoproteins that inactivate the pRB family of tumor suppressor proteins, strongly suggest that pRB and its relatives are intimately involved in muscle cell differentiation. These studies have been substantiated with demonstrations that induction of the SV40 large T antigen in myotubes allows these normally quiescent cells to re-enter the cell cycle. To further clarify the interdependence of myogenic differentiation on pRB function, we have studied myogenesis in isogenic primary fibroblasts derived from mice in which one or both copies of the *RB* gene have been inactivated. We have found that expression of retroviral encoded MyoD induces skeletal muscle differentiation in the absence of pRB. However, in contrast to wild-type differentiated myocytes that are terminally withdrawn from the cell cycle, differentiated myocytes lacking pRB initiate new DNA synthesis when challenged with serum. These results indicate that pRB is not necessary for skeletal muscle gene expression, yet is apparently required to inhibit aberrant DNA synthesis in differentiated skeletal myocytes. In addition, we have preliminary data suggesting that these pRB deficient myocytes, though capable of entering S phase, do fully progress through the cell cycle to metaphase. These findings indicate that pRB may specifically prevent DNA synthesis in skeletal myocytes, while other pRB related proteins that are inactivated during T antigen induction may function as check point controls after the initiation of S phase.

**W 458 INTERLEUKIN-1 BETA STIMULATES GROWTH OF NEONATAL RAT CARDIAC MYOCYTES THROUGH A TYROSINE KINASE SECOND MESSENGER PATHWAY.** JN Palmer, WE Hartogensis, and CS Long. Cardiology Section, VA Medical Center and the University of California, San Francisco, San Francisco, CA. 94122

Cytokines such as IL-1 have been implicated as playing a major role in the inflammation associated with ischemia/reperfusion injury. Further, IL-1 has been recently reported to affect beating rate in high density cultures of neonatal rat cardiocytes; however, the direct effects of IL-1 on cardiac myocyte (MC) growth have never been addressed. To answer this question, we treated isolated rat ventricular MCs in culture with purified murine IL-1β (0.1pg-10ng/ml), finding a maximal 1.38 ±.004-fold increase in myocyte protein synthesis as measured by $^{14}$C phenylalanine uptake (EC50 of ~250pg/ml, n=4, p≤ .005). Since IL-1 has been reported to act via prostaglandin, nitric oxide and protein kinase C second messenger pathways in other cell types, we repeated individual growth experiments in the presence of Indomethacin (1-100 uM), the inducible NO synthase inhibitor Nω-Nitro-L-Arginine Methyl Ester (1-100 uM), or Staurosporine (5nM). None of these agents was found to block the growth-producing effect of 1 ng/ml of IL-1β (n=3-6, p=ns in all cases of inhibitor added with IL-1β). In contrast, the Tyrosine Kinase inhibitor Genistein, (10 uM) blocked the growth effect of IL-1β by ~50% (n=6, p≤ .001 of growth with inhibitor vs. IL-1β growth). To test the hypothesis that IL-1β induces a direct growth effect through the high affinity IL-1 receptor, the 163-171 amino acid fragment of IL-1β that binds to the IL-1 receptor and causes T cell activation was added in concurrent treatment with IL-1β. The fragment was also able to block the growth effect by ~50% (n=3, p≤ .001). To our knowledge this is the first evidence indicating that the cytokine IL-1β can stimulate an increase in myocyte protein synthesis, and suggests a possible role for this cytokine in the process of myocardial recovery from ischemia/reperfusion.

**W 459 EFFECT OF GROWTH FACTORS ON PROLIFERATION AND DIFFERENTIATION OF SATELLITE CELLS DERIVED FROM SINGLE MUSCLE FIBRES OF MOUSE,** **David J. Parry and Alison I. Lunt, Dept of Physiology, University of Ottawa, Ottawa, ON, K1H 8M5 CANADA.**

