GENE EXPRESSION IN NEUROMUSCULAR DEVELOPMENT

Organizers: Helen Blau, Alan Kelly and Bruce Paterson

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Gene Expression in Neuromuscular Development

Keynote Address (joint)

F 001 REGULATION OF REGULATORS, David Baltimore, The Rockefeller University, New York, New York 10021 and the Whitehead Institute for Biomedical Research, Cambridge, MA. 02142. The most conceptually challenging issue of differentiation is not how gene expression is regulated. -- Jacob and Monod largely solved that problem -- but how the regulators are regulated. To achieve a differentiated, stable phenotype requires that a cell program numerous genes in quite precise ways, using robust regulatory strategies to assure long-term stability. In no cellular system is there a satisfactory description of regulation, but as more regulators are isolated, and the genes encoding them are cloned, elements of regulatory strategies are emerging. One is that regulators occur in structurally-related families and that often members of a family interact among themselves. The leucine zipper, helix-loop-helix and rel-related proteins (especially NF-κB) are examples. Also regulators may be sequestered by inhibitors (as IκB for NF-κB or Id for helix-loop-helix proteins) and therefore, the regulation of the inhibitor becomes a crucial component of the system. Certain regulators positively activate themselves, contributing to stability. Also, when their products are not required, many, maybe all, genes are maintained in the non-expressed state by active silencing methods and counteracting silencing may be a crucial aspect of allowing gene expression. Individual genes are affected by a medley of regulators, thus the ensemble of gene products that define any one differentiated state is produced by a multitude of regulators, each maintained by its own particular set of interactions.

Cell Fate Decisions (joint)

F 002 MYOGENESIS IN THE MOUSE, Margaret Buckingham, Gary Lyons, Marie-Odile Ott and Françoise Catala, Department of Molecular Biology, Pasteur Institute, 25 rue du Dr. Roux, 75724 Paris Cedex 15, France.

We have examined the temporal and spatial expression of muscle genes during myogenesis in the mouse embryo, using *in situ* hybridization on tissue sections. The first striated muscle to form is the heart. In the cardiac tube from 7.5 days the myosin and actin isoforms of the adult heart are already co-expressed. Once the heart has acquired atrial/ventricular compartments different myosin gene transcripts begin to show spatial restriction. This is an asynchronous process (1). At no time are transcripts of the MyoD1 family detectable in the heart. The first skeletal muscle to form is the myotome, in the central region of the somites. Myotomal cells, as well as skeletal muscle cells in muscle masses elsewhere in the body originate from the dermomyotome of the somite. In contrast to the heart, different muscle structural gene transcripts appear asynchronously over a period of several days in the myotome (2). Transcripts of myogenic regulatory genes also accumulate with distinct kinetics (3). Myf-5 (in collaboration with E. Bober, H. Arnold) is the only muscle gene sequence detected in the dermomyotome, prior to formation of the myotome (4). Transcripts are present in some cells throughout the dermomyotome, but are concentrated in the dorsal lip region from which myotomal cells originate. Myf-5 transcripts are also present precociously in premuscle masses in the limb buds and elsewhere. At later stages of muscle development myf-5 is no longer detectable; myogenin, MyoD1 and myf-6 are the major myogenic regulatory sequences in foetal skeletal muscle. These results will be discussed in the context of muscle cell lineage determination and of the transcriptional regulation of muscle genes. The myosin alkali light chain (MLC1A) gene (5) expressed in the heart and also in embryonic skeletal muscle, provides an example of a regulatory element containing tandemly arranged consensus sequences binding myogenic factors of the MyoD1 family located in the proximal promoter. However examination of the central CArG-like sequence of this element indicates that myogenin (in collaboration with W. Wright) may be implicated in an additional regulatory phenomenon during skeletal myogenesis.

1) Lyons et al., 1990, J. Cell Biol., 111:1465-1476; 2) Lyons et al., 1990, J. Cell Biol. In press; 3) Sassoon et al. (1989) Nature, 341:303-307; 4) Ott et al. (1990) submitted; 5) Barton et al., 1988, J. Biol. Chem., 263:12669-12676.

F 003 **THE ROLE OF THE ACTIVIN FAMILY IN MESODERM FORMATION IN XENOPUS.** J.C. Smith, J.B.A. Green, G.-D. Guex, G. Howes, H.V. New, M.G. Sargent. National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, U.K.

The mesoderm of amphibian embryos, including muscle, notochord, kidney and blood, is formed in response to an inductive interaction in which cells of the vegetal hemisphere of the blastula act on overlying equatorial cells. The activins, members of the TGFβ superfamily, may be involved in this process. In the adult, the activins are known to modulate the release of follicle stimulating hormone from the pituitary and to promote erythroid differentiation. In the *Xenopus* embryo, we and others (Asashima *et al.*, *Roux's Arch. Devl Biol.* **198**, 330-335, 1990; Thomsen *et al.*, *Cell* in press) have shown that activins A and B can induce isolated presumptive ectoderm to form mesodermal cell types.

In this report we discuss how the activin family might be involved in specifying different regions of the *Xenopus* mesoderm. As markers we use probes for various mesodermal cell types as well as homeobox and zinc finger genes. Different concentrations of activin activate these genes to different extents. We also consider whether activin protein is present in the early embryo, and whether it can influence the expression of other members of the TGFβ superfamily in a cascade mechanism.

F 004 POSITIONAL INFORMATION IN LIMB MORPHOGENESIS, Lewis Wolpert, Department of Anatomy and Developmental Biology, University College and Middlesex School of Medicine, Windeyer Building, London W1P 6DB, UK.

Pattern formation in chick wing development can be understood in terms of the cells acquiring positional values along the antero-posterior and proximo-distal axes. For the antero-posterior axis there is a signal from the polarizing region which may involve retinoic acid as a morphogen; for the proximo-distal axis the proposed specification of position may depend on time in the progress zone. The relationship of positional values to homeobox gene expression will be discussed.

In addition to positional information there may be a primitive prepattern for both cartilage and muscle.

Muscle cells have an origin different from other cells in the limb, migrating in from the somites. Muscle patterning appears to involve migration of muscle cells and varying adhesive interactions with connective tissue cells. Unlike connective tissue cells, which are non-equivalent, muscle cells seem to be equivalent.

Most of the interactions in limb development can be classified in terms of STOP, GO, STAY and POSITION. The complexity of the limb lies in cellular responses rather than cellular interactions.

Gene Expression in Neuromuscular Development

Cellular and Molecular Biology of Commitment (joint)

F 005 **HIERARCHICAL CONTROL OF MUSCLE GENE EXPRESSION**, Eric N. Olson, Thomas J. Brennan, Tushar Chakraborty, T.-C. Cheng, Peter Cserjesi, Diane Edmondson, Li Li, Department of Biochemistry and Molecular Biology, The University of Texas M. D. Anderson Cancer Center, Houston, TX 77030

Establishment of a skeletal muscle phenotype appears to involve a regulatory cascade that culminates with the induction of a battery of genetically unlinked muscle-specific genes. We have used the muscle creatine kinase (MCK) enhancer as a target to study the mechanism whereby the muscle regulatory factor myogenin activates muscle-specific transcription. Myogenin, like other members of the MyoD family, binds to the concensus sequence CANNTG, which is present in the core of the MCK enhancer and in the control regions of numerous other muscle-specific genes. The affinity of myogenin for DNA increases dramatically in the presence of the widely expressed helix-loop-helix (HLH) protein E12, with which it forms heterodimers. Full activity of the MCK enhancer appears to require cooperative interactions between myogenin (or other members of the MyoD family) and other muscle-specific, as well as ubiquitous enhancer-binding factors. One such factor may be the myocyte-specific factor MEF-2, which binds an A+T-rich element in the MCK enhancer that is conserved in other muscle-specific enhancers. MEF-2 expression is activated by myogenin, suggesting that it may function at an intermediate level in the myogenic regulatory cascade. To define the domains of myogenin responsible for muscle-specific transcription, DNA-binding, and cooperative interactions with other enhancer-binding factors, we have generated an extensive series of myogenin mutants by site-directed mutagenesis and domain-swapping. These mutants have revealed regions in the amino and carboxy termini of myogenin that contribute to transcriptional activation and have defined two amino acids (alanine^{86}-threonine^{87}) in the basic region that impart muscle specificity. Mutagenesis of either of these residues converts myogenin from an activator to an inhibitor of myogenesis. The role of E12 in muscle-specific gene expression has also been investigated by creation of E12 mutants that dimerize with myogenin and abolish its ability to bind DNA. These mutants, which inhibit the ability of myogenin to activate myogenesis in transfected fibroblasts, are being examined for their effects on differentiation of cell types from other lineages. In addition to the positive roles played by myogenin and E12, myogenesis is also subject to negative control by growth factors, which can inhibit myogenin expression. Analysis of the 5' flanking region of the myogenin gene has revealed regions that confer muscle-specificity and growth factor responsiveness. Growth factors also can suppress myogenin's actions in cells that express it constitutively. Mechanisms through which growth factor signals may silence myogenin as an activator of muscle-specific genes include, induction of inhibitory HLH proteins such as Id, induction of immediate early genes like *fos* and *jun* that can trans-repress muscle-specific genes, repression of other cellular factors with which myogenin cooperates to activate target genes, changes in protein phosphorylation, and suppression of myogenin's transcription activating properties independent of DNA-binding. The involvement of each of these mechanisms in growth factor-dependent repression of myogenesis will be discussed.

Regulation by Growth Factors, Hormones, and Oncogenes

F 006 *ski* CAN CAUSE SELECTIVE GROWTH OF SKELETAL MUSCLE IN TRANSGENIC MICE, Pramod Sutrave*, Alan M. Kelly† and Stephen H. Hughes*, *NCI-Frederick Cancer Research and Development Center, ABL-Basic Research Program, P.O. Box B, Frederick, MD 21702-1201, †University of Pennsylvania School of Veterinary Medicine, Department of Pathology, Philadelphia, PA 19104.

Viruses that contain the v-*ski* oncogene are not only capable of causing morphological transformation in vitro, but can also induce myogenic differentiation. Viruses that express c-*ski* cDNAs also induce foci and myogenic differentiation. This suggests the possibility that the *ski* oncogene is bifunctional since the two known functions of *ski*, transformation and differentiation, would appear to be contradictory properties. Comparisons of the properties of forms of c-*ski* that are related by alternative splicing, and of several v-*ski* and c-*ski* deletion mutants, have shown that the portions of *ski* required for transformation and differentiation are quite similar; suggesting that the ability of c-*ski* and v-*ski* to cause transformation and induce differentiation may be related aspects of a single property of *ski* rather than two separate functions.

All of the biologically active forms of c-*ski* and v-*ski* that have been studied are localized primarily in the nucleus and overexpressed c-*ski* proteins are associated with condensed chromatin during cell division.

None of the available data make it possible to infer the normal function of c-*ski* either in terms of its role in growth and development (if any) or to have any direct insight into its mode of action. In an attempt to gain some insight into the c-*ski* function, we have introduced several alternatively spliced forms and deleted forms of chicken c-*ski* cDNA into transgenic mice under the control of an MSV LTR. Independent lines of mice that have greatly increased skeletal muscle mass were obtained.

Three lines of muscular mice that carry a truncated form of c-*ski* selectively express the transgene in skeletal muscle. The muscles of one of these three lines were examined in more detail. Increased muscle mass was due to selective hypertrophy of type IIb and IIx fast fibers of virtually every muscle in the animal. Type I and IIa fibers were unaffected and the cardiac muscle was normal. Research sponsored by the National Cancer Institute, DHHS, under contract No. NO1-CO-74101 with ABL.

Gene Expression in Neuromuscular Development

Genes that Regulate Myogenesis

F 007 SIGNALS AND GENES CONTROLLING MYOGENESIS IN VERTEBRATES, H.H. Arnold, T.Braun, A. Salminen, E. Bober, B. Winter, Department of Toxicology, Medical School, University of Hamburg, Grindelallee 117, D-2000 Hamburg F.R.G.
The muscle regulatory factor Myf5, originally isolated from human skeletal muscle, can first be detected in the dermomyotome of 8 day old mouse embryos by in situ hybridization, clearly prior to MyoD1 and myogenin. Its expression in the myotomal muscle is transient suggesting that it may be involved in early muscle cell determination. Forced Myf5 expression in non-muscle cells results in transcriptional activation of muscle-specific reporter genes containing consensus motifs of the MEF1 binding site. MEF1 motifs are present in the human MLC_{1emb} gene promoter and the MLC1/3 gene enhancer. They show various degrees of Myf5 binding in vitro. Myf5 acquires its capacity to bind to DNA with high affinity by forming hetero-oligomeric complexes with other HLH proteins such as E12, E2-2 and E2-5, but not with other muscle regulatory HLH factors (Myf3, 4 and 6) or the lymphatic factor 1yl-1. Mutations in the putative HLH domain of Myf5 indicate that this structure is involved in the protein as well as protein-DNA interactions. Although the natural target genes for Myf5 are unknown, it appears to be a "bona fide" transcription factor. This is concluded from Gal4-Myf5 hybrid proteins which carry the DNA binding domain of the yeast transcription factor GAL4 fused to the entire or parts of Myf5. These chimeric proteins activate transcription of reporter genes containing the Gal4 binding site, therefore Myf5 contains a transcriptional activator domain. In Myf5 deletion mutants we show that the activator region lies outside of the conserved basic region and putative HLH domain. It is associated primarily with the C-terminal half of the molecule, although the N-terminal sequence adds to the activation. Myogenin, another muscle regulatory factor, is absent in cultured myoblasts and begins to accumulate at the onset of myocyte differentiation. The activation of the myogenin gene precedes that of other muscle markers such as MCK activity, myosin synthesis and cell fusion. A reporter construct containing the myogenin gene promoter directing CAT expression is inhibited by bFGF and TGFß, both potent mitogens for myoblasts which block myotube formation. Pertussis toxin, an inhibitor of G_i proteins, obliterates the bFGF effect and upregulates myogenin expression in growth- and differentiation medium. Cholera toxin, forskolin and cAMP inhibit the expression of myogenin and also suppress differentiation. The significance of our observations for possible signal transduction pathways regulating myoblast differentiation will be discussed.

F 008 **MRF4 AND THE REGULATION OF MUSCLE-SPECIFIC GENE EXPRESSION**, T. J. Hinterberger, H. Lin, K. L. Mak, J. L. Mays, S. J. Rhodes, K. E. Yutzey, and S. F. Konieczny, Department of Biological Sciences, Purdue University, West Lafayette, IN 47907.

Skeletal muscle determination and differentiation is controlled in part by the b/HLH muscle regulatory factors MRF4, MyoD, myogenin, and Myf-5. Expression of each factor converts C3H10T1/2 fibroblasts to stable myogenic lineages which can be induced to differentiate and express the contractile protein gene set. Although structurally and functionally related, the specific role that each factor has in regulating skeletal myogenesis remains unclear. To begin defining differences in each b/HLH muscle regulatory factor, we have concentrated our efforts on characterizing the transcriptional activity and protein properties associated with MRF4. In primary and established rat myogenic cell lines, MRF4 is detected after the cells are induced to differentiate. Transcription of the MRF4 gene follows expression of MyoD and myogenin, suggesting that MRF4 controls developmental decisions that occur predominantly during terminal differentiation. MRF4, when complexed with the immunoglobulin enhancer binding protein E12, binds to the related Muscle Regulatory Factor binding site (MRF site) present within the troponin I (TnI), M-creatine kinase and myosin light chain gene enhancer elements. Identical TnI enhancer binding patterns also are obtained with MyoD/E12, myogenin/E12 and Myf-5/E12 complexes. Although MyoD and myogenin efficiently *trans*-activate co-transfected TnI genes, MRF4 produces only a weak *trans*-activation response. The unique transcriptional specificities of each muscle regulatory factor suggest that their diverged amino and carboxy regions dictate specific protein:protein interactions. In support of this hypothesis, we report that the muscle regulatory factors and the TnI MRF site are essential for muscle-specific expression, but are not sufficient to generate the complete TnI enhancer activity. Mutagenesis of two additional regulatory regions, Site I and Site II, dramatically inhibits the muscle regulatory factors from *trans*-activating the TnI gene. Interestingly, the proteins that bind to Sites I and II are found in both muscle and non-muscle cell types. Since MRF4, MyoD, myogenin, and Myf-5 bind to the same MRF site but exhibit different *trans*-activation properties, it is possible that each muscle regulatory factor interacts with different ubiquitously expressed proteins to generate unique, muscle-specific, transcription complexes.

F 009 DMD1, A DROSOPHILA HLH PROTEIN SIMILAR TO VERTEBRATE MyoD, Bruce M. Paterson[1], Juanita Eldridge[1], Claude Dechesne[1], Andreas Dubendorfer[2] and Walter J. Gehring[3].
We have isolated a cDNA clone from a Drosophila library that encodes a protein with characteristics similar to the members of the MyoD family. DMD1 encodes a polypeptide of 332 amino acids with 82% identity to MyoD in the 41 amino acids of the helix-loop-helix region and 100% identity in the essential amino acids of the basic domain proposed to contain a recognition code for muscle-specific gene activation. The gene is single copy and maps to 95AB on the right arm of the third chromosome. Low stringency hybridizations suggest DMD1 is not a member of a multigene family similar to MyoD in vertebrates. DMD1 is a nuclear antigen in both Drosophila and 10T1/2 mouse fibroblasts, consistent with its role as a nuclear regulatory factor. It can activate weakly the cardiac actin promoter in cotransfections into mouse fibroblasts and forms an E12 dependent complex with the MCK enhancer, similar to MyoD. DMD1 will not support myogenic conversion in 10T1/2 cells or the activation of endogenous myogenic factors. DMD1 RNA is first detected at three hours of embryonic development and reaches a maximum level of expression around 9-12 hours. DMD1 RNA expression is not seen in 12-24 hour embryos. In situ antibody staining of whole mount embryos with affinity purified antibody reveals a similar pattern of expression. Unlike the myogenic factors in vertebrates, DMD1 is not expressed in primary cultures of embryonic Drosophila muscle.
[1] Laboratory of Biochemistry, National Cancer Institute, NIH, Bethesda, MD 20892
[2] Zoologisches Institut der Universitat Zuich, Kunstlergasse 16, 80006 Zurich, Switzerland.
[3] Biozentrum, Klinglebergstrasse 70, CH4056 Basel, Switzerland.

F 010 MONOMERS, DIMERS AND TETRAMERS: ASSEMBLY PATTERNS AND THEIR EFFECTS ON MYOGENIN FUNCTION. Woodring E. Wright, Walter D. Funk and Karen J. Farmer, Dept. of Cell Biol. and Neurosci., U.T. Southwestern Med. Ctr., 5323 Harry Hines Blvd., Dallas, TX, 75235

Muscle gene regulation is sufficiently complex that it is likely that myogenin homomers, myogenin/E12 heteromers, and complexes with unidentified additional factors all play important roles. The structure of myogenin homomers was determined using sucrose gradients and HPLC. Most of the protein is tetrameric and not active in EMSA using E-box oligonucleotides. Two types of dimers recognize E-box sequences: the more asymmetric HLH dimer and a more globular D2 dimer. Tetramers are formed by the pairwise association of HLH and D2 dimerization motifs. A small amounts of protein is present as monomomers. Individual monomers can recognize wild-type but not mutant E-box sequences. The consensus binding site for myogenin homomers was determined from an unbiased 270 million-fold degenerate oligonucleotide using a new procedure (CASTing: Cylic Amplification and Selection of Targets). A very strong E-box that represents a distinct subset of the MEF-1 consensus binding site is present. The sequence is nearly palindromic, but the central pair of nucleotides is frequently the non-palindromic dinucleotide GT. The selected sequences showed equal affinities for the two forms of myogenin dimers. Similarly, the same pattern of bases was effected in methylation protection experiments using the HLH and D2 dimers, indicating that the two conformations had little effect on DNA binding activity or specificity.

Gene Expression in Neuromuscular Development

Molecular Basis of Muscle Cell Diversity

F 011 THE AVIAN MYOSIN HEAVY CHAINS, Everett Bandman, Laurie Quinn, Bruce Kerwin, Maria Arrizubieta, and William Tidyman, Department of Food Science and Technology, University of California, Davis, CA 95616.

In the chicken, as in other vertebrates, myosin heavy chain (MHC) is encoded by a multigene family. As a result of biochemical, immunological, and cDNA studies, at least 9 distinct sarcomeric MHCs have been reported. The differential expression of distinct MHC isoforms in different muscle cells during differentiation and development results in a diverse population of muscle fibers. While it has been possible to characterize this diversity using monoclonal antibodies and cDNA probes, the functional significance of MHC isoform diversity remains unclear. In order to address this question we have been studying the properties and the intracellular distribution of the MHCs expressed in the developing pectoral muscle. These studies have illustrated that while different isoforms can co-assemble into the same thick filament and myofibril, different fast skeletal MHC subunits are not found within a single myosin molecule. The inability of different MHCs to form a stable dimer is not the result of compartmentalization of the different isoforms, but results from the thermodynamic instability of the α-helical coiled-coil rod composed of different MHCs. Using a monoclonal antibody library to MHCs we have isolated clones encompassing the majority of the MHC rod for the different isoforms. We have compared the amino acid sequence of the LMM of these isoforms to other published sequences and mapped a number of our monoclonal antibody epitopes. The structural information provided by these sequences as well as the homology between the avian fast isoforms and those expressed in mammalian fast skeletal muscle will be discussed.

F 012 EARLY MYOBLASTS IN MOUSE SOMITES. Giulio Cossu, Elisabetta Vivarelli, Luciana De Angelis, M. Gabriella Cusella De Angelis and Mario Molinaro. Istituto di Istologia ed Embriologia generale. Università di Roma "La Sapienza". Italy.

Previous work from several laboratories had established the existence of phenotypic heterogeneity among myotubes that are formed in colture by different classes of myoblasts.

However, no direct approach has been adopted so far to understand the molecular basis of such heterogenity and the role that it might play in muscle histogenesis.

The recent identification of at least four myogenic regulatory genes has offered a possible key to the understanding of this phenotypic heterogeneity in mammals. Different myogenic programs might in fact depend upon differential expression of different regulatory gene products (or combinations of them). If this was the case, the availability of specific probes and antibodies to the products of these genes, would allow the investigation of the emergence of different classes of myoblasts during embryogenesis.

Myoblasts isolated from 11 d.p.c. or older embryos, as well as satellite cells, co-express MyoD1 and Myogenin proteins before the onset of terminal differentiation. This suggests that phenotypic differences among these classes on myoblasts are not directly related to differential expression of MyoD1 or Myogenin.

On the other hand, myoblasts, isolated from somitomers or early forming somites, undergo terminal differentiation in vitro, without expressing detectable levels of MyoD1 or Myogenin proteins in their nucleus. These cells, whose in vitro differentiation is nerve dependent, assemble slow and fast myosin heavy chains into forming sarcomeres but do not fuse into multinucleated cells. The possible embryological significance of these early myoblasts will be discussed.

F 013 THE HUMAN SKELETAL MYOSIN HEAVY CHAIN GENE FAMILY H. Stedman, J. Shrager, Zhizhong Fei, M. Narusawa, N. Todi, N. Rubinstein, A. Kelly. Departments of Anatomy and Pathobiology, Schools of Medicine and Veterinary Medicine, University of Pennsylvania, Philadelphia, PA 19104

Major limitations to research on myosin isoform diversification in development and disease include uncertainty as to the number and taxonomy of mammalian isomyosins, the scarcity of specific probes for the individual myosin isotypes, and limited acess to the purified, native isozymes for structur/function studies. To address these problems, we have undertaken the complete cloning of the human skeletal myosin heavy chain (MHC) locus. We have isolated 30 independent human genomic DNA fragments in the cosmid cloning vector pWE15. Restriction and cDNA hybridization analyses place most of these cloned fragments into contiguous regions representing an estimated 250 kb of human chromosome 17. There appear to be at least six independent rod encoding regions in these contiguous domains. Nucleotide sequence from carefully selected coding regions has facilitated the assignment of individual genes to their cognate transcripts. This information has facilitated the specific detection and quantitation of these transcripts by numerous means, including the PCR-based amplification of first strand cDNA. In parallel we have isolated cDNAs encoding, as single cloned fragments, the full length of the human embryonic and neonatal MHCs. We have used structural information from the human MHC gene family to develop a strategy for rapidly establishing a comparably specific series of probes for use with any other mammalian genome.

An extension of this approach has facilitated the analysis of genetic polymorphism within the skeletal MHC locus. We have identified polymorphic sites at distinct positions spanning the human locus, thereby establishing a foundation for haplotype analysis in human pedigrees.

F 014 EFFECTS OF NEUROMUSCULAR ACTIVITY UPON THICK AND THIN FILAMENT PROTEIN EXPRESSION IN ADULT MUSCLE FIBERS, Dirk Pette, Fakultät für Biologie, Universität Konstanz, D-7750 Konstanz, Federal Republic of Germany

Neuromuscular activity and specific hormonal signals play a dominant role in the control of gene expression in skeletal muscle fibers. Chronic low-frequency stimulation (CLFS) of fast-twitch muscles in small mammals has been successfully used in our laboratory for studying effects of altered neuromuscular activity on the expression of specific myofibrillar protein isoforms: myosin light (MLC) and heavy (MHC) chains as major components of the thick filament and the troponin (Tn) subunits as major regulatory proteins of the thin filament. Increased neuromuscular activity as induced by CLFS shifts the expression of these proteins at both the mRNA and protein levels towards slower isoforms. The changes at the mRNA level, which are reversible after cessation of stimulation, are rapidly transmitted to the translational level. Here they result in altered synthesis rates of the different isoforms. The finding that the newly synthesized myofibrillar proteins are inserted with some delay into the sarcomeres, points to the existence of still unidentified posttranslational control mechanisms. During the fast-to-slow transition, the MHC isoforms are exchanged in the rabbit in the order MHCIIb -> MHCIId -> MHCIIa -> MHCI. The expression of minor amounts of slow MLC isoforms seems to be coupled to the appearance of MHCIIa. It increases as MHCIIa is exchanged with MHCI. Due to apparent differences in the affinities of the fast MHC isoforms for the fast alkali MLC isoforms, the transition from MHCIIb -> MHCIId -> MHCIIa is accompanied by an increase in the MLC1f/MLC3f ratio. Whereas in the euthyroid rat the fast-to-slow transition does not exceed the step of MHCIIa, it is further shifted towards the slow MHCI isoform in the hypothyroid rat. These and other results suggest that thyroid hormone and increased neuromuscular activity affect the expression of myosin isoforms in an antagonistic manner. The rearrangement of the thin filament regulatory proteins displays specific time courses. Single fiber analyses reveal that the coordinated expression of MHC and TnT isoforms is maintained during the transformation process. The fast-to-slow isoform transition is incomplete for TnC and TnI which points to the existence of hybrid Tn molecules composed of slow TnT and fast TnI/TnC subunits.

F 015 THE BASIS OF DIVERSITY AMONG THE FIRST FIBERS TO FORM IN EMBRYONIC MUSCLES, Frank E. Stockdale, Stanford School of Medicine, Stanford, CA 94305-5306

During development cells not only become determined, or committed to differentiate into distinctive types, they also become committed to differentiate into specific subtypes of cells. For example, mesenchymal cells become committed to differentiate not just into cardiac muscle cells, but into atrial, ventricular, or conducting system cardiac cells which manifest different sets of gene activity. Likewise, in skeletal muscle development, there is not a single type of fiber, but distinct skeletal muscle fiber subtypes such as fast, slow, and fast/slow fibers that are recognized on the basis of different patterns of gene expression and correspondingly different functional properties. There is a family of different myoblasts, the precursors to muscle fibers, that appear during the formation and maturation of all skeletal muscles which provide the cellular basis for the observed diversity among skeletal muscle cells. We and others have shown that myoblasts differ in the fiber types they form because of the constellation of muscle specific proteins the fibers synthesize. We have recently focused on the types of myosin heavy chains (MHC) contained in the first fibers to form in the embryonic avian embryo, the primary fibers. Immunocytochemistry, immunoblotting, and immunopeptide mapping with monoclonal antibodies that distinguished between slow and fast MHC isoforms were used to study the expression of slow MHC isoforms in the avian limb at embryonic, fetal, and adult stages of development. In the embryo and fetus, slow MHC 1 was expressed in future fast, as well as future slow muscles, whereas, in the adult, only the slow muscles retained expression of slow MHC 1. Those embryonic muscles destined in the adult to contain slow fibers or mixed fast/slow fibers not only expressed slow MHC1, but also a MHC expressed in the atria which was designated as slow MHC 3. Slow muscles, later in development, lost the expression of slow MHC 3, initiated the expression of slow MHC 2 (the predominant slow MHC isoform in the adult) and continued expression of slow MHC 1. Thus diversity among fiber types can be detected when fibers first form, prior to division of the dorsal and ventral muscle masses into specific muscle groups and innervation (stage 25 of chicken hind limb development). This diversity is manifest by the expression of a newly described MHC.

Neuromuscular Interactions in Development

F 016 ASSEMBLY OF THE POSTSYNAPTIC CELL SURFACE AT THE NEUROMUSCULAR JUNCTION, Zach W. Hall, Yong Gu, John R. Forsayeth, Department of Physiology, University of California, San Francisco, San Francisco, CA 94143.

We have examined the assembly of the mouse muscle acetylcholine receptor (AChR), an oligomeric membrane protein, in COS cells transiently transfected with cDNAs for α, β, γ and δ subunits. The physiological and pharmacological properties of the AChR expressed on the surface closely resemble those found in C2 myotubes. When cells were transfected with incomplete combinations of AChR subunit cDNAs, toxin-binding activity was not seen on the surface, except in the case of $\alpha\beta\gamma$, which gave about 15% of normal activity. When pairs of subunits were expressed, $\alpha\delta$ and $\alpha\gamma$ heterodimers were formed, but $\alpha\beta$ heterodimers were not. Variation of the ratios of the four cDNAs used in the transfection mixture showed that surface AChR expression was decreased by high concentrations of $\delta\gamma$ cDNAs in a mutually competitive manner. These results are consistent with a scheme for assembly in which $\alpha\delta$ and $\alpha\pi\pi\alpha\gamma$ heterodimers are formed first, followed by association with the β subunit and with each other to form the complete AChR. (Supported by NIH grants to JFR and ZWH and a grant from the Muscular Dystrophy Association to ZWH).

F 017 SYNAPTIC COMPETITION AT THE NEUROMUSCULAR JUNCTION, Jeff W. Lichtman, Rita Balice-Gordon, Peter van Mier, Junichi Nabekura. Department of Anatomy and Neurobiology, Washington University School of Medicine, St. Louis, MO 63110.
During development, vertebrate skeletal muscle fibers undergo a transition from polyneuronal to single innervation. This process also occurs following nerve regeneration in adult animals. In order to better understand the mechanism underlying synapse elimination we have followed neuromuscular junctions over time in living mice. Both in development and in adults we find that axon withdrawal from an endplate site is not accompanied by the occupation of the vacated synaptic sites by the remaining axon terminal. Rather as the nerve terminal withdraws, the postsynaptic acetylcholine receptors underlying it are also permanently eliminated from the site. Interestingly, the first signs of postsynaptic receptor loss occur prior to the structural elimination of the overlying terminal. Moreover, electrophysiological studies show that the nerve is still functional at the time receptors begin to disappear and that the synaptic efficacy of the soon-to-be-eliminated input is attenuated by the loss of postsynaptic receptors. The early postsynaptic changes that precede nerve terminal withdrawal suggests that the postsynaptic cell is involved in the process perhaps as intermediary between competing axons. We are presently studying the fate of the receptors eliminated from one site to see whether metabolism or migration of receptors explains their rapid disappearance. As a start we have used laser illumination to bleach small regions of fluorescently labelled receptors at normal adult neuromuscular junctions to see if bleached areas recover fluorescence due to migration of unbleached receptors. Surprisingly, fluorescent recovery after photobleaching was found to occur over intervals of 3-6 days indicating that receptors may be mobile at the neuromuscular junction. Similarly, when we selectively labelled a portion of a junction's receptors with a micropipette filled with fluorescent α-bungarotoxin, we found that over several days the labelled receptors migrated into other regions of the endplate. Both these results argue that receptors are not entirely fixed at the neuromuscular junction allowing the possibility that receptors that disappear during synaptic competition are actually migrating from one synaptic site to another.

F 018 BASAL LAMINA MOLECULES THAT MEDIATE THE NEURON-INDUCED FORMATION OF POSTSYNAPTIC APPARATUS IN SKELETAL MUSCLE FIBERS.
U.J. McMahan. Department of Neurobiology, Stanford University School of Medicine, Stanford, California 94305.

The basal lamina in the synaptic cleft of the neuromuscular junction contains molecules that direct the formation of cell surface specializations on regenerating axon terminals and muscle fibers. Some of these specializations are directly involved in synaptic transmission. For example, the synaptic basal lamina induces regenerating axon terminals to form active zones, which are involved in the release of the transmitter, acetylcholine, and it directs regenerating muscle fibers to form aggregates of acetylcholine receptors and acetylcholinesterase. Over the last several years my colleagues and I have conducted experiments aimed at identifying and characterizing the basal lamina molecules that induce the aggregation of AChRs and AChE. Our results have led to the following hypotheses. 1) A single basal lamina molecule causes the aggregation of both AChRs and AChE as well as other components of the postsynaptic apparatus, 2) the active molecule is synthesized by motor neurons and released by their axon terminals to be incorporated into the basal lamina, and 3) the basal lamina molecule that directs formation of the postsynaptic apparatus on regenerating muscle fibers is the same molecule that mediates the nerve-induced formation of postsynaptic specializations on developing muscle fibers in the embryo and it helps maintain those specializations on mature muscle fibers in the adult. Our studies indicate further that the active molecule is identical, or very similar, to agrin, a protein we have purified from the electric organ of Torpedo californica. I will discuss evidence that supports these hypotheses and present results of studies that bear upon the steps and mechanisms involved in the formation of the AChR aggregates.

F 019 DIFFERENTIATION AND INNERVATION OF EARLY MUSCLE FIBER TYPES IN THE RAT, Wesley J. Thompson, Keith W. Condon, and Stephanie H. Astrow, Department of Zoology, University of Texas, Austin, TX 78712

Fiber types emerge during fetal development of rat crural muscles through a predictable sequence of expression of sarcomeric myosin heavy chain (SMHC) isoforms. Primary fibers, whose formation begins on E14, all initially express embryonic and, shortly thereafter, slow SMHC. Within a few days of their formation some of these fibers switch from slow SMHC to expression of a neonatal SMHC. The primary fibers which retain the expression of slow SMHC become slow fibers in the young adult, whereas those which switch to neonatal SMHC become fast fibers. The formation of a second generation of fibers, the so-called secondary fibers, begins on E18; these fibers initially express embryonic and neonatal SMHC. A small minority of these secondary fibers in certain muscles later switch from neonatal to slow SMHC expression, but the vast majority become fast fibers in the adult. In many muscles, the primary fibers which switch to the expression of neonatal SMHC are located in particular muscle regions; in other muscles there is no such regionalization. Therefore, the geometry of the pattern of early fiber types is difficult to reconcile with any simple morphogenetic gradient in the limb. Further, as fast and slow fibers are derived from both the primary and secondary fiber generations, fiber fate is imperfectly predicted by the time of fiber formation. To investigate the role of the nervous system in early fiber differentiation, we rendered the musculature aneural by injection of a presynaptic neurotoxin, β-bungarotoxin, on embryonic days 13-15. This toxin produced aneural limbs as judged by examinations of the ventral horn of the spinal cord and of muscle sections stained with antibodies to neurofilaments and synaptic vesicle antigens. While aneural limbs had greatly reduced numbers of muscle fibers (i.e. the course of myotube development was slowed, secondary myogenesis was greatly reduced, and many fibers degenerated following their formation) the basic pattern of fiber types was the same as in normal hindlimb muscles. However, the aneural muscles were found to have a decreased frequency of slow fibers. Closer examination revealed that many of these fibers, although initially expressing their "appropriate" slow differentiation switched to expression of neonatal SMHC. This suggests that innervation may be required to maintain the expression of slow myosin in slow primary fibers. However, the initial differentiation of the fibers appears to be independent of the nervous system. To examine how the nervous system apportions its innervation to these early fiber types, we and others have used glycogen depletion and dye injection into individual muscle fibers to mark the fibers innervated by single motor neurons. We find that early in development motor neurons appear to innervate one or the other of the two types of fibers present in the soleus muscle. These experiments, taken together with experiments examining the reinnervation of fiber types in the early neonate, suggest that somehow motor axons are able to recognize and form selective synaptic connections with early muscle fiber types.

Molecular Biology of Synaptogenesis

F 020 ACETYLCHOLINE RECEPTOR GENES REGULATION DURING ENDPLATE DEVELOPMENT, J.P. Changeux, A. Bessis, J. Cartaud*, A. Devillers-Thiéry, A. Duclert, J.L. Galzi, B. Jasmin*, A. Klarsfeld, R. Laufer, C. Mulle, H.O. Nghiêm, J. Piette and F. Revah, Laboratoire de Neurobiologie Moléculaire, Institut Pasteur, Paris, France, *Laboratoire de Microscopie Electronique, Institut Jacques Monod, Paris, France.

In the adult motor endplate, the AChR is strictly localized under the nerve ending while in the non innervated myotube it is distributed all over the surface of the cell. In situ hybridization with α-subunit probes, containing or not exonic sequences disclose high levels of unspliced and mature mRNA in mononucleated myotomal cells and differentiating myotubes. After the entry of the exploratory motor axons, the clusters of grains located outside the endplate decrease in number. In 15-day old chicks, AChR α-subunit mRNAs become restricted to the subneural "fundamental" nuclei. Denervation causes a reappearance of unspliced and mature mRNA in extrajunctional areas.

Chronic paralysis of the embryo by flaxedil interferes with the disappearance of extrajunctional AChR which, thus, represents an electrical activity-dependent repression of AChR genes. This process has been analysed with a chicken α-subunit genomic probe in primary cultures of chick myotubes. Blocking their spontaneous electrical activity by tetrodotoxin (TTX) causes increases of both precursor and mature α-subunit mRNA levels while α-actin mRNA levels do not change. The entry of Ca^{++} ions and possibly the activation of protein kinase C contribute to the repression of α-subunit gene transcription.

The maintenance and late increase in AChR number at the endplate level requires the intervention of an anterograde signal from neural origin. Calcitonin gene-related peptide (CGRP), a peptide shown to coexist with acetylcholine in chick spinal cord motoneurons, increases surface AChR and α-subunit unspliced and mature mRNA respectively by 1.5 and 3 fold. CGRP stimulates membrane-bound adenylate cyclase in the range of concentration where it enhances AChR α-subunit gene expression.

The data are interpreted in terms of a model which assumes that : 1) in the adult muscle fiber, nuclei may exist in different stages of gene expression in subneural and extrajunctional areas, 2) different second messengers elicited by neural factors or electrical activity regulate the state of transcription of the nuclei via trans-acting allosteric proteins binding to cis-acting DNA regulatory elements.

To look for such components, the 5'-end and part of the upstream flanking region of the α-subunit gene was isolated and sequenced in the chick. This α-subunit promoter, including 850 bp of the 5' flanking sequence, was inserted into a plasmid vector in front of a chloramphenicol acetyltransferase (CAT) gene. This construct directed high CAT expression in transfected mouse C2.7 myotubes but not in unfused C2.7 myoblasts or non myogenic mouse 3T6 cells. DNAse I foot-printing and gel retardation assays show that nuclear factors bind to three distinct domain AR I, II and III located within the most proximal 110 nt domains. Levels of several of these factors change during fusion of myoblasts into myotubes (AR IIb and III) and as a consequence of denervation (AR IIb and III).

Finally, multiple post-transcriptional processes involving, in particular the Golgi apparatus, proteins from the basal lamina and from the cytoskeleton (the 43 KD protein among others) contribute to the clustering, and stabilisation of the AChR in the post-synaptic membrane.

F 021 CLUSTERING OF ACETYLCHOLINE RECEPTORS STABLY EXPRESSED IN FIBROBLASTS,
Toni Claudio, Deborah S. Hartman, Neil S. Millar and Himi T. Kim, Department of Cellular & Molecular Physiology, Yale University School of Medicine, New Haven, CT 06510

Nerve-induced clustering of acetylcholine receptors (AChRs) is one of the key steps involved in formation of the neuromuscular junction. We propose to study this complex process by simplifying both the nerve and muscle cell. The nerve cell has been replaced with putative clustering agents derived from neural tissue or basal lamina from muscle cells or *Torpedo* electric organ. The muscle cell has been replaced by cultured mouse fibroblasts that stably express functional cell surface *Torpedo californica* AChRs (AChR-fibroblasts) (1). The distribution of AChRs on the surface of AChR-fibroblasts without the addition of any external agents is similar to that of AChRs expressed in muscle cells in terms of expression levels, stability and surface distribution. When antibodies specific to the AChR are incubated with AChR-fibroblasts or cultured muscle cells, "micro"clusters of AChRs with average surface areas <0.5 μm^2 are formed and rapidly internalized. In contrast, neural derivatives (media conditioned from the cholinergic neuroblastoma-glioma hybrid cell line, NG108-15) or extracellular derivatives (*Torpedo* extracellular matrix, ECM) produce clusters 2- to 20-fold larger than antibody-induced clusters (1-4 μm^2) and AChR surface levels are not altered. These results suggest that the mechanism by which ECM and NG108-15 conditioned media induce AChR clustering is not the same as that of antibody-induced clustering, which is believed to occur through a direct crosslinking of AChRs.

Both lateral migration and the directed insertion of new AChRs have been implicated as mechanisms of AChR clustering. In our system of *Torpedo* AChRs expressed in mammalian cells, we can separate these two mechanisms because of the temperature-sensitive nature of *Torpedo* AChR assembly (2). Although *Torpedo* AChR subunits are synthesied at 37°C, no assembly occurs unless the temperature is lowered ~10°. In these studies, cells are first induced to express surface AChRs (requiring a shift in temperature from 37°C to 26°C or 20°C), then the cells are shifted back to 37°C where clustering agents or antibodies are added. During this incubation period (only 2-3 hours), AChRs are mobile in the plane of the membrane, however, no new AChRs are either assembled or inserted into the plasma membrane. We observe 1-4 μm^2 clusters after 2-3 hours of treatment with clustering agents at 37°C and can thus conclude that their formation is due to lateral migration of existing cell surface AChRs. Interestingly, cultured muscle cells incubated in this manner with clustering agents reveal the presence of 1-4 μm^2 clusters within one large cluster.

(1) Claudio, T., Green, W.N., Hartman, D.S., Hayden, D., Paulson, H.L., Sigworth, F.J., Sine, S.M. and Swedlund, A. (1987) Genetic reconstitution of functional acetylcholine receptor channels in mouse fibroblasts. *Science* **238**:1688-1694.
(2) Paulson, H.L. & Claudio, T. (1990) Temperature-sensitive expression of all-*Torpedo* and *Torpedo*-rat hybrid AChR in mammalian muscle cells. *J Cell Biol* **110**:1705-1717.

F 022 TRANSGENIC ANALYSIS OF ACETYLCHOLINE RECEPTOR GENE EXPRESSION.
J.P. Merlie, D. McKinnon and J.R. Sanes*. Departments of Pharmacology and of Anatomy and Neurobiology. Washington University Medical School, St. Louis, Missouri 63110

Acetylcholine receptor subunit mRNA's, α,β,γ and δ are more abundant in embryonic and denervated than innervated muscles. By contrast, the level of ϵ subunit mRNA is most abundant in 15d post-natal mouse muscle, and is unchanged by denervation. All 5 subunit mRNA's are more abundant in synaptic compared to extrasynaptic areas of muscle fibers. To study the transcriptional basis of these nerve-dependent phenomena we produced transgenic mice containing a variety of different AChR subunit gene promoters fused to chloramphenicol acetyltransferase (CAT). In 4 of 6 mouse lines transgenic for an 850bp chicken α-promoter-CAT fusion, CAT expression was both muscle specific and increased 100X by denervation. The distribution of α-CAT mRNA transcripts within muscle fibers was studied by *in situ* hybridization. Although no signal could be detected in innervated fibers, denervation led to an increased signal throughout the fiber with a distinctly stronger signal in the synaptic areas. Thus, the α subunit promoter fragment can direct differential expression of the CAT transgene within muscle fibers. To define the minimal cis-acting sequences required for these unique characteristics we have prepared transgenic mice with deletions in the α subunit promoter. One of these, leaving only 116bp proximal to the transcription start site, retains muscle specific expression and a robust response to denervation. We have not yet analyzed expression of this deletions by *in situ* hybridization. In contrast to the relatively compact nature of the chicken α-promoter, the mouse γ-subunit regulatory sequences appear to be far more dispersed. A 750bp γ-promoter-CAT fusion, although active at promoting muscle specific expression in C2 cells *in vitro* was inactive in 20 lines of transgenic mice. An additional construction incorporating 3kb of 5' flanking sequence was similarly inactive. Interestingly, a further 7kb of upstream sequences including a third DNAase I hypersensitive site resulted in strong muscle specific expression in 7 of 10 transgenic mouse lines. It will be interesting to determine the mechanism of action of this distal element and how it interacts with the tissue specific enhancer located nearer the transcription start.

F 023 NEURITE OUTGROWTH PROMOTING ACTIVITY OF N-CAM AND N-CADHERIN. Frank S. Walsh, Patrick Doherty, C. Howard Barton and Lewis H. Rowett. Department of Experimental Pathology, U.M.D.S., Guy's Hospital Campus, London Bridge, London SE1 9RT, U.K.

The interaction of neuronal growth cones with guidance cues in the microenvironment contributes to the high degree of specificity in synaptogenesis. In recent years, a number of different classes of membrane associated proteins have been found to be important in neuromuscular synaptogenesis and these include N-CAM, N-cadherin and the Integrins. We wished to assess the role of N-CAM and N-cadherin in axonal growth and have cloned the respective cDNAs and expressed them in 3T3 fibroblasts. Neurons from both the peripheral and central nervous system as well as a neuronal cell line (PC12 cells) extended longer neurites when cultured on monolayers of 3T3 cells that express human N-CAM. This differential neurite growth could be abolished by enzymatic removal of transfected lipid-linked N-CAM from 3T3 cells or by treatment with an antibody that binds exclusively to human N-CAM. No difference was found in a neuron's ability to respond to NCAM irrespective of whether N-CAM was expressed as a transmembrane or lipid-linked isoform. Similarily, expression of a muscle specific grouping of 37 amino acids in the extracellular domain, termed the MSD region, also had no obvious effect on N-CAM function. A critical threshold value of N-CAM expression was required for neurite outgrowth. Above this value, relatively small increases in N-CAM expression were associated with substantial increases in neurite outgrowth. The ability of neurons to respond to N-CAM in the monolayer was also dependent on N-CAM, but not Integrin, function in the neuron and could also be modulated by removal of polysialic acid from neuronal N-CAM. In contrast to a highly co-operative relationship between transfected N-CAM levels and neurite outgrowth, transfected N-cadherin promoted an increase in neurite outgrowth with a linear relationship found between the level of N-cadherin immunoreactivity and neuronal response. A "sub-threshold" level of human N-CAM which does not support neurite outgrowth on its own acted synergistically with N-cadherin to promote neurite outgrowth. These data demonstrate that there are qualitative differences in the nature of the dose-response curves for cell adhesion molecules (linear and highly co-operative), and suggest that changes in the expression of one adhesion molecule can modulate the function of a second separate adhesion molecule.

Molecular Control of Muscle Gene Expression - I (joint)

F 024 REGULATION OF ALTERNATIVE SPLICING OF CONTRACTILE PROTEIN GENES. Bernardo Nadal-Ginard, Christopher W.J. Smith, James G. Patton and Mary Mullen. Howard Hughes Medical Institute. Department of Cardiology, Children's Hospital. Departments of Pediatrics, Cell and Molecular Physiology, Harvard Medical School. Boston, MA. U.S.A.