Muscle fibres were mechanically dissociated from mouse flexor digitorum brevis muscles with the aid of collagenase (cf Bischoff, 1986, Dev Biol 115:129-139). Single fibres were plated in individual, Matrigel-coated, wells of Primaria tissue culture plates. Under these conditions the majority of fibres adhered to the substratum. Medium containing 20% horse serum with or without various growth factors was added. Within 48 hrs spherical cells could be seen in the vicinity of most fibres. These cells divided over the next few days, became spindle-shaped and eventually fused to form myotubes which stained positively with MF-20, an antibody to sarcomeric myosin heavy chains indicating that the initial cells were satellite cells (SC) which, presumably, detached from the fibres. The effect of growth factors on proliferation and differentiation of SC from individual muscle fibres was assessed after 7 days. The cultures were fixed, stained with MF-20 and hematoxylin, and the number of nuclei in both MF-20-positive and negative cells in representative fields was counted. Addition of 0.5% chick embryo extract resulted in a mean of 1123+/-98 nuclei, 40% of which were in MF-20-positive cells, whereas in the absence of any added growth factor there were only 178 nuclei, of which 65% were MF-20-positive. Addition of bFGF to the basal medium resulted in a dose-dependent increase in the number of nuclei, reaching a maximum of 505 at 1 ng/ml of which only 45% were MF-20-positive. This is consistent with the expected effect of bFGF to inhibit differentiation. Addition of a neutralizing antibody to bFGF prevented the proliferation induced by bFGF. Since an extract of crushed muscle (CME) has been shown to stimulate SC proliferation, the effect of added CME will be tested on this system using fibres from both normal and dystrophic mice. (Supported by MDAC).

**W 460 CONTROL OF MYOGENESIS BY MyoD-like FACTORS AND INSULIN-like GROWTH FACTORS.** Christian PINSET, Frédéric AURADE, Joseph ILAN° & Didier MONTARRAS, Institut PASTEUR, unité de Biochimie, 28 rue du Docteur ROUX, 75724 PARIS. (FRANCE)
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We are studying the contribution of Insulin like Growth Factors (IGF) and factors of the MyoD family to differentiation by forcing or inhibiting their expression in cultured muscle cells.

1 - The pattern of MRF4 expression suggests a role in adult muscle gene expression. However, we observed that forced expression of MRF4 in embryonic mesenchymal 10T1/2 cells is not sufficient to promote adult muscle gene expression as it occurs in myotubes derived from myosatellites cells. Trying to teach the myogenic derivatives of 10T1/2 cells how to express adult muscle gene could reveal crucial regulatory events responsible for expression of late muscle functions.

2 - We observed that expression of MyoD at hight level at the myoblast stage and expression of IGFII are associated with a higher probability to undergo differentiation: inhibition of IGFII gene expression in permissive C2 myoblasts by antisense RNA results in the loss of the ability to differentiate spontaneously and the down regulation of MyoD. Reciprocally, forced expression of MyoD in inducible C2 myoblast (these myoblast do not express MyoD) results in an increased expression of IGFII and spontaneous differentiation. These observations suggest that MyoD and IGFII are involved in a positive regulatory loop promoting differentiation .

3 - We observed that down regulation of MyoD in permissive C2 myoblasts by antisense RNA directed against MyoD or IGFII mRNAs is associated with a marked up-regulation of Myf5. In spite of this, Myf5 expressing cells, unlike MyoD expressing cells, fail to differentiate autonomously. These results indicate that MyoD and Myf5 may not be strictly interchangeable and that additional factors could be responsible for the apparent complementation observed in vivo. What would be the result of a cross between MyoD and IGFII deficient animals ?

**W 461** SELECTIVE ENHANCEMENT Of MYOBLAST PROLIFERATION OR DIFFERENTIATION BY OVEREXPRESSION OF THE TYPE-1 IGF RECEPTOR. LeBris S. Quinn, Brian Steinmetz and Adolf Maas, Department of Biological Structure, University of Washington, Seattle, WA 98195.