Alternative pre-mRNA splicing has emerged in recent years as a widespread device for regulating gene expression and generating protein diversity. In addition, it has provided new insights into some fundamental aspects of splicing. This mode of regulation is particularly prevalent in muscle cells, where genes such as Troponin T are able to generate up to 64 different isoforms from a single transcriptional unit. The α-tropomyosin gene has proven particularly useful for the analysis of the mechanisms involved in this type of regulation. Exon 2 and 3 of this gene are a mutually exclusive pair that are sterically inhibited from splicing together by the presence of an abnormally positioned upstream branch point. Exon 3 is incorporated constitutively as the default choice in all tissues except in smooth muscle cells where it is replaced by exon 2.

Analysis of a large variety of constructs containing this region of the α-TM gene in different cell types and in vitro cell-free system demonstrated that the default exon selection is determined through competition between the two exons *rather than* down-regulation of exon 2. Exon 3 out-competes exon 2. This behavior is determined by the nature of the polypyrimidine tract in front of exon 3 as well as by the sequence of the branch point. Thus, the relative quality of the pyrimidine tracts is the primary determinant of default exon 3 selection, with the relative branch point sequences also playing a role. This role of the pyrimidine tracts is mediated by trans-acting factors. Using U.V. cross-linking a 55 kd protein has been identified, partially sequence and cloned, that binds to the branch point/polypyrimidine tract. The binding of this protein to a large number of different intron sequences demonstrates a perfect correlation between affinity for this factor and splice site strength. These results suggest that the binding of this factor plays an important role in determining splice site selection and, at least in part, determines the default splicing pattern of α-TM.

How is the regulated pattern determined? In vivo expression studies demonstrate that the switch to the regulated selection of exon 2 involves inhibition of the splice site elements of exon 3 and the sequences involved in this down-regulation have been identified.

F 025 TRANSCRIPTIONAL REGULATION IN EMBRYONIC CARDIAC AND SKELETAL MUSCLE

Janet H. Mar, Rocco C. Iannello# and Charles P. Ordahl.
University of California, San Francisco, San Francisco, CA 94143-0452

Cardiac and skeletal myocytes transcriptionally activate the gene encoding cardiac troponin T (cTNT) early in embryonic development. During mid-fetal development, the cTNT gene is up-regulated in cardiac cells and repressed in skeletal muscle cells. We have used transfection and DNA-protein binding experiments to elucidate the cis elements and trans factors which govern expression of this gene in these two related cell types. In embryonic skeletal muscle cells cTNT promoter activity is dependent upon both copies of a conserved M-CAT motif (5'-CATTCCT-3') residing between nucleotides -101 and -60 of the "minimal" cTNT promoter (-101 to +38; all numbers relative to transcription initiation site at +1). This minimal cTNT promoter is inactive in embryonic myocardial cells unless an additional 47 nucleotide "cardiac element" is included. The cardiac element resides 200 nucleotides upstream and is capable of conferring activity to the cTNT minimal promoter in cardiac cells in an orientation- and position-independent manner. An AT rich motif within the cardiac element may be important for its activity. Expression in cardiac cells is also dependent upon both M-CAT motifs indicating that activity in cardiac cells requires collaboration between these motifs and the upstream cardiac element. Sequence motifs which appear to be similar to other muscle regulatory motifs such as CArG (serum response factor binding site) or MEF-1 (Myfkin binding site) can be deleted without significant loss of cTNT promoter in either cardiac or skeletal muscle cells. Thus, cell-specific expression of the cTNT promoter in cardiac and skeletal muscle appears not to require direct interaction with any of the known myogenic determination factors. Two novel factors, the M-CAT binding factor (MCBF) and the cardiac element binding factor (CEBF), bind to their respective cis elements in a sequence specific fashion. These results indicate that both common and different cis elements and trans factors are required for expression of the cTNT promoter in embryonic cardiac and skeletal muscle.

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Present Address: The Murdoch Institute, Royal Children's Hospital, Melbourne, Victoria, Australia 3052.

F 026 DIFFERENTIAL REGULATION OF MYOSIN LIGHT CHAIN EXPRESSION BY SPECIFIC MYOGENIC FACTORS. Nadia Rosenthal, Bruce M. Wentworth, James C. Engert and Uta Grieshammer, Department of Biochemistry, Boston University School of Medicine, Boston MA 02118.

Expression of the myosin light chain 1/3 (MLC1/3) gene locus is regulated both developmentally and fiber-specifically during mammalian skeletal myogenesis. The two transcripts encoding MLC1 and MLC3 are activated sequentially from two widely spaced promoters during embryogenesis, and persist in adult fast fibers, whereas transcription of MLC1 is repressed in adult slow fibers. Activation of MLC transcription appears to be dependent on a strong muscle-specific enhancer element, located 3' to the locus in both rats and humans, over 24 kb away from the upstream MLC1 promoter. Analysis of transgenic mice carrying multiple copies of an MLC1 promoter-CAT transcription unit driven by the MLC enhancer has demonstrated the ability of the MLC enhancer to activate gene expression at the appropriate developmental stage, exclusively in the skeletal muscles of these animals. The MLC enhancer includes three CANNTG consensus sites (A, B and C) which bind MyoD, as defined by bacterial fusion protein-DNA interactions in vitro. Specific pairs of the three sites (A and C, or B and C) are required for enhancer function, both in muscle cells and in non-muscle cells cotransfected with a MyoD or myogenin expression vector. The MLC enhancer is less responsive to trans-activation by a myf5 expression vector, and is not trans-activated by a MRF4/myf6 expression vector. Analysis of the interaction of individual sites with muscle and non-muscle nuclear proteins reveals multiple, distinct protein complexes formed with each site. Only two of the sites (A and C) interact with native MyoD or myogenin present in differentiated muscle extracts. This suggests a hierarchy of function among the three sites within the MLC enhancer that involves known myogenic factors, as well as other nuclear proteins yet to be characterized. We are currently investigating whether different protein interactions with the MLC enhancer may influence promoter choice, resulting in the differential transcription of MLC1 and MLC3 during muscle development and in various adult fiber types.

Molecular Control of Muscle Gene Expression - II

F 027 REGULATION OF THE SODIUM PUMP IN CHICK SKELETAL MUSCLE, Douglas M. Fambrough, Department of Biology, The Johns Hopkins University, Baltimore, MD 21218 Tissue cultured chick skeletal myotubes express the α1 and β1 isoforms of the two subunits of the Na,K-ATPase. During myogenesis the level of Na,K-ATPase increases about 20-fold to reach a steady-state. From this state, myotubes can be stimulated to up-regulate their Na,K-ATPase by any means that should result in an increased cytosolic sodium ion concentration. We have used veratridine to open the voltage-sensitive sodium channels that also appear during myogenesis, and find a near doubling of sodium pump molecules in the sarcolemma within about 24 hours. Studies of the underlying mechanisms for this up-regulation at the protein and nucleic acid levels lead to the following interpretation of the molecular events. The rise in intracellular sodium selectively stimulates increased transcription of the gene encoding the β1-subunit, leading to an accumulation of β1-mRNA and promoting a parallel increase in β1-subunit biosynthesis. The increased production of β1-subunits drives the assembly of more α-β subunit complexes (ie., Na,K-ATPase molecules). Assembly of subunits occurs in the endoplasmic reticulum and these assembled units move through the Golgi apparatus before incorporation into the sarcolemma. The β-subunit is normally N-glycosylated on three asparagines, but glycosylation is not necessary. As the up-regulatory response is coming to completion, the level of β-mRNA falls rapidly, apparently mediated by a large decrease in mRNA stability which may be signaled by a conserved sequence in the 3'-untranslated region of the β-mRNA. Meanwhile, at the protein level, the degradation rate of the sodium pump slows so that with basal levels of mRNAs and translation rates, the myotubes maintain a doubled level of Na,K-ATPase expression. If the stimulus for up-regulation is removed, the myotubes interiorize "excess" sodium pump molecules, bringing their surface expression back to the basal level. Recent experiments have focussed upon (a) the α-β assembly process, defining which portions of each subunit are involved in subunit interactions and (b) analysis of β1-gene structure and regulation.

F 028 ALTERNATIVE RNA SPLICING IN THE CONTROL OF GENE EXPRESSION, David M. Helfman, Wei Guo, George J. Mulligan, Toshifumi Tsukahara, and Steven Wormsley, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724

We are using the rat β-tropomyosin (TM) gene as a model system to investigate the molecular basis for developmental and tissue-specific RNA splicing. The β-TM gene expresses both skeletal muscle β-TM and fibroblast TM-1 by an alternative RNA splicing mechanism. The gene contains 11 exons. Exons 1 through 5 and exons 8 and 9 are common to all mRNAs expressed from this gene. Exons 6 and 11 are used in fibroblasts, as well as smooth muscle cells, whereas exons 7 and 10 are used exclusively in skeletal muscle. Using both *in vitro* and *in vivo* systems we have identified a number of cis elements involved in alternative splice site selection. We recently identified two distinct elements in the intron upstream of exon 7 involved in splice site selection. The first element is a polypyrimidine tract located 89-143 nucleotides upstream of the 3' splice site, which specifies the location of the lariat branchpoints located an unusually long distance (144-153 nt) upstream of exon 7. The second element is comprised of intron sequences located between the polypyrimidine tract and 3' splice site of exon 7. This element contains an important determinant in alternative splice site selection because deletion of these sequences resulted in the use of the skeletal muscle-specific exon in nonmuscle cells. To further study how these sequences contribute mechanistically to tissue specific splicing we have introduced linker-scanning substitutions in the 3' end of intron 6 and within exon 7. These experiments have identified a number of critical regions in the intron and exon that, when mutated lead to the use of exon 7 in nonmuscle cells. These mutations appear to act, in part, by disrupting the formation of an RNA secondary structure which sequesters exon 7. We have also found that transfection of tropomyosin mini-genes containing mutations in the 5' or 3' splice sites of exon 6 (fibroblast-type splice) do not result in the increased use of exon 7 (skeletal muscle-type splice) in nonmuscle cell systems. These results suggest that splice site selection *in vivo* is not regulated via a simple cis-competition mechanism, but rather by a mechanism that inhibits the use of the skeletal muscle exon in nonmuscle cells. In addition, we have initiated studies using ultraviolet photochemical crosslinking, native gel electrophoresis and binding competition experiments in order to identify RNA-binding proteins that interact with the pre-mRNA which may influence the use of exon 7 in nonmuscle and muscle cells.

F 029 MOLECULAR GENETIC MANIPULATION OF CARDIAC MYOSIN, Leslie A. Leinwand, Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, N.Y. 10461. The myosin composition of mammalian muscle has profound effects on its function, but no detailed model of those myosin sequences that determine its behavior have emerged. Additionally, the complex nature of the myosin multigene family in vertebrates has prohibited more classical genetic analysis. To understand the function and modulation of the myosin gene family in the heart, we have used both in vivo and in vitro molecular genetic approaches. In the first approach, myosin subunits have been expressed in E. coli in various combinations and several functions have been assessed. Co-expressed segments of the α cardiac myosin heavy chain (MHC) and the ventricular myosin light chain 1 are capable of associating with appropriate stoichiometry, indicating that post-translational modifications and other eukaryotic proteins are unnecessary for their interaction. The sequences of the MHC head that are necessary for this interaction have been identified. Futhermore, a small segment of the myosin tail has been identified that is both necessary and sufficient for the assembly properties of the intact myosin molecule. In order to complement these in vitro studies we have asked whether a muscle myosin rod can functionally substitute for a nonmuscle myosin rod in the complex motile processes of Dictyostelium. In collaborative experiments with J.A. Spudich (Stanford), we have introduced a chimeric myosin molecule consisting of a Dictyostelium myosin head and the β cardiac myosin rod into Dictyostelium myosin null cells. The parental null cells are defective in cytokinesis, development and the capping of cell surface receptors. Cells expressing the chimeric myosin remain defective in cytokinesis and development, but are capable of capping cell surface receptors. These results suggest that muscle and nonmuscle myosin rods are functionally interchangeable in some motile process, but not in others. The third approach we are using is direct gene transfer into cardiac and skeletal muscle in vivo. An intriguing report in which virally promoted reporter genes could be expressed following direct injection into mouse skeletal muscle prompted us to examine the feasibility of achieving gene transfer in the heart. We have achieved these goals and have made several observations. We find that the heart expresses injected genes even more efficiently than skeletal muscle. A reporter gene driven by the α cardiac MHC promoter and 613 base pairs of upstream sequence is expressed in heart but not in skeletal muscle. Additionally, the expression of this injected gene can be modulated by the thyroid status of the animal. DNA injection into cardiac muscle thus provides a novel means of studying the regulation of cellular genes.

F 030 Developmental Regulation of the Chicken Alkali Light Chain Gene Expression

Yo-ichi Nabeshima, Taichi Uetsuki, Thoru Komiya, Yoko Nabeshima, Atsushi Asakura, Yoko Hosoda and Astuko Fujisawa-Sehara Division of Molecular Genetics, National Institute of Neuroscience, Ogawahigashi-cho 4-1-1, Kodaira-shi, Tokyo 187, Japan

Myosin alkali light chain (MLC) isoforms are encoded in a multigene family, the expression of which is developmentally regulated in a tissue specific manner. To study how the transcription of skeletal muscle MLC gene is dramatically increased and continuously expressed in skeletal muscle myotubes, and how cardiac MLC and embryonic MLC (L23) genes are transiently expressed during myogenesis, we have analyzed the regulatory elements involved in the expression of above three genes and identified the MLC box, muscle specific enhancer and negative regulatory element.

MLC box--A conserved common element in MLC genes

MLC box is an essential element for the expression of MLC genes located at aporoximately 100 bp upstreame from mRNA start sites. The core sequence of MLC box is similar to the consensus of actin CArG box and SRE of c-fos oncogene. The protein complex bound to the MLC box might be identical with one of protein complexes bound to CArG and/or SRE.

Negative Regulatory Element--A homologue of the MLC box

A cis element identified at about 150 bp upstream from cap site of cardiac MLC gene suppresses the cardiac MLC gene expression in skeletal muscle cells but not in cardiac muscle cells. The core sequence of negative element fulfills the sequence of MLC box core with the exception of one base delation. The protein(s) bound to negative element might be identcal with one of proteins bound to SRE. However, negative element binding protein(s) does not recognize the MLC box and the CArG box.

Skeletal Muscle Enhancer--The roles of myogenic regulatory genes

An enhancer sequence located about 2 kb upatream from the transcriptional initiation site of skeletal MLC1f gene is composed of two subelements P and D, co-operative action of them is required for sufficient enhancer activity. The expression of enhancer-CAT fusion gene activated by the co-transfection with each of three chick myogenic regulatory genes and the natures of DNA (core elements)-protein (three factors) complexes will be discussed.

F 031 REGULATION OF SARCOMERIC α-ACTIN GENES BY MYOGENIC COMMITMENT FACTORS, SERUM RESPONSE FACTOR AND F-ACT1, Robert J. Schwartz, King-Lau Chow, Te-Chung Lee and Brent French, Department of Cell Biology, Baylor College of Medicine, Houston, TX 77030

Gene transfer experiments have indicated that the capacity of selective genetic expression of the sarcomeric α-actin gene resides within its 5' promoter and immediate DNA flanking sequences. Cardiac α-actin transcripts appear in the myotomal compartments coincident with myogenin and MyoD constitute some of the earliest markers for skeletal muscle development. We examined the possibility that myogenin and/or MyoD might directly transactivate the cardiac α-actin promoter. Heterodimers of myogenin and E12 (or MyoD and E12) specifically bound a restriction fragment extending from -200 to -103 relative to the start of cardiac α-actin transcription. Methylation interference footprints pinpointed the site of interaction to an E-box immediately adjacent to a previously identified CArG-box (CArG3). Site-directed mutations to the DNA-binding site revealed that either an intact E-box or an intact CArG3 is required for induction of the cardiac α-actin promoter CAT hybrids in myoblasts and for transactivation by myogenin in cotransfected fibroblasts. These results suggest that direct and indirect pathways may be involved in the induction of the cardiac α-actin promoter by myogenin and MyoD. With respect to the closely related skeletal α-actin promoter, we identified four positive cis-acting elements in which each site is required for transcriptional activity. These elements, conserved across vertebrate evolution (between amphibians, birds and mammals), includes the ATAAAA box (-24 bp), paired CCAAT Box Associated Repeats (CBARs at -83 bp and -127 bp), and the upstream TA-rich (UTA at -176 bp) regulatory sequence. Each of the three upstream elements contain the CArG-box core motif $CC(A/T)_6GG$, which is also a binding site for serum response factor (SRF). All three CBAR elements, like the serum response element (SRE), are capable of specifically binding SRF, albeit with different avidity. For example the central UBAR site has the poorest binding to SRF. Mutagenesis of either the UTA or DBAR sites which eliminates binding to SRF and also reduces binding over the wild type UBAR site. Binding isotherms of SRF also suggests that occupancy of the UTA and DBAR sites also influences the binding of the central UBAR. Thus, SRF binds to the skeletal promoter though classic cooperative interactions and utilizes each of the positive regulatory sites that are absolutely required for transcription. The most proximal CBAR is unique in that it is also recognized by another nuclear factor which can be biochemically and immunologically distinguished from SRF. The factor described as F-ACT1 contacts the GG doublet of the core sequence on the coding strand and therefore is likely to compete for CBAR-binding with SRF, which symmetrically contacts the GG doublet on each strand. The factor binding data coupled with our previous transcriptional analyses in vivo suggests that SRF may facilitate skeletal α-actin promoter function by forming a stable multiprotein-promoter complex and that F-ACT1 may impede the SRF-mediated complex formation through a competitive binding to the most proximal CBAR element.

Cell and Molecular Biology of Neuromuscular Disease

F 032 SYNAPTIC STRUCTURE AND REGULATION, Steven J. Burden, James E. Yeadon, Stephen M. Dyer, Helen Lin, Amy Ravin and Alex M. Simon, Biology Department, Massachusetts Institute of Technology, Cambridge, Mass. 02139

We have shown that a peripheral membrane protein of Mr~300 kd copurifies with postsynaptic membranes isolated from *Torpedo* electric organ and is concentrated at neuromuscular synapses. We have now isolated cDNAs encoding the *Torpedo* electric organ 300 kd protein and show that this subsynaptic protein is homologous to dystrophin. The 300 kd protein has repeat units that have 60-70% amino acid sequence identity with the repeat units in human dystrophin and a carboxy-terminal region with 90% homology to the dystrophin b isoform. Antibodies against murine dystrophin react with the *Torpedo* 300 kd protein, and monoclonal antibodies against the *Torpedo* 300 kd protein react with neuromuscular synapses in normal, but not dystrophic, mouse muscle. Dystrophin is likely to appear at synapses early during development, since it is associated with clusters of acetylcholine receptors (AChRs) in non-innervated myotubes in cell culture. These results demonstrate that dystrophin is concentrated at neuromuscular synapses and raise the possibility that loss of dystrophin from synapses may perturb the structure and/or function of the synapse and contribute to muscular dystrophy. AChR gene expression is influenced during myogenesis by multiple pathways including those associated with myotube differentiation and those associated with special aspects of muscle differentiation including synapse formation and membrane depolarization. We have analyzed the murine AChR delta subunit gene and have shown that this gene is controlled by four regulatory elements which collectively limit activation of the gene to myotubes. The first two elements constitute an enhancer which is active in all cell types. The other two elements limit enhancer activity to myotubes: one element represses expression in cell types other than myotubes, but is not required for gene activation in myotubes and the other element, a binding site for myogenic helix-loop-helix proteins, is required both for activating the gene in myotubes and repressing the gene in other cell types.

F 033 THE DYSTROPHIN-GLYCOPROTEIN COMPLEX: IDENTIFICATION AND BIOCHEMICAL CHARACTERIZATION, Kevin P. Campbell, James M. Ervasti, Kay Ohlendieck, Oxana Beskrovnaya-Ibraghimov, Steven D. Kahl and Cynthia J. Leveille, Howard Hughes Medical Institute and Dept. of Physiology and Biophysics, University of Iowa College of Medicine, Iowa City, IA 52242

Dystrophin, the high-molecular weight protein product of the Duchenne muscular dystrophy (DMD) gene, is localized to the sarcolemma of normal skeletal muscle but is absent from the skeletal muscle of patients with DMD and *mdx* mice. The predicted amino-acid sequence of dystrophin suggests that it is involved in the anchoring of sarcolemmal proteins to the underlying cytoskeleton. However, the sarcolemmal proteins which are associated with or bound to dystrophin are not known and the status of these proteins in muscle where dystrophin is absent is also unknown. We now report the purification of a dystrophin-glycoprotein complex and the identification of five dystrophin-associated proteins. The dystrophin-glycoprotein complex was isolated following digitonin-solubilization of rabbit skeletal muscle membranes using WGA-Sepharose and DEAE-cellulose chromatography and further purified by sucrose density gradient centrifugation. In addition to dystrophin, the complex contains a 59 kDa protein triplet and four glycoproteins of 156 kDa, 50 kDa, 43 kDa and 35 kDa, all in stoichiometric concentrations relative to dystrophin. Indirect immunofluorescence with monoclonal antibodies specific for dystrophin, the 156 kDa glycoprotein or the 50 kDa glycoprotein demonstrated a restricted localization of the dystrophin-glycoprotein complex to the sarcolemma of skeletal muscle. Immunoaffinity beads specific for dystrophin or the 50 kDa glycoprotein selectively adsorb the dystrophin-glycoprotein complex indicating that the complex is tightly associated. A marked reduction (~90%) of the 156 kDa dystrophin-associated glycoprotein was also observed in muscle from mice and DMD patients. Thus, the absence of dystrophin may lead to the loss of a dystrophin-associated glycoprotein(s) which may be an initial step involved in the molecular pathogenesis of muscular dystrophy. The elucidation of the function of dystrophin-associated glycoproteins should help to define the function of dystrophin and explain how its absence results in DMD. K.P. Campbell is an Investigator of the Howard Hughes Medical Institute. This work was also supported by MDA.

F 034 REGULATION OF EXPRESSION OF THE GENES CODING FOR DYSTROPHIN AND SKELETAL MUSCLE ACTIN, Uri Nudel, Efrat Barnea, Adina Makover, Dorit Zuk, Sara Bar, Hilla Ben-David, Valerian Nakar, Doron Lederfein, Debora Rappaport, Zehava Levy, Ora Fuchs, Sara Neuman and David Yaffe, Department of Cell Biology, The Weizmann Institute of Science, Rehovot, 76100 Israel

Three mRNA products of the Duchenne Muscular Dystrophy gene were identified. The 14 Kb muscle and brain dystrophin mRNAs and a newly identified 6.5 Kb mRNA. The 6.5 Kb mRNA contains the 3'-untranslated region of dystrophin mRNA and the regions coding for the c-terminal and cysteine-rich domains. It lacks most or the entire regions coding for the actin binding domain and the spectrin like repeats. The expression of the three mRNAs is regulated by three different promoters with different tissue- and cell-type specificities. The muscle dystrophin mRNA is expressed in striated and smooth muscle and in glia cells. The brain dystrophin mRNA is expressed in neurons. The 6.5 Kb mRNA is expressed in brain, testis, liver and several other tissues, but not in skeletal muscle. Its amount in some tissues is comparable to the amount of dystrophin mRNA in skeletal muscle.

The muscle and brain dystrophin promoters and 5' regions were cloned and sequenced. The muscle dystrophin promoter contains several elements characteristic for muscle-specific genes, such as a CarG box and an M-CAT box. In transfection experiments it confers muscle-specific expression on a reporter gene. The brain dystrophin promoter does not contain any obvious cis-transcription elements; nevertheless, in transfection experiments it confers a high level of expression on a reporter gene in neuronal cells. Experiments are in progress to identify functional elements in the muscle and brain dystrophin promoters.

The skeletal muscle actin gene promoter region is highly complex and contains several elements involved in the regulation of expression of the gene. The functional activity and the interaction of some of these elements with nuclear proteins will be discussed.

F 035 THE NATURE OF MUTATION AND THE PROSPECT FOR THERAPY IN DUCHENNE MUSCULAR DYSTROPHY, Ronald Worton, Suman Gangopadhyay, Xiuyuan Hu, Dennis Bulman, Henry Klamut, and Peter Ray, Department of Genetics and Research Institute, Hospital for Sick Children, Toronto, Ontario, Canada, M5G 1X8. The Duchenne muscular dystrophy (DMD) gene is over 2000 kb in size and produces a 13.9 kb mRNA that encodes a high molecular weight cytoskeletal protein (dystrophin) of over 400 kD. The extremely large size of the gene, its message and its protein product has several important implications. It impacts, for example, on the nature of the mutations that are found in the gene, and on the strategies used to detect those mutations. Furthermore the large size of the gene may prove to be a significant impediment to the development of gene replacement strategies for DMD. Initial studies in several laboratories revealed a deletion of one or more exons in 60% of affected boys; quantitative analysis in our laboratory detected duplication of exons in another 6%. As predicted, the severe DMD phenotype is associated frequently with deletions or duplications that shift the reading frame of the message, whereas the milder Becker muscular dystrophy (BMD) phenotype is most often associated with deletions or duplications that maintain the reading frame for those exons downstream of the alteration. Western blot analysis of muscle biopsies confirmed that the C terminal end of the protein is intact in boys with unaltered reading frame. In DMD patients with frame-shift alterations there is truncated dystrophin present in about half of the muscle biopsies. Patients who have neither deletion nor duplication may have nonsense mutations, one of which has been detected by predicting the site of mutation from the size of the trucated protein, and then confirming the presence of a stop codon by sequencing the appropriate PCR amplified exon. Exceptions to the frame-shift rule are of considerable biological interest, and PCR analysis of mRNA from a group of BMD and DMD patients with deletion of exons 3-7 has confirmed the predicted frame-shift in the message. How this message gives rise to dystrophin with an apparently intact C terminus in a mildly affected boy is still a mystery. Duplications studied to date are all tandem in nature and sequence analysis of three duplication junctions demonstrates that the exchange in each case is between non-homologous segments of the gene. This non-homologous recombination is intrachromosomal, taking place between sister chromatids in the single X chromosome of the maternal grandfather (3 cases) or in one X of the maternal grandmother (1 case). Correction of the defect in muscle of affected boys may be possible by the introduction of a normal dystrophin gene. Myoblast transplantation from a related donor may by the simplest way to achieve this, but it is too soon to know results of the first feasibility studies. A potentially more powerful approach is the introduction of a cloned dystrophin cDNA into the muscle, either directly or via a transfected myoblast. The 13.9 kb cDNA is too large to insert into the "traditional" retroviral vectors, but the existence of a very mildly affected male with a deletion of 44% of his DMD gene indicates that it may be possible to construct a trimmed down mini-gene that may produce a functional dystrophin molecule of reduced size. Such constructs are currently being prepared and tested for their ability to produce dystrophin that localizes to the membrane of transfected myogenic cells.

Cell-Cell Interactions in Cell Fate Decisions; Cell and Molecular Biology of Commitment; Genes that Regulate Myogenesis

F 100 GENE STRUCTURE AND FUNCTIONAL ANALYIS OF THE MYOGENIC FACTOR *BMYF* A BOVINE HOMOLOG OF *MYF-5*, Todd E. Arnold, Jeremy L. Barth, Robert A.Worrell, Julie A. Morris, and Robert Ivarie, University of Georgia, Genetics Department, Athens, GA., 30602.
Bmyf is a bovine homolog of human myogenic factor *myf-5*. It encodes a protein 255 amino acids in length which is 96% homologous to the predicted amino acid sequence of *myf-5* and transforms C3H10T1/2 cells to determined myoblasts at high frequency when expressed from a retroviral promoter. Using primers derived from the cDNA sequence, we have determined the structure of the *bmyf* gene. The first of two introns is ~1 kb in length located between nucleotides 569 and 570. A second intron ~.3 kb long occurs between nucleotides 621 and 894 as determined by restriction analysis. Restriction analysis of a clone isolated from a genomic library agrees with the PCR results. This overall structure is similar to that reported for *herculin*, a myogenic factor in the *myoD* gene family, which is located adjacent to a *myf-5* homolog in mouse. Inverse PCR was used to clone 203 bases of 5' flanking DNA. A canonical TATA element is located 100 bases from the 5' end of two independently cloned cDNA copies. Protection assays are in progress to determine if this TATA element is utilized. Stable transformants have been obtained with *bmyf* under the control of a steroid inducible promoter. Results obtained from these lines as well as additional analysis of the genomic clone will be reported.

F 101 **DIMERIZATION CHARACTERISTICS AND SEQUENCE-SPECIFIC DNA BINDING BY PEPTIDES THAT CONTAIN THE HELIX LOOP HELIX MOTIF** **Pamela A. Benfield, Stephen L. Brenner, Spencer J. Anthony-Cahill, Rita K. Steed, Robert Fairman, and William F. DeGrado.** Central Research and Development Department, E.I. DuPont de Nemours and Co., Wilmington, DE 19880-0328

A family of transcriptional regulatory proteins contain a conserved helix loop helix (HLH) structural motif adjacent to a basic region. The HLH motif is believed to constitute a dimerization domain that also orients the adjacent basic regions appropriately for them to interact with DNA. However, the exact structure of these motifs is currently unknown. Among these regulatory proteins are E12 and E47 originally described as important for immunoglobulin gene expression, and the MyoD family of proteins that has been shown to play a critical role in differentiation of muscle cells and expression of muscle specific genes. Each of these regulators bind to a conserved E box motif that contains the sequence CANNTG. E47 and E12 have been shown to form dimers with the MyoD family of regulators and it is the heterodimeric species that is believed to be active in muscle-specific gene activation. We have synthesized peptides, 60 to 75 amino acid residues in length, that contain the basic and HLH motifs from E47 and MyoD. Circular dichroism measurements have been used to examine the structure of these peptides and their ability to form oligomeric complexes both with themselves and with each other. In addition the ability of these peptides to bind specifically to the E box sequences in the rat muscle creatine kinase enhancer has been assessed using gel retardation and footprinting assays. We have used these data to evaluate potential structural models for the helix loop helix family of proteins and to determine dissociation constants for E47 and MyoD peptide dimers.

F 102 SEQUENCE SPECIFICITY OF DNA BINDING BY MYOD, E2A, AND C-MYC BASIC-HELIX-LOOP-HELIX PROTEINS, T. Keith Blackwell, Leo Kretzner, Elizabeth M. Blackwood, Robert N. Eisenman, and Harold Weintraub, Fred Hutchinson Cancer Research Center, 1124 Columbia Street, Seattle, WA 98104. We have developed a novel strategy for studying the DNA-sequence specificity of protein-DNA binding. From oligonucleotides in which specific binding-site positions are random in sequence, those that are bound by a protein complex are isolated in an electrophoretic mobility-shift assay, amplified by the polymerase chain reaction, and finally sequenced directly as a pool. The nucleotide sequence patterns of these "Selected and Amplified Binding-sites" (SaAB)s provide a characteristic "imprint" of protein binding. We have used SaAB imprinting to compare and contrast the sequence-preferences of DNA binding by homo- and heterooligomers of the basic-helix-loop-helix (bHLH) proteins MyoD and E2A. These different protein species preferentially bind a consensus CA - - TG motif, but at internal and surrounding positions select different sequence patterns that suggest half-site recognition. Thus, combinatorial interactions among dimeric proteins can, in fact, define new binding-sequence preferences. With SaAB imprinting we have also derived a CA -- TG site that is specifically bound in vitro by a purified C-terminal fragment of human c-Myc that contains the bHLH domain and adjacent leucine zipper. Our results suggest that some of the biological functions of Myc-family proteins involve sequence-specific DNA binding that is mediated by this region of the protein. We are currently using this approach to study structural aspects of DNA binding by bHLH proteins, to identify new putative bHLH factors that recognize the CA -- TG consensus, and to explore the relationship between DNA binding and transcriptional activation by MyoD.

F 103 POSITIVE AND NEGATIVE REGULATORS OF MYOGENESIS. Bruce T. Blakely and Helen M. Blau, Department of Pharmacology, Stanford University School of Medicine, Stanford, CA 94305.

The mechanisms underlying the expression of the differentiated state can be studied in a variety of cell types by comparing (1) the activation of muscle genes following transfection of cloned myogenic regulators, and (2) the activation observed following fusion with muscle cells in heterokaryons. The stable expression of the mouse cDNA encoding the muscle regulatory protein MyoD induces myogenesis in fibroblasts. In contrast, MyoD, alone or in combination with cDNA encoding the related protein myogenin, fails to activate expression of muscle genes, or to extinguish expression of liver genes, when stably expressed in HepG2 liver cells (Schäfer et al., 1990, Nature 344, 454-458). Fibroblasts and liver cells also differ in their ability to express endogenous myogenic regulators: stable expression of the MyoD cDNA results in expression of the endogenous MyoD and myogenin genes in MRC5 fibroblasts, but not in HepG2 liver cells. By contrast, in heterokaryons muscle genes are activated in both fibroblasts (MRC5) and liver cells (primary hepatocytes and HepG2 cells) and liver proteins are efficiently extinguished. These results indicate that regulators in addition to MyoD are required for muscle gene activation in liver cells. In addition, liver cells express regulators that are inhibitory to myogenesis. Further analysis of the expression of the MyoD family of proteins and characterization of additional positive and negative regulators in transfected cells and in heterokaryons will provide insight into the regulatory networks that control myogenic differentiation. (Supported by NIH, MDA).

F 104 REGULATORY NETWORKS IN MYOGENESIS. Helen M. Blau, Department of Pharmacology, Stanford University School of Medicine, Stanford, CA 94305.

The differentiated state appears stable. Yet, heterokaryon experiments indicate that it results largely from an active, not a passive process, that depends on continual regulation. Muscle genes are accessible and can be expressed in a wide range of cell types that normally never express them. Studies of gene dosage indicate that the stoichiometry, or balance, of positive and negative regulators is critical in determining whether genes are activated or repressed. A characterization of myogenic mutant cell lines indicates that these decisions depend, in part, on the concentration of regulatory components that act as heterodimers, and either promote or inhibit gene expression. Further evidence for plasticity derives from studies of muscle cell fate in vivo using retroviral lineage markers. Taken together, these experiments suggest that both gene activation and gene repression are actively controlled in the course of differentiation during development.

F 105 **BIPHASIC EXPRESSION OF THE MYOGENIC DETERMINATION FACTOR MYF6 DURING MOUSE DEVELOPMENT** Bober,E.; Lyons,G.; Ott, M-O.;Braun,T.; Buckingham, M. and Arnold,H.H., Dept. of Molecular Biology, Univ. of Hamburg, Medical School, D-2000 Hamburg 13, Grindelallee 117, FRG

Four distinct myogenic determination factors have been identified in mammals. These proteins are indistinguishable in their ability to initiate the myogenic program in cultured non-muscle cells, transactivate muscle-specific reporter genes and interact with other HLH proteins to acquire high affinity DNA binding capacity in vitro. MyoDI and Myogenin are located on different chromosomes and are expressed in a specific temporal pattern during mouse development (Sassoon et al., 1989). In contrast Myf5 and Myf6 are closely linked on one chromosome. To study their expression pattern during early mouse myogenesis. we have performed in situ hybridizations on mouse embryos from day 8 until birth. Recently, we have shown that myf5 expression initiates in the dorsal dermomyotome of immature somites of day 8 and follows an antero-posterior gradient. Its expression is transient in somites, the visceral arches and limb buds with maximal levels around day 10.5, subsequently decreasing until undetectable after day 13. Accumulation of myf6 transcripts in somites starts about 1 day after myf5 expression. It follows the same rostro-caudal gradient and disappears clearly before myf5. In limbs and in body musculature myf6 is only activated at about day15 and represents the predominant muscle specific regulatory factor in adult, when myf5 is not expressed any more. The time of myf6 activation in developing skeletal muscle is approximately coordinated with innervation.

F 106 THE MYOGENIC DETERMINATION FACTOR MYF5 ACTS AS A POSITIVE TRANSCRIPTION FACTOR.
Thomas Braun, Barbara Winter, Eva Bober, Dagmar Arnold, and Hans-H. Arnold, Univ. of Hamburg, Dept. of Toxicology, D-2000 Hamburg 13, Grindelallee 117.
Human myf5, like myoD, myogenin and myf6 (MRF4, herculin) belongs to a group of muscle determination factors capable to initiate myogenesis in a wide variety of cells. The four determination factors share a concerved sequence motif for a putative helix-loop-helix structure preceded by a cluster of basic amino acids. Several mutants were analyzed to determine a sequence-specific DNA binding activity which resides in this conserved sequence segment. We furthermore recognized that all four factors can form heterodimers with the DNA protein E12 and other helix-loop-helix proteins, i.e. intl and int2, thereby increasing their DNA binding affinity by several orders of magnitude. Myf5, like the other members of this family, is able in transient transfection assays to activate the gene expression of endogenous muscle genes as exogenously introduced muscle gene reporter constructs like the MLC1-promotor/enhancer. To investigate whether the transactivating capability of myf5 can be atributed to distrinct parts of the myf5 protein we analyzed N-terminal and C-terminal deletions for muscle specific gene activation. Several chimeric molecules with the DNA binding part of the GAL4-protein were constructed to discriminate between the DNA binding properties and transactivating functions of myf5. The main activating domain was located in the C-terminal half of the molecule containing two clusters of serine/threonine amino acids. Successive deletions of the C-terminal-half of the protein resulted in a gradual loss of transactivation capability, whereas the steady state level of the deleted proteins remained constant as measured by western bloting.

F 107 **REGULATION OF NICOTINIC ACETYLCHOLINE RECEPTOR GENE TRANSCRIPTION BY MYOGENIN AND MYOD,** Andres Buonanno and Laura Lautens, Unit on Molecular Neurobiology, LDN, NICHD, NIH, Bethesda, Maryland 20892

Transcription of the nicotinic acetylcholine receptor subunit (nAChR) genes is coordinately activated during terminal differentiation of C2C12 mouse myoblasts. In order to understand the regulation of α and γ subunit gene transcription during muscle development, we have begun to identify the cis- and trans-elements controlling expression of both genes. Herewith we present evidence that myogenin and MyoD regulate transcription of nAChR subunit genes. Expression of myogenin and MyoD transcripts increase in C2C12 myotubes during differentiation; the levels of myogenin transcripts begin to accumulate rapidly before receptor mRNAs. When 3T3 fibroblasts are co-transfected with a construct containing the chick nAChR α subunit enhancer linked to the reporter gene chloramphenicol acetyltransferase (CAT), plus a vector expressing either myogenin or MyoD, transcription of the CAT gene is activated. Results obtained using gel retardation assays and DNA footprinting demonstrated that myogenin and MyoD bind directly to the nAChR α subunit enhancer. There are two consensus E boxes (CANNTG) or "MyoD binding sites" in the enhancer, we found that the site nearest to the transcription initiation site is bound preferentially by both factors. The introduction of point mutations in either binding site practically abolished expression of the CAT reporter gene in transfected C2C12 mouse myotubes; these cells express myogenin and MyoD endogenously. We have begun to delineate the regions regulating transcription of the mouse nAChR γ subunit gene by using progressive deletions. We found that the region between -202 and the initiator methionine confer developmental regulation to a CAT construct transfected into C2C12 myotubes; the regulatory region contains two putative E boxes. Deletion of the first base of the upstream E box represses CAT expression approximately 20-fold. Experiments are in progress to determine if myogenin and MyoD bind directly to the E boxes, and if they activate transcription from the nAChR γ subunit upstream regulatory region. It is interesting to speculate from our results that myogenin and/or MyoD coordinately activate transcription of nAChR genes during myogenic differentiation, and may also modulate transcription of a repertoire of other skeletal muscle genes that are preferentially expressed at the neuromuscular synapse.

F 108 **DISSECTION OF DNA-BINDING AND TRANSCRIPTION ACTIVATING FUNCTIONS OF MYOGENIN,** Tushar Chakraborty, Thomas J. Brennan, Eric N. Olson, Department of Biochemistry and Molecular Biology, The University of Texas M. D. Anderson Cancer Center, Houston, TX 77030

Myogenin belongs to a family of muscle-specific regulatory factors that includes MyoD, myf-5 and MRF4, each of which share homology within a basic region which is thought to contact DNA and a helix-loop-*helix* (HLH) motif which mediates dimerization. Upon oligomerization with the widely expressed HLH protein E12, myogenin binds to E-box (CANNTG) elements present in the muscle creatine kinase (MCK) enhancer and numerous other muscle-specific genes, resulting in transcription activation. By site-directed mutagenesis, we show that the basic domain of myogenin contains a segment of 12 amino acids that is important for DNA binding and activtion of myogenesis. The basic region of E12 is conserved at eight of these twelve residues and can mediate DNA binding when placed in myogenin but cannot activate myogenesis. Replacement of each of the four non-conserved residues of the myogenin basic domain with the corresponding residues from E12 revealed two amino acids that impart muscle specificity. Several point mutations within the basic region show that transactivation is dependent on, but separable from, DNA binding. We and others have recently shown that MRF4, unlike myogenin, MyoD or myf-5, lacks the capacity to transactivate the MCK enhancer. Domain-swapping experiments between myogenin and MRF4 indicates an important role for the carboxy terminus of myogenin in transcription activation of the MCK enhancer. The properties of the myogenin activation domain(s), defined through gene fusions with the DNA-binding domain of the yeast transcription factor GAL4, will be presented.

F 109 FOS MEDIATED INHIBITION OF MYOGENIC DIFFERENTIATION. J.R.Coleman and A. Mendelsohn, Division of Biology and Medicine, Brown University, Providence, RI, 02912
The basic objectives of this research are to characterize the inhibition of differentiation that accompanies oncogenic transformation and to begin to delineate the mechanisms of inhibition at the molecular level in cultured mammalian myogenic cell lines with special emphasis on the fos oncogene. Rat L6 and mouse C2C12 myoblasts were transfected with a variety of oncogene constructs, using G418 resistance for selection of transfectants. Cells transformed by E1a, V-Fos, Ha-Ras, V-Src and V-Mos generally did not form myotubes. Conversely, V-Myb, P-53, V-Erb B, and V-Sis did not inhibit myogenesis. There seems to be a correlation between transforming activity of the oncogene clone in NIH 3T3 cells and the ability of an oncogene to suppress differentiation. Expression of MyoD1 and Myogenin, putative regulators of muscle cell differentiation, was dramatically lowered in E1a, Ha-Ras, and V-Fos transformed C2C12 cells, but not in erbB transformed C2C12 cells. This correlates with the ability of these lines to differentiate. Conditioned media from v-Fos transformed cells did not affect normal C2 cell differentiation, suggesting that a secreted factor was not involved in the mechanism of inhibition. Low serum inhibited proliferation of v-Fos transformed cells, but did not stimulate differentiation, nor did it re-establish normal levels of MyoD1, or Myogenin expression. This suggests that suppression of differentiation by these oncogenes is probably not due to growth stimulation. The forced expression of MyoD, Myogenin, Myf-5, or Mrf-4 partially restores mouse MCK promoter activity in v-fos transformed C2 cells as assayed by CAT assays performed after transient transfection of an MCK-CAT fusion vector. This suggests that Fos suppresses muscle differentation by down regulating expression of muscle transcription factors and probably not by induction of inhibitory factors with greater affinity for muscle specific DNA binding domains. (Supported by NSF Grant Number DCB-8609560).

F 110 INHIBITION OF DIFFERENTIATION IN MYOBLASTS TRANSFORMED BY VIRAL ONCOGENES IS NOT NECESSARILY MEDIATED BY DOWN-REGULATION OF MyoD AND MYOGENIN GENE EXPRESSION, Germana Falcone, Stefano Alemà and Franco Tatò[1], Istituto di Biologia Cellulare, CNR, Rome, Italy, and [1]Dipartimento di Biologia Cellulare e dello Sviluppo, Università "La Sapienza", Rome, Italy
Quail myogenic cells infected with temperature sensitive mutants of Rous sarcoma virus exhibit a temperature-dependent transformation and block of differentiation. When the cells are allowed to differentiate at the restrictive temperature (41°C) and then shifted back to the permissive temperature (35°C), a sharp reduction in the accumulation of muscle-specific mRNAs is observed, following reactivation of the transforming protein pp60v-*src*. This down-regulation is the consequence of a transcriptional inhibition exerted by the oncogene on muscle-specific genes. On the contrary, transcription of the muscle regulatory gene MyoD is not significantly affected by the oncogene both in proliferating myoblasts and in myotubes shifted back to 35°C. Transcription of myogenin gene, instead, is strongly inhibited in proliferating myoblasts, but is not affected following shift down of the differentiated cultures. These findings suggest the existence of an alternative pathway independent of MyoD and myogenin transcription through which the v-*src* oncogene exerts its effect. Block of differentiation induced in the same cells by the v-*myc* oncogene, instead, might be mediated by MyoD or myogenin genes, since transcription of both these genes is strongly inhibited in v-*myc* transformed cells.

F 111 POSITIVE CONTROL OF MYOGENESIS BY THE INSULIN-LIKE GROWTH FACTORS (IGFS) VIA ELEVATED EXPRESSION OF MYOGENIN AND Myf-5. J. Florini*, K. Magri*, D. Ewton*, S. Roof*, P. James§, K. Grindstaff§, and P. Rotwein§, *Biology Dept., Syracuse U., Syracuse, NY 13244, and §Dept. of Medicine, Washington U. School Med., St. Louis, MO 63110
Although many investigators view control of myogenic differentiation in terms of *negative* control by inhibitors such as FGF and TGF-ß (or ill-defined "mitogens"), we showed some time ago that myogenesis is stimulated by the IGFs. More recently, we have studied control of this process in C2 cells, which show a rapid rate of "spontaneous" differentiation. We find that *positive* control by the IGFs occurs even when the cells are not treated with exogenous hormone. When shifted to low-serum medium, C2 cells secrete large amounts of IGF-II as well as some IGF-I and IGF binding proteins, and it appears that differentiation in these cells results from autocrine/paracrine stimulation by the IGFs. Two observations lead to this conclusion: (1) Addition to the cells of antisense oligodeoxynucleotides complementary to the N-terminal sequences of IGF-I and IGF-II mRNAs suppresses "spontaneous" differentiation in C2 cells, and (2) Comparisons among cell lines that exhibit different rates of differentiation show that the rate of "spontaneous" differentiation is paralleled by the formation of IGF-II mRNAs by cells in low-serum medium. The stimulation of differentiation in L6A1 myoblasts by the IGFs involves substantial increases in expression of the myogenesis controlling gene, myogenin, with smaller increases in myf-5. The peak in myogenin mRNA accumulation occurs well before detectable fusion or creatine kinase elevation, and the IGF-I concentration dependency of myogenin expression closely parallels that of creatine kinase elevation and cell fusion. Antisense oligodeoxyribonucleotides complementary to the first 15 nucleotides in myogenin or myf-5 suppress the stimulation of differentiation by IGF-I. We conclude that the IGFs stimulate myogenic differentiation by substantially increasing expression of the myogenesis controlling genes. These observations mandate revision of broad generalizations about the inhibitory role of mitogens/growth factors in controlling muscle cell differentiation.

F 112 HETERODIMERS OF MYOGENIN AND E12 BIND A COMPLEX ELEMENT GOVERNING MYOGENIC INDUCTION OF THE AVIAN CARDIAC α-ACTIN PROMOTER, Brent A. French, King-Lau Chow, Eric N. Olson*, and Robert J. Schwartz, Dept. of Cell Biology, Baylor College of Medicine; and *Dept. of Biochemistry and Molecular Biology, M.D. Anderson Cancer Center; Houston, Texas 77030.

The cardiac α-actin transcripts which appear in the myotomal compartments of the somites constitute one of the earliest known markers for skeletal muscle development. However, two genes regulating myogenesis (MyoD and myogenin) are also expressed early during muscle development. Myogenin and MyoD are members of a family of regulatory proteins which share a helix-loop-helix (HLH) motif required for dimerization and DNA-binding. Myogenin and MyoD form heterodimers with the ubiquitous HLH protein E12 which bind cis-acting DNA elements that have an E-box (CANNTG) at their core. Heterodimers of myogenin and E12 (or MyoD and E12) specifically bind a restriction fragment from the cardiac α-actin promoter. Methylation interference footprints pinpoint the site of interaction to an E-box immediately adjacent to a previously identified CArG-box (CArG3). Site-directed mutations to the binding site reveal that either an intact E-box or an intact CArG3 is required for induction of the cardiac α-actin promoter in myoblasts and for transactivation by myogenin in cotransfected fibroblasts. These results suggest that direct as well as indirect pathways may be involved in the induction of cardiac α-actin promoter by myogenin and MyoD.

F 113 CLONING AND CHARACTERIZATION OF cDNAS FOR CHICKEN MYOGENIC FACTORS, **Atsuko Fujisawa-Sehara, Yoko Nabeshima, Tohru Komiya, Taichi Uetsuki, Atsushi Asakura, Yoko Hosoda, and Yo-ichi Nabeshima, Division of Molecular Genetics, National Institute of Neuroscience, NCNP, 4-1-1 Ogawahigashi, Kodaira, Tokyo 187, Japan.**

Using mouse MyoD and myogenin cDNAs as probes, we have isolated their chicken homologues. Polypeptide sequence of our chicken MyoD was found to be identical with that reported previously (CMD1; Lin et al., 1989) except several amino acid residues. Chicken myogenin cDNA encodes a polypeptide of 227 amino acids the sequence of which is highly homologous to that of mouse myogenin. When these cDNAs were expressed in C3H10T1/2 cells, the cells were converted to myoblasts, which subsequently gave rise to myotubes. Furthermore, both the MyoD and myogenin produced in bacteria were found to bind to the enhancer of chicken LC1 gene (a skeletal myosin alkaline light chain gene) which contains two consensus sequences for MyoD binding sites. Transient expression of MyoD cDNAs in chicken primary fibroblasts activated the promoter of the chicken LC1 gene fused with chloramphenicol acetyltransferase gene. The activation of the gene was dependent on the existence of the enhancer. These results suggest that MyoD and myogenin are involved in the activation of the LC1 gene by direct binding to its enhancer during avian muscle development.

F 114 IDENTIFICATION OF MUSCLE PRECURSOR CELLS *IN VIVO* USING MYOD1 AND MYOGENIN PROBES. Kerryn L. Garrett, May C. Lai, Manfred W. Beilharz and Miranda D. Grounds*, Departments of Microbiology and Pathology*, University of Western Australia, Nedlands, Australia, 6009.

The activation of mononuclear muscle precursor cells following crush injury to the mouse tibialis anterior muscle was monitored *in vivo* by *in situ* hybridization with MyoD1 and myogenin probes. These genes are early markers of skeletal muscle differentiation and have been extensively studied *in vitro*. The *in vivo* role of these regulatory proteins during myogenesis of mature muscle has not been studied previously. MyoD1 and myogenin expression was present in occasional mononuclear cells of uninjured muscle. Elevated co-expression of MyoD1 and myogenin mRNA sequences in mononuclear cells was detected as early as 6 hours after injury. Expression of the genes peaked between 24 and 48 hours and thereafter declined to pre-injury levels at about 8 days. The mRNA's were not detected in newly formed myotubes. The activation of mononuclear cells occurred throughout the muscle fibres with the majority of cells located some distance from the site of crush injury. The sustained presence of MyoD1 and myogenin transcripts suggests that expression of these genes is occurring at the same time as replication of muscle precursor cells prior to fusion *in vivo*. The rapid activation and repression of MyoD1 and myogenin expression indicates that these events are tightly regulated *in vivo*. MyoD1 and myogenin provide precise markers for the very early identification and study of mononuclear skeletal muscle precursor cells in muscle regenerating *in vivo*.

F 115 MYOBLASTS AND MYOFIBERS HAVE DISTINCT TROPONIN I ENHANCER BINDING COMPLEXES THAT CONTAIN MYOD1, David J. Goldhamer, YongWon Nham, and Charles P. Emerson, Department of Biology, University of Virginia, Charlottesville, Va. 22901

The expression of troponin I as well as many other muscle-specific genes is controlled by enhancers, which are activated by unknown regulatory proteins when myoblasts differentiate. Although the TnI gene is transcriptionally silent in myoblasts, we show by gel shift assays that both 23A2 myoblasts and myofibers contain nuclear protein complexes that bind a consensus MyoD1 binding site in the TnI enhancer. Using MyoD1-specific, anti-peptide antibodies, we demonstrate that TnI enhancer binding complexes isolated from both myoblasts and myofibers contain MyoD1 protein. Bases on different electrophoretic mobilities of these DNA/protein complexes, it is concluded that enhancer binding complexes that contain MyoD1 are non-identical in myoblasts and myofibers. Immunoprecipitation studies show that *in vitro* synthesized MyoD1 does not heterodimerize with the myogenic regulatory proteins, myogenin and myf5, suggesting that the different MyoD1 complexes detected in myoblasts and myofibers reflect regulated changes in the association of MyoD1 with other proteins, or post-translational modifications of invariant components. These data imply that DNA binding activity of MyoD1 *per se* is not the limiting factor for muscle gene transcription; rather the interaction of MyoD1 with other regulatory proteins may control accessibility of MyoD1 complexes to muscle enhancers *in vivo*.

F 116 EXPRESSION OF THE MUSCLE REGULATORY FACTORS MRF4, MyoD1, MYOGENIN, AND Myf-5 DURING MUSCLE DIFFERENTIATION *IN VIVO* AND *IN VITRO*, Timothy J. Hinterberger, Simon J. Rhodes, David A. Sassoon* and Stephen F. Konieczny, Department of Biological Sciences, Purdue University, West Lafayette, IN 47907, and *Department of Biochemistry, Boston University School of Medicine, Boston, MA 02118

The four muscle regulatory factors MRF4, MyoD1, myogenin and Myf-5 possess a similar basic/helix-loop-helix domain that is essential for myogenesis and for converting fibroblasts into determined muscle lineages. To begin to examine why four separate, similar factors are involved in skeletal muscle development, we have characterized their expression patterns in rat and mouse embryos, in primary rat muscle cell cultures, and in permanent myogenic cell lines. Northern analyses showed that MRF4 and myogenin mRNAs are restricted to differentiated myofibers in the rat cell line L6J1-C, whereas Myf-5 mRNA is present constitutively and MyoD1 transcripts are not observed. In primary cultures, myogenin and Myf-5 mRNAs increase and MyoD1 mRNA decreases upon differentiation, while MRF4 mRNA again is restricted to myofibers. Although Northern analysis first detects MRF4 in fetal rat muscle at 17 days , *in situ* hybridization reveals MRF4 expression in early rat and mouse somites at approximately the same time that myogenin is detected. Polyclonal antisera currently are being raised against each muscle regulatory factor to examine the steady-state levels of the proteins during development. These studies will help define the specific role of each factor during different developmental stages, in particular cell types, and in the activation of individual contractile protein genes.

F 117 A SHEEP MUSCLE cDNA CLONE WITH HOMOLOGY TO MyoD1

Huynen, L.J.H. & Bass, J.[1]
Dept. of Cell.& Molec. Biol., University of Auckland, New Zealand.
[1]Ruakura Agric. Centre, MAFTech, Hamilton, New Zealand.

MyoD1 belongs to a family of muscle determination genes, all of which share homology and are specifically expressed in skeletal muscle. Members of the family are able to interact with other proteins via a helix-loop-helix motif shown to be important for activity. Using a mouse MyoD1 probe, pVZCIIα (Davis *et al*, Cell 51 p987, 1987) we have isolated a 1.6kb cDNA clone from a λgt10 cDNA library prepared from the thigh muscle of a 7 month old sheep. The frequency of the clone within the library suggests a low rate of expression in adult sheep muscle. Southern analysis indicates that the homology to the mouse cDNA is largely restricted to a 480bp SmaI fragment. Nucleotide sequence analysis and isolation of the promoter region from a sheep genomic library is now in progress. Supported by MAF grant 361009 Ruakura, New Zealand.

F 118 MYOGENIC GENES IN XENOPUS LAEVIS, Charles G.B. Jennings, Douglas A. Melton* and Steven J. Burden, Department of Biology, MIT 16-820, Cambridge, MA 02139, and *Department of Biochemistry and Molecular Biology, Harvard University, 7 Divinity Avenue, Cambridge, MA 02138.

We have used PCR with degenerate primers to search for MyoD-related genes in Xenopus. In addition to MyoD itself, we have isolated a partial sequence related to Myogenin, and a full length cDNA closely related to MRF-4/Herculin/Myf6. Xenopus MRF-4 shares about 75% similarity with its mammalian homologs, and is completely identical within the B-HLH domain. These data indicate that the myogenic gene family arose early in vertebrate evolution. Xenopus MRF-4 is expressed in skeletal muscle but not smooth or cardiac muscle in adult frogs. It appears relatively late in embryonic development, reaching maximal levels at around stage 24, well after the appearance of MyoD, and coincident with the onset of innervation. MRF-4 expression may therefore be induced by innervation; consistent with this possibility, the RNA level falls in response to denervation of adult frog muscle. Preliminary results indicate that MRF-4 is not concentrated at the synaptic region of rat muscle, and is therefore unlikely to regulate synaptic gene expression. It may, however, regulate extrasynaptic gene expresssion in response to electrical activity, and this possibility is currently being investigated.

F 119 NEUTRALIZATION OF MYOD1 BY MONOCLONAL ANTIBODIES BINDING THE CYSTEINE/HISTIDINE-RICH REGION, D. Stave Kohtz, Kimberly S. Timo and Jon Gordon*, Department of Pathology and *Brookdale Center for Molecular Biology, Mount Sinai Medical School, New York, NY 10029.
Monoclonal antibodies were generated that bind an epitope in the cysteine/histidine-rich segment (residues 63-99) of MyoD1. These antibodies neutralize the site-specific DNA-binding activity of MyoD1, as determined by gel retardation assay. One of the antibodies (mAb 1-8) was used to study the effect of transient neutralization of MyoD1 in vivo on myoblast phenotype. Myoblasts (C2C12) at clonal density were microinjected with mAb 1-8, grown into colonies, and induced to differentiate. Only 10% fewer normally differentiating colonies formed from founder cells microinjected with mAb 1-8 than did from controls, suggesting that neutralization of MyoD1 alone is not sufficient to extinguish the myoblast phenotype. This conclusion was supported by the occurrence of mosaic colonies among the population injected with mAb 1-8. Transient neutralization of MyoD1 (and putatively its autoregulated expression) is sufficient to destabilize the myoblast phenotype, but apparently a secondary event (such as expression of an inhibitory trans-acting factor, or methylation of the MyoD1 gene) is required for its complete abrogation.

F 120 **BETA ENOLASE IS AN EARLY MARKER OF MYOGENESIS IN THE MOUSE,** M.Lazar, A.Keller, M.Lucas, N.Lamandé, S.Brosset, D.Butler and F.Gros, Biochimie Cellulaire, Collège de France, 75005 Paris, FRANCE.
In higher vertebrates, the glycolytic enzyme enolase is a dimeric protein formed from 3 subunits - alpha, beta and gamma (A,B,G) - encoded by distinct genes.In adult tissues, AA isozyme is widely distributed,whereas BB and GG are specific to striated myofibers and neurons,respectively.We have investigated the developmental expression of A and B genes in the mouse using subunit-specific probes and antibodies.Data obtained in culture led us to the working hypothesis that transcriptional activation of B gene occurs in newly determined myoblasts,i.e. at very early steps of the myogenic pathway *in vivo*.We have undertaken,therefore,to examine the temporal and spatial pattern of expression of B gene in mouse embryo.In developing hindlimbs,B transcripts were detected by Northern blot analysis at embryonic day 15 (E15),the major accumulation of transcripts occuring during secondary fibers genesis.By *in situ* hybridization on whole embryos,B transcripts were visualized in limb buds already at E12.Most interestingly,B transcripts were accumulated in myotomes and in heart at E9 (18 somites).At the same stage,A enolase transcripts were ubiquitously present at high levels.The specific expression of B transcripts in embryonic striated muscle was observed at all stages (E9 to E20).Studies at earlier stages of development are underway.The present data indicate that an understanding of the molecular mechanisms controlling beta enolase gene expression will be of particular interest.

F 121 REPRESSION OF MYOGENIN'S TRANSCRIPTION ACTIVATING FUNCTION BY fos AND jun Li Li and Eric Olson Department of Biochemistry and Molecular Biology, The University of Texas M. D. Anderson Cancer Center, Houston, TX 77030

Myogenin, a member of the helix-loop-helix family of muscle regulatory factors, binds a concensus sequence, referred to as E box, which is present in the regulatory regions of a number of muscle-specific genes. We have used the muscle creatine kinase (MCK) enhancer as a target to study the influence of growth factor signals on myogenin's transcription activating properties. TGF-β, a potent inhibitor of myogenesis, can repress the ability of myogenin to trans-activate the MCK enhancer through a mechanism independent of DNA binding. Similarly, the TGF-β-inducible early gene products fos, c-jun and junB can prevent trans-activation of the MCK enhancer by myogenin. In contrast, junD, which is expressed constitutively and does not respond to TGF-β, has little effect on the action of myogenin. Analysis of a series of the MCK enhancer mutants supports the conclusion that trans-repression by fos and jun is directed at the E-box concensus sequence within the enhancer core. Since neither fos nor jun interact directly with the MCK enhancer, their inhibitory actions appear to be mediated through interaction with myogenin or through an indirect mechanism. The domain of myogenin that responds to fos and jun is currently being defined by mutagenesis and the possibility that fos and jun interact directly with myogenin is under investigation.

F 122 EFFECT OF *DROSOPHILA* NEUROGENIC GENES ON EMBRYONIC MUSCLE DEVELOPMENT. A. M. Michelson[1], S. M. Abmayr[1], V. Corbin[2], M. Young[2], M. Bate[3] and T. Maniatis[1]. [1]Dept. of Biochemistry and Molecular Biology, Harvard University, Cambridge, MA 02138, [2]Laboratory of Genetics and Howard Hughes Medical Institute, Rockefeller University, New York, NY 10021, and [3]Dept. of Zoology, University of Cambridge, Cambridge, UK CB2 3EJ.

The first morphological evidence of myogenesis in *Drosophila* embryos is the appearance of fused muscle precursors in the mesoderm overlying the developing central nervous system. We previously cloned *nautilus* (*nau*), a gene whose earliest embryonic expression occurs in small clusters of mononucleate mesodermal cells that may represent the founders of particular muscles. We have now examined muscle pattern and *nau* expression in neurogenic mutant embryos to determine if neurogenic genes play a role in myogenesis. In *Notch* (*N*) and *Delta* (*Dl*) mutants, muscle cell fusion does not occur. Furthermore, the early domain of *nau* expression is markedly expanded. Despite the absence of fusion, myosin heavy chain is expressed in neurogenic mutants and these embryos exhibit weak birefringence under polarized light. Thus, two steps in the myogenic differentiation program, cell fusion and the expression of terminal gene products, are distinct processes that can be genetically separated. We further suggest that neurogenic genes may influence muscle development in a manner analogous to their role in the ectoderm, that is, by mediating the choice between alternate cell fates through the process of lateral inhibition. In the case of muscle development, the decision would be between founder cells that determine muscle identities but are incapable of fusing with each other, and cells that are competent to fuse with founders. According to this model, the expanded *nau* domain represents an increased number of muscle founders that develop at the expense of the fusion-competent cell population. The fusion defect is thus a consequence of this change in cell fate resulting from the absence of neurogenic gene function.

F 123 ECTOPIC EXPRESSION OF MyoD IN TRANSGENIC MICE, Jeffrey H. Miner, Jean-Paul Revel and Barbara Wold, Division of Biology 156-29, California Institute of Technology, Pasadena, CA 91125

We have produced transgenic mice by microinjection of a MyoD cDNA under the control of the mouse muscle creatine kinase (MCK) enhancer-promoter. This ~3 kb regulatory sequence is very active in differentiated skeletal and cardiac muscle cells. Thus, these mice are expected to express MyoD at above normal levels in skeletal muscle and at appreciable levels in heart. Heart normally lacks expression of MyoD and its three known relatives. The gross phenotype associated with the transgene is death of transgenic fetuses at 16-18 days of embryonic development. While the hearts of these fetuses are misshapen, they appear to beat normally until near death. Analysis of RNA from these transgenic hearts reveals that cardiac α-actin is aberrantly down-regulated, and skeletal α-actin and myogenin (but not Myf-5 or herculin) are aberrantly activated. These results demonstrate that ectopically expressed MyoD can activate skeletal muscle-specific genes in a non-skeletal tissue in the developing animal, and in this case the phenotype is ultimately lethal. Other transgenic experiments to study the activities and regulation of MyoD family members are currently under way.

F 124 THE POSSIBLE ROLE OF PHOSPHORYLATION IN THE FUNCTION OF CMD1, Seiji Nakamura, Bruce M. Paterson, Laboratory of Biochemistry, National Cancer Institute, NIH, Bethesda, MD 20892.

The molecular mechanisms of action of myogenic determination factors is still not completely understood. Purification of the MyoD family of proteins expressed in eukaryotic cell systems would help to understand a certain aspect of this issue.
We have expressed CMD1 protein in the Baculovirus expression system and purified it to almost homogeneity by using the combination of hydroxylapatite column chromatography and anti-CMD1 antibody affinity column chromatography. Labelling experiments with ^{32}P showed that the protein is phosphorylated in the host insect cells (Sf9).
When combined with E12 protein, made in either reticulocyte lysate or wheat germ system, CMD1 protein binds to the CPK enhancer as shown by gel retardation assay. When CMD1 protein was treated with CIP (calf intestinal phosphatase) which was bound to agarose and combined with E12 protein, the binding affinity of the mixture to the CPK enhancer was remarkably reduced. This finding suggested the possibility that phosphorylation of CMD1 protein could play an important role in skeletal muscle differentiation.

F 125 MOLECULAR CHARACTERIZATION AND EXPRESSION PATTERN OF THE MYOPLASMIN-C1: A POLYPEPTIDE LOCALIZED IN THE MYOPLASM OF ASCIDIAN EGGS.

T. Nishikata, K. W. Makabe and N. Satoh, Dept. of Zoology, Kyoto Univ., Kyoto 606, JAPAN.
Although evidence exists for morphogenetic determinants that are responsible for the regulation of early developmental programs, little is known about their molecular identity. In the ascidian egg, muscle determinants are thought to be sequestered in the so-called myoplasm and play an important role for the larval muscle cell differentiation. In order to explore the molecular identity of the muscle determinants, we established 12 monoclonal antibodies (Mabs) that recognize the myoplasmic components of Ciona intestinalis eggs. Antigenic polypeptides recognized by these Mabs were named "Myoplasmin" after their specific localization. We characterized myoplasmin-C1 as a candidate for muscle determinants because the myoplasmin-C1 antibody suppresses the muscle differentiation when injected into fertilized eggs (Nishikata et al, Development 100, 1987). Myoplasmin-C1 was identified as a single 40-kDa polypeptide at pI 5 on the Western blot membrane using 2D-gel electrophoresis. This polypeptide is bounded tightly to the cortex of the unfertilized egg. We isolated partial myoplasmin-C1 cDNA by screening cDNA library of the ovary using the specific Mab. Myoplasmin-C1 transcript appeared as a single band of about 3kb by Northern blot analysis. The in situ hybridization analysis on the sectioned specimens of Ciona ovary revealed that the myoplasmin-C1 mRNA were abundant in the small oocytes, which also have considerable amount of myoplasmin-C1. We are now trying to isolate full length myoplasmin-C1 cDNA. Analysis of the myoplasmin-C1 molecule will provide us a cue to the molecular understanding of the muscle determinants in the ascidian egg.

F 126 ROLE OF NEURAL INPUT ON THE EXPRESSION OF CALCIUM-BINDING PROTEINS IN *XENOPUS LAEVIS* LEG MUSCLE, Bronwen K. Nishikawa and Brian K. Kay*, Curriculum in Neurobiology and Department of Biology*, University of North Carolina at Chapel Hill, Chapel Hill, NC 27514

Following excision of the sciatic nerve, the *Xenopus* gastrocnemius leg muscle differentially expresses the ancestrally related Calmodulin (CM) and Parvalbumin (PV) genes. Levels of both PV transcripts and proteins decrease, while CM transcript and protein levels increase. Our PV studies have confirmed previous reports (Leberer, E. and D. Pette, 1986) which support the hypothesis that PV expression is under positive control of fast-type motorneuron activity, however, little is known about the control of CM expression in leg muscle. Northern blots of CM transcripts reveal a major 1.4 kb band, which is common to ovary, testes, brain (Chien, Y.-H. and I.B. Dawid, 1984) and leg muscle. By day twelve post-denervation, this CM transcript increases six fold higher than control. Primer extension analysis indicates that CM mRNA from control and denervated muscle have the same transcription start site, which differs from ovary. To determine whether CM may be activated by the muscle's regenerative response, we injected the myotoxin, bupivacaine, into control and denervated gastrocnemius and measured transcript levels by Northern blot analysis. Preliminary data suggest that CM gene expression is transiently enhanced with the combined denervation/toxin treatment. We are currently constructing promoter-CAT chimeras for injection into *Xenopus* oocytes. By subjecting them to various conditions which mimic excitation-contraction coupling, we will begin to dissect the molecular mechanisms governing CM/PV gene expression.

F 127 NEGATIVE CONTROL OF MYOGENIC DIFFERENTIATION. Charlotte A. Peterson and Helen M. Blau, Department of Pharmacology, Stanford University School of Medicine, Stanford, CA 94305

Analysis of a mutant muscle cell line has revealed the presence of molecules that negatively regulate the differentiated state (Peterson et al., 1990, Cell 62, 493-502). The non-differentiating phenotype of the NFB mutant results from a repression of the activity of the helix-loop-helix family of myogenic regulators: MyoD, myogenin, and myf-5. Repression appears to occur both at the level of RNA and protein accumulation. Stable expression of cDNAs encoding MyoD, myogenin, or myf-5 in NFB cells overrides the inhibition of differentiation. Thus, the stoichiometry of these regulators and the negatively acting molecules controls myogenesis. These transfection experiments also demonstrate a complex interplay between the helix-loop-helix family of proteins. The molecular basis for the repression observed in the NFB mutant is currently being determined. (Supported by American Heart Association, Stanford Katharine McCormick Fund, NIH, MDA).

F 128 MYOGENIC FACTORS AND REGULATION OF THE ACETYLCHOLINE RECEPTOR α-SUBUNIT GENE, J. Piette, A. Duclert, J.-L. Bessereau and J.-P. Changeux.

Neurobiologie Moléculaire, Institut Pasteur, 75724 Paris Cedex 15, France

Two adjacent MyoD1-binding sites were identified in the 36-bp muscle-specific enhancer of the α-subunit gene of the acetylcholine receptor (AChR) by gel retardation and methylation interference experiments with purified MyoD1 fusion protein. Mutation of both sites strongly affected transactivation of the promoter by MyoD1 or myogenin in fibroblasts as well as the activity of the promoter in chicken myotubes. These mutations also interfered with binding of nuclear factors present in C2.7 muscle cells; some of these nuclear factors are recognized by anti-myogenin antibodies. mRNA levels of some myogenic genes decreased during post-natal mouse development or increased after muscle denervation of adult mouse, concomitant with changes in AChR α-subunit mRNA. In contrast, M-CK mRNA levels did not vary under these conditions. These results, together with the very early appearance of the α-subunit gene messenger during myotome differentiation in chicken embryos, point to a crucial role of myogenic factors in AChR gene expression.

F 129 ISOLATION AND CHARACTERIZATION OF THE *MYD* GENE. Deborah F. Pinney, Brian P. Brunk and Charles P. Emerson Jr. Department of Biology, University of Virginia, Charlottesville, Va 22901.

A myogenic determination gene, *myd*, was identified by transfection of the 10T1/2 mouse embryo cell line with cloned human genomic DNA. Southern blot analyses suggest that *myd* is not one of the members of the MyoD helix-loop-helix family of genes. Furthermore, RNA analyses demonstrate that transfection of *myd*, under the control of its own promoter, results in the expression of MyoD, myf5 and myogenin. A cosmid (gH001) containing the presumptive *myd* gene locus has been isolated from a genomic library made from one of the primary myogenic transfected cell lines (MydI) and identified by it's ability to convert 10T1/2 cells to a stable myogenic lineage. Fragments from cosmid gH001 hybridize to human sequences transfected into an independently derived primary myogenic cell line (MydII), providing further evidence that this cosmid contains the *myd* gene. Preliminary Northern analyses indicate that cosmid gH001 contains sequences expressed specifically in cultured primary quail muscle cells. We are sequencing pertinent fragments from cosmid gH001 and using these fragments as probes to screen cDNA libraries from a variety of species. Isolation of *myd* gene sequences will enable us to test the hypothesis that *myd* is an early acting gene in the myogenic determination pathway.

F 130 POSSIBLE DISTINCT ROLES OF THE MYOGENIC REGULATORY FACTORS, Myf5, MyoD, Myogenin AND MRF4 IN DETERMINATION, DIFFERENTIATION AND MATURATION EVENTS. Christian Pinset and Didier Montarras. Department of Molecular Biology, Pasteur Institute, 25, rue du Dr. Roux - 75724 Paris Cedex 15

Following recent identification of 4 myogenic regulatory factors (MyoD1, Myogenin, Myf5 and MRF4) we have been studying their role(s) in myogenesis. One central question being, do these factors act redundantly or do they cooperate in a network to control determination and/or differentiation ? We have observed that Myf5 is always expressed in all the determined muscle cells that we have tested. Expression of MyoD1 is optional at the myoblast stage : MyoD1 is constitutively expressed in permissive myoblasts and absent from inducible myoblasts. However, in these cells expression of MyoD1 accompanies terminal differentiation. A similar situation was observed in all the types of myoblasts with Myogenin which also accompanies terminal differentiation. No trace of MRF4 (Myf6, Herculin) was found in myoblasts. Expression of MRF4 follows expression of MyoD1 and Myogenin and occurs after the initiation of terminal differentiation. These observations, together with results of transfection experiments and studies performed on developing embryos lead us to propose that Myf5 would be initially involved in determination events while MyoD1 and Myogenin would participate to the control of terminal differentiation in already determined muscle cells, MRF4 being mobilized later in the differentiation process, may be, to control expression of a particular subset of muscle functions.

F 131 EXPRESSION OF THE QMF GENES DURING AVIAN MYOGENESIS, **Mary E. Pownall, Fabienne Charles de la Brousse and Charles P. Emerson, Jr., Department of Biology, University of Virginia, Charlottesville, VA 22901.**

We have recently isolated and begun to characterize three quail myogenic regulatory cDNAs. Sequence comparison to the previously described myogenic cDNAs indicates that qmf1 likely represents quail MyoD1 while qmf2 and qmf3 represent quail myogenin and myf-5, respectively. RNA hybridization studies demonstrate distinct expression patterns of these genes in primary cultures of myogenic cells as well as in quail embryonic tissues. We have also analyzed the expression of the qmf genes in clonal myoblast populations. In addition, we have used an *in situ* hybridization approach to examine the differential expression of the qmf genes in the developing quail. We have already shown that qmf1 gene expression occurs in cells of the dorsal medial lip of compartmentalized somites. The lack of contractile protein gene expression in this region of the somite suggests that determined myoblasts that localize to this region represent a source of proliferative myogenic stem cells that are used to populate and expand the growing muscle forming regions of the developing embryo. We will thus present evidence for the temporal and spatial expression of the qmf2 and qmf3 genes with respect to qmf1. Finally, to begin to understand the molecular mechanisms underlying qmf gene activation during myogenesis, we have also analyzed the transient expression of qmf1 promoter-CAT gene fusions in cultured myogenic cells. Results of transient and stable transfection assays will be presented.

F 132 EXPRESSION OF MYOGENIC FACTORS IN DEVELOPING CHICKEN SKELETAL MUSCLES, Osamu Saitoh and Muthu Periasamy, Department of Physiology and Biophysics, University of Vermont, Burlington, VT 05405

Using basic-myc region of mouse myogenin cDNA as a probe, we screened a cDNA library prepared from chicken embryo skeletal muscle to isolate avian myogenic factors. Several putative MyoD1 related clones were isolated. In addition to the avian MyoD1 homologue (called CMD1, Lin et al 1989), one of the cDNA clones was identified as the avian myogenin. Using these two types of cDNAs, we have studied the expression of MyoD1 and myogenin in differentiating fast and slow-twitch muscles during chicken embryogenesis. Northern analysis indicated that avaian MyoD1 was expressed in all skeletal muscles tissues including fast and slow-twich muscles at all stages tested. In addition, embryonic fast skeletal muscle and adult slow twitch muscle (ALD) contained an additional mRNA species of ~1.0 kb which hybridized to the MyoD1 probe, whereas adult fast muscle showed only 1.5 kb MyoD1 transcript. The presence of additional stage specific- and fiber type specific myogenic factor(s) is suggested. In comparison with high level expression of MyoD1, myogenin mRNA was not present in abundance in any skeletal muscle tissues tested. Although myogenin mRNA was detected in 12 day embryonic skeletal muscle and cultured myotubes, myogenin expression was low in both late embyonic (17day) and adult skeletal muscles. Denervated fast muscle which expressed neonatal/slow muscle phenotype did not show any increase in the expression of myogenic factors (MyoD1 and myogenin), suggesting that regeneration may not be involved in the re-expression of neonatal/slow muscle phenotype.

F 133 DIFFERENTIAL EXPRESSION AND ACTIVITY OF TWO DISTINCT XENOPUS MYOGENIC FACTORS, Jon B. Scales, Eric N. Olson, and W. Michael Perry, Department of Biochemistry and Molecular Biology, The University of Texas M.D. Anderson Cancer Center, Houston, TX 77030

The isolation of two *Xenopus* myogenic factors, Xlmf1 and Xlmf25, representing amphibian homologs of MyoD has recently been reported (Scales, J.B. et al, Mol. Cell. Biol., 10:1516-24). These two genes have surprisingly different kinetics of expression. Xlmf1 transcripts are initially detected around the time of the mid-blastula transition and reaches maximal levels between stages 20 and 30. In contrast, Xlmf25 transcripts are present in oocytes and early embryonic stages peaking around stage 20. Forced expression of both cDNAs will convert mouse 10T1/2 fibroblasts to a myogenic phenotype shown to express myosin heavy chain. Removal of the carboxy terminal 110 amino acids of Xlmf1 prevents the protein from converting 10T1/2 cells to the myogenic phenotype. The resulting protein, however, still contains the entire BHLH region indicated to be sufficient for myogenic conversion of fibroblasts by MyoD. Xlmf1 and Xlmf25 activate transcription from muscle specific genes (MCK) in the absence of MyoD and myogenin in 10T1/2 cells and frog oocytes. The relative ability to transactivate varies between the two frog genes in the two systems. Preliminary analysis of transcriptional regulatory elements of both Xlmf1 and Xlmf25 have been performed and indicate the presence of regions capable of driving expression of a fused lacZ gene when these constructs are injected into *Xenopus* oocytes and embryos. The results of experiments to further define these elements and their roles in temporal and spatial expression in *Xenopus* embryos will be presented. (Supported by HD 27246, ACS CD343A, and HD 39849-01)

F 134 EXPRESSION OF HOMEOBOX CONTAINING GENES IN THE C2C12 MUSCLE CELL LINE, Beat W. Schäfer and Eva Wey, Department of Pediatrics, University of Zürich, Steinwiesstrasse 75, CH-8032 Zürich, Switzerland

Understanding the molecular mechanism underlying determination and differentiation requires that all necessary factors are being identified first. Although a lot of progress in this direction has been made by the isolation of 4 different regulators, all containing a helix-loop-helix motif, it could be shown that additional proteins are required for the trans-activation of muscle specific genes (Schäfer et al., Nature 344, 454-458, 1990). One approach to identify possible additional components involved in determination is to search for proteins homologous to known regulators of cell fate.

We therefore choose to identify the genes containing a homeobox domain which are expressed in the C2C12 muscle cell line. Towards this end we synthesized degenerated oligonucleotides derived from the conserved homeobox of known hox genes. These were then used to amplify specific sequences by PCR in a cDNA library made from C2C12 cells. A specific band could be obtained by this technique indicating that there are in fact homeobox containing genes expressed during muscle development. The corresponding cDNA clones are currently being sequenced and their expression patterns analyzed.

F 135 **THE CONSEQUENCES OF A CONSTITUTIVE *MYOD1* EXPRESSION IN ES CELLS AND MOUSE EMBRYOS.** **Moshe Shani[1], Charles Emerson[2], Yonat Magal[1], Itzhak Dekel[1], Alexander Faerman[1], and Sonia Pearson-White[1]. Institute of Animal Science[1], The Volcani Center, Bet Dagan 50250, ISRAEL, and Department of Biology[2], University of Virginia, Charlottesville, VA 22901.**

A variety of Differentiated cell types can be converted to skeletal muscle cells following transfection with the myogenic regulatory gene *MyoD1*. To determine whether multipotent embryonic stem (ES) cells respond similarly, cultures of two ES cell lines were electroprated with a *MyoD1* cDNA driven by the ß-actin promoter. Although all transfected clones expressed high levels of *MyoD1* mRNA, none were converted to skeletal muscle cells. However, this ectopic expression was associated with a low level of activation of the endogenous myosin light chain 2, but not that of the skeletal muscle actin or myosin heavy chain genes.

No skeletal muscle fibers could be detected when the transfected ES cells were induced to differentiate under normal conditions. In contrast, preferential myogenesis was observed when the these cells were allowed to differentiate in the presence of muscle-specific differentiation media. Surprisingly, the continued expression of MyoD1 was not required for skeletal myogenesis. About 50% of transfected ES clones no longer expressed the exogenous or the endogenous *MyoD1* genes. These results indicate that multipotent ES cells do not respond to the *MyoD1* unless they are induced to differentiate under specific culture conditions, that *MyoD1* expression is required for the establishment of the myogenic program but not for its maintenance, and that MyoD1 is a weak trans-activator.

Microinjection of the ß-actin-*MyoD1* fusion gene into mouse fertilized eggs resulted in embryonic lethalities. The time and cause of embryonic death, analyzed by in situ hybridization will be presented and discussed.

F 136 CONSTITUTIVE EXPRESSION OF A CELL DIVISION CONTROL-PROTEIN KINASE IN SKELETAL AND CARDIAC MUSCLES, Regina P. Shiau, Paul E. Wolkowicz, Vincent J. Kidd and Patrick K. Umeda, Departments of Medicine, Biochemistry, and Cell Biology, University of Alabama at Birmingham, Birmingham, AL 35294.

Cell proliferation depends not only on the activation of DNA synthesis but also on transitions through phases of the cell cycle that are mediated by cell division control(cdc)-protein kinases. Among the latter is a recently identified kinase, $p58^{GTA}$, which is expressed coordinately with the cdc-protein kinase, $p34^{cdc2}$. Overexpression of $p58^{GTA}$ slows down passage through the cell cycle, whereas suppression of this kinase accelerates cell division. Therefore, $p58^{GTA}$ may act as a negative regulator of the cell cycle. To evaluate the involvement of $p58^{GTA}$ in the irreversible withdrawal of skeletal myoblasts from the cell cycle during terminal differentiation, and the cessation of cardiac myocyte division during development, we have isolated and characterized cDNA clones for the rat and avian homologues of $p58^{GTA}$. The rat cDNA shows at least 90% identity in the nucleotide and amino acid sequence to the previously characterized human cDNA, thereby establishing the identity of the clones. In both species, Northern blot analyses indicate that the expression of $p58^{GTA}$ mRNA in muscle is elevated when compared to that in liver. Indirect immunofluorescence of primary skeletal and neonatal cardiac muscle cultures using $p58^{GTA}$ antibodies demonstrates high level expression of the protein only in myogenic cells, comparable to that in non-muscle cells during late metaphase. The increased and constitutive expression in muscle of a protein kinase that is normally expressed in late metaphase may suggest a role for $p58^{GTA}$ in muscle development. We are defining the function of this gene by suppression in myogenic lines stably transformed with an anti-sense $p58^{GTA}$ gene.

F 137 **Myo D activates Xenopus skeletal actin gene transcription by interaction with two binding sites.**

Georges Spohr and François Levrat. Département de Biologie Cellulaire, Université de Genève, 30, quai E. Ansermet 1211 Genève 4, Switzerland.

Striated muscle actins in *Xenopus laevis* are expressed in the myotome after the beginning of accumulation of Myo D transcripts. Myo D activates the transcription of some muscle specific genes. We show, by co-injection in Xenopus oocytes, that Myo D is also able to activate transcription from skeletal actin α III promoter constructs. 153 bp of 5' sequence (-125 to +28) encompassing the first two CArG boxes are sufficient for the transactivation. By in vitro DNA-binding experiments with purified Myo D protein, we find two Myo D binding sites, one each flanking the transcription start site. These recognition sequences share only partial homology to the consensus.

F 138 CADHERIN-RELATED MOLECULES IN DIFFERENTIATING SKELETAL MUSCLE CELLS. Anna Starzinski-Powitz and Michael Donalies, Institut für Genetik, Universität zu Köln, Zülpicherstr. 47, D-5000 Köln 1, FRG.

Cadherins are molecules involved in the control of morphogenetic processes (e.g. neurite outgrowth, compaction of early embryos, establishment of cell polarity) which may also control muscle histogenesis. They are a multigene family of integral membrane molecules which function in Ca^{2+}-dependent cell adhesion. To date three subclasses of cadherins have been identified: N- (neuronal), E- (epithelial), and P- (placental) cadherin of which N-cadherin is transiently expressed in developing skeletal muscle.

We have investigated whether in addition to N-cadherin other novel cadherins are expressed in developing muscle cells. In order to do this, polymerase chain reactions (PCR) were performed with RNA from mouse C2 myoblasts and myotubes using a pair of oligonucleotide primers derived from sequence stretches which are conserved between N, E and P-cadherin. Nucleotide sequence analysis of the PCR products revealed that the nucleotide sequence-derived amino acid sequence of one of these cDNA clones showed 60% identity and 75% similarity (including isofunctional amino acids) to N-cadherin. This indicated that we had isolated a novel member of the cadherin family which we designated M-cadherin. Characterization of the almost full-length M-cadherin cDNA clone confirmed this assumption insofar as the whole cDNA-derived protein sequence shows significant homologies to the cadherins. M-cadherin mRNA (about 3.6 kb) is induced in differentiating C2 cells and is encoded by a single-copy gene.

F 139 A NOVEL MUSCLE-SPECIFIC ENHANCER IN THE β CARDIAC MYOSIN HEAVY-CHAIN GENE IS REGULATED BY AN AP5-BINDING PROTEIN BUT NOT BY MYOD1 OR MYOGENIN,
W. Reid Thompson, Bernardo Nadal-Ginard, and Vijak Mahdavi, Department of Cardiology, Children's Hospital, Harvard Medical School, Boston, Massachusetts 02115
The β cardiac myosin heavy-chain (β-MHC) gene codes for the major contractile protein of ventricular myocardium and slow-twitch skeletal muscle. Its expression is influenced by tissue- and species-specific developmental and hormonal factors. Mutations in this gene locus are responsible for certain forms of familial hypertrophic cardiomyopathy. However, the regulatory mechanisms that control expression of this gene have not been elucidated. Using transient and stable transfection assays, we have identified a sequence at -354 to -180 in the rat and -357 to -180 in the human gene that functions as a muscle-specific enhancer, conferring to heterologous promoters the characteristic features of β-MHC regulated expression. DNase I protection and gel mobility shift assays have revealed several protein binding sites that appear to be critical for the activity of the enhancer. Deletion and point mutation analyses have identified a sequence homologous to a regulatory element in the SV40 enhancer, AP5/GT-II, that is necessary for high-level expression and sufficient to confer skeletal and cardiac muscle specificity. We also show that, in contrast to the enhancers in other muscle-specific genes, the β-MHC enhancer is unresponsive to the muscle lineage determining factors MyoD1 and myogenin, indicating that activation of this enhancer may involve a novel myogenic pathway.

F 140 STUDY OF THE REGULATION OF HUMAN α-CARDIAC ACTIN GENE EXPRESSION IN YEAST. Fu-Yun Xu, and Larry Kedes. Institute for Genetic Medicine and Department of Biochemistry, University of Southern California School of Medicine, Los Angeles, CA. 90033, U.S.A.
We have tested the ability of the human cardiac α-actin (HCA) gene promoter to function in yeast. When HCA promoter-CAT gene chimeric constructs were transformed into yeast in the presence of the myogenic determinant factor MyoD1 produced from a yeast expression vector, HCA-CAT was functionally activated more than 50 fold. Three protein factors, MyoD1, Sp1, and SRF or a SRF-like factor are required for muscle-specific expression of HCA gene in skeletal muscle cells. Since neither SRF nor Sp1 have been detected in yeast, we are studying whether the same cis-sequence requirements for activation of HCA gene in skeletal muscle cells are also involved in yeast.

F 141 INHIBITION OF MUSCLE DIFFERENTIATION BY SRC IS ASSOCIATED WITH THE DOWN REGULATION OF THE MYOD1 EXPRESSION, Heeyong Yoon and David Boettiger, Department of Microbiology, University of Pennsylvania, Philadelphia, PA 19104
MyoD1, one of muscle regulatory factors, is known to be down-regulated in myogenic cells whose differentiation is blocked by ras-, fos-oncogene, fibroblast growth factor, transforming growth factor-β or BUdR. Here we show that MyoD1 expression is inhibited in src-transformed cells. Primary chicken muscle cells are infected with temperature sensitive mutant of Rous sarcoma virus, tsLA24A, and passaged at 36°C until they are fully transformed. These cells do not fuse to make myotubes and do not produce muscle contractile proteins (tropomyosin, titin, nebulin and troponin T) and RNAs (cardiac α actin, myosin heavy chain, myosin light chain and troponin T) but produce cytoskeletal RNAs (γ-actin, α-tubulin). When cells are grown at 41°C, cells begin to fuse and express muscle contractile genes both transcriptionally and translationally. MyoD1 mRNA is expressed at very low level at 36°C, however induced up to 13 fold when cells are grown at 41°C. This induction is time-dependent, which highest level occurs at 1 day after temperature shift to 41°C and then decreases. This highest level occurs before cells are fused to myotubes and make muscle contractile RNAs. This suggests that MyoD1 regulates the expression of other muscle genes and decrease in MyoD1 expression is responsible for the inhibition of muscle differentiation in src transformed cells.

Regulation by Growth Factors; Hormones and Oncogenes; Genes that Regulate Myogenesis; Cell and Molecular Biology of Neuromuscular Disease

F 200 A DEVELOPMENTAL AND COMPARATIVE ANALYSIS OF GLYCOSAMINOGLYCAN SYNTHESIS IN NORMAL AND DYSTROPHIC PRIMARY MUSCLE CELL CULTURES, Karen Allen, Nancy Crisona and Richard Strohman, Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720

Earlier reports from this laboratory showed that basic fibroblast growth factor (bFGF) is stored in the basal lamina of skeletal muscle fibers in vivo, and suggested that this storage was augmented in fibers of mdx mouse muscle. We also show elsewhere that increased bFGF content of mdx muscles is not associated with either increased bFGF receptor number or increased receptor affinity in mdx compared with control muscle cells in vitro. There is however an increase in mdx muscle cultures of FGF extracellular matrix (ECM) binding sites when matrices of mdx satellite cells are compared with similar matrices from control cells. In this abstract we report on relative rates of synthesis of specific extracellular matrix glycosaminoglycan (GAG) molecules and their appearance (a) as soluble components of the medium, (b) as cell associated components and (c) as components of a deposited ECM. Included in our assays are measurements of total sulfated GAGs, heparan sulfate and chondroitin sulfate, and total FGF-binding of these GAG fractions from mdx and normal muscle satellite cell and fibroblast cultures.

F 201 BASIC FIBROBLAST GROWTH FACTOR DISTINGUISHES DEGENERATING AND REGENERATING MYOFIBERS IN MDX DYSTROPHIC MUSCLE, Judy E. Anderson[1] and Elisavetta Kardami[2], Department of Anatomy[1] and St. Boniface General Hospital Research Centre[2], University of Manitoba School of Medicine, Winnipeg, MB R3E 0W3.

Basic FGF is known to be involved in stimulating muscle mitogenesis, and in promoting expression of embryonic contractile protein isoforms. Mdx mouse skeletal muscle is known to undergo dystrophic insult due to dystrophin deficiency. Subsequent myosatellite cell proliferation maintains myofiber number, and muscles compensate for ongoing weakness with hypertrophy. Fluorescence labelling of bFGF distinguished between intact, damaged, and regenerating areas of mdx muscle during early, maximal and later dystrophy. Myofiber necrosis in mdx cardiac and skeletal muscle was indicated by homogeneous intracellular staining for bFGF, rather than pericellular and myonuclear staining of intact fibers. Regenerating skeletal myofibers had intense centronuclear staining. Results suggest a role for bFGF in muscle regeneration.

F 202 REGULATION OF ALPHA ACTIN GENE EXPRESSION IN THE QM MYOGENIC CELL LINE. Parker B. Antin and Charles P. Ordahl. Department of Anatomy, UCSF San Francisco, CA 94143

We have recently isolated an avian muscle cell line (QM), which has many of the essential features of mammalian muscle cell lines. Northern and immunoblot analysis has shown that following differentiation QM cells express many muscle-specific genes, including MHC, MLC2, sTNT, cTNT, cTNC, sTNI, α-TM, MCK and desmin; however, QM myotubes fail to express any isoform of α-actin. Southern analysis using a chicken skeletal α-actin-specific cDNA probe showed that the skeletal α-actin genes were present in the QM genome. Northern analysis showed that QM muscle cells express high levels of chicken $MyoD_1$. Expression of mouse $MyoD_1$ in QM cells did not induce expression of α-actin in QM myotubes. To determine whether QM muscle cells contain transfactors necessary for expression of the skeletal α-actin promoter, a 2000 nucleotide 5' fragment of the chicken skeletal α-actin promoter linked to CAT (sACT-2000-CAT) was tested by transient and stable transfection for activity in QM cells. Transient transfection analysis showed that sACT-2000-CAT was as active in differentiated cultures of QM7 as in differentiated cultures of chick primary breast muscle. Analysis of pooled clones containing stably introduced sACT-2000-CAT revealed that CAT activity increased three fold following shift of cells to differentiation medium, while CAT activity from clones containing 550nt of the cardiac troponin T promoter linked to CAT showed more than thirty fold induction during the same time period. We conclude that QM cells contain the appropriate trans factors for transcription of the skeletal α-actin promoter, suggesting that the absence of appropriate trans factors alone is unlikely to explain the absence of sarcomeric actin mRNA and protein in QM muscle cells. However, the poor induction of stably transfected sACT-2000-CAT indicates possible defects in the developmental upregulation of this promoter.

F 203 Y-CHROMOSOME SPECIFIC PROBES AS CELL MARKERS FOR MYOBLAST TRANSPLANTATION STUDIES. Manfred W. Beilharz, May C. Lai, Ying Fan, Kerryn L. Garrett, and Miranda D. Grounds*, Departments of Microbiology and Pathology*, The University of Western Australia, Nedlands, Australia, 6009.