A retroviral expression vector (LISN) was utilized to overexpress the human type-1 insulin-like growth factor (IGF) receptor in mouse C2 myoblasts. C2-LISN myoblasts expressed 30 times higher levels of IGF receptors than control (C2-LNL6) myoblasts infected with a retroviral vector that did not code for the type-1 IGF receptor. In high serum conditions, neither C2-LNL6 nor C2-LISN myoblasts underwent terminal differentiation, but C2-LISN myoblasts expressed 10-fold higher levels of myogenin. In low serum conditions with either low or high (500 ng/ml) IGF concentrations, C2-LISN myoblasts underwent terminal differentiation more rapidly than C2-LNL6 myoblasts and expressed 10-20 times higher levels of sarcomeric myosin heavy chain. Treatment of both C2-LNL6 and C2-LISN myoblasts with 10 ng/ml transforming growth factor-$\beta$ (TGF-$\beta$) in low or high (500 ng/ml) IGF concentrations inhibited differentiation. However, treatment of C2-LISN myoblasts with the combination of TGF-$\beta$ and 500 ng/ml IGF-I induced proliferation to form multilayers, whereas C2-LNL6 myoblasts in the same conditions remained contact-inhibited. When TGF-$\beta$ was removed from multilayered C2-LISN myoblast cultures, numerous fused and sarcomeric myosin heavy chain-positive myotubes appeared within 48 hours, indicating that the multilayered C2-LISN myoblasts were *differentiation-competent*. These findings support the idea that the type-1 IGF receptor mediates both differentiation and proliferation responses to the IGFs. Additionally, overexpression of the type-1 IGF receptor may represent a strategy to expand populations of differentiation-competent myoblasts for experimental or clinical applications.

**W 462 A Novel Growth Factor for Muscle--rhGGF2,** Robert M. Sklar, Cassandra Kirk, David Gwynne, Robert McBurney, Mark Marchionni, Cambridge NeuroScience, One Kendall Square, Bld.700, Cambridge, MA 02139
Recently, the expression of one or more product of the neuregulin gene in peripheral neurons has been described by *in situ* hybridization (Marchionni, et al., Nature 1993;362:312-318). A secretable form of neuregulin, rhGGF2, has been cloned and expressed in CHO cells. The possibility that rhGGF2 may have a trophic effect on muscle cells was investigated. Primary human myoblasts can be differentiated into multi-nucleate myotubes *in vitro* over a period of eight days. Differentiation of myoblasts in the continuous presence of rhGGF2 results in greater numbers of nuclei in myotubes after 6 days of differentiation. This effect appears to be unique to rhGGF2, and is not observed with a variety of other growth factors that have effects on muscle cultures, such as, PDGF and IGF-I. rhGGF2 is mitogenic for subconfluent quiescent myoblasts.

W 463 RESPONSE OF RABBIT SMOOTH MUSCLE MYOSIN HEAVY CHAIN (MHC) GENE TO TGFß IN CULTURED VASCULAR SMOOTH MUSCLE CELLS. Chandra Somasundaram, Robert C Kallmeier and Philip Babij. Dept. Physiology, University College, London WC1E 6BT, England.

TGFß has been shown to be a potent growth inhibitor of vascular smooth muscle cells (VSMC) and is also associated with the development of cellular hypertrophy in VSMC. To study the role of TGFß in regulating differentiation in VSMC we have used the rabbit smooth muscle MHC gene as a model since its expression is tissue-specific and developmentally regulated. Primary cultures of VSMC from rabbit aorta were grown to confluency in 10% FCS and then synchronized with respect to the cell cycle by maintainance in serum-free medium (SFM). After 48hr in SFM, cells were incubated with 0.2ng/ml TGFß1 for 60hr and cells harvested for mRNA analysis. Compared to control, TGFß1 increased by 10-20% the level of smooth muscle MHC mRNA (SM1) when measured by RNase protection analysis. Determination of [$^3$H]thymidine incorporation into TGFß treated cells confirmed that TGFß did not stimulate DNA synthesis. The effect of TGFß was also studied following transient transfection into VSMC of 5' end deletion mutants of the rabbit smooth muscle MHC promoter linked to the reporter gene pCAT Basic. Following DNA transfection, VSMC were switched to SFM and incubated with TGFß for 60hr. When deletion mutant pRSMHC-1,328 was used for transfection, TGFß stimulated a 2.3 fold increase in CAT activity compared to SFM alone. We recently showed that this mutant gave the highest relative CAT activity (24-58 fold) in transfected VSMC cultured in 10% FCS. A similar 2.3 fold increase in CAT activity was observed from the longer mutant pRSMHC-2,301. These increases in CAT activity were not observed when shorter mutants (-1,221 and -754) were transfected into VSMC. These results indicate that TGFß may participate in control of smooth muscle MHC expression in VSMC.