The lack of suitable specific cell markers is a limiting factor in many experiments in transplantation and cell biology. The ideal cell marker is specific, permanent, non-transferable and can be identified *in situ*. In addition, it is desirable that transplanted cells are immunologically compatible. Experiments on Myoblast Transfer Therapy in animals require a nuclear marker to follow the fate of (donor) skeletal muscle precursor cells (myoblasts) transferred into host muscles. The use of specific DNA sequences on the Y-chromosome fulfil all of the above criteria, and tissues can be transplanted between male (donor) and female (host) animals of a single strain rather than across strains. Probes specific for DNA of the Y-chromosome are precise nuclear markers and have the advantage that the Y-chromosome can be identified even without expression of the appropriate genes: this is important for experiments on muscle transplantation as donor muscle nuclei can be readily identified even after fusion with host cells to form multinucleated muscle cells. We have tested four Y-chromosome probes for specificity to male and female DNA extracted from mice, rats, dogs and humans. We demonstrate that the Y-1 probe (1) is highy specific for *in situ* hybridisation with tissue sections in muscle transplantation studies in mice.

1. Nishioka Y. & Lamothe E. (1986). Genetics 113: 417-432.

F 204 INDUCTION OF INSULIN-LIKE GROWTH FACTOR-I PEPTIDE IN SOME MUSCLE FIBERS AFTER ECCENTRIC EXERCISE, Frank W. Booth and Ray B. Biggs, Department of Physiology and Cell Biology, University of Texas Medical School, Houston, TX 77225

Eccentric exercise is defined as the stretching of skeletal muscle as it contracts. In humans, eccentric exercise in untrained skeletal muscle leads to muscle soreness and leakage of muscle enzymes into the blood one to three days post exercise. In rats, myofibrillar protein synthesis rates are increased 41-52% in the tibialis anterior muscle 12-41 hours after a single bout of 192 eccentric contractions. To test whether insulin-growth factor-I (IGF-I) is involved in the protein synthetic response, immunocytochemical analysis was performed at the following times after 160 eccentric contractions to the plantaris muscle of the rat. Two days post exercise, a small percentage of fibers showed IGF-I expression. Most expressing fibers showed diffuse expression, but an occasional fiber only showed expression at the sarcolemma. Three days post exercise, a smaller percentage of fibers than at two days showed IGF-I expression and the muscle exhibited marked edema and extracellular infiltration. Four days post exercise, some muscle fibers demonstrated multiple central nuclei. Some of these fibers were smaller than the basal lamina. IGF-I was present in a few of these regenerating fibers as well as in and around an occasional blood vessel. Edema was gone. Five days post exercise, bunches of adjacent muscle fibers had IGF-I expression. These observations indicate that regeneration of previously untrained muscles fibers after eccentric exercise is a complex set of events. Supported by NIH AR 19393.

F 205 TPA INDUCES MYOGENIC DIFFERENTIATION IN HUMAN RHABDOMYOSARCOMA CELLS WITHOUT INTERFERING WITH THE EXPRESSION OF THE MYF FACTORS.
M. Bouché, M.I. Senni, A.M. Grossi, G. Cossu and M. Molinaro. Institute of Histology and General Embryology, University of Rome "La Sapienza". Italy.

We have recently shown that the tumor promoter TPA inhibits DNA synthesis and induces myogenic differentiation when administered to the human rhabdomyosarcoma cell line RD (Cancer Res. 50,3377,1990). By norhern blot hybridization we now demonstrate that the myf3 and the myf4 genes are expressed at comparable levels in both control and in differentiated TPA-induced RD cells. The myf5 gene is not detectable either in the control or in TPA-treated RD cells. Immunofluorescence analysis was carried out with specific anti-MyoD1 and anti-Myogenin antibodies. The results obtained show that 100% of the nuclei of control RD cells are stained with the anti-MyoD1 antibody and some of them coexpress Myogenin as revealed by double immunofluorescence experiments. After 8-10 days of TPA treatment, the percentage of myosin positive, differentiated RD cells increased up to 70-80% of the total population, without changes in the expression of MyoD1 or Myogenin proteins. These data show that TPA-induced differentiation of RD cells does not result in changed expression of the myogenic determination genes myf3 and myf4. It is likely that different mechanisms, activated by TPA treatment, are required to trigger myogenic differentiation in RD cells.

F 206 DOMAINS OF Ad5 E1A PROTEIN THAT ARE INVOLVED IN THE SUPPRESSION OF MYOGENIC DIFFERENTIATION. Maurizia Caruso, Antonio Giordano*, Fabio Martelli and Armando Felsani. Istituto Tecnologie Biomediche and Istituto Biologia Cellulare, CNR, Roma, Italy; *Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Myogenic differentiation is obligatorily coupled to withdrawal of myoblasts from the cell cycle and is inhibited by the expression of the adenovirus 5 E1A gene product (Webster et al., Nature 1988, 322:553-557). A number of cellular factors specifically associate with this transforming protein, the most prominent among which are proteins with molecular weight of 300 k, 107 k, 105 k and 60 k. The 105 k and 60 k proteins have been identified as pRB, the product of the retinoblastoma susceptibility gene, and cyclin-A respectively. Since these proteins are both involved in cellular growth regulation, they might be important in mediating the E1A repression of myogenic differentiation. To test this hypothesis, we stably transfected into the C2 murine myoblasts expression constructs coding for E1A mutants impaired in the ability to bind one or more of the E1A associated proteins; such mutations interfere with the transforming function of E1A. For each transfected mutant, clonal derivatives are being analyzed for E1A expression, proliferative rate, accumulation of muscle specific mRNAs and proteins, expression of regulatory genes such as MyoD1 and myogenin.

F 207 PHORBOL ESTERS SELECTIVELY AND REVERSIBLY INHIBIT A SUBSET OF MYOFIBRILLAR GENES RESPONSIBLE FOR THE ON-GOING DIFFERENTIATION PROGRAM OF CHICK SKELETAL MYOTUBES. J. Choi, S. Holtzer, S.A. Chacko, H. Holtzer, Department of Biochemistry & Anatomy, Univ. of Pennsylvania, Philadelphia, PA 19104.

During skeletal myogenesis, over a dozen myofibrillar genes are activated. The expression of these genes produces the proteins that form the contractile apparatus. Previously, we have demonstrated that phorbol esters selectively and reversibly disassemble the contractile apparatus as well as inhibit the synthesis of many muscle contractile proteins without inhibiting that of housekeeping proteins. We now demonstrate that phorbol esters reversibly decrease the transcription rates and the message levels of at least six myofibrillar genes: myosin heavy chain, myosin light chain 1/3, myosin light chain 2, cardiac and skeletal α-actin, and skeletal troponin T. The steady state message levels of these six genes decrease 50 to 100 fold upon 48 hours of exposure to phorbol esters. These decreases can be attributed at least in part to decreases in transcription rates and to decreases in message stabilities. In contrast, phorbol esters do not decrease the expression of the housekeeping genes, α-tubulin, β-actin, and PA3. Phorbol esters do not decrease the steady state message levels of MyoD1, a gene that has been shown to be important in the activation of many of the skeletal muscle specific genes. We conclude: 1) part of the continuous expression of many myofibrillar genes is linked at the steady state message and transcriptional level; 2) this linkage is independent of those maintaining some of the housekeeping genes; and 3) the mechanism of this linkage does not involve MyoD1 message levels.

F 208 AFFINITY PURIFICATION OF MYOGENIC CELLS FOR MYOBLAST TRANSFER, Mark G. Coleman*, Susan Prattis+, Joe N. Kornegay+, Gareth E. Jones# Terence A. Partridge†, Diana J. Watt*, Departments of *Anatomy and †Histopathology, Charing Cross & Westminster Medical School, London, U. K., +College of Veterinary Medicine, North Carolina State University, North Carolina, U.S.A., #Department of Anatomy, King's College, London, U. K.

For the preparation of myogenic cells from bulk sources for their subsequent use in myoblast transfer therapy, affinity purification methods seem the most promising means of achieving pure populations of myogenic cells. We have already developed a method[1] to segregate contaminating cells from precursor cells using an antibody panning protocol. We are now attempting to further develop and refine this method by the use of magnetic beads as the vehicle for affinity separation.

In this procedure, cells cultured with primary antibody are mixed with magnetic beads coated with secondary antibody. Muscle N-CAM positive cells are then separated by application of a magnetic field. We are now studying the characteristics of the cells separated in this way.

1. Jones, G. E., Murphy, S. J., Watt, D.J. (1990). J. Cell Science *97(4)* in press.

Supported by Action Research for the Crippled Child and the Muscular Dystrophy Group of Great Britain.

F 209 MYOBLAST IMPLANTATION INTO DYSTROPHIC SKELETAL MUSCLE: ANALYSIS BY NON-RADIOISOTOPIC IN SITU HYBRIDISATION TO A Y CHROMOSOME-SPECIFIC DNA PROBE. Gary Coulton, Jenny Morgan, Charles Pagel and Terry Partridge. Departments of Biochemistry and Histopathology, Charing Cross and Westminster Medical School, London, UK.

Implantation of normal myoblasts may prove useful in the treatment of Duchenne Muscular Dystrophy (DMD). Myoblasts implanted into mdx mouse (genetic homologue of human DMD) muscles survive and express normal gene products, including dystrophin. The positional fate of donor myoblast nuclei and their spatial relationship with expressed dystrophin have been analysed by a combination of immunohistochemistry and in situ hybridisation. Male, donor-derived, nuclei were injected at various times into female mdx host tibialis anterior muscles and subsequently detected by in situ hybridization using a DNA probe (pY353/B) specific for repeat sequences (Tdy locus) on the mouse Y chromosome. The probe was labelled by random primer incorporation of digoxigenin-deoxyUTP.

Male, control, muscle sections (8μm cryostat), exhibited hybridisation in 65 to 70% of nuclei whereas all nuclei within female muscles were negative. Hybridisation conditions could be controlled such that a single spot, presumably indicative of the single Y chromosome, was visible within each male nucleus. Varying numbers of nuclei were detected in sections from muscles following myoblast implantation. The frequency and distribution of donor nuclei within muscle fibres was consistent with that of dystrophin-positive fibres in the regenerated muscles.

This approach provides us with a powerful tool for continued analysis of muscle implantation protocols with respect to the number of myoblasts needed for muscle rescue, the migratory capacity of donor myoblasts and the temporal and spatial relationship with the expression of other genes involved in the regenerative process.

F 210 STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF A PROMOTER REGION OF THE AVIAN MYOGENIC REGULATORY GENE CMDI.

Claude A. Dechesne*, Juanita Eldridge and Bruce M. Paterson, Laboratory of Biochemistry, NCI, NIH, Bethesda, MD 20892, * INSERM U.300, 34060 Montpellier, France.

CMDI belongs to the family of genes such as MyoD able to induce myogenesis. In order to analyse the regulation of CMDI gene, a 1.1 kb genomic fragment upstream the ATG initiation codon has been isolated. This fragment has structural and functional characteristics of a promoter. Expression of chloramphenicol-acetyl-transferase gene, under the control of this promoter fragment, shows that this sequence contains both species and muscle specific regulatory elements. The promoter activity is inhibited in presence of BrdU. Deletion analysis suggest the presence of successive negative and positive *cis*-acting regulatory elements.

F 211 CONSTRUCTION AND *IN VITRO* EXPRESSION OF A FULL LENGTH HUMAN DYSTROPHIN cDNA.

George Dickson, Kay E. Davies*, D. Love* and Frank S. Walsh. Department of Experimental Pathology, U.M.D.S., Guy's Hospital Campus, London Bridge, London SE1 9RT, U.K. and *Institute of Molecular Medicine, John Radcliffe Hospital, Headington, Oxford OX3 9DU, U.K.

Based on predicted domain structure and immunocytochemical localisation, dystrophin has been postulated to be an actin-binding component of the sub-sarcolemmal cytoskeleton involved in membrane stabilisation and binding to membrane glycoproteins. As an initial step towards the study of structure-function relationships within the predicted sub domains of dystrophin, we have prepared eukaryotic expression vector constructs of a full-length human dystrophin cDNA and a truncated cDNA encoding the N-terminal putative actin-binding domain of molecule. The constructs have been used to transfect mouse fibroblast cells in culture with expression of appropriate mRNAs. In both cases, however, expression at the protein level using immunostaining techniques was restricted to an intracellular vesicular pattern in a perinuclear location, associated with a faint filamentous cytoplasmic network. These studies indicate that specific accessory proteins absent in fibroblasts may be involved in sub-plasma membrane integration of the dystrophin molecule in skeletal muscle.

F 212 REGENERATION IN OVERSTRETCHED CHICKEN MUSCLE IS ACCOMPANIED BY EXPRESSION OF cMYC AND MUSCLE REGULATORY FACTORS. Brenda R. Eisenberg, Elizabeth Guise, Nick Riccardo and James Piskura. Department of Physiology, MC 901, University of Illinois, Box 6998, Chicago, IL 60680.
We have studied chicken anterior latissimus dorsi (ALD) muscle during regeneration after damage caused by over-stretching and looked at re-expression of myogenic factors and proto-oncogenes. In our earlier morphometric studies, we found the volume of the muscle almost doubles after 14 days. This is largely due to an increase in the number of unclassified cells some of which appear to be migrating satellite cells which proliferate at least 25-fold in the chicken (Kennedy et al., 1988 and 1989). ALD muscles were loaded for time intervals from 30 minutes to 72 hours. Total RNA was extracted from rapidly frozen tissue. Northern and slot hybridizations were made on filters with ^{32}P nick-translated probes for chick c-myc and mouse myogenin (Edmondson and Olson, 1989; mouse probe cross-hybridize to avian mRNA). Slot results were standardized by hybridization of 18S ribosomal RNA probe and results quantified by beta scanning. Several fold increases in the expression of both c-myc and myogenin were found as ratios of stretched to control ALD muscle. The time course for c-myc expression rises rapidly and is maximum by 30 minutes and has fallen considerably by 3 hours. Myogenin increases are detected in some muscles by 3 hours but is consistently high in all muscles by 6 hours. Supported by HL 40880.

F 213 Protooncogene, c-fos, Mediated Regulation of Chicken Cardiac Myosin Light Chain 2 (MLC2) Gene Expression. Shyamal K. Goswami, Ana Maria Zarraga and M.A.Q.Siddiqui. Department of Anatomy and Cell Biology, SUNY-Health Science Center at Brooklyn, 450 Clarkson Avenue, Brooklyn, New York, NY 11203
The chicken cardiac myosin light chain 2 (MLC2) promoter containing 1.3Kb of 5'-flanking DNA fused to the chloramphenicol acetyl transferase (CAT) coding sequence is expressed in a tissue specific manner following transfection of the primary chicken cardiac muscle cell. Expression of MLC2/CAT recombinant in skeletal muscle cell requires the removal of a 89 bp sequence element (CSS) located at -281 relative to the transcription initiation site. When these two constructs are cotransfected with the expression vector containing the fos gene, the MLC2 promoter activity is strongly reduced in a dose-dependent manner in both muscle cells. Mutants,in which the 5'-distal sequences of the promoter are deleted, do not respond to the fos-mediated inhibition of transcription. The putative fos responsive element (FRE) lies between -1130 to -1203 in the distal MLC2 promoter. The FRE element is not required for the cardiac muscle tissue specific expression, as the promoter without the FRE sequence still retains the cardiac tissue specificity. Therefore, fos protein is important in regulation of MLC2 gene expression, but is not involved in mechanisms underlying tissue specific transcription.

F 214 EXPRESSION OF MYOD1 AND MYOGENIN IN THYMIC CELLS *IN VIVO*.
Miranda D. Grounds*, Kerryn L. Garrett and Manfred W. Beilharz, Departments of Pathology* and Microbiology, University of Western Australia, Nedlands, Australia, 6009.
When thymic cells from mice are grown in tissue culture, myotubes are formed which have the characteristics of conventional skeletal muscle cells. Cells with striated filaments can also be demonstrated in sections of thymus, particularly from lower vertebrates, and these skeletal muscle-like cells have been termed myoid cells. The role of these cells is unknown. The expression of the skeletal muscle specific regulatory genes MyodD1 and myogenin was examined by *in situ* hybridisation in sections of thymus from adult mice. Some mRNA positive mononuclear cells were located throughout the thymus, but were concentrated in the medulla which is where myoid cells are mainly observed. MyoD1 and myogenin gene expression was also examined in either irradiated or transplanted thymuses. The co-expression of mRNA for MyoD1 and myogenin in normal thymus shows that myoid cells are activated with respect to the myogenic pathway. The presence of these mRNA positive cells in the thymus raises interesting questions about the role of myoid cells and the role of these skeletal muscle specific regulatory genes *in vivo*.

F 215 RECOMBINANT PDGF-BB STIMULATES GROWTH AND INHIBITS DIFFERENTIATION OF RAT MYOBLASTS, Pei Jin, Thomas Sejersen and Nils R. Ringertz
Department of Medical Cell Genetics, CMB, Karolinska Institutet, S-104 01Stockholm, Sweden

We previously found that L6 myoblasts and skeletal muscle isolated from developing rats express the PDGF β-receptor gene (Jin et al., *J. Cell Biol.* (1990) 110:1665-1672). We now report that recombinant human PDGF-BB is a mitogen for L6 myoblasts and also a potent inhibitor of myogenic differentiation. Treatment of L6J1 myoblasts with PDGF-BB increased the rate of DNA synthesis and stimulated cell proliferation. In differentiation medium (DME/0.5% FCS or DME/insulin), PDGF-BB prevented fusion of confluent myoblasts and suppressed biochemical differentiation in L6J1 cells. Inhibition of myoblast differentiation was, however, reversible. Withdrawal of PDGF-BB from the medium allowed myoblast fusion to occur. Northern blot hybridization showed that the PDGF β-receptor mRNA was downregulated to an undetectable level when confluent cultures of L6J1 myoblasts in growth medium (DME/5% FCS) were shifted to differentiation medium. Receptor binding assays further indicated that binding of PDGF-BB to its receptors on L6J1 myoblasts declined rapidly before creatine kinase activity rose. Similar effects of PDGF-BB on cell growth and differentiation were also observed in rat L8 myoblasts. Our results provide the first demonstration that PDGF-BB is a potent regulator of myogenesis of rat myoblasts and suggest that it may regulate muscle differentiation in vivo.

F 216 MACROPHAGE-COLONY-STIMULATING FACTOR (CSF-1) STIMULATES PROLIFERATION OF MYOGENIC CELLS, Gareth E. Jones*, Susan J. Murphy*+, Clare Wise+, Diana J. Watt+, Departments of Anatomy, *King's College London, & +Charing Cross & Westminster Medical School, London U.K. Myoblast transfer requires the implantation of pure myogenic cells into the recipient myopathic muscle. We have developed an antibody panning protocol[1] to produce such pure populations and are presently investigating ways of amplifying such cell populations in order to increase the number of cells available for implantation. Cultures of murine mononuclear muscle precursor cells (mpc), segregated from contaminant fibroblast using the antibody panning protocol[1], and the murine C2 myoblastic cell line, were both assessed for proliferative capacity under a regime of growth factor supplementation. Cell proliferation was measured, using standard techniques, from parallel cultures established in 96-well plates. The addition of 1.32 nM human recombinant CSF-1 (Cetus Corp) to both C2 and mpc cultures led to significant stimulation of the proliferative capacity compared with control cultures. Polyclonal antibodies directed against murine CSF-1 and the plasma membrane receptor for CSF-1, CSF-R (both kind gifts from Dr. J. Pollard, Albert Einstein College of Medicine) detected significant expression of surface-located CSF-R in both C2 and mpc cultures using standard immunofluorescence methods[2]. Here we present data illustrating these findings.

1. Jones, G.E., Murphy, S.J., Watt, D.J. (1990). J. Cell Science 97(4) in press.
2. Boocock, C.A., Jones, G.E., Stanley, E.R., Pollard, J.W. (1989) J. Cell Science 93 447-456.

Supported by the Muscular Dystrophy Group of Great Britain.

F 217 IDENTIFICATION OF CIS-ACTING REGULATORY ELEMENTS INVOLVED IN MUSCLE-SPECIFIC EXPRESSION OF THE DUCHENNE MUSCULAR DYSTROPHY GENE. Henry J. Klamut, Lucy O. Bosnoyan, Ronald G. Worton and Peter N. Ray, Department of Genetics and Research Institute, The Hospital for Sick Children, Toronto, Ontario, M5G 1X8, Canada. The Duchenne muscular dystrophy (DMD, dystrophin) gene is expressed primarily in skeletal and cardiac muscle, and to a lesser extent in smooth muscle and brain. Expression of this gene in neuronal tissue is regulated independently by a second promoter. In our initial studies of the muscle-specific promoter region, we have demonstrated that 150 bp of upstream sequence is capable of regulating expression of chimeric reporter gene constructs in a muscle-specific manner when transfected into both clonal human myoblast cultures and the H9C2(2-1) rat myogenic cell line. Furthermore, we have found within this 150 bp fragment a number of regions of strong homology to previously defined muscle-specific regulatory elements such as the CarG box and the MEF1 (MyoD) binding site. In order to determine whether these elements actually play a role in the regulation of this gene, PCR-generated oligomer cassettes corresponding to each of these regions have been prepared and assembled in various combinations in the pBLCAT3 vector. By comparison of CAT activity in differentiating H9C2 myoblasts transfected with each of these constructs to that of the wild-type promoter construct, we have found that deletion of a 39 bp region (-12 to -51 bp) containing the ATA box essentially abolishes CAT gene expression in this cell line. Exclusion of 11 bp (-51 to -62 bp) containing a GC box and a portion of a MEF1 homology region results in a 50% reduction in CAT activity, while removal of 69 bp (-81 to -150 bp) containing the CarG box dramatically increases CAT activity relative to the wild-type promoter construct. These results suggest that muscle-specific regulation of DMD gene expression involves an interplay between one or more negative-regulatory elements located between -82 and -150 bp, and one or more positive regulatory elements located within -81 bp of the transcriptional start site. Current studies are aimed at precisely defining trans-acting factor binding sites within these regions.

F 218 FETAL RAT MYOBLASTS DEMONSTRATE A DISTINCTIVE PATTERN OF MYOSIN HEAVY CHAIN EXPRESSION FOLLOWING DIFFERENTIATION IN VIVO AND IN VITRO, Peter A. Merrifield and Maggie M. Sopper, Department of Anatomy, The University of Western Ontario, London, Ontario N6A 5C1.
The development of crural muscles in the hindlimb of embryonic rats is characterized by the sequential appearance of primary (1°) and secondary (2°) myotubes, which first differentiate around embryonic day 14 (ED14) and ED18, respectively (Condon et al, Dev. Biol. 138:256. 1990). Immunolocalization of myosin heavy chain (MHC) isoforms using monoclonal antibodies (Mabs) specific for embryonic (Mab 47A), slow (Mab 10D10) and fast Type IIB (Mab 212F) MHCs demonstrates that 1° myotubes co-express embryonic and slow MHCs while 2° myotubes express exclusively embryonic MHC. This suggests that 2° myotubes may develop from a distinct population of fetal myoblasts. To test this hypothesis, primary cultures were prepared from the hindlimb muscles of ED16-ED20 Sprague-Dawely rats, grown at clonal and mass density and analysed for MHC expression following differentiation. Western blot and ABC-AP immunohistochemistry detect the expression of only embryonic MHC in these cultures. Slow and fast Type IIB MHCs cannot be detected under conditions used to detect these isoforms in total embryonic and adult extraocular muscles. These data suggest that, as in the mouse (Vivarelli et al, J.Cell Biol. 107:2191. 1988), fetal rat myoblasts represent a distinct myoblast population with a limited potential for MHC expression in vivo and in vitro. To examine the fate of fetal myoblasts and established cell lines with similar phenotypes (ie. L6 cells) in vivo, myoblasts were infected with a retroviral vector containing a β-galactosidase gene (ΨBAGα, Price et al, PNAS 84:156. 1987), cloned and injected into muscles of neonatal rats. The differentiated progeny of these genetically marked myoblasts are currently being examined for muscle fiber type using X-GAL and ABC-AP immunohistochemistry. (Supported by MDAC)

F 219 THE IMMEDIATE AND LONG-TERM FATES OF MYOGENIC CELLS IMPLANTED INTO MDX MOUSE MUSLES. Jennifer E. Morgan, Charles N. Pagel and Terence A. Partridge. Department of Histopathology, Charing Cross & Westminster Medical School, London W6 8RF, U.K.

In earlier investigations we have shown that implantation of normal muscle precursor cells (mpc), obtained from newborn mouse muscle, into the growing or regenerating muscles of the mdx mouse, partially alleviate the dystrophin-deficiency (Partridge *et al.* ,1989, Nature 337, 176-179). More recently, we have attempted to simplify the mdx mouse as an experimental model by blocking proliferation of the endogenous myogenic cells with high doses of X rays, thus preventing regeneration and producing a progressive atrophy (Wakeford *et al.*, 1990, Muscle & Nerve in press). In such muscles, implanted normal mpc rapidly permeate repair degenerating host muscle fibres, forming mosaic mdx/normal muscle fibres, and also form new fibres of purely donor origin. Muscles restored in this way, assume an almost normal histological appearance.

This takeover of the host muscle by implanted cells appears not to progress beyond 3-4 weeks. We are now investigating the longer-term fate of implanted mpc. In particular, we are examining the capacity of such cells to remain in the muscle as a source of "resting" myogenic cells capable of responding to future episodes of muscle fibre degeneration and thus of sustaining the muscle in the face of more chronic demands on its regenerative capacity.

Supported by the Muscular Dystrophy Group of Great Britain.

F 220 A PROLACTIN/GROWTH HORMONE-LIKE PEPTIDE REPRESSES MYOGENIC-SPECIFIC TRANSCRIPTION BY SUPPRESSION OF AN ESSENTIAL TRANSCRIPTION FACTOR.

George E.O. Muscat *, Kari Gobius and Jacqueline Emery
Centre for Molecular Biology and Biotechnology,
University of Queensland, St Lucia , Queensland 4072, AUSTRALIA

A protein which has homology to prolactin and growth hormone is not expressed in rodent myogenic cell lines. Transient and stable expression of PLF repressed the transcription of the myogenic specific isoforms, but did not effect the transcription of the cytoskeletal isoforms in the actin multigene family that is developmentally regulated during myogenesis. Stable co-transfection analyses of 5' unidirectionally deleted actin promoters and a PLF expression vector identified the cis-acting region that mediated this transcriptional repression. Analyses of cells stably transfected with PLF showed reduced expression of myogenic helix-loop-helix genes. However, this did not account for the transcriptional repression of muscle specific genes. Electrophoretic mobility shift analysis indicated that this prolactin-like peptide suppressed the level of an essential transcription factor(with respect to the ubiquitous Oct-1), that is necessary for high level myogenic specific transcription.

F 221 MUSCLE ISOACTIN EXPRESSION IN MURINE EMBRYONIC STEM CELLS, Willie A. Ng, Thomas C. Doetschman, Jeffrey Robbins, James L. Lessard, Departments of Pediatrics and of Molecular Genetics, Biochemistry, and Microbiology, University of Cincinnati, Cincinnati, OH,45229

Embryonic stem (ES) cells are pluripotential cells obtained from the inner cell mass of mouse blastocysts that can be maintained *in vitro* in an undifferentiated state when cultured on a layer of embryonic fibroblasts. Upon removal from the feeder layer, the cells spontaneously differentiate into complex embryoid bodies (EB) which exhibit many of the characteristics of 4-10 day embryos, including heart-like areas which rhythmically contract. We examined the expression of the muscle isoactins in this system using transcript-specific probes for all four muscle actin mRNA's. Total cellular RNA was isolated for Northern blot analysis from undifferentiated ES cells and EB's after 5, 10, 15 and 20 days in suspension culture. No muscle actin expression could be detected in ES cells. Day 5 EB's display very low to undetectable levels of the muscle actin mRNA's. By day 10, there were significant amounts of α-vascular and γ-enteric actin mRNA's, a lower level of α-cardiac actin mRNA, and a very low level of α-skeletal actin mRNA. The α-vascular and α-cardiac actin expression decreased through days 15-20, whereas the γ-enteric actin mRNA level remained constant from days 10-20. No skeletal actin mRNA was detected at days 15 or 20. Polymerase chain reaction analyses also were carried out using individual EB's. These data again demonstrated that EB's transcribe all four muscle-specific actin genes. The pattern of muscle actin expression suggests that both striated and smooth muscle cells are present in the differentiating embryoid bodies. The appearance of α-vascular, α-cardiac, and α-skeletal actin mRNA reflects the normal events of early cardiogenesis and is consistent with the presence of primitive cardiomyocytes in EB's. Moreover, the detection of γ-enteric actin mRNA suggests that smooth muscle cells are also present in EB's since this isoactin has not been found in striated muscle.

F 222 GROWTH-PROMOTING ACTIVITIES FOR NORMAL HUMAN SKELETAL MUSCLE SATELLITE CELLS (HMSC), Zetan Nie and Richard G. Ham, Department of Molecular, Cellular, and Developmental Biology, Campus Box 347, University of Colorado, Boulder, CO 80309

The serum-free medium developed specifically for clonal growth of HMSC (Ham et al., In Vitro 24:833-844, 1988) consists of optimized nutrient medium MCDB 120 plus supplement SF (EGF, dexamethasone, insulin, fetuin, and serum albumin). The amounts of insulin, fetuin and albumin are large enough for impurities to have growth-promoting roles and growth is further enhanced by adding serum to the serum-free medium. We have now extracted the growth-promoting activity from a zinc insulin preparation into neutral saline under conditions where very little of the insulin dissolves. The recovery of the activity is almost 100 % with a 7-fold purification. We have also replaced the growth requirement of HMSC for fetuin with a fraction from fetal bovine serum (50 PEG 2X) prepared by sequential ammonium sulfate and polyethylene glycol precipitation. This fraction contains very little fetuin or albumin. Thus, the growth-promoting activity of commercial fetuin is due to a minor contaminant. The additional serum activity is enriched together with the fetuin-replacing activity in fraction 50 PEG 2X. Furthermore, the fetuin-replacing activity from serum binds to a heparin-Sepharose column and is eluted by NaCl at an unusually high concentration (approximately 2 M). This property is different from that of all known heparin-binding growth factors. We are now in the process of further purification and characterization of the insulin and fetuin contaminant activities. In the process of these studies, we have also found that protamine stimulates serum-free clonal growth of normal HMSC above that obtained in MCDB 120 plus SF. The optimal concentration of protamine is 10 μg/ml. The presence of protamine enhances growth responses to EGF, insulin and albumin, but does not alter the amounts needed for an optimal response. These data suggest that protamine has an independent mitogenic effect on HMSC (Supported by NIH grant AR39860).

F 223 MYOGENESIS AND PROLIFERATION OF THYMUS-DERIVED MYOGENIC CELLS *IN VIVO*. Terence A. Partridge, Charles N. Pagel, Jennifer E. Morgan, Karin Muller*, Karl-Heinz Westphal*. Department of Histopathology, Charing Cross & Westminster Medical School London W6 8RF, U.K. *Laboratorium fur Molekulare Biologie-Genzentrum, Martinsried, Germany. Tissue cultures of mouse thymus contain cells which differentiate into skeletal muscle. A number of clonal lines of such cells, designated AM1-AM6, have been selected to undertake spontaneouse myogenic differentiation in high growth medium. These cells are of interest because they can be grown clonally in culture without undergoing the neoplastic transformation commonly seen in murine cells in such conditions. Thus, cells of these lines have the ability to become incorporated into developing skeletal muscle when injected into mouse blastocysts or embryos and do not give rise to tumours. We have examined the behaviour of these cells when injected into mature skeletal muscle of the mdx mouse. When injected into degenerating muscles of the mdx mouse, AM1 cells become myogenic and give rise to dystrophin-positive muscle fibres. No tumours were formed in such muscles. If the mdx muscle is irradiated with 18Gy 3 days prior to the injection of AM1 cells, then these cells proliferate and invade the muscle, rapidly forming a large tumour. We are now investigating the nature of this switch between differentiation and proliferation of this cell-type in these two conditions. These cells are of interest for myoblast transfer studies because they appear to have some stem-cell properties, raising the possibility of extensive proliferation *in vitro*. In addition control of the switch between proliferative and myogenic pathways *in vivo* could be used as a means of improving the dispersal of myogenic cells from an injection site and of increasing the amount of muscle formed by injection of a given number of myogenic cells.

Supported by the Muscular Dystrophy Group of Great Britain.

F 224 CONTINUAL CYCLOSPORINE A TREATMENT IS REQUIRED FOR LONG TERM SURVIVAL OF ALLOGENEIC MYOBLASTS. Grace K. Pavlath, Marilyn A. Travis and Helen M. Blau, Department of Pharmacology, Stanford University School of Medicine, Stanford, CA 94305.

Mature muscle fibers express little or no class I or class II major histocompatability antigens. Determining whether there is an immune response to myoblast cells transplanted to treat human muscle diseases is critical to the design of proposed therapeutic strategies. To address this question, we used as a model mouse C2C12 myoblasts injected into the leg muscles of syngeneic, allogeneic, immunosuppressed and immunodeficient (scid) mice. To monitor the fate of individual muscle cells following injection into mouse muscle tissues, the cells were marked with β-galactosidase using retroviral infection. The introduced C2C12 cells fused both with endogenous muscle fibers and with themselves and were maintained for at least 6 months in the syngeneic C3H host ($H\text{-}2^k$). In contrast, C2C12 cells were rapidly eliminated by 10 days after injection into allogeneic C57 mice ($H\text{-}2^b$). Daily i.p. injections of cyclosporine A prevented this rapid loss and the cells were maintained during the 30 day treatment period and appeared comparable to cells in syngeneic or scid mice. During this time, the fibers containing injected myoblasts matured, as evidenced by the loss of expression of fetal myosin heavy chain and H-2 of the donor haplotype. Two weeks after cyclosporine A treatment was stopped, significant fiber loss and infiltration of the muscle by immune cells was observed. At 5 weeks, donor-containing fibers were scarce. These findings, in conjunction with our studies that demonstate that γ-interferon induces class I and class II major histocompatability antigens on myoblasts (Mantegazza et al., Neurology, in press), indicate that the use of allogeneic myoblasts in cell therapy of human muscle disorders may be complicated by the need for continual immunosuppression of patients. (Supported by MDA)

F 225 **THE *SKI* PROTO-ONCOGENE FAMILY IN MUSCLE DEVELOPMENT,** Sonia H. Pearson-White, Departments of Biology and Hematology-Oncology, MR4 Building Box 1131 Room 1127, University of Virginia Medical Center, Charlottesville, Virginia 22908.

A recently described proto-oncogene family, the *ski* family, may have important functions in early embryonic development. The chicken v-*ski* oncogene can convert quail embryo fibroblasts to the myogenic (muscle) pathway, as well as transform them to tumorigenicity. Transgenic mice expressing *ski* have unusually large skeletal muscles, causing hypertrophy of Type II fast fibers, rather than hyperplasia. Members of the *ski* family of genes, *ski* and *sno*, are expressed during skeletal muscle development in determined myoblasts and in differentiated myofibers, and at lower levels in adult muscle. Several regulatory genes have been identified that convert fibroblasts to the myogenic pathway: MyoD1, myogenin, myf5, and MRF4, all of which share a helix-loop-helix *myc* homology region crucial for the conversion function. *Ski* shows no homology to the MyoD1 family of genes. To investigate the role of *ski* family genes in muscle development, I have isolated two novel *ski*-related cDNAs from primary human myoblasts. Sequence, expression and transfection data will be presented as part of the preliminary characterization of the role of the *ski* family genes in muscle development.

F 226 EFFECT OF HYPOTHYROIDISM ON MYOSIN HEAVY CHAIN (MHC) EXPRESSION IN PHARYNGEAL MUSCLES. Basil J. Petrof, Alan M. Kelly, Neal R. Rubinstein, Allan I. Pack, Departments of Medicine, Anatomy and Pathobiology, University of Pennsylvania, PA 19104.

Altered function of pharyngeal dilator muscles has been implicated in the loss of upper airway patency that occurs in obstructive sleep apnea syndrome (OSAS). The association between this clinical disorder and hypothyroidism is also well established. The purpose of this study was to examine the effect of hypothyroidism on MHC expression in pharyngeal dilator muscles. Twelve 6-week old male, Sprague-Dawley rats were randomized to control and hypothyroid (PTU for 6 weeks) groups. Monoclonal antibodies specific to types I, IIA, and embryonic MHC were used to perform immunostains and ELISA's of the geniohyoid, sternohyoid and genioglossus muscles. Control muscles were predominately composed of fast fibers with <10% of fibers expressing type I MHC. Hypothyroidism induced significant increases in types I and IIA MHC in the 3 muscles studied. Embryonic MHC was not detected in either control or hypothyroid muscles. In view of the expected reduction in force-generating capacity of type I and IIA fibers, these changes may play a role in the pathogenesis of OSAS in hypothyroid patients.

F 227 IGF-BINDING PROTEINS PRODUCED BY MUSCLE AND DERMAL FIBROBLASTS LeBris S. Quinn[1], Luan D. Ong[1] and Richard A. Roeder[2], [1]Department of Biological Structure, University of Washington, Seattle, WA, and [2]Department of Animal Science, University of Idaho, Moscow, ID.

IGF-I and IGF-II binding proteins (IGF-BPs) are secreted by cultured bovine fibroblasts derived from fetal skeletal muscle and dermis. ^{125}I-IGF ligand blots of media conditioned by these cells reveal that several forms of IGF-BPs are produced. Binding proteins of apparent MW 29-32 KD and 42-44 KD are produced by both dermal and muscle fibroblasts. Addition of IGF-I to the cells, but not addition of bFGF or growth hormone, causes an upward shift in the mobility of the lower MW forms. Dermal fibroblasts produce relatively more of the low MW BPs than do muscle fibroblasts, and muscle fibroblasts produce relatively more of the higher MW BPs than do dermal fibroblasts. Different forms of IGF-BPs may modulate the availability of the IGFs to myogenic cells in developing muscle tissue.

F 228 EXPRESSION OF PRENATAL MYOSIN HEAVY CHAIN IN MYOFIBERS OF A DISTINCT SUBGROUP OF PATIENTS WITH CENTRONUCLEAR MYOPATHY. J.A. Sawchak, J.H.Sher[1], R.W. Kula, S. A. Shafiq, M.G. Norman[2], A.L. Pestronk[3], M. Ambler[4], Departments of Neurology and Pathology[1], State University of New York, Health Science Center at Brooklyn, Brooklyn, New york, Department of Pathology[2], BC's Children's Hospital and University of British Columbia Vancouver, British Columbia, Department of Neurology[3], Washington University School of Medicine, St. Louis, Missouri, 63110, and Department of Pathology[4], Rhode Island Hospital, Providence, Rhode Island, 02903.

We have previously reported (Neurology 40 [S/1]:204,1990) the results of immunocytochemical studies using myosin heavy chain (MHC) specific monoclonal antibodies (MAbs) of muscle from 3 patients with centronuclear myopathy (CNM) which showed that the muscle from 2 brothers with severe, neonatal CNM strongly expressed prenatal isoform of MHC while the muscle from a woman with childhood onset of CNM did not. We have subsequently had the opportunity to similarly study muscle from 9 additional cases of CNM including both severe neonatal as well as childhood onset types of CNM. We found expression of a developmental myosin heavy chain only in a subset of the patients with severe, neonatal, X-linked CNM. This suggests that those exhibiting this abnormal expression of a developmental isoform of a muscle contractile protein may suffer from a distinct type of muscle disease with a different pathogenesis than the other patients with CNM. It remains to be seen if this heterogeneity in myosin expression in X-linked CNM is related to the genetic heterogeneity reported by Starr et al. J Med Genet, 27:281-3, 1990.

F 229 MYOGENIC DIFFERENTIATION IS A COMMON FEATURE OF WOUND-DERIVED NEOPLASMS IN v-jun TRANSGENIC MICE. Andre C. Schuh[1], Peter K. Vogt[2] and Martin L. Breitman[1,] [1]Division of Molecular and Developmental Biology, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, CANADA and [2] Department of Microbiology, USC School of Medicine, Los Angeles, CA, USA.

V-jun is the transforming gene of avian sarcoma virus 17. This viral oncogene, as well as the related cellular genes -- c-jun, junB, and junD -- encode transactivating DNA-binding proteins, which recognize both the AP-1 consensus sequence TGACTCA, a response element which confers sensitivity to the tumor-promoting phorbol ester TPA, and the related cyclic AMP responsive element, TGACGTCA. We have recently generated transgenic mice carrying v-jun driven by the promoter of the widely-expressed H-2K^K class I MHC antigen gene [Schuh, A.C. et al., Nature 346:756-760 (1990)]. Animals carrying H-2K/v-jun are initially phenotypically normal, but following full-thickness wounding exhibit abnormal wound repair consisting of hyperplastic granulation tissue. These lesions are often slowly progressive, and over 2-5 months a fraction gives rise to malignant sarcomas. By histological and immunohistochemical analysis, these wound-derived neoplasms lie on a continuum between fibro- and rhabdomyosarcoma. In contrast, similar analyses of benign precursor lesions are negative for all muscle markers tested, suggesting that myogenic differentiation occurs as a relatively late event during tumor development. Clonal cell lines derived from these sarcomas often give rise spontaneously to myotubes. However, several non-differentiating cell lines have also been isolated. The phenotypes of the sarcomas and their derived cell lines, and the relationship between myogenic differentiation and transformation by v-jun will be presented.

F 230 DELETED MITOCHONDRIAL GENOMES AT LOW FREQUENCY IN MYOBLAST CLONES FROM PATIENTS WITH PROGRESSIVE EXTERNAL OPHTHALMOPLEGIA, Eric A. Shoubridge, Louise Boulet and George Karpati, Department of Neurology and Neurosurgery, Montreal Neurological Institute, Montreal, Canada H3A 2B4.
Heteroplasmy for large mtDNA deletions is a common feature in skeletal muscles of patients with progressive external ophthalmoplegia and/or Kearns-Sayre Syndrome. Myoblast clones cultured from KSS patients are reported to be either heteroplasmic or to contain wild-type genomes only. Using PCR we tested whether deleted mtDNAs were present at low frequency in myoblast clones from 4 patients with large mtDNA deletions detectable in skeletal muscle on Southern blots (50-90% of total genomes deleted) and in one KSS patient in which the deleted mtDNA could only be detected by PCR. Deleted mtDNAs were rare in uncloned myoblasts compared to adult muscle in all patients suggesting that selection for deleted molecules occurs in postmitotic muscle cells. Deleted mtDNAs were not detected in any myoblast clone by Southern blot analysis. PCR analysis showed that some, but not all, myoblast clones contained deleted genomes at low frequency. Suprisingly, all of the clones examined in the patient with the low-frequency deletion in adult muscle contained deleted mtDNAs. These results suggest that factors such as the intermitochondrial distribution of mutant genomes or nuclear genomic background may be important determinants of the fate of deleted mtDNAs during muscle growth and development.

F 231 CHARACTERIZATION OF CANINE MUSCLE PHOSPHOFRUCTOKINASE DEFICIENCY,
Bruce F. Smith, Hansell Stedman, and Urs Giger, Section of Medical Genetics, Veterinary Hospital, University of Pennsylvania, Philadelphia, PA 19104-6010
In humans, muscle type phosphofructokinase (M-PFK) deficiency causes a metabolic myopathy and hemolytic disorder (Glycogenosis Type VII). Recently, a canine model has been described which represents a close homologue of the human disease. M-PFK deficient dogs lack muscle PFK activity and have reduced erythrocyte and brain PFK activities. On SDS-PAGE stained with coomasie blue, affected tissues lack a protein band of approximately 85 kdal, while carrier dogs show a band of approximately half the density of normal dogs. An M-PFK specific antibody (guinea pig anti rabbit M-PFK) hybridizes to this band in normal subjects but fails to show binding in affected animals. Immunoprecipitation with this antibody removes nearly all PFK activity from normal muscle homogenates but does not affect the residual activity in homogenates of muscle from affected individuals. Normal canine muscle cells express M- and L-type PFK in culture. However, cultured myoblasts from deficient dogs do not express M-PFK, even after differentiation to myotubes. Northern blot analysis of mRNA from affected dogs indicates the presence of normal amounts of a normal length transcript. Overlapping clones of this transcript have been isolated from canine muscle cDNA libraries and from PCR amplifications of bulk canine muscle cDNA, utilizing primers from areas of known interspecific homology. Preliminary sequence analysis indicates a high degree of homology with human and rabbit M-PFK. The study of M-PFK deficient dogs may increase our knowledge of the relationship between the molecular defect and the resultant phenotype as well as provide useful information for the development of theraputic strategies for myopathies.

F 232 LOSS OF EPIDERMAL GROWTH FACTOR AND FIBROBLAST GROWTH FACTOR RECEPTORS DURING SKELETAL MUSCLE DIFFERENTIATION. T. J. Templeton and S. D. Hauschka, Dept. of Biochemistry, University of Washington, Seattle, WA 98195.
Prior studies in this laboratory have shown that cell surface receptors for epidermal growth factor (EGFR) and fibroblast growth factor (FGFR) are completely lost during the differentiation of mouse skeletal muscle MM14 myoblasts. MM14 cells require FGF, in addition to serum factors, for proliferation and repression of differentiation. To study the regulation of receptor loss Northern blot analysis was performed using a human EGFR ligand-binding domain cDNA probe. Two EGFR transcripts (6 kb and 10 kb), present in proliferating myoblasts, are absent 24 h following switching the cells to low-mitogen differentiation medium. Using a mouse FGFR-family cDNA (*Bek*) as a probe on Northern blots we could readily detect a transcript in mouse 3T3 fibroblasts but only faintly in MM14 myoblasts, possibly the result of cross-hybridization to a transcript from a distinct FGFR gene. To clarify the identity of the MM14 FGFR we used degenerate FGFR oligonucleotide primers and the polymerase chain reaction (PCR) to isolate a FGF receptor cDNA from reverse-transcribed MM14 myoblast RNA template. This receptor is identical to a putative murine bFGFR (Reid *et al.* PNAS. USA, 87,1596, 1990). Using the PCR-derived cDNA as a probe we detect a 4.5 kb transcript in MM14 myoblasts which decreases markedly, but does not disappear completely, following myocyte differentiation. We are determining whether the residual FGFR transcript expression in differentiated cultures can be ascribed to a sub-population of differentiation-defective cells. If so, the loss of the FGFR transcript in myocytes would correlate with the loss of FGFR cell surface receptors during muscle differentiation. This would suggest that the PCR-derived FGFR cDNA represents the physiologically-relevant FGF receptor in skeletal muscle myoblasts.

F 233 EXTRINSIC CONTROL OF FAST AND SLOW MUSCLE FIBER DEVELOPMENT: SUPPRESSION OF SLOW MYOSIN HEAVY CHAIN IN DEVELOPING HUMAN MUSCLE.
Marilyn A. Travis, Mildred Cho, Steven G. Webster, and Helen M. Blau, Department of Pharmacology, Stanford University School of Medicine, Stanford, CA 94305.

Vertebrate muscles are composed of fibers with different contractile properties, designated as fast and slow. The question remains whether the heterogeneity of differentiated myofibers is due to extrinsic factors that act on the differentiated myofiber, or is derived from myogenic precursors (myoblasts) that differ intrinsically. To address this question, we determined whether changes in the proportion of slow fibers during human muscle development were attributable to inherent differences in the myogenic precursors from which they derive. We analyzed myoblast clones derived from muscle at six stages of human development. Slow myosin heavy chain (MyHC) was expressed by each muscle colony derived from tissues at each stage of development tested. In contrast, the proportion of slow fibers *in vivo* differed greatly during these stages of human limb development: during the first trimester of development 100% of fibers expressed slow MyHC, at midgestation only 3% expressed this isoform, and toward the end of gestation through adulthood approximately 50% of fibers were slow. These results suggest that the expression of slow MyHC *in vivo* is not the result of commitment to a distinct slow myoblast lineage, but is controlled exogenously by myoblast-extrinsic factors that suppress its expression. (Supported by NIH, MDA, March of Dimes).

F 234 THE INHIBITION OF MYOGENESIS BY THE H-*ras* ONCOGENE INVOLVES A PROTEIN KINASE C DEPENDENT PATHWAY, T.B. Vaidya, C.M. Weyman[1], C.L. Ashendel[1] and E.J. Taparowsky, Department of Biological Sciences and [1]Department of Medicinal Chemistry and Pharmacognosy, Purdue University, West Lafayette, Indiana 47907.

Although the inhibition of skeletal muscle differentiation by serum, purified growth factors and oncogenes is well documented, the intracellular signal transduction pathways used by these inhibitors are poorly understood. We have demonstrated that an activated ***ras*** oncogene inhibits myogenesis in the mouse myoblast cell line, 23A2. This inhibition is associated with the down-regulation of the myogenic regulatory gene, MyoD1, and can be reversed by the constitutive expression of the MyoD1 cDNA. We also have found elevated levels of the calcium- and phospholipid-dependent protein kinase, PKC, in differentiation-defective 23A2-***ras*** cells. In addition, while the wild-type myofibers exhibit a 50 % reduction in PKC activity as compared to the undifferentiated myoblasts, the differentiation-defective 23A2-***ras*** cells retain at least as much PKC activity as the normal myoblasts, suggesting that levels of this kinase may be critical to the control of myogenic differentiation and the regulation of MyoD1 gene expression. To examine the role of PKC in wild-type 23A2 myoblasts and differentiation-defective 23A2-***ras*** cells, we have used the tumor-promoting phorbol ester, TPA, to manipulate PKC activity in both cell types. Treatment of wild-type 23A2 myoblasts with a concentration of TPA that causes complete down-regulation of PKC activity inhibits fusion, but does not affect the biochemical differentiation of the cultures. Treatment of the ***ras*** cultures with a concentration of TPA that similarly down-regulates PKC does not restore biochemical or morphological differentiation in these cells. Interestingly, when 23A2 myoblasts are treated with TPA prior to the introduction of the H-***ras*** oncogene, the resultant cultures are differentiation-competent, despite the presence of high levels of the oncogenic H-***ras*** mRNA. We conclude from these studies that protein kinase C activity is essential for ***ras*** to initiate a differentiation-defective phenotype in 23A2 myoblasts, but that continued activation of PKC is not required to sustain the non-muscle phenotype of the 23A2-***ras*** cells.

F 235 REGULATION OF THE HUMAN CARDIAC ß-MYOSIN HEAVY CHAIN GENE AND CHARACTERIZATION OF RECOMBINANT ß-MHC FRAGMENTS, Hans-Peter Vosberg, Annette Wettstein and Martin Pfordt, Dep. of Cell Physiology, Max-Planck-Institute for Medical Research, D-6900 Heidelberg

Two cardiac myosin heavy chain genes, α and ß, on chromosome 14 are differentially expressed in cardiac muscle. The ß-heavy chain is mainly found in the ventricles and the α-chain in the atria. We have sequenced the ß-gene in its entire length plus flanking regions (about 28 kb). A regulatory region controlling the expression of the gene in muscle cells has been identified by gene transfer, band shift and footprint analysis. We have demonstrated that a protein present in skeletal muscle nuclei (rabbit) and presumably related to the family of myoD1 regulatory proteins binds specifically to a signal region which controls the activity of the promoter. The signal region is located about 110 bp upstream of the CCAATT box. We have further constructed cDNA clones which express fragments of the ß-S1 domain. Our interest is in particular focussed on a biochemical characterization of a mutated fragment which contains an arginine to glutamine exchange in position 403. This mutation has recently (Geisterfer-Lowrance et al., Cell 62:999, 1990) been implicated in the etiology of a case of familial hypertrophic cardiomyopathy.

F 236 EXTENT OF MIGRATION OF DONOR PRECURSOR CELLS INTRODUCED INTO RECIPIENT MYOPATHIC MUSCLE, Diana J. Watt* and Marjorie A. England+, Departments of Anatomy, *Charing Cross & Westminster Medical School, London W6, +University of Leicester, Leicester, U.K.

Success in myoblast transfer therapy depends on the extent of migration of introduced donor cells through the recipient muscle in order to disseminate normal gene products. We already know[1] that donor muscle precursor cells are capable of both entering into and participating in the formation of new muscle fibres in the host. The present work was designed to establish how far and by which route precursor cells can infiltrate.

An enriched myogenic cell population prepared from mouse muscle by an antibody panning method[2] was infected with the MoMLV retroviral vector carrying the lacZ gene and implanted into one muscle of the lower hind limb of the mdx mouse. Nuclepore membrane was inserted between the implanted and adjacent recipient muscles. At varing times after implantation, adjacent implanted and non-implanted muscles and intervening membrane were removed and examined by electron microscopy in order to visually detect cells in transit through the membrane. Analysis of origin of migratory cells and extent of movement of donor cells was ascertained using the isoenzyme allotypes of glucose-6-phosphate isomerase characteristic of donor and recipient tissues, B-galactosidase staining and the presence or absence of dystrophin.

1. Jones, G.E., Murphy, S. J., Watt, D.J. (1990). J. Cell Science *97(4)* in press.
2. Watt, D. J., Morgan J. E., Clifford, M. A., Partridge, T. A. (1987) Anat. Embryol. *176 :* 527-536

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F 237 RETROVIRUS VECTOR-MEDIATED GENE TRANSFER TO MUSCLE CELLS
John Wolfe, Rene Morris, Bruce Smith, Laboratory of Pathology and Section of Medical Genetics, University of Pennsylvania School of Veterinary Medicine, Philadelphia, PA 19104

Retrovirus vectors are useful for gene transfer experiments since they can transfer single copy genes into a high percentage of target cells compared to other DNA transfection techniques. We are using somatic cells deficient in β-glucuronidase (GUSB), a lysosomal hydrolase, as a model to investigate vector-mediated gene transfer and expression in various somatic tissues. We have developed retroviral vectors expressing rat or human GUSB, under control of viral or natural promoters, which produce normal levels of GUSB in deficient cells. Enzyme correction completely restores normal lysosomal function to affected cells. We also have recently developed a GUSB vector in the double copy (DC) configuration which places one minigene transcriptional unit upstream of the retroviral transcriptional unit in the target cell to overcome promoter interference from the viral LTR sequences. This vector expresses 8-10 fold higher levels of GUSB compared to an N2-based vector with the minigene located within the vector transcriptional unit. Myoblasts have many features which make them attractive as a target tissue for gene transfer (accessibility, mitotic activity, repopulation in vivo). We have shown that, unlike bone marrow cells, retroviral vectors can infect nearly 100% of myoblasts in primary cultures. The myoblasts differentiate into myotubes (cross-striations, multiple nuclei, active contraction in tissue culture, and expression of myosin heavy chains) expressing the transferred gene. Furthermore, infected myoblasts grown in proliferative conditions retain the ability to differentiate into myotubes in culture through at least 20 divisions while stably expressing the transferred gene. The ability to transfer and express genes in a high percentage of myoblasts should make the retroviral vector system useful for differentiation studies and correction of some classes of genetic myopathies such as enzyme deficiencies.

F 238 REGULATION OF MYOBLAST PROLIFERATION AND DIFFERENTIATION BY SPECIFIC PDGF ISOFORMS, Zipora Yablonka-Reuveni*, Ronald A. Seifert^ and Daniel F. Bowen-Pope^, *Department of Biological Structure and ^Department of Pathology, School of Medicine, University of Washington, Seattle, WA 98195

The expression of receptors and the mitogenic response to platelet-derived growth factor (PDGF) by rodent and avian myoblasts was investigated. C2 myoblasts, derived from adult mouse skeletal muscle (presumably from satellite cells) bind at saturation $165x10^3$ molecules/cell of ^{125}I-PDGF-BB and only bind $6\text{-}12x10^3$ molecules/cell of ^{125}I-PDGF-AA and ^{125}I-PDGF-AB. This indicates that C2 myoblasts express high levels of β-PDGF receptor subunits and low levels of α-subunits. PDGF-BB enhances the proliferation of C2 cells maintained in 2% fetal calf serum by 5-10 fold, while PDGF-AB has a less than 2 fold effect, and PDGF-AA has no effect. Conversely, PDGF-BB suppresses myoblast differentiation, but PDGF-AA and PDGF-AB have no effect. Similar studies were conducted on clonally derived myoblasts from the breast muscle of chicken embryos. Myoblasts from 19-day embryos (E19) demonstrate high saturation binding of PDGF-BB ($75\text{-}100x10^3$ molecules/cell) and much lower saturation binding of PDGF-AA and PDGF-AB ($3\text{-}7x10^3$ molecules/cell). Preliminary studies indicate that PDGF-BB promotes the proliferation of E19 myoblasts. Initial experiments with chicken E10 myoblasts indicate that, unlike the E19 myoblasts, they bind only low levels, if any, of the three PDGF isoforms. PDGF thus may participate in late stages of muscle development and in muscle regeneration by stimulating proliferation and inhibiting differentiation of myogenic cells. (Supported by NIH and AHA).

F 239 PROGRESSIVE MUSCLE DEGENERATION IN THE MDX MOUSE DIAPHRAGM

A. Kelly, J. Shrager, M. Narusawa, R.Panettieri, H. Maguire, J. Sladkey, L. Sweeney, H. Stedman. Depts. of Anatomy, Neurology and Pathobiology, University of Pennsylvania, Philadelphia, PA. 19104

The mdx mouse has appeared to be of limited utility as a model of Duchenne dystrophy (DMD) since limb muscles exhibit minimal degeneration and fibrosis. We have focused on the respiratory muscle of the mdx mouse since respiratory failure is the leading cause of death in DMD. In contrast to limb muscles, the mdx diaphragm exhibits a series of histological changes that closely parallel those of DMD. Focal areas of myofiber degeneration, necrosis and regeneration emerge in the diaphragm between 20 and 30 days of age as in limb muscles. However, degeneration of the mdx diaphragm is progressive so that by one year there is extensive replacement fibrosis. Hydroxyproline assays indicate a six fold increase in collagen compared to controls. As a result, there is a profound loss of force generating potential and increase in muscle stiffness in the mdx diaphragm. This is accompanied by a 40% reduction in length of the diaphragm. Muscle regeneration is sustained throughout the course of disease and relative proportions of embryonic myosin progressively increase with age. Changes in intercostal muscles compare to those of limb muscles. Why the diaphragm uniquely exhibits these changes is being investigated.

Molecular Basis of Muscle Cell Diversity; Neuromuscular Interactions in Development; Contractile Protein Assembly; Cell and Molecular Biology of Neuromuscular Disease

F 300 INTRACELLULAR LOCALIZATION OF COFILIN IN CULTURED MUSCLE CELLS. Hiroshi Abe, Rie Nagaoka, Katsumi Yabusaki, and Takashi Obinata. Department of Biology, Faculty of Science, Chiba University, Yayoi-cho, Chiba 260, Japan.

At early phase of myofibrillogenesis, actin in microfilament bundles may be redistributed in sarcomeric thin filaments, and during this process, considerable amount of G-actin is pooled in the cytoplasm. Low Mr-actin binding proteins seems to be involved in the actin redistribution. Among these, cofilin is of particular interest, since it binds to both G- and F-actin, regulates actin-assembly in a pH dependent manner, and is expressed more abundantly in muscle tissues than in non-muscle tissues. In this study, we examined location of cofilin in cultured muscle cells with a specific monoclonal antibody (MAB-22). In the normal myotubes, cofilin is mostly diffuse in the cytoplasm, but it was occasionally detected in the ruffled areas, microspikes of growing myotubes and associated with sacomeric structures. In degenerating myotubes with vacuoles, actin-cofilin-rods were sometimes generated abundantly along myofibrils. Treatment with DMSO, cycloheximide, or a heat-shock produced intranuclear and cytoplasmic rods and vesicles in myotubes, where cofilin and actin were co-localized. The dynamic changes in cofilin distribution may suggest that cofilin is involved in redistribution and turn-over of actin molecules in muscle cells.

F 301 EXPRESSION OF INTRACELLULAR CALCIUM RELEASE CHANNELS DURING MYOGENESIS, J.A. Airey, M.D. Baring, and J.L. Sutko, Dept. Pharmacology, Univ. Nevada, Reno, NV 89557

To investigate the role of intracellular calcium release in myogenesis, we have started to identify the factors controlling the expression of the ryanodine (R) and inositol trisphosphate (IP_3) receptors, two intracellular calcium release channels. In embryonic chick skeletal muscle, the RR is present in day E9 muscle prior to mature muscle organization. These findings were extended using C2C12 and the nonfusing, MyoD1 deficient BC_3H1 myogenic cell lines. The RR is not detectable prior to induction of differentiation and increases in abundance during differentiation in both fusing and nonfusing (50 uM Ca_o) C2C12, and in the nonfusing BC_3H1 cells indicating that neither fusion nor MyoD1 is required for RR expression. The addition of growth factors, TGF-ß1 (5 ng/ml) or bFGF (90 ng/ml), which block differentiation, prevents RR expression. The IP_3R is expressed prior to differentiation in both cell types, and in contrast to the RR, the level of IP_3R does not increase during differentiation and is not blocked by the growth factors tested. These results suggest that the temporal aspects of the expression of the RR and IP_3R are consistent with a possible role for these intracellular calcium release channels in myogenesis.

F 302 SYNAPTIC BASAL LAMINA ACETYLCHOLINESTERASE AT NEUROMUSCULAR JUNCTIONS. Lili Anglister, Department of Anatomy and Embryology, Hebrew University-Hadassah Medical School, Jerusalem 91010, Israel

Acetylcholinesterase (AChE) in skeletal muscle is highly concentrated at the neuromuscular junctions, where it is found in the synaptic cleft, associated with the synaptic portion of the myofiber's basal lamina. Muscle contains several molecular forms of AChE. The amount of AChE and its molecular forms in muscle in general, and at the junctions in particular, are highly dependent on the integrity of synaptic structure and function. Our studies were aimed at learning the individual role of nerve, muscle and basal lamina in producing and directing the accumulation of AChE at synaptic sites. By using surgical manipulations in frog muscles we demonstrate that nerve terminals, in the absence of myofibers, produce synaptic AChE, and a substantial fraction of it becomes adherent to the synaptic basal lamina. The nerve carries high concentrations of the asymmetric (A_{12}) AChE molecular form towards its terminals, where some of it is extrenalized. In contrast, in the absence of nerve, denervated adult myofibers do not produce synaptic AChE and the production of asymmetric AChE is drastically diminished. However, regenerating myofibers do produce significant amounts of asymmetric AChE, even in the absence of nerve. The newly made AChE reaccumulates at the old synaptic sites, in the synaptic basal lamina, which contains components directing this process. Our results demonstrate that both myofibers and nerve terminals can produce synaptic basal lamina AChE; Nerve-muscle-basal lamina interaction and additional factors regulate its production and maintenance. (Sponsored by grants from Israel-US BSF, Israel Acad. Sci., Bat-Sheva de Rothschild and Bruno-Goldberg Foundations).

F 303 REGULATION OF ACTIN DEPOLYMERIZING FACTOR IN DEVELOPING MUSCLE, James R. Bamburg, Todd E. Morgan and Marvin E. Adams, Department of Biochemistry, Colorado State University, Fort Collins, CO 80523

Actin depolymerizing factor (ADF) is an 18.5 kDa protein capable of regulating actin assembly by severing actin filaments and sequestering monomers. It is present in many embryonic and adult avian and mammalian tissues. We have investigated the developmental expression of ADF and its mRNA in chick muscle and in cultured myocytes. ADF levels in muscle decline from 0.2% of the total tissue protein at 10 days embryonic development to undetectable levels by 14 days posthatching, remaining at this low level throughout subsequent development. The ADF mRNA follows the same pattern, suggesting that the regulation is transcriptionally controlled. In myocyte cultures, ADF mRNA levels do not decline as *in vivo*, and levels of immunoreactive ADF increase over a period of two weeks, a time of intense myocyte fusion and sarcomere formation. The increase in immunoreactive ADF is due to the formation of a new isoform that we isolated and characterized as inactive in depolymerizing filamentous actin *in vitro*. This isoform contains an incorporated phosphate and it represents a posttranslationally modified form of ADF, produced, we believe, by calcium/calmodulin dependent protein kinase II. The levels of active ADF found in both muscle tissue and cultured myocytes correlate well with the levels of total actin remaining in the unassembled state. Thus ADF is an important actin assembly regulatory protein in developing muscle and its activity is controlled by both pre- and posttranslational mechanisms. Supported by NIH grants GM35126, NS28338 and GM43398.

F 304 ISOLATION AND ANALYSIS OF *DROSOPHILA* PARAMYOSIN PROTEIN AND cDNA: PRELIMINARY EVIDENCE FOR PRODUCTION OF ALTERNATIVE ISOFORMS, K. David Becker, William A. Kronert, Patrick T. O'Donnell, Maja Vito, Judith M. Heitz and Sanford I. Bernstein, Biology Department and Molecular Biology Institute, San Diego State University, San Diego, California 92182.

We have utilized standard biochemical techniques to isolate paramyosin from *Drosophila melanogaster* larvae and adults. Identification of the protein was aided by the use of an antibody to *Caenorhabditis elegans* paramyosin (provided by R. Waterston and R. Barstead). The protein is ≈106 kD, migrates at a pI of 6.3 and corresponds to overlapping spots 19, 20 and 21 of Mogami *et al.* (J. Biochem. 91:643-650, 1982). Interestingly, we have found that paramyosin accumulates in adult thoracic muscle lacking myosin heavy chain protein. This is in contrast to the myosin light chains, which fail to accumulate under these conditions (Chun and Falkenthal, J. Cell Biol. 107:2613-21, 1988). Using an antibody made against *Lethocerous* paramyosin (provided by B. Bullard), we have isolated *Drosophila* paramyosin cDNAs from adult and embryonic libraries. DNA sequencing of a full length embryonic cDNA has identified a single open reading frame with 48% identity and 79% conservation compared to *C. elegans* paramyosin. Like the *C. elegans* molecule, computer analysis predicts non-helical N- and C-terminal regions separated by an alpha-helical structure capable of forming a coiled-coil. We have determined the genetic location of the paramyosin gene to be 66E1 on the left arm of the third chromosome. Analysis of genomic DNA via Southern blot hybridization, also indicates that only a single paramyosin gene per haploid genome exists in *D. melanogaster*. However, four size classes of paramyosin mRNA can be identified by hybridization to total cellular RNA. These can be divided into two sets. The size difference within each set is due to choice of polyadenylation signal. The two larger mRNAs encode the 110 kD (size from DNA sequence) paramyosin protein, whereas the two smaller mRNAs (found only in the pupal and adult stages) may encode a smaller form, ≈70 kD, of the paramyosin protein. We are currently isolating cDNAs that represent the smaller transcripts for further characterization of their coding potential.

F 305 OVEREXPRESSION OF CREATINE KINASE B, UNDER THE CONTROL OF THE SKELETAL α-ACTIN PROMOTER, REDUCES LEVELS OF THE HOMODIMERIC MM ISOZYME IN TRANSGENIC MOUSE MUSCLE. J. Brosnan; S. Pattabhiraman, T. Van Dyke* and A. Koretsky. Dept. Biol Sci, Carnegie Mellon Univ. and *Univ. of Pittsburgh, Pittsburgh, PA 15213.

Expression of the different genes (M, B and mitochondrial) of creatine kinase is regulated in a developmental and tissue specific manner. The protein products of the M and B genes dimerize to form the active enzyme (MM, MB and BB) whereas the mitochondrial form is believed to function as an octomer. The existence of the various isozymes within skeletal muscle has resulted in the proposal of a creatine kinase shuttle. This shuttle is believed to couple the efficient formation and utilization of energy within the working muscle. This hypothesis requires that the different active units of creatine kinase have different subcellular localizations. The CK_{mito} form is located between the mitochondrial membranes and the dimeric CK_{mm} at the M line of the myofibril. In vitro studies on stripped myofibrils indicate that neither CK_{BB} nor CK_{MB} can bind at the M-line in mammalian systems. In order to investigate the role and validity of the creatine kinase shuttle we have produced transgenic mice which overexpress the B isozyme under the control of the skeletal α-actin promoter. Positive mice were identified using PCR and the integrity of the transgene determined by Southern blot analysis. Isozyme analysis reveals that there are elevated levels of MB and BB within the skeletal muscle of the transgenic animals. The level of MM is reduced. These results show that the α-actin promoter is sufficient to direct expression of high levels of the B monomer and that the protein is stably incorporated into active creatine kinase in muscle. These mice are currently being investigated to determine the effects of this isozyme shift on the physiology, biochemistry and developmental profile of the transgenic muscle.

F 306 EXPRESSION OF MLC1EMB IN THE HUMAN MASSETER MUSCLE.

G.S.Butler-Browne[1], N. Soussi-Yanicostas[1], C. Laurent-Winter[2].
INSERM U 262, Clinique Universitaire Baudelocque, Paris, FRANCE[1].
Institut Pasteur, 25 rue du Dr Roux, Paris, FRANCE[2].

In the limbs, muscle fibers can be divided into 3 sub-types (I, IIA and IIB) with roughly equivalent diameters. In the masseter muscle, however, there is a large heterogeneity both in fiber size and in enzyme activities. The type II fibers are much smaller than the type I fibers in this muscle and following preincubation at pH 10.2, 4.6 and 4.3 the fibers can be divided into 4 different sub-types (I, IIB, IIC and intermediate staining fibers IM). Previous biochemical and immunochemical studies carried out in our laboratory (1,2) have demonstrated in the adult masseter muscle a persistance of two proteins, fetal MHC and MLC1emb, usually only detected during fetal development . One of these, fetal MHC, was found to be mainly localized to the small diameter IM and IIC fibers. In the present study we have looked at the expression and localization of the second of these proteins, MLC1emb, using 2D-gel electrophoresis, northern blot analysis and *in situ* hybridization. We show that MLC1emb is expressed in all fiber types during development of both the quadriceps and masseter muscles.After 30 weeks of gestation the expresion of MLC1emb is repressed in the quadriceps whereas in the masseter it persists during postnatal development. In the adult masseter MLC1emb is only expressed in type II fibers.

F 307 INTRINSIC CONTROL OF PRIMARY AND SECONDARY MUSCLE FIBER DEVELOPMENT.

Mildred Cho, Steven G. Webster, Marilyn A. Travis, Helen M. Blau, Department of Pharmacology, Stanford University School of Medicine, Stanford, CA 94305.

Developing vertebrate muscles are formed from fibers that arise at different times during development, designated as primary and secondary. We have examined whether differences between primary and secondary fibers are determined by intrinsic differences between populations of myoblasts or by factors extrinsic to the muscle fibers. To distinguish between these possibilities, we isolated human myoblasts from muscle tissues when either primary (week 7 of gestation) or secondary (week 9 of gestation) fibers are formed and characterized the isoforms of MyHC (MyHC) expressed by individual myogenic clones in culture. To characterize MyHC profiles, we used antibodies generated in our laboratory that distinguish embryonic and neonatal myosin isoforms from adult fast MyHCs in developing muscles (Silberstein et al., 1986 Cell 46, 1075-1081). We found that myoblasts from both stages synthesize embryonic and slow MyHCs but only week 9 myoblasts synthesize neonatal MyHC upon differentiation. Similarly, *in vivo*, primary fibers express embryonic and slow MyHC, whereas secondary fibers express first embryonic and then neonatal MyHC. These results suggest that primary and secondary fibers derive from distinct myogenic precursors. Whether these precursors comprise distinct lineages is currently under investigation using retroviral lineage labeling. (Supported by NIH, MDA, March of Dimes).

F 308 MECHANISMS OF SLOW FIBER LOSS IN A MOUSE FAST-TWITCH MUSCLE, Keith Condon[†], Laura Silberstein[§], Helen M. Blau[§] and Wesley J. Thompson[¶], [†]Dept. Cell Biology & Neuroscience, Univ. of Texas Southwestern Medical Center, Dallas, TX 75235, [§]Dept. Pharmacology, Stanford Univ. School of Medicine, Stanford, CA 94305 and [¶]Dept. Zoology, Univ. of Texas, Austin, TX 78712

The extensor digitorum longus (EDL) muscle in mice undergoes a progressive loss of slow fibers during early adult life, from ca. 160 fibers at 18 days of age to 0-9 fibers at 3-6 months. Immunocytochemistry using antibodies to myosin heavy chain isoforms shows that during this period many fibers which express slow myosin also co-express fast 2a myosin, suggesting the slow fibers are being transformed to fast type 2a fibers. Based on the response to denervation, this transformation is innervation dependent. Two mechanisms can be hypothesized to account for this transformation: 1) this muscle possesses no slow motor neurons and the slow fibers, innervated by fast motor neurons, are converted to fast; 2) the slow fibers are selectively innervated by slow motor neurons but these slow motor neurons transform to fast and in turn convert the fibers they innervate. The first hypothesis predicts mixed motor units, the slow fibers being distributed among the fast units based upon their relative frequency (approximately 5-6 fibers per unit). The second hypothesis predicts slow motor units in varying stages of transformation to a fast 2a type. Individual motor units in C57BL/6J mice between the ages of 20-30 days were isolated from teased ventral roots and identified using the glycogen depletion technique. Biasing our sample to units of slow twitch rise time, we found 3 units where greater than 90% of the fibers stained for slow myosin. Each of these units contained fibers transforming to 2a. In contrast, fast twitch units were homogeneous with respect to myosin isoform (either 2a or 2b). Thus, slow fibers in mouse EDL appear to be selectively innervated and their loss results from the transformation of slow units to type 2a.

F 309 A NEW MUSCLE PROTEIN GENE IN *DROSOPHILA*, Richard M. Cripps* and John C. Sparrow, Department of Biology, University of York, Heslington, York. YO1 5DD U.K. *Present address: Department of Biology and Molecular Biology Institute, San Diego State University, San Diego, CA 92182-0057.

We have carried out a mutagenesis screen to identify new EMS-induced dominant flightless mutations of *Drosophila*. The mutants isolated mapped to three regions of the genome. Some were in the muscle myosin heavy-chain gene, Mhc, and some in the indirect flight muscle-specific actin gene, Act88F. The remaining five mutations all mapped to the tip of the third chromosome left arm, in a region previously unidentified as containing a muscle protein gene. We have named these mutants *lethal (3) laker, l(3)lkr*.

All five *l(3)lkr* mutants show dominant flightless and recessive lethal phenotypes. Trans-heterozygote combinations of each of the mutants are lethal, which indicates that they are alleles of the same gene. Adult wild-type heterozygotes have an abnormal myofibrillar structure very similar to that of mutants in other muscle protein genes: the hexagonal arrangement of filaments is normal towards the centre of the myofibril, but becomes progressively more disrupted at the periphery. Heteroallelic mutant embryos develop normally, but are unable to break out of the eggshell. They show a highly abnormal body wall muscle structure.

We have mapped the gene to five polytene chromosome bands at 62A10,B1-B2,4. Wild-type heterozygotes for a deficiency in this region show the dominant flightless and myofibrillar defect phenotypes similar to heterozygotes for both *l(3)lkr* mutants and deficiencies of other muscle protein genes.

There is clearly strong evidence that *l(3)lkr* is a major muscle protein gene. The next stage in our analysis of *l(3)lkr* will be to clone the gene, identify the product, and determine the changes that have taken place in the mutants.

F 310 THE *ERECT WING* LOCUS OF *DROSOPHILA MELANOGASTER* IS IMPORTANT FOR PROPER DEVELOPMENT OF THE NERVOUS SYSTEM AND INDIRECT FLIGHT MUSCLES, Susan M. DeSimone and Kalpana White, Department of Biology, Brandeis University, Waltham, MA, 02254

The *erect wing* (*ewg*) gene in *Drosophila melanogaster* is required for development of the indirect flight muscles (IFM), and for viability. Mutations in this locus result in: missing or severely reduced IFMs, embryonic lethality, aberrant axonal projections in the developing embryonic CNS, or hypoactivity in the adult. In order to understand the mechanism of gene action, a molecular analysis was begun. Transcription from *ewg* is complex with respect to size, temporal, and spatial pattern. Several cDNAs have been cloned and sequenced. This analysis has revealed that there is differential splicing in both coding and non-coding regions of the transcripts. A search of protein data bases has not revealed homology to any previously identified protein. *In situ* hybridization experiments show that the gene is expressed in the CNS throughout development. Transcripts were also detected in the developing PNS in embryo, larval and pupal stages but were not detected in adult PNS structures. Finally, transcripts are present in the myocytes that will form the IFMs. However, no transcripts were detected in muscle cells at any stage of development. An antibody has been raised against an open reading frame that contains common and cDNA-specific sequences. Western blot analysis suggests that there is one dominant protein species, and perhaps several minor species. Preliminary immunocytochemical studies have shown that the antigen(s) is nuclear and present in the pattern predicted by *in situ* hybridization studies. Work in progress will address the expression of *ewg* in flies that carry the mutation effecting IFM development.

F 311 COMMITMENT AND DIFFERENTIATION OF INJECTED CLONAL MYOBLASTS IN VIVO. Joseph X. DiMario and Frank E. Stockdale. Stanford University School of Medicine, Stanford, California 94305-5306.

The role of myoblast commitment in the origin of muscle fiber type diversity in avian skeletal muscle is being examined *in vivo*. Fiber type heterogeneity, as demonstrated by differential myosin heavy chain (MHC) gene expression, is influenced by extrinsic factors such as innervation, hormones, and activity. However, developing avian skeletal muscle contains myoblasts committed to the formation of myotubes which exclusively synthesize fast, slow, or both fast and slow MHCs in clonal cultures. To more critically evaluate the extent to which fiber type diversity *in vivo* is established by autonomous myoblast commitment and by modulatory factors such as innervation impinging on maturing myofibers, myoblasts were allowed to differentiate under several sets of developmental signals *in vivo*. Clonal myoblasts which form myotubes expressing fast, slow, and fast/slow MHCs in differentiated cultures were isolated from embryonic, fetal, and adult skeletal muscle, transfected with a reporter gene, and injected into musculature at several developmental stages. These myoblasts contributed to the host musculature by forming muscle fibers which are often morphologically distinguishable from host fibers. Preliminary analysis of MHC content of fibers formed by injected myoblasts indicates that these myoblasts are committed to the formation of specific fiber types *in vivo* despite their differentiation in a developmentally heterologous environment.

F 312 THE TEMPORAL APPEARANCE OF SATELLITE CELLS DURING DEVELOPMENT,
Jeffrey L. Feldman and Frank E. Stockdale, Dept. of Medicine, Stanford University School of Medicine, Stanford, CA 94305-5306.

Myogenesis is characterized by a continuum of fiber formation from myoblasts, the precursors to fiber formation, that begins early in embryogenesis and continues through adult life as fibers are regenerated. However, there is a discontinuum in myoblast types. When myoblasts of differing types first appear or disappear has only been partially resolved, especially with regard to satellite cells (adult myoblasts). Embryonic myoblasts can be isolated from limb buds at their earliest formation, but rarely after 7-8 d of development. They are replaced by fetal myoblasts that appear late in the first week of development. It is not known when satellite cells first appear and replace fetal myoblasts. To determine when this occurs, three distinguishing features *in vitro* between satellite and fetal myoblasts were used- the type of fibers they form, the size of the myoblasts in early cultures, and the rate at which they initiate DNA synthesis or become activated. We have demonstrated that satellite cells isolated from adult chicken PM form only fast myotubes, while fetal myoblasts from the same muscle form myotubes that express fast and slow myosins in prolonged primary cultures. Using immunofluorescence and competitive ELISA, the production of slow myosin heavy chain can be determined with a monoclonal antibody (S46) in same aged cultures derived from myoblasts isolated from different developmental stages. Fetal and adult myoblasts also differ significantly in size in early cultures, with fetal myoblasts averaging almost 3X the length of satellite cells even after 24hr *in vitro*. Therefore the appearance of a smaller sized population of myoblasts indicates the appearance of satellite cells in a muscle. Further, using ^{3}H-TdR uptake to measure DNA synthesis, fetal cells become active within 8hr of plating and peak at 24hr, while satellite cells are not activated 32hr after plating and peak at 64hr. Using these criteria, it has been determined that fetal myoblasts disappear and a distinct population of satellite cells appears prior to hatching.

F 313 *Abstract Withdrawn*

F 314 VIMENTIN mRNA LOCATION IN MUSCLES AND FIBROBLASTS DETECTED BY CONFOCAL MICROSCOPY.
L. Cripe* and A.B. Fulton. *Pediatrics, Biochemistry, Univ. of Iowa, Iowa City, IA .

The cytoskeleton is a complex fibrillar structure in eukaryotic cells. Many cytoskeletal proteins make stable associations with the Triton-resistant cytoskeleton during translation: i.e. undergo "cotranslational assembly". Vimentin, an intermediate filament protein, assembles both cotranslationally and after translation. Others have shown that mRNAs for some cytoskeletal proteins are localized in cells; vimentin mRNA is perinuclear in young muscle cells. These results suggest that mRNA for cytoskeletal proteins assembling cotranslationally is located in cells at a site appropriate for assembly. To test this hypothesis we detected vimentin mRNA in fibroblasts and skeletal muscle cells by *in situ* hybridization. Chicken muscles and fibroblasts were fixed, hybridized with a biotinylated DNA probe to a vimentin specific sequence, and the probe detected by confocal microscopy and fluorescently labeled avidin. We observed three patterns of mRNA. In fibroblasts, vimentin mRNA was perinuclear. In muscle, vimentin mRNA was bipolar in myoblasts and diffuse in young myotubes. A newly developed micromass method will allow us to extend these studies to mature myotubes, in which vimentin is located at costameres. These studies increase our knowledge of the role of mRNA location in cotranslational assembly. Cotranslational assembly may prove most important for maintaining differentiated cytoskeletal structures such as the myofibril.

F 315 CGRP, NERVE ACTIVITY AND SYNAPTOGENESIS, Guido Fumagalli, Jacopo Andreose, Carlo Sala, Sergio Balbi and Terje Lømo*,CNR Ctr Cytopharmacol, Dept Pharmacol, Univ of Milan, Italy and * Inst of Physiol, Oslo, Norway.

CGRP, a peptide that induces expression and insertion of AChR in chick muscle cells in vitro, is present at the motor nerve endings of the rat end-plates at birth only. To determine whether CGRP expression reflects a function required during synaptogenesis, the tibialis nerve of adult rats was crushed and CGRP immunoreactivity was followed during reinnervation of the soleus muscles. At day 5, 60% of the endplates were contacted by growing axons and almost all of them were positive for CGRP. The same endplates were not stained by antibodies against Secretogranin I and II, two secretory proteins which are expressed at the neonatal rat neuromuscular junction. The maximal proportion of peptide containing endplates (80%) was reached at day 10 corresponding to a time when most ot the original end plates had been reinnervated. Later on the proportion of CGRP containing neuromuscular junctions gradually decreased and was undistinguishable from controls three weeks later. To determine whether this transient accumulation of CGRP immunoreactivity was due to either reexpression of embryonic features associated with nerve regeneration or the loss of activity-dependent intercellular signal(s) between nerve and muscle, intact endplates were examined after chronic block of evoked nerve activity. Accumulation of CGRP at the nerve endings and of its mRNA in the soma of the motor neurons occurred as long as evoked activity of the sciatic nerve was blocked with TTX. Nerve endings could be depleted of their CGRP content by electrical stimulation; one week after TTX withdrawal, peptide content was still abundant periferally but its mRNA had returned to control values. Similar results were obtained when Botulinum toxin (BoTx) was injected subcutaneously. With both TTX and BoTX extensive sprouting of the nerve ending and enlargment of the postsynaptic area were prominent. These data suggest that CGRP plays a specific role during synaptogenesis and that its expression is controlled by activity.

F 316 THE EXPRESSION OF H36 AND DESMIN DURING PRIMARY AND SECONDARY MYOGENESIS *IN VIVO*, Mindy George-Weinstein, Jacquelyn Gerhart, Rachel Foster* and Stephen Kaufman,* Department of Anatomy, Philadelphia College of Osteopathic Medicine, Philadelphia, PA 19131 and *Department of Cell and Structural Biology, University of Illinois, Urbana, IL 61801

The membrane protein, H36, is a marker of myogenic precursor cells *in vitro* (see Kaufman and Foster, abstract, this meeting). To determine when H36 is expressed relative to the terminal differentiation of muscle cells *in vivo*, cryosections of days 10 to 18 rat embryos were labeled by the immunofluorescence localization method with monoclonal antibodies against H36, desmin and skeletal muscle myosin. These proteins were not detected in the compact somites of day 10 embryos. By 11 days of development, desmin and myosin, but not H36, were expressed in cells of the dermomyotome. On the 12th day of development, the H36 antibody faintly labeled cells of the dermomyotome. Only desmin was detected in 11 and 12 day limb buds. The developing muscle masses of the day 14 hindlimb contained cells which expressed desmin but not H36 or myosin, as well as cells which expressed all 3 proteins. Cells which expressed H36 also expressed myosin during the period of primary myogenesis. Labeling with H36 remained weak until the 17th day of development. Between days 17 and 18, at the onset of secondary myogenesis, there was a dramatic increase in the intensity of labeling with the H36 antibody. At this time, cells that express desmin and H36 but not myosin were present in the hindlimb. These results indicate that two populations of myoblasts arise during the development of the limb and these can be distinguished by the expression of H36. Primary myoblasts express desmin but not H36 prior to terminal differentiation. Both desmin and H36 are expressed in myoblasts that develop towards the end of primary myogenesis *in vivo*, and presumably these develop into secondary myofibers.

F 317 MITOCHONDRIAL DEVELOPMENT DURING CARDIAC DIFFERENTIATION: A MOLECULAR AND BIOCHEMICAL STUDY IN VIVO AND IN VITRO IN THE RAT, Mariana Gerschenson, Robert L. Low, James Loehr, Laurie Miller and Kathryn L. Houmiel, Department of Pathology, University of Colorado, School of Medicine, Denver, CO 80262

The mitochondria (mt) of the cardiac myocyte contain multiple copies of a small circular DNA genome that encodes a few essential polypeptide components of the oxidative phosphorylation pathway of the inner membrane. Our goal is to study the control of mtDNA replication during cardiac development. Two model systems have been developed: 1) A cultured cell line of rat cardiac myoblasts which provide cells at different stages of cell growth and differentiation, and 2) an *in vivo* system which follows cardiac growth in the rat from 18th day of gestation through adulthood. In this study, the levels of mtDNA are being quantitated relative to the activities of the respiratory complex enzymes, cytochrome b, DNA polymerase-gamma (the mtDNA replicase) and the mt endonuclease. The latter enzyme is a phospholipid stimulated DNase that selectively nicks a site just upstream from the origin of mtDNA replication. Preliminary data suggest that *in vivo* most mt enzymes peak within the first 48 hours after birth relative to both mt protein concentrations and mtDNA levels. Similar changes occur during differentiation of myotubes *in vitro*. These data indicate that there is a relationship between the expression of mt genes and cardiac developmental changes soon after birth.

F 318 DOMAINS OF CLUSTERED ACETYLCHOLINE RECEPTOR IN HYBRID MYOTUBES, Herman Gordon†, Evelyn Ralston, and Zach W. Hall. Dept. of Physiology, Univ. of Calif., San Francisco, CA 94143. †Present address: Dept. of Anatomy, Univ. of Arizona, Tucson, AZ 85724

Cultured myotubes of the C2 mouse muscle cell line exhibit spontaneous clustering of acetylcholine receptor (AChR) on their surface. Two variant sublines, S27 and 1R$^-$, fail to exhibit such clustering for different reasons. S27 was originally selected on the basis of a defect in glycosaminoglycan biosynthesis but was later found to not cluster AChR. S27 makes normal amounts of AChR, both internally and on the cell surface. 1R$^-$ makes mRNA for the AChR subunits, but synthesis of the alpha subunit is decreased by more than 90%, resulting in a reduced level of AChR. When co-cultured, the two variant lines fuse spontaneously into hybrid myotubes. AChR clusters comparable in size and morphology to those in wild type myotubes form in the hybrids. In myotubes containing only a few 1R$^-$ nuclei, the AChR clusters appear near the 1R$^-$ nuclei. In hybrid myotubes containing mostly S27 and only a few avian nuclei, mouse AChR from the S27 nuclei is found clustered near the avian nuclei. We suggest that AChR made by S27 nuclei diffuses into traps near the 1R$^-$ or avian nuclei and that glycosaminoglycans may play a role in the trapping mechanism. Such a dissection of nuclear functions provides a useful model of the specialization that occurs in vivo near the neuromuscular junction.

F 319 DYSTROPHIN EXPRESSION IN FUSION-ARRESTED MUSCLE CULTURES. Orla Hardiman, Robert M. Sklar, Robert H. Brown, Jr., The Cecil B. Day Laboratory for Neuromuscular Research, Building 149 13th St., Charlestown, MA 02129

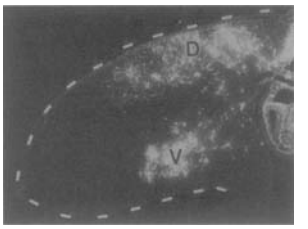
The addition of the glucocorticoid methylprednisolone to most mixed or clonal normal muscle cultures increases myogenesis as assessed by the fusion index at 10 days after initiation of fusion. By contrast, in some normal and most dystrophic muscle cultures glucocorticoids inhibit fusion. We have observed that some normal myoblasts which are serum-deprived but fusion-arrested by glucocorticoids express dystrophin but may fail to express myosin heavy chain. Analogously, cultures of Becker dystrophy muscle from a patient with a deletion of exons 45-47 of the dystrophin gene express dystrophin of subnormal size independently of fusion and myosin heavy chain expression. In this experimental paradigm, the expression of dystrophin and myosin heavy chain is independently regulated. Dystrophin thus appears to be another example of a muscle-specific protein which does not require fusion for expression (Endo T, Nadal-Ginard B, Cell 49:515-526, 1987).

F 320 FETAL AND ADULT MYOBLASTS EXHIBIT DIFFERENT PROGRAMS OF MYOSIN ISOFORM PROGRESSION, Rebecca S. Hartley, Everett Bandman* and Zipora Yablonka-Reuveni, Department of Biological Structure, University of Washington, Seattle, WA 98195 and *Department of Food Science and Technology, University of California, Davis, CA, 95616

The expression of early myosin isoforms by cultured myoblasts from avian pectoral muscle was studied. Using isoform specific monoclonal antibodies in indirect immunofluorescence assays we found that terminally differentiated fetal (embryonic day 10) myoblasts which simultaneously express ventricular and embryonic fast myosin heavy chain are detectable by 24 hrs post-plating. Contrarily, adult myoblasts initially express ventricular myosin only, but not until the third day of culture. Beginning on the fifth day in adult cultures, myotubes and occasional mononucleated cells expressing embryonic myosin are also present. Likewise, passaged adult myoblasts and myoblasts isolated from cold-injured adult muscle also display a lag between expression of ventricular and embryonic myosin. Since few embryonic myosin-positive myoblasts are observed in adult cultures ($<1\%$) we investigated whether fusion is a prerequisite for embryonic myosin expression in adult myogenic cultures. To block fusion but not terminal differentiation, EGTA was added to fetal and adult cultures 24 hrs post-plating. EGTA had no effect on ventricular and embryonic myosin expression in fetal cultures, other than a slight reduction in myosin levels. In contrast, EGTA blocked expression of embryonic but not ventricular myosin in adult cultures. These results suggest that fetal and adult myoblasts from avian pectoral muscle undergo distinct developmental programs upon terminal differentiation in culture.
(Supported by NIH)

F 321 FLUORESCENT TRACING OF MYOGENIC CELL-MIGRATION IN THE CHICK LIMB BUD. Kensuke Hayashi and Eijiro Ozawa. Division of Cell Biology, National Institute of Neuroscience, NCNP, 4-1-1,Ogawahigashi, Kodaira, Tokyo 187, Japan

The first step of pattern formation of the limb musculature is the formation of dorsal and ventral myogenic masses in limb buds. However, the migration pattern of myogenic cells in this process has not been well documented. We developped a new method to detect myogenic cells in chicken limb bud, and described their migration. We labeled somitic cells by injecting a fluorescent dye (DiI or DiO) into the cavity of a somite. Myogenic cells derived from it can be observed with a fluorescent microscope. At first, the labeled cells diffusely distributed in the proximal region of the limb bud. At about st.21, the pre-chondrogenic core region became free of labeled cells, and the dorsal and ventral masses were formed (Figure). At st. 26, the labeled cells differentiated and expressed myosin. This labeling method has some advantages over the commonly used chicken/quail nuclear marker system. It does not include surgical operations that may disturb the normal development. And the cells are labeled intesely enough to be detected in a whole mount preparation.



F 322 EXPRESSION OF JAW-SPECIFIC MYOSIN HEAVY CHAIN AND TROPOMYOSIN ISOFORMS IN REGENERATES OF CAT JAW-CLOSING MUSCLE, Joseph F.Y. Hoh, Rosa Bestak, Lucia H.D. Kang and Ajita Rughani, Department of Physiology, University of Sydney, NSW, 2006, Australia.

Jaw-closing muscles of the cat contain two types of fibres: superfast fibres co-expressing superfast myosin and a jaw-specific tropomyosin, and slow fibres which co-express a jaw-specific isoform of slow myosin and α and β tropomyosins (Hoh et al., 1989, Proc Aust Physiol Pharmacol Soc 20, 191P-192P). Jaw muscle fibres are committed to express a distinct subset of myofibrillar proteins from those of limb muscle fibres. Regenerated jaw muscle cells express superfast myosin even in the absence of innervation (Hoh & Hughes, 1990, Muscle & Nerve, in press), but nerves to limb fast and slow muscles, through their impulse patterns (Hoh et al., 1989, Proc Aust Physiol Pharmcaol Soc 20, 190P), can modulate the expression of superfast and jaw-specific slow myosins in jaw muscle regenerates (Hoh & Hughes, 1988, J Musc Res Cell Motil, 9, 59-74). In this work, we produced monoclonal antibodies against superfast myosin heavy chain and jaw-specific tropomyosin, and used them to characterize the expression of these proteins in fibres of cat jaw muscles regenerated in limb muscle beds. Using immunocytochemical techniques, we show that jaw-specific tropomyosin is co-expressed with superfast myosin while β tropomyosin is co-expressed with jaw-specific slow myosin in jaw muscle regenerates innervated by limb muscle nerve fibres.

F 323 SECONDARY MYOTUBE FORMATION IN THE SOLEUS MUSCLE OF THE MOUSE FOLLOWING FETAL MOTOR NEURON ABLATION, Donna S. Hughes and Marcia Ontell, Department of Neurobiology, Anatomy and Cell Science, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261

Fetal mouse lumbosacral spinal cord was laser ablated, permitting the evaluation of the effects of the absence of nerve on myogenesis. Soleus muscles (S) of 20-day in utero (20E) mice whose cords were ablated at 14 days in utero (14E20E) were examined with EM and morphometry. At ablation, S contained 1° but no 2° myotubes and no neuromuscular junctions. 14E20E S contained 477±19 fibers at its widest girth (WG), more than the ~300 1° myotubes in normal 14-day in utero S, but fewer than the 728±14 fibers at the WG of normal 20E S. That the additional fibers which are present in 14E20E S are a result of 2° myotube formation rather than enhanced 1° myotube formation is evidenced by numerous clusters. To determine whether the reduced number of fibers found at the WG was a result of a decrease in the total number of fibers found in 14E20E S or whether it reflected the fact that many fibers were too short to pass through the WG, the total number of fibers at 14E20E was determined in closely spaced, ultrathin sections along the length of S. 14E20E S contained ~70% of the myofibers of normal 20E S (20E S contained ~837 fibers). In the absence of innervation, mammalian muscle produces significant numbers of 2° myotubes. Contractile protein gene expression in 14E20E S is being studied with in situ hybridization. Supported by NICHD 25630, MDA, and March of Dimes.