W 464 THE INDUCTION OF XENOPUS VENTRAL MESODERM BY BMP2 AND BMP4 IS CODEPENDENT ON A FUNCTIONAL ACTIVIN RECEPTOR SIGNALING SYSTEM. Gerald H. Thomsen#*, Ali Hemmatti-Brivanlou#, and Douglas A. Melton#. #Department of Biochemistry and Molecular Biology, Harvard University, Cambridge MA 02138, and *Department of Biochemistry and Cell Biology, SUNY Stony Brook, Stony Brook, NY 11794-5215.

Xenopus blastula animal caps expressing BMP 2 or 4 proteins from injected mRNAs display proper temporal expression of early and late ventral mesodermal markers such as Xwnt8 and alpha globin. These caps can also differentiate into characteristically ventral, vesiculated tissue. Induction of ventral mesoderm by the BMPs is, however, inhibited when a dominant-negative (C terminally-truncated) activin receptor is coexpressed in the animal cap. These results suggest that the ventral mesoderm-inducing activity of BMP proteins is codependent on a signal from endogenous activin receptors. Additional experiments aimed at blocking BMP signaling in whole embryos are in progress using dominant negative ligands, and the latest results will be presented.

W 465 **bFGF ACTIVATES MAP KINASE KINASE (MEK) IN PROLIFERATING MYOBLASTS.** Mary Pat Wenderoth, #Jean S. Campbell, Stephen D. Hauschka, and #Edwin G. Krebs. Dept. Biochem. & #Dept. of Pharm., University of Washington, Seattle,WA
Growth factors are key regulators of proliferation and differentiation in myogenesis. In MM14s, a mouse skeletal muscle cell line, FGF is required during the G1 phase of the cell cycle to repress differentiation and to promote proliferation. Removal of FGF from the media results in differentiation into myocytes with the concomitant up regulation of the muscle specific gene program. In these myoblasts, bFGF interacts with FGFR-1 (*flg*), which is a receptor tyrosine kinase that is down regulated during differentiation. It has been proposed that the phosphorylation of muscle determination factors (MDFs) and/or the ubiquitous helix-loop-helix transcription factors with which they form heterodimers alters the affinity of the complexes for the E-Box (CAnnTG). In Swiss 3T3 fibroblasts, it has been shown that epidermal growth factor (EGF) initiates the MAP kinase cascade by binding to its specific receptor tyrosine kinase, EGFR. Currently, this cascade consists of the sequential activation of MAP Kinase Kinase (MAPKK or MEK), MAP Kinase (MAPK or ERK) and S6 kinase. We are investigating whether a similar kinase cascade is involved in FGF repression of muscle differentiation in myoblasts. Preliminary results demonstrate that at 3-5 hrs. post mitogen withdrawal both bFGF and PMA stimulate the activation of MAPKK. However, only PMA, **not** bFGF stimulate MAPK activity. MAPK isoforms, erk-1 and -2, are present in MM14 myoblasts and are activated *in vitro* by MAPKK. We are currently investigating the following possibilities: 1) the presence of an MAPK phosphatase, 2) presence of a novel isoform of MAPK or 3) presence of a novel substrate for MAPKK in myoblasts. It will be of interest to dissect how bFGF signals both repression of differentiation and stimulation of proliferation and to determine whether the specificity of the FGF signal lies at the level of specific kinase cascades or the activation of specific transcription factors or both.

**W 466** REGULATION OF MUSCLE TRANSCRIPTION BY THE MAP KINASE PATHWAY, Jumin Zhou, Keith L. Ligon and Eric N. Olson, The Department of Biochemistry and Molecular Biology, The University of Texas M. D. Anderson Cancer Center, Houston, TX 77030

Terminal muscle differentiation is negatively regulated by serum and growth factors which inhibit the expression of muscle specific genes. Myogenic bHLH proteins activate most muscle specific genes by binding to the E-box consensus sequence in the promoters and enhancers of these genes. To activate these genes, myogenic bHLH proteins must heterodimerize with members of the E protein family, such as E12 and E47. The activation domains from both myogenic factors and the E proteins in these heterodimers are important for the full transcriptional activity, making myogenic factors, as well as E proteins, possible targets for growth factor repression. Our observations suggest that MAP and raf-1 kinases, which are activated by growth factors, can inhibit transactivation of E-box dependent reporter genes by myogenic bHLH proteins and E47. This inhibition is seen for myogenin and E47 in their native form and for fusions of their activation domains to the GAL4 DNA binding domain. In vitro kinase assays show that MAPK can phosphorylate serine residues of GST-myogenin and both serine and threonine residues of GST-E47 at high efficiency. In gel assays have also shown that there is a 42kd kinase activity in C2 myoblast extracts, but not in differentiated C2 myotubes, that can phosphorylate GST-E47. These data suggest that growth factors may repress the activity of myogenic bHLH protein and E proteins through the MAP kinase signalling pathway.