F 324 RETROVIRAL LINEAGE MARKING IN LIMB MUSCLE OF POSTNATAL RATS: MYOBLASTS CONTRIBUTE PROGENY TO ALL FIBER TYPES. Simon M. Hughes and Helen M. Blau, Department of Pharmacology, Stanford University School of Medicine, Stanford, CA 94305.

Muscle fibers specialized for fast or slow contraction are arrayed in characteristic patterns in diverse muscles. This diversity could derive from distinct precursors (myogenic lineages) or be specified at the level of the differentiated multinucleated muscle fiber. To distinguish between these possibilities, we first characterized the distribution of fiber types within muscles of the limb using a battery of monoclonal antibodies to myosin heavy chain isoforms (Webster et al., 1988, Cell 52, 503-513). The cellular origin of diverse fiber types was determined using a retroviral vector to label cells and monitor their fate. This method permits an analysis of the progeny of a single myoblast, or clone, *in vivo* (Hughes and Blau, 1990, Nature 345, 350-353). Our results show that myoblasts in postnatal rat muscle contribute progeny to fibers of diverse fast and slow types. This evidence of plasticity of myogenic precursors was corroborated in experiments using cells that were labeled in tissue culture and injected into diverse rat muscles: these cells also contributed to all fiber types. Taken together, these results demonstrate that the myogenic precursors in postnatal muscle do not comprise distinct lineages that are restricted in their choice of fiber type or pattern of gene expression. (Supported by NIH, MDA, March of Dimes).

F 325 MYOSIN ISOFORM mRNA CO-EXPRESSION IN MUSCLE FIBERS DURING TRANSFORMATION BY CHRONIC STIMULATION. Jamil Jacobs-El, Jennifer Kim, David J. Dix and Brenda R. Eisenberg, Department of Physiology, MC 901, University of Illinois, Box 6998, Chicago, IL 60680.

Chronic stimulation via an implanted electrode alters isomyosin heavy chain (MHC) expression and provides a model for examining transcriptional regulation by single myonuclei in rabbit tibialis anterior muscle. After 2 post-surgical days, a stimulator was turned on at 10 Hz for 24 h/d with sacrifice of the rabbit from 4 - 21 d later. Fiber type composition was assayed on serial frozen sections by in situ hybridization with biotinated isoform-specific riboprobes (cDNAs from Umeda, Wittinghofer and Maeda), by immunochemistry (with slow-specific antibody from Zak), and with histochemistry for ATPase activity and oxidative enzymes (NADH). Isomyosin switching is not synchronized among individual fibers from the population. About 5% of control fibers co-express both fast and slow MHC mRNAs apparently within one myonuclear domain. Transforming fibers co-express both MHCs more frequently. By 4 d of stimulation most fibers show increases in oxidative capacity. Transforming fibers with low amounts of slow protein have higher slow mRNA levels than normal slow fibers. Not until 10 d do the first fibers show some slow MHC gene expression and these all have very high oxidative capacity. We conclude myonuclei can simultaneously express two MHCs and that oxidative enzymes are up-regulated before slow MHC mRNA expression. MDA support.

F 326 ASSEMBLY OF TRANSVERSE TUBULES (TTs) AND TRIADS IN RABBIT SKELETAL MUSCLE DEVELOPING IN SITU. Annelise O. Jorgensen, Shaohua Yuan and Wayne Arnold, Department of Anatomy, University of Toronto, Toronto, Canada M5S 1A8

The temporal appearance and subcellular distribution of the 1,4-dihydropyridine receptor (DHPR), the ryanodine receptor (RR) and TS28 (a marker of adult (Jorgensen et al J. Cell Biol. 110: 1173 (1990) and forming TTs (Yuan et al, ibid 110: 1187(1990)) were compared by double immunofluorescence labeling of cryosections of developing rabbit skeletal muscle during the formation of TTs and triads. Images of the results were obtained by conventional and confocal microscopy. The results showed that the DHPR and RR first appear and continue to codistribute in discrete foci in the outer zone of the cytosol of all myotubes before the formation of TTs begin on fetal d 17 and until 1-2 days after birth. By contrast on fetal d 17 TS28 like TTs first appear in a few myotubes where it is confined to discrete foci at the cell periphery. Later TS28 is distributed in wavy strands originating from the sarcolemma and 1-2 days after birth it is distributed in a chicken wire-like pattern throughout the cytosol. Assuming that TS28 represents the forming TTs, the results presented are consistent with the idea that DHPRs complexed to RRs accumulate in the cytosol before the onset of TT formation but are only incorporated into discrete regions of TTs subsequent to the onset of TT formation. The codistribution of DHPR and RR throughout development suggest that DHPR incorporation into TTs also results in the formation of a junctional complexes between TTs and the sarcoplasmic reticulum (i.e. triads).

F 327 *IN VITRO* DEVELOPMENT OF PRECURSOR CELLS IN THE MYOGENIC LINEAGE IS INDEPENDENT OF MYOD1 AND MYOGENIN. Stephen J. Kaufman and Rachel F. Foster, Department of Cell and Structural Biology, University of Illinois, Urbana, IL 61801.
Expression of the muscle-specific membrane protein H36 and intermediate filament protein desmin, detected by immunofluorescence, was used to identify cells at distinct stages in the skeletal myogenic lineage. These proteins were coordinately expressed in cultures of rat hindlimb myoblasts from 17 and 19 day fetuses and newborn pups, and in satellite cells from juveniles. Both $H36^+$ and $desmin^+$ cells were present in cultures from 15 day fetal hindlimb, but desmin expression was more prevalent: $H36^-/desmin^+$ myoblasts predominate during this early stage and develop into $H36^-/desmin^+$ myotubes. These results indicate that two populations of myoblasts can be distinguished based on expression of H36 and their temporal appearance during embryonic development, and that H36 is a marker for satellite cells. H36 was not initially expressed in limb bud cells from 12 day embryos, and only 1% expressed desmin, but when these cells were serially passaged every 3 - 4 days $H36^+/desmin^-$, $H36^+/desmin^+$ and $H36^-/desmin^+$ cells developed. There was a progressive increase in the frequency of $H36^+$ cells, with 75% of cells positive by passage 6. The maximum frequency of cells that expressed desmin occurred in passage 5. These results demonstrate that precursors to the cells that express H36 and desmin are present in the 12 day embryo hindlimb bud and that the transition from $H36^-/desmin^-$ precursors to cells with a myogenic phenotype can occur *in vitro*. Expression of MyoD1 and myogenin did not occur in these cells, indicating that the initial expression of H36 and desmin in the myogenic lineage precedes and/or is independent of these regulatory proteins. The conversion of precursor cells in the 12 day limb bud to a more advanced stage of development serves to define additional cells in the myogenic lineage and affords the opportunity to study how they are regulated.

F 328 HEAVY CHAIN HOMODIMERS ARE THE THERMODYNAMICALLY STABLE FORM OF AVIAN FAST SKELETAL MYOSINS. Bruce Kerwin and Everett Bandman, Department of Food Science and Technology, University of California, Davis, CA, 95616.
Using a double antibody sandwich ELISA we examined the heavy chain isoform composition of myosin molecules isolated from chicken pectoral muscle during different stages of development. At 2 days and 40 days post-hatch, when multiple myosin heavy chain isoforms are being synthesized, we detected no heterodimeric myosins, suggesting that myosins are homodimers of the heavy chain subunit. In order to determine the basis for the homodimeric composition of avian skeletal myosin, chymotryptic rod fragments of embryonic, neonatal, and adult myosins were prepared and equimolar mixtures of embryonic and neonatal rods and neonatal and adult rods were denatured in 8 M guanidine. The guanidine denatured myosin heavy chain fragments were either dialyzed or diluted into renaturation buffer and reformed dimers which were electrophoretically indistinguishable from native rods. Analysis of these renatured rods using double antibody sandwich ELISA showed them to be predominantly homodimers of each of the isoforms. Although hybrids between the different heavy chain fragments were not detected, exchange was possible under these conditions since a mixture of biotinylated neonatal rods and fluoresceinated neonatal rods formed a heterodimeric biotinylated-fluoresceinated species upon renaturation. Therefore, we propose that homodimers are the thermodynamically stable form of skeletal muscle myosin isoforms and that there is no need to invoke compartmentalization or other cellular regulatory processes to explain the lack of heavy chain heterodimers *in vivo*. (Supported by NIH grant AG08573 and a grant from the MDA).

F 329 DEVELOPMENT OF CONNECTIN (TITIN) AND NEBULIN IN CULTURED CARDIAC AND SKELETAL MUSCLE CELLS, Masatoshi Komiyama[1], Zhen-Hua Zhou[1,2], Koscak Maruyama[3] and Yutaka Shimada[1], [1]Department of Anatomy and Cell Biology, School of Medicine, Chiba University, Chiba 280, Japan; [2]Shanghai Institute of Physiology, Chinese Academy of Sciences, Shanghai 20031, China; [3]Department of Biology, Faculty of Science, Chiba University, Chiba 260, Japan.
Development of connectin and nebulin prior to and during myofibril assembly was examined by fluorescence microscopy of embryonic chick cardiac and skeletal muscle cell cultures. In premyofibril areas, proteins constituting I-Z-I brushes (α-actinin, actin and troponin C) were detected first, then nebulin, and finally connectin and myosin. The finding that I-Z-I-like complexes were formed earlier than the appearance of nebulin and connectin seems to indicate that these giant myofibrillar proteins are not necessary for the initial assembly of I-Z-I complexes. We further observed that connectin formed a mature striated pattern after α-actinin, and nebulin after actin and much after α-actinin. This appears to indicate that these large proteins are not a preformed scaffold(s) of myofibrils upon which sarcomeric proteins are accumulated. We propose that there exists a two-step/time-lag development of connectin filaments: the firstly expressed connectin (the embryonic form) deposited in I-Z-I structures would be replaced by the secondly expressed connectin (the adult form) upon the start of sarcomeric differentiation. Another possibility is that the former (the I-Z-I associated form) will fuse with the latter (the myosin associated form), thereby playing some role in the integration of myosin filaments with the preexisting I-Z-I brushes.

F 330 CHARACTERIZATION OF AN EMBRYONIC ISOFORM OF VASCULAR SMOOTH MUSCLE MYOSIN HEAVY CHAIN BY cDNA CLONING AND PROTEIN ANALYSIS

Makoto Kuro-o, Ryozo Nagai, Ken-ichi Nakahara, Hidetsugu Tsuchimochi, Yoshio Yazaki. 3rd Department of Internal Medicine and Department of Laboratory Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo, Tokyo, Japan.

Two types of smooth muscle myosin heavy chain (MHC) isoforms, SM1 and SM2, are generated through developmentally regulated alternative RNA splicing from a single gene. We previously demonstrated that rabbit vascular smooth muscles at the embryonic and neonatal stages express a third type of MHC isoform distinct from SM1 or SM2. In this study we have isolated a cDNA clone encoding an embryonic smooth muscle MHC isoform, SMemb, from a rabbit pup aorta cDNA library, and characterized its protein profiles using specific antibody raised against the predicted carboxyl terminus. SMemb, originating from an MHC gene distinct from that of SM1/SM2, showed 72 % homology with SM1 or SM2 at the nucleotide level. At the protein level, SMemb corresponds to 200 kDa MHC and is clearly distinguishable from 196 kDa nonmuscle MHC as determined by immunoblotting. SMemb mRNA is down-regulated with development but consistenly expressed in adult brain and in the cultured smooth muscle cell. We have confirmed the identity of brain MHC and SMemb by isolating an MHC cDNA clone from a brain cDNA library. These results indicate that SMemb is an important molecular marker for studying the smooth muscle cell differentiation as well as the neuromuscular development.

F 331 EFFECTS OF GROWTH HORMONE ON IGF-1 AND MYOSIN HEAVY CHAIN mRNA CONCENTRATIONS IN RAT MUSCLE, Paul T. Loughna and Peter C. Bates, Royal Veterinary College, Royal College St. London NW1 OTU and London School of Hygiene and Tropical Medicine, 4 St Pancras Way, London NW1 2PE.

The aim of this study was to examine the role of growth hormone in regulating muscle phenotype. Male rats were hypophysectomised (Hx) and 14 days later divided into three groups. The first group were killed (Hx0) and the other two groups were treated with saline (Hx7) or hGH (Hx7GH). A fourth group of animals were non-hypophysectomised controls (Hnorm). Total RNA was extracted from the gastrocnemius muscle using a modified hot phenol method. Concentrations of the mRNAs for IGF-1 and the type I, type IIa, type IIb, embryonic and neonatal myosin heavy chains (MHCs) were estimated by Northern and slot blot hybridisation using isoform specific oligonucleotides. Muscle IGF-1 mRNA concentrations fell in response to Hx though this fall was considerably greater in Hx7 than Hx0. Levels of MHC mRNAs did not generally differ greatly between Hx0 and Hnorm. However in Hx7 rats concentrations of type I and type IIa MHC mRNAS were dramatically reduced and in contrast type IIb MHC mRNA was increased. The expression of the embryonic and neonatal MHC genes also exhibited opposite responses to Hx. Levels of all mRNAs were restored towards Hnorm levels in Hx7GH.

F 332 EXPRESSION AND DISTRIBUTION OF NA^+ CHANNELS IN DEVELOPING RODENT SKELETAL MUSCLE, M. Lupa, D. Krzemien, K. Schaller and J. Caldwell, Department of Cellular and Structural Biology, University of Colorado School of Medicine, Denver, CO 80262

It has recently been shown that voltage-activated sodium channels (NaCh) are concentrated 10-25X in the postsynaptic membrane at the adult neuromuscular junction. We have used the loose patch clamp technique to investigate the development of this NaCh concentration in postnatal mouse muscle. The muscle employed in these experiments was the ear flexor muscle *levator auris longus*, a thin and sheet-like muscle that is advantageous for physiological recording. During the first days after birth the density of NaCh is low and nearly uniformly spread across the muscle cell surface. From the second through the fourth postnatal week, however, NaCh density at the endplate increases about 10-fold, while NaCh density in the extrajunctional membrane (100-200μ away) approximately doubles. At birth about 80% of the NaCh are resistant to 300nM tetrodotoxin (TTX). The percentage of TTX-sensitive channels at the endplate increases nearly linearly until the adult stage, when only 8% of the Na^+ current is insensitive to the blocking action of 300nM TTX. We conclude that the entire increase in NaCh density at the endplate can be attributed to localized insertion or trapping of TTX-sensitive Na^+ channels. We are presently using RNAase protection assays to study mRNA levels for these channels during development, in order to understand the role of NaCh gene regulation in this process.

F 333 DEVELOPMENTAL REGULATION OF METABOLIC ENZYME GENE TRANSCRIPTS IN EMBRYONIC MOUSE MUSCLE. Gary Lyons*, Yvonne Edwards°, Jean-Claude Perriard+ and Margaret Buckingham*, *Dept. of Molecular Biology, Pasteur Inst., Paris, France; °MRC Human Genetics Unit, Univ. College of London, London, U.K.; +Inst. of Cell. Biology, Swiss Fed. Inst. of Technology, Zurich, Switzerland.
Three metabolic enzymes, B-creatine kinase (BCK), M-creatine kinase (MCK) and carbonic anhydrase III (CAIII), have different patterns of expression in striated muscles of developing mouse embryos. Using antisense cRNA probes to each of these gene transcripts for *in situ* hybridization, we show that BCK is an early marker for myogenesis. It is expressed at high levels in both cardiac and skeletal muscle as they form between 7.5 and 8.5 days *post coitum* (*p.c.*). MCK, the striated muscle-specific isoform is not expressed until 13 days *p.c.*, when it is detected in both cardiac and skeletal muscle. It has been proposed that MyoD1 directly regulates MCK gene expression in skeletal muscle by binding to its enhancer. We show that MCK transcripts are not detected until two days after MyoD1 mRNAs are expressed, suggesting that MyoD1 by itself is not sufficient to upregulate MCK gene expression. CAIII is first detected in skeletal muscle at 10 days *p.c.* Initially CAIII appears to be expressed in all skeletal muscle cells. At 15 days *p.c.*, CAIII begins to be restricted to developing slow muscle fibers. This change in the pattern of expression is correlated with the formation of neuromuscular junctions and may be directly related to the pattern of innervation of developing muscle fibers.

F 334 NERVE-MUSCLE INTERACTIONS FOLLOWING FETAL DENERVATION, Rosa L. Mallonga and Marcia Ontell, Department of Neurobiology, Anatomy and Cell Science, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261
Sciaticotomy was performed on 5 Swiss Webster mice on day 17 of gestation (E17), and the cut ends of the nerve were apposed to facilitate reinnervation. The contralateral side served as a control. At 6 wks the extensor digitorum longus (EDL) muscle on each side was injected with HRP. Examination of spinal cord sections showed that axotomized fetal motor neurons survived and innervated distal muscles. The size of the motor neuron pool on the denervated/reinnervated (D/R) side was ~47% of that seen on the intact side. The control EDL motor neuron pool was located at L3-L5. However, the location of the D/R EDL motor neuron pool was shifted cranially to L2-L4. D/R EDL also was analyzed immunohistochemically to determine the effect of fetal axotomy on N-CAM expression. The segregation of N-CAM from along the entire length of the muscle fiber to the neuromuscular junction was almost complete in normal muscle at 2 wks. However, this segregation did not occur in fetally denervated muscle, suggesting that innervation is responsible not only for maintenance of the pattern of N-CAM expression (Covault and Sanes, 1986), but also for its establishment. By 6 wks the D/R muscle showed the same distribution of N-CAM as age-matched, control muscle. Supported by NICHD 25630 and grants from MDA and March of Dimes.

F 335 INHIBITORS FOR COLLAGEN SYNTHESIS PREVENT MYOGENESIS OF C2C12 MUSCLE CELLS BY BLOCKING THE EXPRESSION OF MYOD1 AND MYOGENIN, Ryoichi Matsuda*, Osamu Saitoh** and Muthu Periasamy**, *W. Alton Jones Cell Science Center, Lake Placid, NY 12946 and **Dept of Physiol. & Biophys., Univ. of Vermont, Burlington, VT 05405
Cis 4-hydroxy L-proline, a proline analogue which disrupts hydroxylation and glycosylation of procollagen, or ethyl-3, 4-dihydroxybenzoate, a specific inhibitor for prolyl 4-hydroxylase, prevented myogenesis of C2C12 muscle cells. Both inhibitors prevented myoblast fusion and biochemical differentiation in a dose-dependent manner. Northern blot analyses showed that inhibitor-treated cells failed to express muscle regulatory genes MyoD1 and myogenin. The C3H10T1/2 cells stably transfected with myogenin cDNA, did express exogenous myogenin but failed to express endogenous myogenin and to undergo myogenesis when the cells were cultured in differentiation medium containing *cis* 4-hydroxy L-proline. These results suggest that the activation of muscle regulatory genes is tightly coupled with the formation of extracellular matrix. (This work was supported by grants from NIH and W. Alton Jones Cell Science Center)

F 336 CELL LINE MODELS OF MYOGENIC DIVERSIFICATION: MHCS AND MYODS. Jeffrey B. Miller and Nancy E. Block, Neuroscience Center, Massachusetts General Hospital, Boston, Massachusetts, 02129

Multiple types of myoblasts, as well as multiple types of fast and slow myotubes, are formed during myogenesis in birds and mammals. It is not known how the diverse types of myogenic cells arise during development. One possibility is that different combinations of the MyoD family of myogenic regulatory proteins (MyoD1, myogenin, Myf-5, MRF4) control distinct subsets of muscle-specific genes, and thus control myogenic diversity. As one approach to testing this hypothesis, we have examined the expression patterns of myosin heavy chain (MHC) isoforms and MyoD family mRNAs in mouse muscle cell lines. After 7d in low mitogen medium, differentiated cells of different cell lines expressed widely varying ratios of embryonic MHC, slow MHC, and perinatal or adult fast MHCs (see table). The cell lines also expressed widely varying ratios of mRNAs encoding different MyoD family members. Expression of MyoD1 in BC3H-1 cells did not alter the pattern of MHC isoform expression, though the resulting cell line (termed BD) did form multinucleated myotubes. Thus, different mouse muscle cell lines were found to express distinct patterns of MHC isoforms and MyoD family mRNAs. No simple correlation was found between the pattern of MHC protein expression and the patterns of MyoD1 and myogenin mRNA expression. MRF4, expressed only by MRF4-7 cells, may perhaps bias MHC expression towards those MHCs expressed in later fast muscle development. This possibility can be tested by expressing MRF4 in other cell lines. The different mouse muscle cell lines may serve as models for examining the molecular basis of differential MHC isoform expression. (support by AHA, NIH, and W. R. Hearst Foundation)

Cell line	emb	slow	peri or fast
BC3H-1	+++	±	±
C_2C_{12}	+++	++	+++
MRF4-7	+	+	+++
Sol8	+++	+++	+

F 337 RSV TRANSFORMED EMBRYONIC MYOBLASTS AND SATELLITE CELLS EXPRESS IN VITRO DISTINCT MYOGENIC PROGRAMS. V. Mouly, G. Butler-Browne°, E. Bandman†, M. Lemonnier & M.Y. Fiszman. Unité de Biochimie, Institut Pasteur, 25-28 rue du Dr Roux, 75724 Paris Cedex 15, France. °INSERM U262, 123 bd Port Royal, 75674 Paris Cedex 14, France. † Dpt of Food Science and Technology, UC Davis, Davis, Ca 95616, USA.

The analysis of muscular markers expressed in primary cultures of quail or chicken muscles during development and after hatching shows that there are four different types of myogenic cells appearing successively: three embryonic appear at respectively 4 days, 7 days and 9 days in ovo, and the 4th one is satellite cells appearing around hatching. These different populations have been transformed by a ts mutant of the RSV, and still express when differentiated distinct myogenic programs. These programs are characterized by the differential expression of myosin light chains and cholinergic receptor. Moreover, while embryonic myoblasts express the embryonic form of myosin heavy chain, satellite cells coexpress this form along with the neonatal form. The analysis of tropomyosins expressed in these cultures show that the level of the β isoform is decreased in transformed satellite cells, as compared to embryonic myoblasts. This last result should be analysed as regard to the expression during general muscle maturation during development, and particularly the one of pectoralis muscle, where this isoform disappears after hatching under the control of transcriptional regulation. These transformed cells thus represent an interesting model to study the nuclear factors involved in muscle maturation, particularly concerning the regulation of tropomyosin genes expression.

F 338 TRANSITION IN THE EXPRESSION OF THREE TYPES OF SMOOTH MUSCLE MYOSIN HEAVY CHAIN ISOFORMS DURING VASCULAR DEVELOPMENT AND IN ARTERIOSCLEROSIS Ryozo Nagai, Makoto Kuro-o, Ken-ichi Nakahara, Yoshio Yazaki. 3rd Department of Internal Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo, Tokyo.

Smooth muscles express at least three types of myosin heavy chain (MHC) isoforms; SM1 (204 kda), SM2 (200 kDa) and embryonic MHC (SMemb; 200 kDa). In this study we determined the expression of SM1, SM2 and SMemb in smooth muscles during vascular development and in arteriosclerosis. Immunofluorescence histology using specific antibodies against each MHC isoform and mRNA analysis revealed the predominant SMemb expression in fetal and neonatal aortas and its disappearance with development. SM1 was consistently expressed since the embryonic stage, but SM2 appeared only after the neonatal stage. In experimental arteriosclerosis produced by either endothelial denudation or high-cholesterol feeding, proliferating neointimal cells strongly expressed both SM1 and SMemb but not SM2, indicating a remarkable resemblance in the phenotype with embryonic smooth muscles. However, after 8 weeks of the endothelial denudation, the neointimal smooth muscles undergo redifferentiation as shown by reexpression of SM2 and disappearance of SMemb, whereas in the hyperlipidemic model they did not redifferentiate as long as being fed with the high-cholesterol diet. Our results indicate that dedifferentiation of the smooth muscle cell towards the embryonic phenotype is involved in the mechanisms underlying the pathogenesis of atherosclerosis.

F 339 **CHARACTERIZATION OF A MYOSIN HEAVY CHAIN GENE EXPRESSED IN EMBRYONIC MUSCLE CELLS** William Nikovits, Jr. and Frank E. Stockdale
Stanford University, Dept. of Medicine, Stanford, CA. 94305-5306

We are examining the genetic regulation of a myosin heavy chain (MHC) gene, expressed during early stages of myogenesis in the limbs, to study the mechanism by which precursor myogenic cells become committed to form fibers of specific types. During the embryonic phase of vertebrate myogenesis multiple types of muscle fibers, each arising from different myoblast precursors, can be identified. Commitment of one type of embryonic skeletal muscle myoblast is characterized by the expression of a slow myosin (SM) isoform, recognized by the monoclonal antibody (mAb) S58. An expression library made from quail skeletal muscle fibers was screened with mAb S58 and a cDNA clone, encoding primarily a portion of the S2 region of this slow MHC protein, was isolated. A quail genomic Southern blot probed with an oligonucleotide (3-2a) demonstrated that this MHC cDNA is derived from a single gene and, under the conditions used, is specific for a single MHC isoform. Immunopeptide mapping of MHC proteins present in early muscle development suggested the interesting result that the slow MHC protein present in embryonic muscle fibers is identical to that found in atrial tissue of the heart. Consistent with protein data, RNA analysis has demonstrated that the slow MHC cDNA-encoded isoform isolated from skeletal muscle is present in atrial tissue of the neonatal heart. This MHC isoform appears to be different from previously identified slow MHCs present in neonatal (SM 1) and adult (SM 2) slow muscle fibers, and has been designated SM 3. A portion of the gene encoding the SM 3 slow MHC cDNA has been isolated from a quail genomic library. The restricted expression of this gene in embryonic skeletal muscle myoblasts is being studied to better understand how particular muscle fiber types are established. The expression of the embryonic slow MHC isoform in skeletal muscle is limited to, and characteristic of, a subset of embryonic muscle fibers, substantiating the idea that activation of only certain genes within a larger gene family occurs to define unique skeletal muscle fiber types.

F 340 DIFFERENTIAL EXPRESSION OF ACTIN-BINDING PROTEINS, COFILIN AND ADF, IN MUSCLE AND NON-MUSCLE TISSUES DURING DEVELOPMENT. Takashi Obinata, Hiroshi Abe, Kan Saiga, Masatoshi Tsukahara and Narihiro Minami, Department of Biology, Faculty of Science, Chiba University, Yayoi-cho, Chiba 260, Japan.

Cofilin and ADF, actin-binding proteins of low molecular weight, are involved in the regulation of actin assembly in embryonic skeletal muscle; the former is a pH-dependent actin modulator and the latter is an actin depolymerizing factor. We recently determined whole sequence of cofilin and ADF in embryonic chicken muscle and found that the functionally distinct two proteins exhibit high sequence homology (Abe et al., Biochemistry 29, 7420-7425, 1990). Here, we demonstrate the expression of these two proteins in non-muscle and muscle tissues during development by using specific immunological and cDNA probes. While ADF is most abundant in brain through developmental ages, its expression is also remarkable in embryonic smooth, cardiac and skeletal muscles, but declines as the muscles develops. In contrast, cofilin is expressed in both embryonic and adult muscles. Smooth muscle is the tissue which expresses cofilin most abundantly. Interestingly, immunocytochemical studies showed that cofilin distribution in adult skeletal muscle is closely related with the fiber-type: it is enriched in slow fibers and associated with the sarcomeric structures.

F 341 DEVELOPMENTAL EXPRESSION OF ALL MHC INCLUDING MHC_{2X}. Marcia Ontell[1], William LaFramboise[1], Molly Daood[1], Robert Guthrie[1], Martin Ontell[1], Stephano Schiaffino[2], Paolo Moretti[2], Gill Butler-Browne[3], Robert Whalen[3], University of Pittsburgh, Pittsburgh, PA 15261[1], University of Padova, Padova, Italy[2], Pasteur Institute, Paris, France[3].

A quantitative analysis of MHC isoforms expression in perinatal and adult rat diaphragm muscles was performed with antibodies which permitted immunohistochemical identification of the 6 MHC isoforms in typical rat muscles. Isoform switching, leading to the emergence of the adult phenotype, was very complex. Perinatally, 4 isoforms could be coexpressed in a single fiber. Elimination of developmental isoforms did not usually result in the myofiber immediately achieving its adult phenotype. Activation of genes for adult isoforms could be delayed to puberty (i.e., MHC_{2A} and MHC_{2X} are expressed perinatally, while MHC_{2B} is not expressed until 30 days). In most fibers MHC_{2B} expression is transient (i.e., ~27% of fibers and <5% of fibers express MHC_{2B} at day 60 and 115, respectively). Most adult IIA fibers expressed MHC_{2X} during development. In fact, the high frequency of fibers containing MHC_{2X} during the perinatal period as compared to the adult suggested that this isoform may be regarded as both a developmental and adult isomyosin in the diaphragm. Adult I fibers emerge from both 1° and 2° myotubes, with 2° fibers being the source of ~70% of the adult type I fibers. Fibers containing $MHC_{\beta/slow}$ during the perinatal period coexpressed MHC_{emb}. A marked increase in fibers with $MHC_{\beta/slow}$ occurred between 4-21 days pn. These fibers arose from a population which expressed MHC_{emb} and MHC_{neo} during their development. The adult myosin phenotype of the diaphragm myofibers [determined immunohistocytochemically (~30% IIX, 31% IIA, 35% I, and <5% IIB) and with 5% SDS-PAGE (which permits resolution of 6 MHC bands)] was not achieved until the rat was ≥115 days old. Supported by NIH AR36294 and HD25630 to M.O.

F 342 DYSTROPHIC SATELLITE CELL SENESCENCE IS PREVENTED BY NEONATAL DENERVATION, Martin Ontell, Donna Hughes, Stephen D. Hauschka* and Marcia Ontell, Department of Neurobiology, Anatomy and Cell Science, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261 and Department of Biochemistry,* University of Washington School of Medicine, Seattle, WA 98105

It is hypothesized that termination of the early regenerative response of dystrophic muscle with age is due to exhaustion of the satellite cell's (SC) mitotic capability. We have demonstrated that transient neonatal denervation (Den) of 129ReJ *dy/dy* (*dy*) muscle modifies the phenotypic expression of *dy* in vivo (i.e., eliminating necrosis, fiber loss, and regeneration). If this hypothesis is correct, it could be expected that SC from older *dy*-Den muscles would fail to demonstrate the reduced mitotic capacity of SC of age-matched *dy* muscle in vitro. Cultures of SC from EDL of ~100 day old *dy*, *dy*-Den and 129ReJ++ (+) (maintained at clonal density, in the presence of FGF to prevent differentiation, and passaged every 4 days), were followed for 16 days (sufficient time to result in *dy* SC senescence). Doubling times, calculated at the end of each passage, were significantly less for the *dy*-Den than for the *dy*, and no significant differences were found between the *dy*-Den and the +. Prior to passaging, cultures were pulse labeled with ^{3}H-thymidine. At all passages, the labeling index of the *dy*-Den was similar to that of the +, and the SC continued to divide throughout the 16 days. Labeling index of the *dy* was < 1/2 of the *dy*-Den on days 4 and 8 in culture, and was even less at later stages. These observations are consistent with the hypothesis that in the *dy* mouse the cessation of regeneration is due to exhaustion of the SC mitotic capability. Supported by NIAM-AR36294.

F 343 DEFINITION OF A MYOSIN HEAVY CHAIN LOCUS WHICH ENCODES THE DIAPHRAGM-SPECIFIC MHC ISOFORM. Janice Parker-Thornburg, Beverly Bauer, James Gulick, Joseph Palermo, and Jeffrey Robbins. Department of Pharmacology and Cell Biophysics, University of Cincinnati, College of Medicine, Cincinnati, OH 45267-0575.

In many organisms, different isoforms of the myosin heavy chain (MHC) protein are encoded by a multigene family. To begin to understand how different isoforms might contribute to the functional differences of various muscle tissues in the mouse, we have identified and partially characterized a number of myosin isoforms. By cloning and sequencing selected portions of the MHC gene isolates, we have identified unique, transcript-specific sequences, and used these as probes to examine the temporal and spatial expression patterns of the genes. Using this strategy, two genomic clones have been identified that encompass a region containing two linked MHC genes. The sequences identified by a cosmid clone, cos7, overlap sequences contained in a bacteriophage clone, λ3A. Northern analysis of transcripts from the 5'-most gene of the linked pair indicates that this gene has a distinct, tissue-specific expression pattern. It is expressed at high levels in mouse diaphragm, at a low level in the rear posterior thigh muscle, and is undetectable in other muscle types. By comparing the transcription pattern of this gene with previously reported patterns of MHC protein expression (Schiaffino et al., 1989; Termin et al., 1989), we conclude that the 5'-most MHC gene encodes the 2X (or, IID) MHC isoform that is found in high concentrations in the diaphragm.

Schiaffino et al. (1989). J. Musc. Res. & Cell Mot. 10, 197-205.
Termin et al. (1989). Histochem. 92, 453-457.

F 344 INTRA-COMPARTMENTAL SORTING OF MYOSIN LIGHT CHAIN ISOPROTEINS DURING BIOGENESIS OF MUSCLE CYTOARCHITECTURE.

Jean-Claude Perriard, Thierry Soldati, and Marius Messerli , Institute for Cell Biology, Swiss Federal Institute of Technology, 8093 Zürich, Switzerland.

During myogenesis, cytoarchitecture changes dramatically as myofibrils emerge, assembled from newly expressed sarcomeric contractile isoproteins. The intra-comparmental sorting of the myosin light chain MLC 1f was investigated. The fate of exogenous proteins was monitored by the newly developed epitope tagging technique. Sarcomeric MLC 1f does not associate equally well with the non sarcomeric cytoskeleton of different non-muscle cell types, however, binds preferentially to the sarcomeric MHC in chicken embryo cardiomyocytes. Detailed analysis of MLC 1f binding to sarcomeres was investigated in adult rat cardiomyocytes (ARCs), where a stress fiber-like cytoskeleton coexists with myofibrils. Upon transfer of tagged MLC 1f cDNA by microinjection, the resulting MLC1f protein was bound almost exclusively to the sarcomeric MHC. Analysis by confocal laser scanning microscopy showed that the sarcomer formation was not uniform throughout the cells and several patterns of myofibrillar association of the newly synthesized proteins could be discriminated for. Deletion mutants and chimeric constructs of sarcomeric and non-sarcomeric MLC showed that there are three functionally distinct domains in the MLC. While the N-terminal domain seems not to be associated with the sorting, the C-terminal third appears to be responsible for the basal MHC binding and the middle third might modulate the preferential interaction with different MHC isoproteins. Studies on the importance of distinct amino acids for the sorting process are in progress.

F 345 SKELEMINS ARE MEMBERS OF A FAMILY OF MYOSIN-ASSOCIATED PROTEINS WITH IMMUNOGLOBULIN SUPERFAMILY C2 AND FIBRONECTIN-TYPE III DOMAINS. Maureen G. Price, Christopher A. Brooks and Richard H. Gomer, Department of Biochemistry and Cell Biology and Howard Hughes Medical Institute, Rice University, Houston, TX, 77051.

Muscle function depends on structural integration of the myofibrils, provided in part by the cytoskeleton via unknown molecular mechanisms. Skelemins are a pair of closely related 200 and 220 kD cytoskeletal proteins localized to peripheral rings around the M-disc, or middle of the myosin bundle, in mammalian striated muscle. Skelemins therefore could link the peripheral cytoskeleton to the myofibrils. To gain insight into skelemin structure and function, we have sequenced cDNA clones that were isolated from a mouse skeletal muscle lambda ZAP expression library by immunoscreening. Overlapping cDNA clones representing over 80% of the total coding region have been sequenced. Surprisingly, the deduced amino acid sequence features two approximately 100 amino acid-long domains having 23% to 40% identity with either the immunoglobulin superfamily C2 domain or the fibronectin-type III domain. Previously found in extracellular adhesion/recognition molecules, these domains were recently discovered in cytoplasmic myosin-associated proteins, including twitchin and titin, 86 kD and C-protein, and smooth muscle myosin light chain kinase. Skelemins by virtue of their primary structure are thus members of this family of myosin-associated proteins. Each member of this protein family exhibits a different arrangement of the two domains. The presence of the immunoglobulin superfamily C2 and fibronectin-type III domains in the myosin-associated proteins suggests the interesting possibility that they may specifically bind to one another via these domains.

F 346 CONTRACTILE PROTEIN EXPRESSION AND CONTRACTILE PROPERTIES IN FROG JAW MYOFIBERS DURING MATURATION. P. J. Reiser[1] and K. E. Alley[2], Physiology and Biophysics[1], Univ. of IL, Chicago, IL 60680 and Oral Biology[2], Ohio State Univ., Columbus, OH 43210.
Recent morphological results are consistent with the complete loss of myofibers from larval (lar) frog jaw muscles and the establishment of an entirely new set of adult (ad) myofibers from satellite cells in the same beds during metamorphosis (Alley, Am. J. Anat. 184:1-12, 1989). This change in myofiber populations is associated with the disparate feeding behaviors between the lar and ad frogs (slow undulations vs ballistic-type movements, respectively). The goal of the present study was to characterize the expression of contractile proteins and to determine if correlations exist between isoform expression and single fiber contractile properties in the jaw muscle levator mandibulae posterior (MLMP) at these two stages. SDS gel results reveal an almost complete switch in myosin heavy chain expression from two isoforms in ~ equal proportions in lar MLMP to only one higher MW isoform in ad MLMP. The myosin light chains (LC) of lar and ad MLMP co-migrate with those of ad tibialis anterior (fast limb muscle) and are present in apparently similar proportions, except for a lower proportion of LC3 in lar MLMP. Differences also exist in the migration of several other proteins, tentatively identified as troponin and tropomyosin subunits based on MW and stoichiometry, between lar and ad MLMP muscles. The lar MLMP is clearly distinct from ad MLMP and from ad fast and slow limb myofibers with respect to protein isoform expression. Preliminary data also indicate that ad MLMP fibers are ~50% faster compared to lar MLMP fibers. We conclude that the short-lived lar MLMP myofibers are specialized fibers that accommodate the larval-type feeding behavior before metamorphosis. Supported by NIH Grants AR39652 and DE07629.

F 347 EXPRESSION OF A COMPLETE MYOSIN HEAVY CHAIN cDNA IN MAMMALIAN CELLS, Hansjorg Rindt, Beverly Bauer and Jeffrey Robbins, Department of Pharmacology and Cell Biophysics, University of Cincinnati, Cincinnati OH 45267-0575.

To initiate studies on the relationship of structure and function of the myosin heavy chain (MHC) we produced enzymatically active MHC protein in an *in vitro* expression system. We constructed a cDNA encoding a skeletal fast white isoform from the chicken that contained 108 basepairs of 3'-untranslated region and was preceded by the leader sequence of the alfalfa mosaic virus. This construct was cloned into the expression vector pMT2 and used to transiently transfect COS-1 cells. Similarly, a truncated cDNA encoding the subfragment-1 (S1) of the MHC was expressed in the COS cells also. Both the full length and the S1 protein were detected in extracts from transfected cells using a monoclonal antibody specific to chicken fast MHC protein. The S1 protein was synthesized more abundantly which could be explained in part by higher relative levels of the S1 mRNA. Both proteins were partially purified by association with actin and demonstrated K^+/EDTA ATPase activity that approached that of native myosin.

F 348 REGULATION OF ACETYLCHOLINESTERASE BIOGENESIS IN SKELETAL MUSCLE, Richard L. Rotundo, Cristina F-V. Bertetta, Anna M. Gomez, Francesca Barton, and William R. Randall, Department of Cell Biology and Anatomy, University of Miami School of Medicine, Miami, Fl. 33101.

Acetylcholinesterase (AChE), the enzyme responsible for terminating neurotransmission at the neuromuscular junction, consists of a family of oligomeric forms encoded by a single gene. In multinucleated skeletal muscle fibers the transcription, translation, and assembly of the AChE molecules is compartmentalized within nuclear domains. Once assembled, the oligomeric forms can undergo post-translational modifications which determine their fate either as secreted, membrane-bound, or extracellular matrix associated molecules. The expression of AChE in skeletal muscle fibers is regulated to a large extent by the activity state of the cells, and appears to involve both transcriptional as well as translational controls. Spontaneously contracting skeletal muscle fibers in tissue culture express the collagen-tailed asymmetric form of AChE which localizes to the cell surface, and this expression can be modulated by agents which inhibit voltage-dependent sodium channels or phosphoinositide metabolism. Furthermore, activation of protein kinase C mimics the effects of spontaneous contraction. These studies suggest a molecular mechanism for the localized transcriptional and translation regulation of genes encoding synaptic components at the neuromuscular junction.

F 349 CLONAL HETEROGENEITY OF GLUCOCORTICOID RESPONSE IN PRIMARY HUMAN MYOBLASTS, Robert M. Sklar, Orla Hardiman, Robert H. Brown, Jr., Cecil B. Day Laboratory for Neuromuscular Research, Building 149, 13th St., Charlestown, MA 02129

Glucocorticoids stimulate myogenesis of primary mixed cultures from normal muscle as assessed by measurement of fusion index. In some mixed cultures from diseased muscle (mitochondrial myopathy, some dystrophies), glucocorticoids inhibit myogenesis. We have designated the glucocorticoid- induced stimulatory and inhibitory responses of myogenesis as "MSG" and "MIG" respectively. Two types of myoblast clones were derived from mixed cultures. One was MSG like the parent mixed culture; the other was MIG. By contrast, MIG mixed cultures yielded only MIG clones. In the former instance, the proportion of derived clones which were MSG correlated with the level of myogenic stimulation by glucocorticoids in the primary mixed cultures. When we subcloned MSG clones, both MIG and MSG subclones were found, suggesting that myoblasts with the MIG phenotype arise from a MSG progenitor cell. The magnitude of myogenic stimulation by glucocorticoids correlated inversely with the age of the patient; the age-related decline in stimulation of myogenesis by glucocorticoids may be enhanced in cultures of Duchenne dystrophy muscle. To our knowledge, clonal heterogeneity of the glucocorticoid response in human myoblasts has not previously been reported; the significance of this finding in normal muscle development and in the pathogenesis or treatment of muscle disease remains to be defined.

F 350 THE CONTRACTILE PROTEIN ISOFORM PROFILE OF DEVELOPING MAMMALIAN MUSCLE: ESTABLISHMENT OF ADULT SKELETAL MUSCLE FIBRE COMPOSITION OCCURS LATE IN MAMMALIAN DEVELOPMENT. Colin J. Sutherland, Vicki L. Elsom, Monica L. Gordon, Sally L. Dunwoodie, Peter W. Gunning[1], and Edna C. Hardeman. Muscle Development Unit and [1]Muscle Genetics Unit, Children's Medical Research Foundation, P.O. Box 61, Camperdown N.S.W. 2050, Australia.

We have determined the pattern of accumulation of mRNAs from 21 contractile protein isoform genes in the hindlimb of developing rats from embryonic day 14 (E14) to birth and in human foetal hindlimbs. We find that there is no fast or slow programme of gene expression during early muscle development but rather that each gene is regulated independently. Only in late skeletal muscle development *in utero* does a coordinated phenotype similar to that in adult fibres emerge. In addition, during early myogenesis a certain group of isoforms comprising some fast and some slow isoforms is preferentially expressed both *in vitro* and *in vivo*. This group of isoforms may have properties that facilitate *de novo* sarcomere assembly. We define a short period late in rat foetal development when it appears that a synchronised burst of myoblast fusion occurs in the limb leading to the rapid establishment of muscle fibre composition in the neonate. Our results strongly suggest that information extrinsic to the muscle cells regulates the coordination of contractile protein gene expression which leads to the appearance of a mature muscle fibre phenotype.

F 351 SCIATIC NERVE LESION AS A MODEL FOR STUDYING INNERVATION DEPENDENT CHANGES IN GENE EXPRESSION, Doris A. Taylor, Howard J. Federoff* and Leslie A. Leinwand*, Department of Microbiology and Immunology and *Department of Medicine, Albert Einstein College of Medicine, Bronx, NY 10461

To define the molecular genetic events that accompany innervation at the neuromuscular junction (NMJ), we have examined the response of two contractile protein genes to sciatic nerve (SN) crush and recovery. SN lesion is associated with a loss of sensory and motor function in the limb. In rats, SN crush produces flexion, decreased ability to perform motor tasks, loss of response to sensory stimulation, and slight atrophy of the hindlimb skeletal muscles within 2 days after the crush. β-myosin heavy chain (MHC) and slow myosin light chain (MLC1s) RNA levels were unchanged in soleus muscle at 2 days post crush. As atrophy progressed, β-MHC and MLC1s RNA rapidly decreased reaching 23% and 9% of control RNA levels respectively, by 17 days. During this period the lesioned limbs continued to show flexion. By 21 days as sensory and motor function improved, β-MHC and MLC1s reached approximately 40% and 10% respectively, of control values. By 30 days β-MHC and MLC1s returned to approximately 90% and 97% control levels as muscle weight increased to about 80% control. This increase in mRNA and tissue weight presumably reflects in part the recruitment of satellite cells to the soleus as reinnervation occurs at the NMJ. Reinnervated soleus muscle may provide an excellent model to identify the cis-acting elements that confer nerve transcriptional regulation. Preliminary data indicate that directly injected chloramphenicol acetyltransferase (CAT) DNA driven by the Rous sarcoma virus (RSV) promoter yields concentration dependent CAT activity in soleus muscle. Future experiments will be directed toward defining those elements of the β-MHC promoter and slow myosin light chain promoter that confer nerve responsiveness.

F 352 NON-UNIFORM EXPRESSION OF SPECIAL ISOMYOSINS IN DEVELOPING RAT MUSCLE SPINDLES, Lars-Eric Thornell[1] Fatima Pedrosa[1] and Thomas Soukup[2], [1]Department of Anatomy, University of Umeå, S-901 87 Umeå [2]Inst Physiol, Czechoslovak Academy Sciences, Prague

Muscle spindle fibres contain slow tonic, slow twitch, fast twitch, neonatal, embryonic and α-cardiac like (α-MHC) MHC. Each spindle fibre type (bag_1, bag_2 and chain) has its own MHC profile and the MHCs are non-uniformly expressed along the length of the fibres. This complexity arises early in development. Spindle primordia which specifically express slow tonic MHC in addition to slow twitch and neonatal MHC are first seen at 17-18F (fetal days) in rat hindlimb muscles. These myotubes are the bag2 fibre precursors. Bag1 precursors appear at 19F expressing neonatal MHC and acquire slow tonic and slow twitch MHC at 20F. The first chain myotube in the spindles appears at 21F and the second at 3 days postnatally both expressing neonatal MHC but not slow MHCs. Expression of α-MHC is first seen in the bag2 fibres at 21F. Variation in MHC expression along the fibre length is seen from the newborn stage onwards in both bag fibres. Three days after birth the bag1 fibres begin to express α-MHC. Expression of slow tonic and α-MHC is restricted to nuclear bag fibres. Neonatal denervation leads to disappearance of the spindle fibres, whereas neonatal de-efferentation affects the regional expression of MHC in bag fibres.
We propose that spindle fibres arise from special cell lineages.Primary myotubes destined to become bag2 fibres attract afferent nerves which induce the expression of slow tonic MHC. Further modulation and regional expression of MHCs is dependent on both sensory and motor innervation. Thus, muscle spindles are an attractive in vivo model to study regulation of MHC expression.