*Late Abstracts*

REGULATION OF MYOGENIC DIFFERENTIATION IN RD CELLS. M. Bouché, F. Zappelli, M.I. Senni and M. Molinaro. Institute of Histology, University "La Sapienza", Rome.

The human rhabdomayosarcoma cell line RD undergoes a very limited and abortive myogenic differentiation despite the fact that these cells express the myf3 and myf4 genes. We have previously shown that the tumor promoter TPA induces myogenic differentiation in these cells (Aguanno et al. Cancer Res. 50; 3377, 1990). Furthermore TPA induces the accumulation of specific muscle products, such as skeletal myosin heavy and light chains and alfa-actin, without altering the myf3 and myf4 expression (Bouché et al. Exp. Cell Res. 208; 209, 1993). However TPA inhibits the expression of the Id trascript within 1 day of treatment. While TPA-induced inhibition of Id message accumulation correlates with differentiation, cell confluence also causes reduction in Id expression, without inducing differentiation. Furthermore the endogeneous myf3 and myf4 are able to bind specific DNA sequences, even in the absence of TPA. Overexpression of any of the myf factors does not induce spontaneous differentiation but enhances the effect of TPA treatment, independently from the level of the expressed exogeneous messages. These data suggest that differentiation of RD cells is likely to depend upon the activity of complexes containing the various members of the MyoD family, which can be regulated by proteins affecting dimerization, such as Id, but also by other mechanisms induced by TPA, such as phosphorylation. We are currently studying the role of protein kinase C isoforms in the differentiation process induced by TPA. We are also developing approaches aimed at the identification of binding activities interacting with myf3/myf4 factors in a positive or negative manner. These binding activities may well be responsible for the suppression of differentiation in RD cells, which is rescued by TPA treatment.

REDUCTION OF UTROPHIN ASSOCIATED WITH ACETYLCHOLINE RECEPTOR DEFICIENCY AT THE NEUROMUSCULAR JUNCTION IN HUMAN DISEASES, Clarke R. Slater*, Carol Young*, Sarah J. Wood*, Louise V.B. Nicholson*, Guy S. Bewick*, Peter Baxter*, Angela Vincent+, Mark Roberts+, John Newsom-Davis+, *Muscular Dystrophy Group Research Laboratories, Newcastle General Hospital, Newcastle upon Tyne NE4 6BE, +Institute for Molecular Medicine, John Radcliffe Hospital, Headington, Oxford, UK.

Utrophin, the product of the autosomal homolog of the dystrophin gene, is present at the internal surface of skeletal muscle fibres at the neuromuscular junction (NMJ) but not in the extrajunctional region. At the NMJ it is precisely colocalised with acetylcholine receptors (AChRs) both in the adult and at all stages of development. So far, no examples of genetically determined utrophin deficiency have been reported which might help to determine its function.

We have recently made immuno-cytochemical studies of 2 patients with congenital myasthenic syndromes with well-documented AChR deficiency. In both cases, the intensity of labelling for utrophin was much less than at normal NMJs. By contrast, the intensity of labelling for dystrophin, a form of $\beta$-spectrin, the 43K dystrophin-associated glycoprotein and the 43K AChR-associated protein were all normal. Initial studies of one of these patients suggest that the gene defect is not in the utrophin gene. One patient with acquired autoimmune myasthenia gravis has also been studied and has reduced utrophin labelling. More than 20 other patients, including some with congenital myasthenic syndromes not involving AChR deficiency, had normal levels of utrophin labelling.

We conclude that utrophin is closely associated with AChRs at the NMJ but that its reduction in the cases we have studied is a secondary consequence of AChR deficiency.