F 353 ANALYSIS OF cDNA CLONES ENCODING THE THICK-FILAMENT ASSOCIATED 86 kD PROTEIN REVEALS SIMILARITY TO MUSCLE C-PROTEIN AND MEMBERS OF THE IMMUNOGLOBULIN SUPERFAMILY, Kevin T. Vaughan, Franz E. Weber, Steven Einheber, and Donald A. Fischman, Department of Cell Biology and Anatomy, Cornell University Medical College, New York, New York, 10021

In addition to myosin, thick myofilaments in chicken striated muscle contain at least 6 other proteins: C-protein, myomesin, M-protein, M-CK, titin and 86 kD protein. Except for M-CK, none of these proteins has a clearly defined function. 86 kD protein is a myosin-binding protein restricted to fast twitch muscles. It is located in the C-region of the sarcomere along nine of the 43 nm stripes, and co-localizes with C-protein in seven of these stripes (Bahler et al., J.Mol.Biol. 186:381,1985). Overlapping clones of 2.1 kb hybridize with a muscle-specific mRNA of 2.3 kb, a message size sufficient to encode the 86 kD protein. Sequence information obtained from these clones shows that the C-terminal 44 kD of the 86 kD protein is remarkably similar to that of skeletal C-protein (Einheber and Fischman, P.N.A.S. 87(6):2157,1990), 49% identity with 17% conservative substitutions. The predicted amino acid sequence shows unusually high proline content (11%), and abundant charged residues (7% lysine and 7% glutamate). Notable features of this amino acid sequence include homology to the C-2 repeats found in the immunoglobulin superfamily and to fibronectin type III repeats. These repeats are restricted to the C-terminal 44 kD. The similarity between 86 kD protein and C-protein at their carboxyl termini suggests a common functional domain which may be related to their co-localization in the A-band.
(Supported by NIH AM32147 and MDA).

F 354 **FUNCTIONAL ANALYSIS OF RECOMBINANT HUMAN CARDIAC MLC2 PROTEIN.** **RAJ WADGAONKAR, S. SHAFIQ, M.A.Q. SIDDIQUI. DEPARTMENT OF ANATOMY AND CELL BIOLOGY, SUNY HEALTH SCIENCE CENTER AT BROOKLYN, NY 11203.**

The physiological role(s) of myosin light chains (MLCs) is not known, although their involvement in myosin ATPase activity and in myosin/actin interaction has been the subject of several studies. Cardiac MLCs are particularly interesting for investigation of regulatory mechanism, since their expression is modulated not only during development, but also in response to specific stimuli that affect the cardiovascular system. In order to study the physiological function of the regulatory phosphorylatable MLC isoform, MLC2, we have isolated a full length human ventricular MLC2 cDNA and cloned it into the bacterial expression vector pT7-7. The recombinant pT7(HMLC2) was expressed into E. coli. BL21 under IPTG-inducing conditions. The recombinant HMLC2 protein was tested for the specificity of binding with myosin subfragment S1 isolated from the rat and chicken cardiac, skeletal and smooth muscles. The human recombinant MLC2 protein interacted specifically with S1 fragment of myosin heavy chain and it bound to all myosin heavy chains tested. The binding site of MLC2 on S1 is being examined by deletion analysis of the recombinant protein. We are also studying the role of MLC2 in MLC2/S1 interaction and in regulation of the ATPase activity by introducing specific mutations in the putative domain(s) of the polypeptide.

F 355 cDNA CLONING AND ANALYSIS OF AVIAN SKELETAL C-PROTEIN, Franz E.Weber, Kevin T. Vaughan, Steven Einheber and Donald A. Fischman. Department of Cell Biology and Anatomy, Cornell University Medical College, New York, New York, 10021.
Although the composition of muscle thick filament accessory proteins has been studied in mammalian and avian systems, the regulation and function of these proteins remains unknown. C-protein, the most abundant of these in rabbit and chicken muscle, is localized in a series of protein-rich stripes which traverse the thick filament. C-protein has been shown to be phosphorylated in cardiac muscle but its role in contraction has not been elucidated. A partial clone encoding 80% of avian C-protein has been described (Einheber and Fischman, P.N.A.S. 87:2157,1990) and sequence analysis indicates that this protein belongs to a family of myosin-associated proteins which share immunoglobulin C2 and fibronectin type III repeats. This family includes: titin, twitchin, smMLCK and skelemin. Overlapping clones obtained from a gene-specific library have provided an additional 500 bp of sequence. Peptide sequence from the purified protein has been identified in the deduced amino acid sequence of these clones. Primer extension experiments have shown the full length mRNA to be 4 kb in length. The C-terminal part of C-protein contains nine regions of internal homology, six of which resemble C-2 repeats of the immunoglobulin superfamily and three of which resemble fibronectin III repeats. New N-terminal sequence does not reveal additional fibronectin III repeats or C-2 repeats.(Supported by the Revson Foundation and grants from NIH AM32147 and MDA).

F 356 CONTROL OF THE EXPRESSION OF THE MYOSIN HEAVY CHAIN GENES DURING REGENERATION OF THE RAT SOLEUS MUSCLE, Robert G. WHALEN, Kathy BOCKHOLD, Christiane GOBLET, Sanjay SESODIA and Christine WINTER, Department of Molecular Biology, Pasteur Institute, 25 Rue du Dr. Roux, F-75015 Paris, France

When the soleus muscle of 100 gram Wistar rats is exposed to a subcutaneous injection of the snake toxin notexin, this muscle degenerates completely and then regenerates in a homogeneous fashion [Whalen et al (1990) *Develop. Biol.*, **141**, 24-40]. If reinnervation of the fibers is allowed to take place, slow myosin accumulates and is the sole form present at 28 days post-toxin; however, at 14 days mRNAs are detected by PCR for the slow, neonatal, fast IIA and fast IIB isoforms. In the absence of innervation, fast myosin (probably IIB and/or IIX) will accumulate instead. Innervated regenerating muscles exposed to high levels of thyroid hormone will still accumulate almost exclusively slow myosin, suggesting that a hierarchy of influences can be established for these factors. Immunohistochemistry and/or PCR are being used to examine the expression of other gene products in this system, including MyoD/myogenin/MRF4/myf5 gene products, thyroid hormone receptors, extracellular matrix proteins, and glucose transporters. The effects of innervation and thyroid hormone levels on the expression of these molecules can be determined using this system.

Molecular Biology of Synaptogenesis; Molecular Control of Muscle Gene Expression; Cell and Molecular Biology of Neuromuscular Disease

F 400 COMPARISON OF NUCLEAR FACTOR BINDING TO THE TWO MEF1/MYOD CONSENSUS SEQUENCES IN THE MUSCLE CREATINE KINASE ENHANCER.
Stephen Apone, Jean N. Buskin and Stephen D. Hauschka, Univ. of Washington, Seattle WA 98195

The mouse muscle creatine kinase (MCK) gene 5' enhancer contains two similar factor binding sites separated by 13 nucleotides. The "right" site binds a myocyte-specific factor, MEF1 which is antigenically related to MyoD. Both sites bind MyoD, although the "left" site has 5-fold lower affinity. The two sites act synergistically and when mutated reduce enhancer activity by 98%. A consensus sequence was derived from comparison of the two sites and sequences of other muscle regulatory regions which bind MEF1 and/or MyoD. The consensus sequence, often in pairs, appears in many muscle genes and could account for the coordinate regulation of the muscle program.

Here we report a left site binding factor which is distinct from MEF1. Oligomers corresponding to either the right or left site were used in mobility shift assays as probes to detect site specific DNA binding nuclear factors. An enhancer with a mutant left site did not compete for binding to the left site oligomer but did compete for binding to the right site oligomer; whereas a mutant right site enhancer competed for left site binding but not right site binding. The left site factor was present in nuclear extracts of myocytes as well as the non-muscle cell line, 10T1/2, suggesting that the left site binds a ubiquitous binding factor.

A mutant enhancer with two right sites has even higher activity than the wild-type enhancer while an enhancer with two left sites has lower activity than wild-type. The muscle-specificity of enhancers with two left or right sites is currently being tested. Two or more right site-binding oligomers do not substitute for the enhancer, suggesting that other elements are important for enhancer function.

F 401 ANALYSIS OF ALTERNATIVE SPLICING OF *DROSOPHILA* MYOSIN HEAVY CHAIN TRANSCRIPTS IN VIVO AND IN VITRO, Sanford I. Bernstein, Dianne Hodges and Norbert K. Hess, Biology Department and Molecular Biology Institute, San Diego State University, San Diego, California 92182.
We are examining the cis-acting signals required for alternative splicing of the muscle myosin heavy chain (MHC) gene of *Drosophila melanogaster*. The 3' penultimate exon (exon 18) of MHC transcripts is excluded from larval muscle mRNA, while it is included in adult thoracic muscle mRNA. By transforming organisms with the MHC gene promoter, linked to the MHC gene 3' end, we were able to generate correct tissue-specific expression of this minigene and stage-specific splicing of exon 18, indicating that all the cis-acting sequences necessary for alternative splicing are contained within the construct. The splice acceptor site that precedes exon 18 is unusually purine-rich and therefore might be a key element in stage-specific alternative splicing of this exon. To test this possibility, we converted it to a consensus splice site. This had no effect on the splicing pathway in vivo, ruling out the possibility that the unusual splice junction is the primary determinant of alternative splicing. We have also used a *Drosophila* cell-free splicing system to examine splicing of MHC 3' end transcripts in vitro. Splicing of transcripts containing exons 17, 18 and 19 and the intervening sequences results in exclusion of alternative exon 18. Exon 17 is not spliced to exon 18 when substrates truncated in exon 18 are used or when exon 18 and exon 19 are ligated prior to the splicing reaction. To test if a constitutively used branchpoint and polypyrimidine tract would mediate splicing of exons 17 and 18, we inserted such a sequence into the junction of intron 17 and exon 18. This permits high levels of splicing of exon 17 to exon 18 in transcripts terminating in exon 18 or in transcripts containing all three exons where the intron preceding exon 19 has previously been removed. However exon 18 is skipped when the intron preceding exon 19 is included in the pre-mRNA containing the constitutive splice acceptor. Recently we have cloned and sequenced the 3' end of the MHC gene of *D. virilis*. Sequence analysis suggests that alternative splicing of exon 18 may involve recognition of a distant polypyrimidine tract and branchpoint, since these elements are conserved in the two distantly related species.

F 402 CHARACTERIZATION OF A MUSCLE-SPECIFIC ENHANCER-LIKE ELEMENT WITHIN INTRON 1 OF THE DUCHENNE MUSCULAR DYSTROPHY GENE. Lucy O. Bosnoyan, Ronald G. Worton, Peter N. Ray, and Henry J. Klamut, Department of Genetics and Research Institute, The Hospital for Sick Children, Toronto, Ontario, M5G 1X8, Canada. The Duchenne muscular dystrophy (DMD, dystrophin) gene locus codes for a 14 kb mRNA generated from approximately 70 exons spread over 2300 kb of the Xp21 region on the short arm of the X chromosome. Introns 1 and 2 together account for approximately 400 kb of this length, and conservation of these large introns at the 5' end of the gene suggests that they play some important role within the DMD gene locus. In order to examine the possibility that these introns contain important regulatory domains involved in DMD gene transcription, we have generated a series of constructs in the pTKGH vector containing Hind III fragments derived from a cosmid clone containing approximately 25 kb of the intron 1 sequence lying immediately downstream of exon 1. These constructs were transfected into the H9C2(2-1) rat myogenic cell line and tested for levels of growth hormone secretion relative to that generated by the vector alone. One of these constructs, containing a 5kb Hind III fragment, generated significanly higher levels of secreted growth hormone upon myoblast differentiation. This potential enhancer activity has been localized to a 3.5 kb Hind III-Xba I fragment subcloned into the pBLCAT2 vector, and appears to behave in a muscle-specific manner based on comparisons of chloramphenicol acetyltransferase activities in prefusion myoblasts and normal human fibroblasts. Experiments are currently in progress to further delimit the sequences responsible for this enhancer-like activity, to examine positional and orientation effects on its activity, and to identify potential binding sites for muscle-specific transcriptional factors.

F 403 A GENETIC SELECTION SYSTEM FOR THE IDENTIFICATION OF A DEVELOPMENTAL STAGE-SPECIFIC ALTERNATIVE SPLICING FACTOR FROM MAMMALIAN MUSCLE, Roger E. Breitbart and Bernardo Nadal-Ginard, Laboratory of Molecular and Cellular Cardiology, Howard Hughes Medical Institute, Deptartment of Cardiology, Children's Hosptial, and Deptartments of Pediatrics and Cellular and Molecular Physiology, Harvard Medical School, Boston, MA 02115

Alternative RNA splicing is an important mechanism for the generation of developmental stage- and tissue-specific protein isoforms in myriad systems, and is particularly highly developed in muscle. While progress has been made in understanding the constitutive splicing machinery, no cell-specific vertebrate alternative splicing factor has yet been isolated. We have developed an experimental system in which genetic selection is exploited to identify sequences encoding a mammalian alternative splicing factor. This strategy employs chimeric gene constructs in which expression of a selectable drug resistance marker, in the form of a fusion protein, is dependent on the alternative splicing of the mutually exclusive exons 16 (adult muscle-specific) and 17 (default) from the rat fast skeletal troponin T gene. Only the developmental stage-specifc exon 16 splice produces a functional mRNA encoding drug resistance. Stable cell lines transfected with these test constructs can be used to select for a putative *trans*-acting factor capable of conferring drug resistance by virtue of a switch in splicing to the adult muscle pattern. The mRNA or gene sequences encoding such a factor are introduced by transfection of these lines with an adult muscle cDNA expression library or genomic DNA, respectively. Such sequences inducing a shift in splicing can then be recovered from drug-resistant clones.

F 404 INHIBITION OF DESMIN EXPRESSION BLOCKS MYOGENIC DIFFERENTIATION. S.K. Choudhary, H. Li, and Y. Capetanaki. Department of Cell Biology, Baylor College of Medicine, Houston, TX 77030.

During terminal differentiation of skeletal muscle, myoblasts cease dividing and fuse to form multinucleate myotubes. This process is associated with down regulation of vimentin and up regulation of desmin. The biological function of these two intermediate filament proteins during myogenesis is unknown. Desmin, like vimentin, seems to interlink the nucleus to the plasma membrane. This, together with their affinity to nuclear constituents, such as lamin B, DNA, RNA, and histones, as well as their presently shown similarities with the DNA binding and dimerization (HLH and leucine repeat) region of myogenic and other transcription factors, respectively, suggest possible functional role in transport processes, signal transduction and modulation of gene expression. It has also been suggested that desmin might play a critical role in the lateral alignment of myofibrils. To study the role of desmin during myogenic differentiation, we have isolated and sequenced the mouse desmin cDNA, which is now used to modulate the normal desmin expression. Transfection experiments of C_2C_{12} myoblasts with constructs designed to express antisense desmin RNA were performed. Expression of the desmin antisense RNA in some of the obtained stable cell lines blocked fusion and further differentiation of these myoblasts. Northern analysis revealed that the endogenous desmin mRNA in these cell lines was significantly reduced as compared to control C_2 cells. In other cases, partial inhibition of desmin expression, only caused a delay in the differentiation process. As shown by Western blot analysis, desmin inhibition does not affect the expression of vimentin, suggesting a distinct role for each of these proteins during myogenesis.

F 405 ANALYSIS OF ALDOLASE A ALTERNATIVE PROMOTER UTILIZATION DURING *IN VITRO* AND *IN VIVO* MYOGENESIS , Melissa C. Colbert and Elena Ciejek-Baez, Department of Biochemistry, University of Rochester School of Medicine and Dentistry, Rochester, New York, 14642.

The gene for aldolase A in mouse has been shown to be regulated by two independent alternative promoters with attendant alternative first exons. The muscle-specific promoter/exon M functions only in muscle while the housekeeping promoter/exon H is ubiquitously active. We have analyzed the developmental expression of M and H promoters in mouse throughout myogenesis both *in vitro* and *in vivo*. In C_2C_{12} cells primer extension and RNase protections assays revealed the M promoter was activated within 24 h of the onset of myogenic differentiation, and both M- and H-specific mRNAs accumulate over 5 days in culture. Nuclear run-on transcription and *in situ* hybridizations suggested that even with transcription from the M promoter, the downstream H promoter remains active in all differentiated cells. These *in vitro* results were then compared to similar RNase protection studies of M and H expression during myogenic development *in vivo*. The data show the M promoter appeared to be similarly regulated *in vivo* as *in vitro* while the H promoter activity *in vivo* varied with developmental stage. *In situ* hybridizations and ATPase histochemistry suggest simultaneous transcription from both promoters occurs at early developmental ages. The M promoter develops fiber-type restriction as maturation proceeds while the H promoter remains constitutively active in all fiber types. These studies together suggest that the alternative promoters of aldolase A are independently regulated *in vitro* or *in vivo*; and neither promoter occlusion nor promoter switching occur.

F 406 MOLECULAR GENETIC STUDIES OF MYOSIN ISOFORM FUNCTION IN *DROSOPHILA* Mary Beth Davis, Gregg A.Hastings, and Charles P. Emerson, Jr. Department of Biology, University of Virginia, Charlottesville, VA 22901

Drosophila has a single muscle myosin heavy chain (MHC) gene. A potential of 480 distinct protein isoforms can be generated from this locus by regulated alternative RNA splicing.[1] *In situ* hybridization studies and cDNA clone analysis have shown that only one MHC isoform is expressed in the indirect flight muscle (IFM) and this isoform is comprised of a unique combination of alternative exons. A "minigene" corresponding to this isoform has been constructed and the ability of this minigene to rescue MHC mutations is being examined in a series of transgenic experiments. In addition, minigenes containing mutant forms of this IFM-MHC isoform have been constructed by replacing the wild type alternative exons with a different alternative exon. Since most of the alternative exons are in functional domains of the protein, these experiments may indicate the ability of different functional domains to substitute for each other and whether only certain combinations of exons can perform as a unit.

These experiments are a prelude to experiments concerning the regulation of MHC alternative splicing in the IFM. A classical *Drosophila* mutagenesis project to identify genes whose products are involved in the IFM-specific splicing of MHC will be described.

[1]George, et al. (1989), Mol. Cell Biol. **9**, 2957.

F 407 A ROSTROCAUDAL GRADIENT OF TRANSGENE EXPRESSION IN ADULT MOUSE SKELETAL MUSCLE. Maria J. Donoghue, John P. Merlie and Joshua R. Sanes. Departments of Anatomy and Neurobiology and of Pharmacology, Washington University Medical School, St. Louis, Missouri 63110

Adult transgenic mice containing the muscle-specific myosin light chain (MLC) one promoter, MLC enhancer and chloramphenicol acetyl transferase (CAT) transcription unit display a graded pattern of transgene expression. Levels of CAT activity correlate with muscles' positions within the body and, thus, with their segments of innervation. For example, muscles innervated by cranial nerves express little transgene product, those innervated by lumbar and sacral nerves express high levels of CAT, and muscles innervated by cervical and thoracic nerves have intermediate levels. The amount of CAT activity is reflected in the abundance of the RNA for the transgene. The correlation between the segment of innervation and the level of transgene expression is valid for axial and limb muscles and does not appear to be due to muscle fiber type, modification of the CAT protein, the site of transgene integration into the chromosome or the presence of the CAT DNA sequences. Therefore, it is likely that the differential transcription is mediated by MLC sequences. Interestingly, there is no gradation in the expression of the endogenous MLC locus. A possible interpretation of these results is that MLC sequences, when taken out of the context of their genomic location, are responding to an endogenous molecule that affects transcription in a graded manner.

F 408 REGULATION OF MCK EXPRESSION IN CARDIAC MUSCLE, Dorit B. Donoviel, Sharon L. Amacher, Cyndy L. Gartside, and Stephen D. Hauschka, Department of Biochemistry, University of Washington, Seattle, WA 98195.

We are analyzing MCK gene expression in three cardiac muscle systems: transgenic mice, primary newborn rat cultures, and a permanent cardiac myocyte line derived from a mouse atrial tumor. Transgenic analysis (Johnson et al., 1989. M.C.B. 9:3393) has shown that MCK-reporter gene constructs containing -3300 MCK and the 206 nt 5'-MCK enhancer are active in skeletal and cardiac muscle. Preliminary data from 3 founder transgenics indicate that a 110 nt fragment of the MCK enhancer which contains two MEF1/MYOD sites **is inactive** in both skeletal and cardiac muscle. This is unexpected since the same construct is active in skeletal muscle cell transient assays. Since a 206 nt fragment containing this 110 nt fragment **is active** in transgenic skeletal and cardiac muscle, this suggests that elements outside the 110 nt piece are crucial for expression in-vivo. Stable transfection assays with a transformed cardiac cell line appear to confirm and extend the transgenic data: i.e., -3300 MCK directs high expression, -1256 MCK exhibits 20-50 fold lower expression, and the 110 nt enhancer fragment is inactive. Preliminary data from transient transfections of primary newborn rat cardiac cultures also indicate high expression of -3300 MCK, whereas the 206 nt enhancer fragment is expressed only slightly above background. Further transgenic analyses and transfection assays with both cardiac cell culture systems are being carried out to confirm these results, as well as to delineate the critical DNA elements for cardiac-specific MCK expression. Additional studies involving DNA binding proteins which interact with these elements will be reported.

The goal of these studies is to decipher the molecular mechanisms responsible for differentially regulating the expression of muscle genes in cardiac and skeletal muscle.

F 409 TRANSGENIC ANALYSIS OF HUMAN SARCOMERIC ACTIN GENE EXPRESSION DURING STRIATED MUSCLE MATURATION, Sally Dunwoodie, Josephine Joya, Karen Brennan, Ruth Arkell, Monica Gordon and Edna Hardeman, Children's Medical Research Foundation, P.O. Box 61, Camperdown, N.S.W. 2050, Australia.

The sarcomeric actins are coexpressed throughout the development of mammalian striated muscles. However, the relative representation of each isoform differs in heart and skeletal muscle. In the heart, cardiac actin consistently is the dominant sarcomeric actin. In contrast, during skeletal muscle development cardiac actin predominates in the early embryo and is replaced by skeletal actin beginning around birth. We examined a detailed timecourse of cardiac and skeletal actin gene expression during mouse skeletal muscle development to determine precisely when this isoform replacement occurs. This then served to set the criteria with which we examined regions of the human cardiac and skeletal actin genes which are involved in correct developmental expression in transgenic mice. We found that the promoter region of the human cardiac actin gene which confers tissue-specificity as defined in cell culture is not sufficient to account for correct relative expression of the gene in the two striated muscle tissues. Also, we observed that the expression of the exogenous cardiac actin gene impacts upon the expression of its endogenous counterpart. This suggests that these mice may prove useful in addressing questions concerning the regions of the sarcomeric actin genes which are involved in coordinating the output from the members of this multigene family.

F 410 In Vivo Analysis of the Rat Myosin Light Chain 1/3 Enhancer.
James C. Engert, Bruce M. Wentworth and Nadia Rosenthal. Boston University School of Medicine, Boston MA 02118

The rat myosin light chain (MLC) enhancer, located 25 Kb downstream of the MLC1 promotor, has three MyoD binding sites. We have previously used site directed mutagenisis to destroy one, two, or all three of these sites (1). Individual mutation of either of the two upstream sites, A or B, has no effect on enhancer activity in muscle cells as determined by CAT assays. However, mutation of the third site, C, weakens the enhancer considerably. In contrast, cotransfection of non-muscle cells with MyoD or myogenin expression vectors plus enhancer constructs results in transactivation of the CAT gene only if site B and C are intact. All three sites bind to MyoD fusion protein in vitro when isolated fragments or oligonucleotides are used, but a footprint is obtained only over site C when a fragment including both sites B and C is used. The MLC enhancer also contains at least two A/T rich regions which increase muscle-specific activity and may constitute binding sites for MEF2. It is clear that multiple myogenic transcriptional factors are involved in activating the MLC enhancer, and that other cellular factors must contribute to effecting muscle-specific gene activation. In order to unify the functional assays with the in vitro binding studies, and to elucidate the pattern of protein binding spatially and temporally, we are comparing the formation of transcriptional protein complexes over the MLC enhancer in vivo during myogenesis.

1) Wentworth BM, Donoghue M, Engert J, Berglund E and Rosenthal N (1990) Proc Natl Acad Sci (USA), In Press.

F 411 PURIFICATION OF A MUSCLE TRANSCRIPTION FACTOR FROM CHICKEN EMBRYONIC SKELETAL MUSCLE, Iain K. Farrance and Charles P. Ordahl, Department of Anatomy, University of California, San Francisco, CA 94143.

The cardiac troponin T (cTNT) gene is expressed during early embryonic development of skeletal muscle but is transcriptionally repressed at mid-fetal development. The promoter regions responsible for governing the expression of the cTNT gene in embryonic skeletal muscle and cardiac cells have been shown to be separate and distinct. A conserved sequence motif (CATTCCT or "M-CAT"), present in two copies in the cTNT distal promoter region, have been shown to be responsible for the transcription of this promoter in embryonic skeletal muscle cells. Because M-CAT motifs are present in other muscle gene promoters it is a good candidate for a sequence element which may be recognized by a regulatory protein(s) affecting the expression of many muscle specific genes. DNA footprint and mobility shift PAGE analyses have shown that a nuclear protein present in embryonic muscle cells binds to the M-CAT motif. This transcription factor has been termed M-CAT binding factor (MCBF). Here we present protocols for purification of MCBF from day 13 embryonic skeletal muscle crude nuclear extracts. Crude nuclear extract was first passed over heparin agarose and MCBF eluted with a salt gradient. MCBF activity, monitored by mobility shift PAGE analysis, eluted at 0.3 M KCl, was recovered quantitatively and was purified 5 to 10-fold. MCBF was also purified on Cibacron blue agarose. MCBF elutes from this resin at 0.6 M KCl with good recoveries and 5 to 10-fold purification. Two approaches for DNA affinity chromatography are being used to purify MCBF from heparin and Cibacron blue agarose enriched extracts. First, concatamerized M-CAT oligomers have been covalently attached to agarose beads to use as an affinity resin for MCBF using the procedure developed by Kadonaga and Tjian. Second, MCBF is bound to free synthetic biotinylated M-CAT oligomer and MCBF:DNA complexes are removed from solution with strepavidin paramagnetic beads. Preliminary results show that both methods result in good purification of MCBF.

F 412 TRANSCRIPTIONAL AND POST TRANSCRIPTIONAL REGULATION OF THE β TROPOMYOSIN GENE EXPRESSION IN THE CHICKEN, Marc Y. Fiszman, Laurent Balvay, Marguerite Lemonnier, Domenico Libri and Madeleine Toutant, Department of Molecular Biology, Pasteur Institute, 25 rue du Dr Roux, 75724 Paris Cedex 15, France

In the chicken, the β tropomyosin gene spans approximately 15kb and consists of 12 exons. This gene gives rise to 3 transcripts through the use of two distinct promoters, two termination signals for transcription and alternative splicing of two mutually exclusive internal exons. In myoblasts, transcription initiates from the internal promoter, splices in exon 6A and terminates at exon 9B while in myotubes, it initiates from the distal promoter, splices in exon 6B and terminates at exon 9A. In order to understand how expression of this gene is controlled, we have started to characterize the two promoter sequences and have defined some of the cis acting elements which control the mutually exclusive alternative splicing of exon 6A and 6B. Various length of sequence coming from either one of the two promoters were cloned upstream of a CAT gene and their potential to promote transcription was assayed in transient expression experiments as well as in stable transformants using quail muscle as well as non muscle cells. A strong silencer is present upstream of the internal promoter and its function is further described. To explain the exclusive use of exon 6A in non muscle cells, we will show that a secondary structure of the primary transcript is the major regulatory element.

F 413 DEVELOPMENTAL REGULATION OF A SECRETED MACROMOLECULE FROM BC3H1 MYOGENIC CELLS. Douglas N. Foster[1,2], Beverly A. Hodgdon[2], Bonhong Min[4], and Arthur R. Strauch[2,3], Dept. of Poultry Science[1], Molecular, Cellular, and Developmental Biology Program[2], Dept. of Cell Biology, Neurobiology, and Anatomy[3], The Ohio State University, Wooster and Columbus, OH, and Dept. of Biochemistry, Kon-Kuk University, Choongji-City, Korea[4]

Differentiation of mouse BC3H1 myogenic cells involves cell shape remodeling. Since cell-cell/cell-matrix contacts are permissive for BC3H1 myoblast cytodifferentiation, we have begun to characterize changes in cell surface moieties that may mediate remodeling. The sulfated glycoprotein SGP-2 (also called clustrin; TRPM-2; SP-40,40) has been implicated in a number of cellular remodeling events. There is good correlation between the reported size of the smaller SGP-2 protein and a 33 Kd polypeptide present in conditioned medium from developing myoblasts. A rat SGP-2 cDNA (Collard and Griswold, Biochemistry 26:3297, 1987) was used to determine if BC3H1 myoblast remodeling includes the developmental expression of SGP-like molecules. High cell density stimulates expression of a 1750 bp SGP-like mRNA in BC3H1 cells. To characterize the mouse SGP mRNA, a BC3H1 early-stage myocyte cDNA library was screened with the rat cDNA probe. The largest cloned cDNA insert was sequenced and was found to be 90% homologous to the rat cDNA. The 1733 bp mouse SGP mRNA encodes a polypeptide that is approximately 51 Kd. This is the first report implicating SGP in myogenic cell differentiation and cellular remodeling. A study of the biosynthesis and processing of mouse SGP in BC3H1 cells may provide new information regarding modulation of cell surface macromolecules during myogenesis. Supported by NIH grant 43370 and the OARDC.

F 414 CIS-ACTING ELEMENTS THAT CONTROL MUSCLE-SPECIFIC EXPRESSION OF THE *DROSOPHILA* TROPOMYOSIN I GENE, Linda Gremke and Robert V. Storti, Department of Biochemistry, University of Illinois College of Medicine, Chicago, Il. 60612.

We have shown previously that expression of the *Drosophila* Tropomyosin I (TmI) gene is controlled by a minimal 5′ promoter and an intron muscle enhancer region required for expression in indirect flight muscle and jump muscle of the adult fly. In work presented here, transcriptional control of the TmI gene has been investigated by P-element transformation of TmI/*lacZ* and hsp70/*lacZ* reporter genes expressed under the control of TmI cis-acting control elements. The *lacZ* encoded β-galactosidase activity serves as a marker for tissue-specific expression. The results of this work have allowed us to identify distinct cis-acting regions within the first intron of the gene that control the temporal regulation of the gene and the tissue-specificity and the levels of expression. The patterns of β-galactosidase activity as determined by X-gal substrate staining for the different elements correlate with endogenous expression of the TmI gene as determined by *in situ* hybridization of TmI gene probes. Furthermore, the control regions as determined by deletion analysis bind stage specific proteins obtained from nuclei of late stage embryos at the time when muscle cells form.

F 415 CHARACTERIZATION OF A NOVEL PROTEIN THAT BINDS TO AN E BOX IN THE MLC 1/3 ENHANCER. Uta Grieshammer, Bruce M. Wentworth and Nadia Rosenthal, Department of Biochemistry, Boston University Medical School, Boston, MA 02118

The 173 bp region of the myosin light chain (MLC) 1/3 enhancer sufficient for muscle-specific gene expression contains several protein binding motifs including four E boxes (consensus sequence CANNTG) which are present in binding sites for helix-loop-helix proteins. Three of these motifs (sites A, B and C) can bind bacterially expressed Glutathione-MyoD fusion protein specifically, whereas the fourth consensus sequence (CATGTG, site D) does not appear to be a MyoD binding site. A 25 bp sequence including site D is 100% conserved between the human, rat, and mouse MLC enhancers, suggesting functional importance. We initiated studies to characterize protein(s) that interact with the MLC enhancer at this site. We screened nuclear extracts of different cell lines for the presence of protein complexes binding to site D using a 15 bp oligonucleotide as a probe in mobility shift assays. A strong sequence-specific band was observed for nuclear extracts from HeLa and pre-B (70Z) cells, a weak interaction was present in C2 myoblasts and myotubes, and was absent in mature B cells (70Z cells treated with LPS) and in FS4 foreskin fibroblasts. The observed cell type distribution suggests that the protein(s) binding to site D might be involved in negative regulation of differentiation-specific enhancers. In a Southwestern analysis of HeLa nuclear extracts, labeled site D oligonucleotides bound specifically to a protein of approximately 30 kDa molecular weight indicating that a single protein is sufficient for the interaction to occur. We are currently cloning the gene which is coding for the site D binding protein.

F 416 ISOLATION AND CHARACTERIZATION OF THE MOUSE CARDIAC MYOSIN HEAVY CHAIN GENE LOCUS, James Gulick and Jeffrey Robbins, Department of Pharmacology and Cell Biophysics, University of Cincinnati School of Medicine, Cincinnati, Ohio 45267-0575

In order to isolate the murine cardiac myosin heavy chain (MHC) genes, we probed a mouse cosmid library and a mouse bacteriophage library with a chicken fast-white skeletal MHC cDNA clone. CosMHC-1, a clone isolated from the cosmid library, was found to encompass the entire α-cardiac gene, it's 5'-flanking region and ~10 kb of the 3' end of the β-cardiac gene which is located ~4 kb upstream of the α-cardiac gene. Two bacteriophage clones, λβ-11 and λβ-12, were isolated that overlap with the cosmid clone and also contain the 5' end of the β-cardiac gene. Primer extension and polymerase chain reactions were used to define the transcriptional start sites of the two genes. Consensus sequences for several putative regulatory elements which are involved in the transcriptional regulation of other muscle genes were located near the transcriptional start sites of the two genes. Sequences postulated to interact with the thyroid hormone receptor were found upstream of the CCAAT and TAATA boxes of the α-cardiac gene. A comparative analysis of the two promoter regions to other MHC promoters was performed in order to facilitate the localization of additional control regions.

F 417 CONTRACTILE PROTEIN GENE EXPRESSION IN MYOBLASTS IS REGULATED BY CELL ARCHITECTURE, Peter Gunning, Galina Schevzov and Catriona Lloyd, Muscle Genetics Unit, Children's Medical Research Foundation, P.O. Box 61, Camperdown N.S.W 2050, Australia

We have characterized the relationship between cell architecture and contractile protein gene expression by creating targeted lesions in the mouse C2 myoblast cytoskeleton. C2 cell architecture was manipulated by introducing γ-actin and normal and mutated β-actin genes and selecting for high level expression. β-actin precipitated an increase in cell size and microfilament content. In contrast, both the β-actin gene carrying a mutation near its tropomyosin binding site and the γ-actin gene produced cells of reduced size with highly disorganized microfilaments. In cells of reduced size, endogenous actin gene expression was regulated such that the total actin mRNA level remained relatively unchanged. However, the larger cells resulting from β-actin gene transfection had induced higher expression of the endogenous β-actin gene such that the actin mRNA level was increased over 2-fold. Steady state protein levels paralleled the changes in total actin mRNA. The expression of the tight-actin-binding tropomyosins in these cells followed that of the endogenous actins whereas that of the loose-actin-binding tropomyosins was largely uneffected. When actin filaments were depolymerized using cytochalasin D, the regulation of mouse β and γ actin mRNA in the smaller cells was abolished. We conclude that the cellular demand and/or function of actin filaments can regulate the expression of genes encoding its actin and tropomyosin constituents.

F 418 ALTERNATIVE RNA SPLICING OF β-TROPOMYOSIN PRE-mRNA: IDENTIFICATION OF EXON AND INTRON ELEMENTS THAT INHIBIT THE USE OF THE SKELETAL MUSCLE-SPECIFIC EXON IN NONMUSCLE CELLS. Wei Guo and David M. Helfman, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724

The rat β-TM gene expresses two isoforms, skeletal muscle β-TM and fibroblast TM-1, by an alternative RNA splicing mechanism. The gene contains 11 exons. Exons 1 through 5 and exons 8 and 9 are common to all mRNAs expressed from this gene. Exons 6 and 11 are used in fibroblasts, as well as smooth muscle cells, whereas exons 7 and 10 are used exclusively in skeletal muscle. Previous studies from our laboratory have shown that the intron sequences upstream of the 3' splice site of exon 7 are important for alternative splice site selection, because deletion of these sequences resulted in use of the skeletal muscle-specific exon in nonmuscle cells. To further study how these sequences contribute mechanistically to tissue-specific splicing we have introduced linker-scanning substitutions in the 3' end of intron 6, and have identified a number of critical regions that when mutated lead to the use of exon 7 in nonmuscle cells. In addition, we have identified several regions in exon 7 that when mutated result in the usage, to different extents, of this skeletal muscle exon in nonmuscle cells. We have also found that transfection of tropomyosin mini-genes containing mutations in the 5' or 3' splice sites of exon 6 (fibroblast-type splice) do not result in the increased use of exon 7 (skeletal muscle-type splice) in nonmuscle cell systems. These results suggest that splice site selection *in vivo* is not regulated via a simple cis-competition mechanism, but rather by a mechanism that inhibits the use of the skeletal muscle exon in nonmuscle cells.

F 419 CHANGES OF N-CAM SPLICING DURING MUSCLE DEVELOPMENT.

Marion Hamshere, George Dickson* and Ian Eperon, Department of Biochemistry, Leicester University, Leicester, England, *Department of Experimental Pathology, Guys Hospital, London, England.

The calcium-independent neural-cell adhesion molecule (N-CAM) is an important mediator of cell-cell interactions. Incorporation of the MSD exons is one of the muscle-specific features of the human cDNA clone which was shown to enhance myogenesis in transfected cell lines (Dickson *et al* (1990) *Nature* **344** 348-351). We have identified the MSD exons in murine RNA and analysed the developmental profile of incorporation of these exons. 1). Exon MSD 1a alone is included in a significant proportion of mature mRNAs from neural cells, but is generally co-spliced with MSD 1b and MSD 1c in muscle. 2). Exons MSD 1b and MSD 1c are expressed in adult muscle but not in adult neural tissue or cells of neural tumour origin. 3). Triplet exon (AAG) usage is low in RNA from adult neural tissue, but is increased in RNA derived from embryonic nerve tissue, neural tumour cells, and muscle. 4). Low levels of unreported combinations of these exons were identified in RNA from a mouse muscle cell line. These previously uncharacterized combinations (MSD 1c without MSD 1b) were also found to be specifically enriched in RNA isolated from denervated muscle. The expression of exons within the MSD appears, therefore, to be regulated in a stage- and tissue-specific manner by alternative splicing not only of but within the MSD region.

F 420 MUSCLE REGENERATION OF INJURED MYOCARDIUM

Race L. Kao, James A. Magovern, Jennifer Y. Tong and George J. Magovern.
Surgical Research, Allegheny-Singer Research Institute, Pittsburgh, PA 15212

Ventricular muscle cells of adult mammals are terminally differentiated cells which lose their ability to multiply by cell division. Myocardial injuries consistently heal by scar formation with hyperplasia of non-muscle cells and hypertrophy of remaining cardiac myocytes. The goal of this study was to utilize myogenic stem cells (satellite cells) from skeletal muscle to generate new muscle in the injured myocardium for improving the function of an ailing heart. Dogs provided with humane care amd under proper anesthesia were used for satellite cell isolation from tibialis anterior muscle. A localized transmural myocardial injury was produced by a 5 cm diameter cryoprobe cooled to -160^{o}C by internally circulating liquid nitrogen. A sharp visual demarcation between injured and normal tissue was observed, and uniformly destroyed cardiac myocytes and fibrous scar formation was documented by succession histology. Satellite cells in culture were pulse labeled with ^{14}C-thymidine before implantation into the subepicardial zone at the center of the cryoinjured myocardium. The implantation sites were marked, and the dogs were allowed to recover for 0, 1, 3, 14, 28, 70, 180, and 300 days before the hearts were fixed by perfusion fixation. Micrographs and electron micrographs of tissue samples procured up to 4 weeks following cryoinjury and satellite cell implantation indicated a transmural fibrous scar, which developed after tissue inflammation and necrosis, with living satellite cells containing radioactive nuclei at the implantation sites. Scar tissue was found at the nonimplantation sites and in the control heart which had cryoinjury only. Ten weeks after cryoinjury, new muscle cells with radioactive nuclei were also observed at the sites of satellite cell implantation. The regenerated muscle cells displayed morphological characteristics similar to cardiac myocytes judging from their mitochondria, glycogen, and intercalated discs.

F 421 DIFFERENTIAL EXPRESSION OF TWO NONMUSCLE MYOSIN HEAVY CHAINS IN CHICKEN AND HUMAN CELLS, Sachiyo Kawamoto and Robert S. Adelstein, N.H.L.B.I., N.I.H., Bethesda, MD 20892

To understand the biological significance of two different nonmuscle 200 kD myosin heavy chain (MHC) isoforms in vertebrate cells, we studied the effect of serum and growth factors on the expression of the two mRNAs (7.3 kb) encoding these two MHCs. The ratio of mRNAs encoding MHC-A and B can be altered in cultures of chicken embryo fibroblasts and primary cultures of aorta smooth muscle cells by re-adding serum to serum-depleted cells. This treatment resulted in a 3-fold increase in the mRNA encoding MHC-A and a 3-fold decrease in the mRNA encoding MHC-B, compared to controls. The effect was maximal at 6 h following serum stimulation (i.e., during the G_1 phase), thereafter returning to resting values. Actinomycin D abolished the serum induced changes suggesting that they occurred at the level of transcription. Using the human cell line A431 (epidermoid carcinoma cells), we studied the effect of epidermal growth factor and found that the addition of this factor increased the mRNA encoding MHC-A approximately 3-fold, without altering the level of the mRNA encoding MHC-B. These results suggest that expression of the two MHC mRNAs can be differentially regulated.

F 422 TRANSCRIPTIONAL REGULATION OF THE β-TROPOMYOSIN GENE. Jeffrey A. Kazzaz and David M. Helfman, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724.

The rat β-tropomyosin (β-TM) gene generates two distinct isoforms, namely skeletal β-TM and fibroblast TM-1, via tissue-specific alternative RNA processing. The skeletal β-TM isoform is expressed in skeletal muscle and fetal heart muscle but is not expressed in adult heart muscle. The fibroblast TM-1 isoform is expressed in fibroblasts, smooth muscle, and a variety of nonmuscle tissues. In addition, the expression of β-TM isoforms differs in normal fibroblasts vs those transformed by DNA and RNA tumor viruses. All tissues and cell types that express the gene use a single transcriptional start site, although the levels of mRNA can differ greatly in different cell types. For example, the levels of skeletal β-TM mRNA is 20-50X higher than TM-1 mRNA in fibroblasts. In order to understand what cis-acting elements are involved in the tissue-specific regulation of this gene we cloned various portions of the 5' region into a CAT expression vector and transfected a number of different cell types. We demonstrate that there is a muscle-specific cis-acting element upstream of the transcription start site. This element is activated in fibroblasts when co-transfected with MyoD. In addition, we present evidence of cis-acting elements downstream of the transcription start site that are required for expression in nonmuscle cells.

F 423 REGULATION OF THE MOUSE DESMIN GENE EXPRESSION DURING MYOGENESIS. H. Li, and Y. Capetanaki. Department of Cell Biology, Baylor College of Medicine, Houston, TX 77030.

Desmin, the muscle specific intermediate filament (IF) protein, is one of the earliest myogenic markers. RNA analysis during *in vitro* myogenesis of C_2C_{12} cells revealed that desmin is expressed in myoblasts as early as myoD in contrast to myogenin which appears at the onset of differentiation. However, during embryonic development of skeletal muscle, desmin seems to proceed myoD expression while it is not clear if myogenin proceeds or follows desmin expression. To study the regulation of desmin expression during myogenesis both *in vitro* and *in vivo*, we have isolated and partially characterized the mouse desmin gene using the corresponding cD A probe. Sequence analysis of 1000 bp upstream region revealed several potential cis-acting regulatory elements: 1) three myoD1-binding sites located at -78, -854, and -961, respectively. 2) A region with homology to MCAT motif, that is important for troponin expression, exists at position -600. 3) A CBAR (or CARG) box which was initially found in the cardiac actin gene is located at position -273. To determine whether the desmin gene expression is under the control of the myogenic master genes and how they interact to each other to control the tissue and stage specific expression of desmin, varying lengths of mouse desmin upstream regions have been fused to the bacterial chloramphenicol acetyl-transferase gene. These constructs are co-transfected into 10T-1/2 and C_2C_{12} cell lines with members of the myoD family. The result of the obtained CAT assays and further gel retardation and footprinting experiments of intact and mutated regions will provide some insight in understanding the regulation of desmin expression during myogenesis.

F 424 INVOLVEMENT OF THE MUSCLE REGULATORY FACTORS MYOD, MYOGENIN, MYF-5, AND MRF4 IN REGULATING TROPONIN I GENE EXPRESSION, Haishan Lin, Katherine E. Yutzey, and Stephen F. Konieczny, Department of Biological Sciences, Purdue University, West Lafayette, IN 47907.

The muscle regulatory factors MyoD, myogenin, Myf-5 and MRF4 have been implicated in establishing myogenic lineages and in regulating expression of the contractile protein gene set. We previously have shown that expression of the quail skeletal troponin I (TnI) gene is regulated by an Internal Regulatory Element (IRE) that contains a single Muscle Regulatory Factor binding site (MRF site). The TnI IRE is differentially *trans*-activated to high levels by MyoD, myogenin and Myf-5 but only minimally *trans*-activated by MRF4. Interestingly, *in vitro* translated MyoD, myogenin, Myf-5, and MRF4, when complexed with the immunoglobulin gene transcription factor E12, bind to identical nucleotides within the TnI MRF site. As expected, nuclear proteins derived from 23A2 myofibers also form multiple complexes with the TnI MRF site. One of these complexes is antigenically related to MyoD while a second complex is related to myogenin, suggesting that both muscle regulatory factors control TnI gene expression. Point mutations within the TnI MRF site inhibit the binding of the muscle regulatory factors and abolish the *trans*-activation by MyoD. Our data suggest that the muscle regulatory factors control expression of the TnI gene by directly binding to the single MRF site located within the IRE. Current studies are focused on comparing the DNA binding properties and *trans*-activation potentials associated with each muscle regulatory factor to determine how TnI gene expression is regulated during myogenesis.

F 425 REGULATION OF MUSCLE GLYCOGEN PHOSPHORYLASE GENE EXPRESSION. Jean M. Lockyer and James B. McCracken, Jr., Human Genetics Program, Tulane University School of Medicine, New Orleans, LA 70112.

Glycogen phosphorylase plays a key role in glycogen metabolism, initiating the breakdown of glycogen to glucose-1-phosphate. Three isozymes (muscle, liver, and brain) have been described, each of which exhibits a unique pattern of tissue-specific expression and is encoded by a separate gene. Muscle phosphorylase is differentially regulated during muscle development. During the final stages of muscle differentiation to myotubes, the brain (or fetal) isozyme is replaced by the muscle isozyme. We have identified the presence of regulatory sequences within 612 base pairs of the transcription initiation start site which appear to be important for the muscle-specfic gene expression of glycogen phosphorylase. Deletion analysis was performed using CAT fusion constructs made with various regions of 5' flanking DNA of the human muscle phosphorylase gene. The plasmids were transfected into C2C12 mouse myoblasts and the resultant CAT activity was analyzed in proliferating and fused muscle cells. Maximal CAT activity in fused cells was obtained with a construct (-612-MGP-CAT) containing 612 base pairs of 5' flanking DNA. Deletion of 43 base pairs decreased CAT activity to values equivalent to those obtained with 96 base pairs of the promoter region. The 10-20 fold increase in CAT activity observed in fused cells transfected with -612-MGP-CAT was not observed in proliferating myoblasts. A low level of CAT activity driven by sequences within 96 base pairs of the transcription start site was present in proliferating cells which had not yet differentiated. A 10 base pair sequence, CTCCAAAAGG, contained within the 43 base pairs from -612 to -570 is repeated at -252. Point mutations made within the 10 base pairs present at -592 result in 50-70% decreased CAT activity compared to controls. The decrease in activity was specific for fused cells. Gel retardation analysis indicates that a factor(s) binds to sequences within the 43 base pairs. We are identifying the precise site of binding within this region and further characterizing the tissue specificity of the factor(s).

F 426 EXPRESSION OF THE *DROSOPHILA* TROPOMYOSIN I AND II GENES DURING DEVELOPMENT, Pat C.W. Lord and Robert V. Storti, Department of Biochemistry, University of Illinois College of Medicine, Chicago, Il 60612

We have determined the pattern of expression of the muscle transcripts of the *Drosophila* tropomyosin I (TmI) and tropomyosin II (TmII) genes during development using northern blot analysis and RNA *in situ* hybridization to whole embryos and tissue sections. No TmI or TmII muscle mRNA is detected in embryos before stage 13 of development (9-10 hrs at 25°C) prior to the onset of myogenesis in the embryo. Both TmI and TmII RNAs are first detected at stages 13-14 which corresponds to the early stages of myoblast fusion and myofibrillar formation. Interestingly, the pattern of expression of the two muscle transcripts differs. TmI RNA is expressed in somatic musculature in two rows per segment which may correspond to expression in individual myogenic cells. This pattern resembles the pattern of cytoskeletal tropomyosin mRNA expression. TmI RNA is also present in visceral musculature. During late embryogenesis (stage 16-17), TmI RNA expression decreases. In contrast to TmI expression, TmII RNA is first detected at stages 13-14 and continues to be expressed at high levels throughout embryonic development. Unlike TmI, TmII RNA appears to be distributed throughout the length of the myofibrils in somatic muscle. TmII is also detected in visceral musculature. Thus, TmI and TmII RNAs are both present during myogenesis in the embryo, however, their pattern of expression and levels of expression differ during development implying different functions for these two proteins during embryogenesis.

F 427 CIS AND TRANS ACTING ELEMENTS REQUIRED FOR ACTIVITY OF THE CARDIAC TROPONIN T GENE PROMOTER IN EMBRYONIC CARDIAC AND SKELETAL MUSCLE CELLS.

Janet H. Mar, Rocco C. Iannello and Charles P. Ordahl. UCSF, San Francisco, CA 94143

Cells of both cardiac and skeletal muscle lineage express the cardiac isoform of the troponin T (cTNT) gene during early embryonic development. At mid-fetal development, transcription of the cTNT gene is upregulated in cardiac cells but is repressed in skeletal muscle cells. We have used transfection assays and DNA-protein binding experiments to define the cis elements and trans factors which are required for cTNT promoter activity in these two related muscle cell types. cTNT promoter activity in embryonic skeletal muscle requires only 99 nucleotides upstream from the transcription initiation site. This minimal promoter, however, is not active in embryonic heart cells. Activity of the cTNT promoter in cardiocytes requires an additional 47-nucleotide region located 200 basepairs upstream of the minimal promoter. This "cardiac element" can confer transcriptional activity to the minimal promoter in cardiac cells and functions independent of both orientation and position suggesting that it may be an enhancer-like element. An AT-rich motif is located at the 3' end of this "cardiac element" and may play a role in its activity . Although the cardiac element is required, it is not sufficient for activity of the cTNT promoter in myocardial cells. Promoter activity in both heart and skeletal muscle is dependent on the presence of two intact copies of an M-CAT motif (5'-CATTCCT-3') within the minimal promoter. Binding assays identified two factors from nuclear extracts, the M-CAT binding factor (MCBF) and the cardiac element binding factor (CEBF), which interact specifically with their respective cis sequences. The requirement for common and different DNA sequences and trans-acting factors in cardiac and skeletal muscle suggests that the overall regulation of the cTNT promoter results from cooperative interactions between MCBF and CEBF.

F 428 CIS-ACTING ELEMENTS REGULATING EXPRESSION OF THE *DROSOPHILA* TROPOMYOSIN II GENE DURING DEVELOPMENT, Jere E. Meredith and Robert V. Storti, Department of Biochemistry, University of Illinois College of Medicine, Chicago, IL 60612.

The *Drosophila* tropomyosin II genes utilizes a 5′ muscle promoter for expression in muscle and a cytoskeletal promoter located within the third intron of the gene for expression in nonmuscle cells. Transcriptional control of both promoters has been investigated by expressing TmII promoter/*lacZ* reporter gene constructs in P-element mediated germline transformants. Flies transformed with a TmII/*lacZ* reporter gene containing 1.4kb of DNA upstream of the muscle transcriptional start site and the first intron express β-galactosidase activity in the visceral and somatic muscles of the late embryo, larva and adult indicating that this region is sufficient for muscle-specific expression. A series of 5′ deletions have allowed us to define the boundaries of promoter function. Furthermore, additional deletions and rearrangements within the first intron of the gene indicate the presence of cis-acting enhancer elements that control muscle expression. This arrangement is similar to that determined for the TmI gene (see Gremke and Storti poster).

A reporter gene containing 1.4kb of DNA upstream of the fourth exon (the first exon of the cytoskeletal mRNA) does not express β-galactosidase activity in early embryo transformants but does express activity in middle and late stage embryos suggesting that additional cis-acting elements for cytoskeletal early embryo (maternal) expression lie outside the 1.4kb upstream region tested. We are presently testing for the presence of separate maternal control elements.

F 429 EXPRESSION OF THE VSM α-ACTIN GENE DURING BC3H1 MYOGENIC CELL DIFFERENTIATION IS GOVERNED BY POSITIVE AND NEGATIVE *CIS*-ACTING REGULATORY ELEMENTS. B.H. Min[1,3], E. Stoflet[4], L.K. Foster[1,3], M.J. Getz[4], A.R. Strauch[1,2], and D.N. Foster[1,3], The Ohio State Biochemistry Program[1], Colleges of Medicine[2] and Agriculture[3], The Ohio State University, Columbus, OH 43210 and the Department of Biochemistry and Molecular Biology[4], Mayo Clinic Fndn., Rochester, MN 55905

A 330 bp sequence located upstream from the transcription start site of the mouse vascular smooth muscle (VSM) α-actin gene is highly conserved in mammalian species. Cell transfection assays using reporter gene fusion plasmids showed that four putative *cis*-acting regulatory elements having the configuration $CC(A/T)_6GG$ were located in this region and together were required for tissue-restrictive core promoter activity in differentiated mouse BC3H1 myocytes and early-passage rabbit aortic smooth muscle cells. The core promoter and larger segments of the 5'-flanking region including portions up to -1064 were not active in undifferentiated BC3H1 myoblasts or mouse AKR-2B fibroblasts. The region between -147 and +1 was inactive as a promoter in both myogenic and non-muscle cell types. Removal of a 33 bp sequence located between -224 and -191 permitted high level reporter gene expression in BC3H1 myoblasts and slightly increased transcription in fully-differentiated myocytes. This 33 bp region may serve as a binding site for a repressor protein which restricts VSM α-actin expression and whose efficacy as an inhibitor of transcription varies as a function of developmental state in BC3H1 myogenic cells. Supported by NHLBI 43370 and the OARDC.

F 430 PROMOTER ANALYSIS OF THE TWO HUMAN SMOOTH MUSCLE ACTIN GENES AND CLONING OF THE CArG BOX BINDING PROTEIN, Takeshi Miwa & Shinji Kamada, Res. Inst. for Micro. Diseases, Osaka Univ., Suita, Osaka 565, JAPAN

Mouse C2 myogenic cells express two smooth muscle actin genes as well as two striated muscle actin genes during differentiation into multinucleate myotubes. Promoter regions of two human smooth muscle actin (enteric γ-actin and aortic α-actin) genes were analyzed with C2 myotubes. The smooth muscle γ-actin gene has several CArG boxes, myoD1 and Ap2 binding sites. The major nuclear factor binding site was the second CArG box, which functioned as a positive regulatory region. The smooth muscle α-actin gene has these sequences in the promoter region and in the first intron region. Nuclear factors bound to the second CArG box and the intron CArG box, each of which functioned as positive regulatory regions. The functional role of myoD1 binding is under investigation.

To clone cDNA of the CArG-binding factor (CBF) gene, λgt11 cDNA expression libraries were constructed from C2 myotubes and screened for *in situ* DNA-binding specific for the intron CArG box in the smooth muscle α-actin gene. The 1.6 kb cDNA encoding 285 amino acids (CBF-A) was cloned and β-galactosidase fusion proteins expressed in *E.coli* bound to DNA fragments containing the CArG boxes. When the level of CBF-A in C2 cells was increased by the CBF-A expression plasmid, gene expression with the CArG boxes in promoter regions was repressed. Therefore CBF-A cDNA must encode a transcriptional factor having repressor function. A predicted amino acid sequence of CBF-A is similar with some single-stranded nucleotide binding proteins in parts and the fusion protein can bind to single-stranded DNA, whereas CBF from C2 cell nuclear extracts cannot. From these results, CBF-A is distinguishable from CBF in C2 cells and is a novel CArG box binding protein.

F 431 A UNIQUE MUSCLE-SPECIFIC ENHANCER IN THE AMPD1 GENE.

T.Morisaki, R.L.Sabina, and E.W.Holmes; Duke Univ Med Center, Durham, NC 27710

AMP deaminase (AMPD) has been found in all eukaryotic cells. Tissue-specific and stage-specific isoforms of this enzyme are found in vertebrates, and multiple genes as well as alternative splicing have been shown to account for this isoform diversity. The AMPD1 gene is expressed predominantly in skeletal muscle where transcript abundance is controled by stage-specific and fiber-type specific signals. The importance of this activity in skeletal muscle is underscored by the metabolic myopathy that developes in individuals with inherited deficiency of AMPD1. To define the cis- and trans-acting factors that control expression of AMPD1 the following studies were performed. 1)Deletion analyses of a chimeric gene containing 6kb of the 5' flanking region of rat AMPD1 fused to a reporter gene demonstrate that bases -100 to -79 relative to the transcription start site are essential for expression in myocytes. 2)This sequence, GTCCGAAAGCTATAAATAGGTC, is conserved in the human AMPD1 gene; an oligonucleotide of this sequence yields a gel retardation complex which is much more prominent with myocyte than non-myocyte nuclear extract; and a segment of this region of the gene exhibits a DNase I footprint (underlined) with myocyte nuclear extract. 3)Ligation of this 22 bp element to a heterologous promotor directs muscle-specific expression. 4)Although the core of this 22 bp element is somewhat similar to the A/T rich sequence in other muscle-specific enhancers, olgonucleotides specific for MEF2, CArG, MCAT and myoD1 do not compete in gel shift assays. We conclude from these analyses that this 22 bp of the AMPD1 gene is necessary and sufficient for muscle-specific expression and it binds a trans-acting factor(s) that is different from that shown to bind to previously identified muscle-specific enhancers.

F 432 ALTERNATIVE RNA SPLICING OF β-TROPOMYOSIN PRE-mRNA, George J. Mulligan, and David M. Helfman, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724

The rat β-tropomyosin (TM) gene expresses both skeletal muscle β-TM and fibroblast TM-1 by an alternative RNA splicing mechanism. The gene contains 11 exons. Exons 1 through 5 and exons 8 and 9 are common to all mRNAs expressed from this gene. Exons 6 and 11 are used in fibroblasts, as well as smooth muscle cells, whereas exons 7 and 10 are used exclusively in skeletal muscle. We are using uv cross linking, gel shifts, and *in vitro* splicing systems to identify cellular factors interacting with the pre-mRNA that are involved in alternative splice site selection. In both HeLa cell and myogenic cell splicing systems we have identified a protein of approximately 60 Kd that binds a long polypyrimidine tract upstream of the skeletal muscle specific exon (exon 7). This polypyrimidine tract is involved in use of the adjacent lariat branch points associated with the splicing of exon 5 to exon 7 (skeletal muscle-type splice). The potential function of this and other factors interacting with the pre-mRNA will be discussed. We are also studying the molecular basis for the ordered pathway of intron removal of β-TM pre-mRNAs. Our previous studies demonstrated that alternative splicing of TM pre-mRNAs follows a sequential pathway in which the splicing of exon 5 to exon 6 (fibroblast-type splice) or exon 7 (skeletal muscle-type splice) required that either alternative exon must first be joined to exon 8. We found that a pre-mRNA containing exons 5 and 6 plus 17 nucleotides of intron 6 was not spliced. Interestingly, mutating the natural 5' splice site of exon 6 (GUAGUA) to a consensus match (GUAAGU), allowed exon 5 to be spliced to exon 6, without downstream exon sequences. These results are in agreement with the exon definition model recently proposed by Berget and co-workers. However, when the consensus 5' splice site of exon 6 was introduced into a pre-mRNA containing exons 5 through 8, there was still a preference for splicing of exon 6 to 8 before exon 5 was spliced to 6. The results of these experiments will be discussed.

F 433 *CIS*-ACTING SEQUENCES THAT DETERMINE ALTERNATIVE SPLICING OF AMPD1.
L. Kristin Newby, Ikuo Mineo, Edward W. Holmes, Departments of Medicine and Biochemistry, Duke University, Durham, NC, 27710

Alternative splicing is commonly employed in skeletal muscle as a mechanism for generating isoform diversity. AMP deaminase (AMPD) exhibits fiber-type and stage-specific isoforms in skeletal muscle. In rat, but not man, the AMPD1 gene exhibits tissue-specific and stage-specific alternative splicing of exon 2 as one mechanism for generating isoform diversity. Alternative splicing of rat AMPD1 is dependent upon the interaction between two cis-acting elements: 1) Sequences in exon 2 and/or the immediate flanking introns 2) Sequences in the 3' branch/acceptor site of intron 2. Since human AMPD1 does not exhibit alternative splicing we have created chimeric minigenes in eukaryotic expression vectors to determine which of the above regions of rat AMPD1 are responsible for alternative splicing. Replacement of the 3' branch/acceptor site in rat intron 2 with the comparable region of human AMPD1 does not affect alternative splicing. We conclude from these studies that sequences in rat exon 2 or the immediate flanking introns are the cis-acting element responible for alternative splicing. Mutation of the first base of rat exon 2 to a C, the same as human, does not result in constitutive retention of exon 2 as in man. Present studies are utilizing site-directed mutagenesis to determine which of the other 3 bases of exon 2 that are not conserved between man and rat are responsible for alternative splicing.

F 434 DIFFERENTIAL REGULATION OF MYOSIN LIGHT CHAIN 1/3 EXPRESSION IN CELL HETEROKARYONS, L. Pajak, S. Hughes, M. Mariappan, and D. Wieczorek, Dept. Mol. Gen., Biochem., & Micro., Univ. Cincinnati Med. Center., Cincinnati, OH 45267.
The transcriptional processes controlling myosin light chain $1_f/3_f$ (MLC 1/3) expression are developmentally regulated with two functional promoters producing two distinct transcripts that are differentially spliced. During myogenesis, myosin light chain 1 expression precedes myosin light chain 3. Studies were initiated to investigate the mechanisms and factors regulating the developmental expression of this alternatively-spliced gene. Results demonstrate that the developmental influences controlling the expression of the MLC 1/3 gene which are present *in vivo* can be uncoupled in long term cultures of differentiated L6E9 myotubes to solely express MLC 3 in the absence of MLC 1. Interestingly, the absence of MLC 1 expression in these cells can be reversed with cell heterokaryon formation using mouse C2 myotubes. In these heterokaryons, the pattern of both rat MLC 1 and MLC 3 expression is altered to resemble the expression pattern exhibited by C2 myotubes cultured alone, showing that the expression of both MLC 1 and MLC 3 is "plastic" and amenable to modification dependent upon the cellular environment. Additional results demonstrate that diffusable factors from C2 myotubes cannot induce rat MLC 1 mRNA production in L6E9 cells suggesting a common cellular environment is essential for this activation and processing. Since MLC 1 transcripts can be produced, these results also illustrate that the MLC 1 promoter of the rat L6E9 cell is not the primary defect leading to the absence of these transcripts.

F 435 ACETYLCHOLINE RECEPTOR CLUSTERING INDUCED IN FIBROBLASTS BY TRANSFECTION OF RECOMBINANT RAPSYN, William D. Phillips[1], Carrie Kopta$_2$, Paul Blount[1], Paul D. Gardner[3], Joe Henry Steinbach$_2$ and John P. Merlie[1], Departments of Pharmacology[1] and Anesthesiology[2], Washington University Medical School, St. Louis, MO 63110 and Department of Biochemistry[3], Dartmouth Medical School, Hanover, NH 03756.
The aggregation of muscle nicotinic acetylcholine receptors (AChR) into high density clusters beneath the motor nerve is a crucial step in synapse formation. We have begun to study this phenomenon by introducing the individual molecular components into a fibroblast cell line. Fibroblast cells were transfected with the four subunits for fetal ($\alpha,\beta,\gamma,\delta$) or adult ($\alpha,\beta,\epsilon,\delta$) type mouse AChR and clonal cell lines stably expressing functional AChR were isolated. Immunofluorescent staining indicated that AChR were dispersed on the surface of these cells. The cells were than transfected with RAPSYN, a 43kD protein normally concentrated under the post-synaptic membrane. AChR became aggregated into clusters ranging in length from less than 1μm to greater than 10μm. These AChR clusters were found to co-localize with aggregates of intracellular RAPSYN as revealed by double labeling, suggesting that a direct interaction is involved. The mechanism by which RAPSYN induces AChR aggregation is unknown. However, RAPSYN transfected into the parent fibroblast cell line formed large plasma membrane aggregates in the absence of AChR, indicating that self-association of RAPSYN is independent of any interactions with AChR. Furthermore, cell surface AChR labeled with α-bungarotoxin prior to transfection with RAPSYN subsequently became co-localized with RAPSYN aggregates, suggesting that RAPSYN induces AChR-clusters by entrapping these diffuse AChR.

F 436 ABERRANT 3'-PROCESSING OF SKELETAL MUSCLE β-TROPOMYOSIN RNA IN MOUSE BC3H1 MUSCLE-LIKE CELLS, P.A. Rubenstein, A.R. Strauch, and Y.-C. Wang,. Dept. of Biochemistry, Univ. of Iowa, Iowa City, IA 52242.
The mRNAs for rat skeletal muscle β-tropomyosin (SK TM-β) and embryonic fibroblast tropomyosin-1 (EF TM-1) are produced by a tissue specific alternative splicing and 3'cleavage/polyadenylation of transcripts encoded by a single gene. To study the differentiation dependence of this phenomenon in more detail, we screened a cDNA library made from the poly(A)$^+$ mRNA from 2-day differentiated mouse BC3H1 muscle-like cells. We isolated a novel 2.1 kb clone containing the coding sequence of mature SK TM-β mRNA including a SK TM-β specific 3'-untranslated region (UTR) with a normal but unused cleavage/polyadenylation site. It also contained an additional 1 kb intron and the last fibroblast specific exon encoding the final 27 residues of EF TM-1. Cleavage and polyadenylation occurred at a polyadenylation site following the end of the fibroblast exon. Northern analysis of mRNA from fully differentiated cells using a probe derived from this clone revealed 1.2 and 2.1 kb TM-β messages, but neither was seen in undifferentiated cells. The amount of the aberrant TM mRNA constituted about 50% of the β-tropomyosin mRNA during early stages of differentiation and decreased in fully mature cells. A similar experiment in mouse C2 muscle cells showed large amounts of 1.3 kb mRNA but only trace amounts of the 2.1 kb species. The 2.1 kb cDNA produced β-tropomyosin in an in vitro transcription-translation system, and the 2.1 kb message was found in polysomes from BC3H1 cells. Thus, tissue-specific exon splicing and 3'processing of β-TM RNA are not necessarily coupled.

F 437 ISOLATION OF HUMAN MUSCLE SODIUM CHANNEL GENES, Kristin L. Schaller and John H. Caldwell, Department of Cellular and Structural Biology, University of Colorado, Denver, Colorado 80262

The voltage-gated (Na) channel is essential for the conduction of action potentials in mammalian muscle cells. Recent cloning of cDNAs encoding the Na channel from invertebrates and vertebrates has shown that the primary amino acid sequences have regions that have been highly conserved during evolution. We have taken advantage of this conservation of amino acid sequence by using gene amplification by the polymerase chain reaction (PCR) to isolate two Na channel genes from human muscle. cDNA was amplified with PCR from RNA isolated from muscle biopsies of normal and diseased muscle. One gene (HSM1) is most homologous to the gene expressed in normal adult rat muscle, and the second gene (HSM2) was found in diseased muscle and is most homologous to that expressed in denervated rat muscle. We have recently begun to apply this technique to human heart muscle.

F 438 TISSUE SPECIFIC TRANSCRIPTION OF CARDIAC MYOSIN LIGHT CHAIN-2 GENE IS REGULATED BY AN UPSTREAM REPRESSOR ELEMENT AND AN ACTIVATOR LOCATED IN FIRST INTRON
Ruoquin-shen, Shyamal K. Goswami, Eduardo Mascareno, Ashok Kumar and M.A.Q. Siddiqui
Department of Anatomy & Cell Biology, SUNY Health Science Center, Brooklyn, NY 11203
The physiological expression of the cardiac muscle myosin light chain-2 (MLC2) gene in chicken is restricted to cardiac muscle tissue only, although the cardiac MLCs appear in other myogenic cells during early chicken embryonic development. The mechanism by which the cardiac MLC2 gene expression is repressed in differentiated non-cardiac muscle tissues is unknown. Using sequential 5'-deletion mutants of the MLC2 promoter introduced into primary skeletal muscle cells in culture, we have demonstrated that a 89 bp region (CSS) is essential for repression of the MLC2 gene transcription in skeletal muscle. The removal of CSS sequence alone allows transcription in skeletal muscle without affecting the promoter activity in cardiac muscle cells. DNase-I footprinting shows that protection of CSS DNA is afforded by proteins from the skeletal muscle, but not from the cardiac muscle suggesting that a negative regulatory mechanism accounts for the lack of expression of the cardiac MLC2 gene in skeletal muscle. Interestingly, an element located in the first intron of the gene is capable of activating the cardiac MLC2 gene transcription in skeletal muscle by overriding the repressor activity of CSS in transient expression assay. It appears, therefore, that the CSS and IRE elements in MLC2 gene are important functional components of a complex regulatory apparatus which presumably functions both to repress and activate transcription and to ensure a developmental program for tissue specific gene expression.

F 439 DNA-BINDING NUCLEAR FACTORS INVOLVED IN MYOSIN HEAVY CHAIN β GENE TRANSCRIPTION, Noriko Shimizu, Evelyn Dizon and Radovan Zak, Department of medicine, University of Chicago, Chicago, IL. 60637
Expression of the myosin heavy chain (MHC)β gene is regulated transcriptionally in a muscle-specific fashion. We have previously shown by using DNA-mediated transfection experiments that at least 5 positive cis-acting elements are involved in the transcription of MHC gene, two of them being essential for muscle-specific activation from both homologous as well as heterologous promoters. We have initiated analysis of nuclear factors that bind to these two elements, labelled A and B. The A element resides between nucleotides -275 and -260, while the B element resides near the putative CAAT box of the gene. The binding activity of nuclear extracts from non-muscle and muscle cells was analyzed by the gel retardation assay. Using the A element as a probe two bands with distinct mobilities were found. One is mainly present in nuclear extracts from undifferentiated muscle and non-muscle cells, while the other is predominantly present in nuclear extracts from differentiated muscle cells. Using methylation interference analysis both bands showed identical nucleotides interaction. Similarly, using B element as a probe two distinct gel-shifted bands were seen. One is predominantly present in nuclear extracts from non-muscle cells and the other is mainly present in nuclear extracts from muscle cells (myoblasts and myotubes). Both bands showed the same methylation interference pattern.

F 440 POSITIVE AND NEGATIVE REGULATION OF THE MURINE ACHR DELTA SUBUNIT GENE, Alexander M. Simon, Stephen M. Dyer, and Steven J. Burden, Department of Biology, Massachusetts Institute of Technology, Cambridge MA 02139
The murine acetylcholine receptor delta subunit gene is expressed at high levels in myotubes and not in myoblasts or fibroblasts. We have found that transfected gene fusions containing 501 bp of 5' flanking DNA confer cell-type and differentiation-dependent expression. Within this cis-acting region, we have identified four important regulatory elements. One element is an enhancer located between nucleotides -95/-53. It does not contain a MyoD1-binding site and is equally active in myotubes, myoblasts, and fibroblasts. The activity of this enhancer is augmented by an element within nucleotides -148/-95, which by itself has no enhancer activity. The activity of this enhancer is rendered myotube-specific by two elements, a silencer element that is 5' to the enhancer and a MyoD1-binding site that is 3' to the enhancer. Deletion of the silencer, located between nucleotides -501/-258, elevates expression in fibroblasts and myoblasts (6-fold), but has no effect in myotubes. We also demonstrate that mutation of the MyoD1-binding site, located between nucleotides -23/-15, results in ectopic activation (6-fold) of the delta subunit gene in myoblasts and fibroblasts and diminished expression in myotubes (6-fold). These results suggest that the MyoD1 binding site acts in two ways: it is critical for suppression of the delta subunit gene in myoblasts and fibroblasts, and for activation of the gene in myotubes.

F 441 STRUCTURAL AND FUNCTIONAL ASPECTS OF *h*-AND *l*-CALDESMONS
Kenji Sobue, Kenichiro Hayashi and Yasushi Fujio, Department of Neurochemistry and Neurophamacology, Osaka University Medical School, Osaka 530, Japan
Caldesmon(CaD) is one of important regulatory proteins in smooth and non-muscle contraction. Judging from SDS-PAGE, two different *Mr* forms of CaD (120-150kDa *h*-CaD and 70-80kDa *l*-CaD) have been identified. *h*-CaD is predominantly expressed in smooth muscles, whereas *l*-CaD widely distributes in non-muscle cells. In this study, the nucleotide and deduced amino-acid sequences of two CaD isoforms have been determined by cloning and sequencing the cDNA. The *h*-and *l*-CaD cDNAs encode sequences of 771 and 517 amino-acids with the calculated *Mrs* of 88,743 and 58,844, respectively. Two isoforms conserve the completely identical sequences in the N- and C-terminal domains except for the insertion of Ala-508 in *l*- CaD. The central repeating sequences of *h*-CaD (residues 201-447) is deleted in the *l*-CaD molecule. The short N-terminals of two CaDs individually show the unique seqences. Northern and Southern blot analyses reveal that two mRNAs (4.8 and 4.1kb) coding for two CaDs isoforms are generated from a single gene by alternative splicing. Using a series of truncated CaDs expressed in *E. coli*, the common calmodulin-, tropomyosin- and actin-binding sites and the minimum regulatory domains, which are involved in the Ca^{2+}-dependent regulation of actin-myosin interaction, have been identified within the limited consensus sequences (residues 381 to 433- for *l*-CaD and residues 636 to 688- *h*-CaD).

F 442 TISSUE-SPECIFIC REGULATION IN TRANSGENIC MICE USING AN α-CARDIAC MYOSIN HEAVY CHAIN PROMOTER, Arun Subramaniam, James Gulick and Jeffrey Robbins, Department of Pharmacology and Cell Biophysics, University of Cincinnati, Cincinnati, Ohio 45267-0575

We have cloned and characterized the gene locus for the murine α- and β -cardiac myosin heavy chains (MHC). These genes are tandemly linked β → α, and are separated by a 6 kb intergenic region. The 6 kb region, which contains a number of putative *cis*-acting regulatory cassettes, was placed in front of the bacterial cat reporter gene and used to generate transgenic mice. Currently, two independent transgenic lines have been developed from which numerous tissues and muscles were examined for CAT activity. As expected, extremely high levels of activity were present in the cardiac tissue, while no activity was observed in striated muscle, which does not normally express the α-cardiac MHC, nor in smooth muscle such as the uterus. Somewhat surprisingly, low levels of CAT activity were found in the aorta, and in the distal lobes of the lung, which had been dissected free of any obvious vasculature. PCR analysis of these tissues using primers specific for the α-cardiac transcript showed corresponding levels of α-cardiac mRNA in the two tissues.

F 443 CLONING AND EXPRESSION OF TWO NONMUSCLE MYOSIN HEAVY CHAINS IN CHICKEN TISSUES, Masayuki Takahashi, Sachiyo Kawamoto and Robert S. Adelstein, N.H.L.B.I., N.I.H., Bethesda, MD 20892

Vertebrate nonmuscle cells contain at least two different myosin heavy chains (MHCs) of approximately 200 kD which we refer to as MHC-A and MHC-B. The sequence of the cDNA encoding MHC-A in chicken intestinal epithelial cells has been published (Shohet et al., Proc. Natl. Acad. Sci. 86: 7726, 1989). We have now isolated cDNA clones encoding MHC-B from a chicken brain library and are studying the expression of the two isoforms at the levels of mRNA and protein. Using specific oligonucleotide probes, we quantitated the amount of the two mRNAs in different tissues. The ratio of mRNA encoding MHC-A vs MHC-B varies from over 9:1 in intestinal epithelial cells and spleen to 6:4 in kidney and 2:8 in brain. Using SDS-polyacrylamide gels, we have separated two nonmuscle MHC isoforms (196 and 198 kD) which can be distinguished from each other by peptide mapping. Intestinal epithelial cells and spleen contain 196 kD MHC, whereas brain contains 198 kD MHC. Kidney contains approximately equal amounts of both 196 and 198 kD MHCs. These results suggest that MHC-A mRNA encodes the 196 kD polypeptide and MHC-B mRNA encodes the 198 kD polypeptide.

F 444 TRANSCRIPTIONAL REGULATION OF THE MOUSE IIB MYOSIN HEAVY CHAIN GENE, Shin'ichi TAKEDA, Daniel NORTH, Melissa LAKICH, Edouard PROST and Robert G. WHALEN, Dépt. de Biologie Moléculaire, Institut Pasteur, 28 Rue du Dr. Roux, 75724 Paris Cedex 15, France

We have cloned the murine fast IIB myosin heavy chain (MHC) gene, which is expressed in adult fast-contracting, glycolytic muscle fibers. A series of deletions of the 5'-flanking region of the promoter was introduced into the pUC118-CAT vector and was transfected into mouse C2 cells and virus-transformed quail myoblasts and myotubes. We have identified a consensus sequence in the MHC-IIB promoter corresponding to a MyoD binding site, located ca. 950 bp upstream from the site of initiation of transcription. We have demonstrated by gel retardation and footprinting that the GST-MyoD fusion protein binds to this sequence *in vitro*. However, a 250 bp fragment containing this sequence does not activate the transcription of an 80 bp mouse creatine kinase (MCK) basal promoter. Between the CArG and TATA boxes of MHC-IIB gene, we have identified three half-site motifs for thyroid hormone binding which overlap with another consensus sequence for MyoD. Oligonucleotides based on these sequences bind to human β T3 receptor (βT3R) and GST-MyoD fusion protein. CAT constructs containing this region coupled to the HSV TK promoter show a T3 response in mouse C2C12 cells when co-transfected with a rat βT3R. A 150 bp fragment of the MHC-IIB promoter containing these sequences and the CArG and TATA boxes gives relatively high CAT activity in quail myotubes. However, a 500 bp fragment has very low activity. Despite the presence of apparent negative regulatory activity, we find a region between -150 and -500 bp, similar to MEF2, which possesses strong, muscle-specific enhancer function when coupled to the MHC-IIB MyoD binding consensus and the MCK basal promoter. Although a 900 bp construct displays relatively high promoter activity, other sequences upstream (up to -2300 bp) confer further negative regulatory activity on the promoter constructs. These different sequences could be responsible for (i) rendering the IIB MHC gene silent in certain muscle fibers, and (ii) could account for the stimulatory influence of T3 on the expression of this gene during post-natal development of IIB fibers.

F 445 EXPRESSION OF CARDIAC/SLOW TROPONIN C GENE IN STRIATED MUSCLES OF THE CHICKEN. Naoji Toyota, *David Bader and Yutaka Shimada, Department of Anatomy and Cell Biology, School of Medicine, Chiba University, Chiba 280, Japan. *Department of Cell Biology and Anatomy, Cornell University Medical College, 1300 York Avenue, New York, N.Y. 10021, U.S.A.

The expression of cardiac/slow troponin C (C/STnC) gene in various tissues was analyzed with the cDNA probe corresponding C/STnC protein. Northern blot and S1 nuclease protection assay analyses showed that C/STnC mRNA was expressed in the ventricular, breast, anterior latissimus dorsi (ALD) and posterior latissimus dorsi (PLD) muscles but was not found in smooth muscles and nonmuscle tissues. Namely, the transcription of C/STnC mRNA showed an unusually high degree of striated muscle specificity.

In our previous immunohistochemical studies, the C/STnC protein was found to exist only in the cardiac and ALD muscles. Therefore, the expression of C/STnC mRNA in the ventricular and ALD muscles corresponded with that of C/STnC protein, but that in the breast and PLD muscles did not. These results indicate that in the ventricular and ALD muscles the C/STnC gene expression is controlled transcriptionally; in the breast and PLD muscles it is regulated at the level of posttranscription. This suggests that the diverse gene regulatory mechanisms direct the expression of C/STnC in different muscles.

F 446 MUSCLE-SPECIFIC EXPRESSION OF THE HUMAN TROPONIN I-SLOW PROMOTER IS MEDIATED BY MULTIPLE COMPONENTS: *MYOD1* EXPRESSION IS NOT SUFFICIENT FOR ITS ACTIVATION IN NONMUSCLE CELLS. Robert Wade[1], Shari Corin[1], Pam Conley[2], and Larry Kedes[3], [1]Department of Biological Chemistry, University of Maryland School of Medicine, Baltimore, MD 21201, Beckman Center, Stanford University School of Medicine, Stanford CA 94305, [3]Program in Molecular Biology and Genetics, USC School of Medicine, Los Angeles CA 90033.

We have cloned and structurally characterized the human slow-twitch skeletal muscle troponin I gene. Transient transfection assays using promoter/CAT fusion constructs have demonstrated that 4.2 kb of TnI-slow 5' flanking sequence exhibits high level promoter activity when introduced into cultures of C2 myotubes and primary cultures of embryonic chick skeletal muscle cells. TnI-slow promoter/CAT constructs are not expressed upon transfection of numerous nonmuscle cell lines. In contrast with several other muscle-specific genes, TnI-slow promoter/CAT constructs are not *trans*-activated in nonmuscle cells by co-transfection of a *MyoD1* expression vector. Detailed examination of the 5' flanking region has identified a muscle-specific enhancer element approximately 1 kb upstream of the transcriptional start site. This muscle-specific enhancer does not contain a consensus *MyoD1* binding site and appears to bind nuclear factors present in muscle and non-muscle cells. The enhancer element does contain a consensus *MEF2* and A/T-rich sequences that may be related to those seen in other muscle genes. Additional transient transfection studies have identified a sequence immediately adjacent to the muscle-specific enhancer which is required for high level expression in muscle cells but does not exhibit enhancer activity by itself. We conclude that high level muscle-specific activity of the TnI-slow promoter is mediated by multiple components, at least some of which require interaction with regulatory factors other than *MyoD1*. We are currently testing whether these sequences might play a role in the fiber-type-specific expression of the human troponin I gene.

F 447 INTERACTION OF REGULATORY ELEMENTS IN THE PROMOTER AND INTRON-1 OF THE MOUSE MUSCLE CREATINE KINASE (MCK) GENE. Mary Pat Wenderoth, Margaret Shield, and Stephen D. Hauschka. Department of Biochemisty, Univ. Washington, Seattle, WA 98195

Three regions of regulatory importance have been identified in the mouse MCK gene: a proximal promoter region, an intron-1 region, and a 5' enhancer which contains the MEF/MyoD binding site(s). The proximal promoter region, from -776 to -80 nt, confers a 13-fold induction of the CAT reporter gene during differentiation of mouse MM14 cells and is sufficient for muscle-specific expression of CAT in transgenic mice. Results of transient transfections of MM14 cells with a series of 5' deletions within this region suggest the presence of both positive and negative regulatory elements. A construct containing 302 nt of 5' sequence exhibits 15-fold higher expression in MM14 myocytes than a construct containing the entire 776 nt region. This activity is decreased to basal levels by further deletion to -179 nt. Gel mobility shift experiments using myocyte nuclear extracts are in progress to examine factors interacting with this promoter region.

Within a 865 nt fragment of intron-1 at least two positive and one negative element have been identified. In transient transfection assays of differentiated MM14 cells the activity of the fragment is found to be position, orientation and promoter dependent. The 865 nt fragment does not increase gene expression when placed 5' of constructs that contain the 5' MCK enhancer. However, when placed 5' of constructs lacking the enhancer or containing an enhancer mutated at the MEF/MyoD site, gene expression is increased 5- to 10-fold. The intron-1 fragment is promoter dependent as it increases CAT activity 5- to 10-fold over the weak -776 MCK-CAT promoter, but only 2-fold over the stronger -302 MCK-CAT construct. The intron-1 fragment is normally found 3' of the transcription start site yet placing it 3' of a -80 MCK-CAT construct gave a 50% reduction in CAT activity. These results indicate that interactions between cis-acting elements within the MCK regulatory regions are capable of modulating gene expression levels.

F 448 TRANSCRIPTIONAL REGULATION OF THE MYOSIN LIGHT CHAIN 1/3 GENE.

Bruce M. Wentworth, James C. Engert, Uta Grieshammer and Nadia Rosenthal.
Boston University School of Medicine, Boston, MA 02118.

Transcription of the myosin light chain 1/3 (MLC) locus is governed by a 173 bp enhancer element located 25 kb downstream of the MLC1 promotor in both rodents and humans. The rat MLC enhancer directs tissue-specific expression of linked reporter genes exclusively in skeletal muscle in both transgenic animal and tissue culture model systems. The sequence of the 173 bp MLC enhancer is highly conserved in both rodents and humans and contains three E-box (CANNTG) motifs (A, B, and C) that bind in vitro to bacterial fusion proteins of the muscle transcriptional regulatory factors MyoD, MRF4 (herculin), myf5, and myogenein (1, 2, 3). Site C is necessary, but not sufficient, to effect muscle-specific expression and requires either an intact site A or B plus a nearby A/T-rich motif for full enhancer activity (2). We are characterizing the ability of protein complexes from muscle and non-muscle cell lines which bind to MLC E-box motifs in order to determine the identity of the natural protein partners establishing muscle-specific gene expression.

1) Donoghue, M., Ernst, H., Wentworth, B., Nadal-Ginard, B. & Rosenthal, R. (1988). Genes and Devel 2:1779-1790
2) Wentworth BM, Donoghue M, Engert J, Berglund E and Rosenthal N (1990) Proc Natl Acad Sci (USA), In Press.
3) Rosenthal N, Berglund EB, Wentworth BM, Donoghue M, Winter B, Braun T, and Arnold H-H (1990) Nucl Acids Res, In Press.

F 449 Disproportionate accumulation of thick and thin filament proteins in the unc-78 mutation of *Caenorhabditis elegans*.

G. E. White, C. M. Petry, W. T. Smith, IV and F. Schachat. Department of Cell Biology, Duke University Medical School, Durham, N.C. 27710.

We have initiated studies to analyze the effect of mutations in the unc-78 locus on *C. elegans* contractile protein expression. The phenotype of unc-78 mutants is disruption of the myofilament lattice and accumulation of thin filaments at the periphery of the body wall muscle cells of adult animals. Using quantitative densitometry, we have determined the levels of expression of four major components of the myofilament lattice: actin, myosin, tropomyosin, and paramyosin. Comparison of myofibrillar preps from CB1217 (the reference allele for unc-78) and the wild type strain N2 by SDS-PAGE shows that the levels of both myosin and paramyosin are significantly reduced in CB1217. Analysis of the myosin heavy chains using the Neville gel system reveals that the unc-78 mutant expresses all four myosin heavy chains. The ratio of the two myosins of the body wall muscle, myosin A and myosin B, is not significantly different from the wild type. This implies that the disruption of the myofilament lattice that occurs in unc-78 mutants results from under-expression of thick filament proteins. Studies are currently in progress to determine whether this under-accumulation is due to a transcriptional or posttranscriptional defect.

F 450 Differential regulation of α and β tropomyosin expression in fast skeletal muscle.

E.K. Williamson and F. Schachat, Cell Biology, Duke University Medical Center, Durham, N.C. 27710

Protein studies show that the physiological and ultrastructural continua in fast skeletal muscle reflects a molecular continuum that arises from the coexpression of different sets of thin filament protein isoforms . We have investigated how one aspect of this continuum is controlled by determining the relationship between α and β tropomyosin (Tm) subunit expression at the protein and mRNA levels. Steady state levels of Tm mRNA and protein subunits have been compared with other thin filament proteins in a variety of fast skeletal muscles, each expressing different levels of subunits: ranging from α_2 to $\alpha\beta$. Using specific oligonucleotide probes, we find that even though the ratio of α Tm subunit to actin or TnC varies over a two-fold range, the ratio of α Tm mRNA to those for either troponin C (TnC) or actin is constant. In contrast to the constitutive levels of α Tm mRNA, the steady-state levels of β Tm mRNA reflects the more than ten fold variation in the levels of β subunit. This indicates that the expression of the α and β Tm mRNAs in fast muscle are regulated differently: the α Tm gene appears to be constitutively expressed, while the β Tm mRNA levels seems to be transcriptionally controlled. Wiezorek et al. (1988) suggested that this α Tm gene is constitutively expressed because it exhibited multiple promoter sites and Ruiz-Opazo et al. (1987) showed that there were two 3' poly-A signals With regard to the α Tm mRNA in fast skeletal muscle, primer extension studies show that only one promoter is used and RNase H studies revealed no preference in 3' poly-A site. Therefore these potential differences in mRNA structure are not the basis for controlling α Tm expression. Protein synthesis studies are underway to determine whether the differences in accumulation of the Tm subunits is due to differential turnover or translational control.

F 451 THE 300kDa SUBSYNAPTIC PROTEIN AND SYNAPTOGENESIS, James E. Yeadon, Helen Lin, Stephen M. Dyer and Steven J. Burden, Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139

The 300kDa (Mr ≈300,000) protein is a peripheral membrane protein associated with postsynaptic membranes from *Torpedo* electric organ and neuromuscular synapses in higher vertebrates. The distribution of the 300kDa protein in these tissues suggests a functional role for the protein in the formation and/or maintenance of synaptic structure. Using monoclonal antibodies (mabs) against 300kDa we isolated a partial cDNA encoding the 300kDa protein from a *Torpedo* electric organ cDNA expression library. The sequence of this cDNA revealed 2.6 kb of coding sequence with an overall 88% amino acid identity with the *b* isoform of human dystrophin. We have demonstrated that mabs against the 300kDa protein react with neuromuscular synapses in normal mouse muscle, but not in muscle from dystrophic *mdx* mice. Moreover, antibodies against murine dystrophin react with the *Torpedo* 300kDa protein in Western blots. These results suggest that the 300kDa protein is the *Torpedo* homolog of dystrophin. Using immunoflourescence staining we have shown that acetylcholine receptors (AChRs) are concentrated at neuromuscular synapses in dystrophic muscle and can cluster in myotubes grown in culture from embryonic *mdx* mouse muscle. Thus, dystrophin is not required for the formation of AChR clusters. Nevertheless, dystrophin may be involved in regulating the structure of the postsynaptic membrane, such as stabilizing AChR clusters once they have formed.

F 452 ACTIVATION OF TROPONIN I GENE EXPRESSION REQUIRES THE INTERACTION OF MUSCLE-SPECIFIC REGULATORY FACTORS WITH MULTIPLE UBIQUITOUS TRANSCRIPTION FACTORS, Katherine E. Yutzey, Haishan Lin, and Stephen F. Konieczny, Department of Biological Sciences, Purdue University, West Lafayette, IN 47907.

Transcriptional activation often is mediated by the binding of multiple activating factors to distinct DNA elements clustered within a regulatory region. The muscle-specific expression of the contractile protein gene, troponin I (TnI), is controlled primarily through a 148 bp internal regulatory element (IRE) located within the first intron. DNA-protein binding studies have revealed at least four protein complexes that interact with distinct regions within the IRE, each of which contributes to the complete activation capacity of the enhancer element. One of these regions, the MRF site, binds the muscle regulatory factors MyoD, myogenin, MRF4, and Myf-5. Analysis of TnI IRE enhancer activity in myofibers reveals that the MRF binding site is necessary for enhancer function but, alone, has only minimal enhancer activity. Two additional regions of the IRE (Site I and Site II) interact with proteins expressed in muscle and nonmuscle cells. Deletion of Site I results in an 80% loss of enhancer activity while deletion of Site II results in a 40% loss of activity. In addition, the ability of MyoD to *trans*-activate TnI gene expression also is greatly reduced when Site I or Site II are mutated. The identities of these proteins are unknown, but the sequences of Site I and Site II are similar to the binding site consensus sequences for AP-2 and SP1, respectively. Our results suggest that the muscle regulatory factors must interact with the proteins that bind to Site I and Site II to fully activate TnI gene transcription during skeletal myogenesis.

Late Abstracts

α-CRYSTALLIN B CHAIN IS LOCATED IN Z-BANDS AND DECREASES DURING DURING MUSCLE ATROPHY, Yoriko Atomi, Shigeru Yamada, Richard Strohmanm*, Tsutomu Nishida+, Yoshiaki Nonomura**, Department of Sports Sciences, Department of Phamacology**,University of Tokyo, Tokyo Japan 153, Ohtsuka Phamaceutical Co., Tokushima, Japan+, Department of Molecular & Cell Biology, University of California, Berkeley, Ca 94720*.

Atrophy of rat soleus muscle was characterized by an early decrease in a 22-kDa protein. This protein is found in the soluble compartment of muscle and also in the myofibrillar Z-band, and shows a 95% sequence homoloty with the B chain of bovine lens α-crystallin (αB-crystallin). αB-Crystallin cDNA was cloned from a rat heart cDNA library and the DNA sequence for the complete coding region and for partial non-coding regions were determined. Immunoblotting and Northern analysis revealed αB-crystallin mRNA and protein expression in slow skeletal and cardiac muscle, but barely detectable expression in fast skeletal muscle. Immunocytochemistry at the light and electron microscope levels revealed localization of mucle αB-crystallin in Z-bands of isolated myofibrils of skeletal muscles. αB-Crystallin gene expression inhibition is one of the early events of induced acute muscular atrophy. Based upon sequence homology with lens αB-crystallin, a functional role is tentatively assigned implicating muscle αB-crystallin as a myofibril-stabilizing protein which is transcriptionally regulated during changes in muscle mechanical state.

BIPHASIC EXPRESSION OF THE MYOGENIC DETERMINATION FACTOR MYF6 DURING MOUSE DEVELOPMENT Bober,E.; Lyons,G.; Ott, M-O.;Braun,T.; Buckingham, M. and Arnold,H.H., Dept. of Molecular Biology, Univ. of Hamburg, Medical School, D-2000 Hamburg 13, Grindelallee 117, FRG

Four distinct myogenic determination factors have been identified in mammals. These proteins are indistinguishable in their ability to initiate the myogenic program in cultured non-muscle cells, transactivate muscle-specific reporter genes and interact with other HLH proteins to acquire high affinity DNA binding capacity in vitro. MyoDI and Myogenin are located on different chromosomes and are expressed in a specific temporal pattern during mouse development (Sassoon et al., 1989). In contrast Myf5 and Myf6 are closely linked on one chromosome. To study their expression pattern during early mouse myogenesis. we have performed in situ hybridizations on mouse embryos from day 8 until birth. Recently, we have shown that myf5 expression initiates in the dorsal dermomyotome of immature somites of day 8 and follows an antero-posterior gradient. Its expression is transient in somites, the visceral arches and limb buds with maximal levels around day 10.5, subsequently decreasing until undetectable after day 13. Accumulation of myf6 transcripts in somites starts about 1 day after myf5 expression. It follows the same rostro-caudal gradient and disappears clearly before myf5. In limbs and in body musculature myf6 is only activated at about day15 and represents the predominant muscle specific regulatory factor in adult, when myf5 is not expressed any more. The time of myf6 activation in developing skeletal muscle is approximately coordinated with innervation.

MOLECULAR CLONING OF CHICKEN VENTRICULAR-LIKE MYOSIN HEAVY CHAIN

B.Camoretti-Mercado, A.F.R.Stewart*, M.Gupta, R.Wiesner, S.Jakovcic, and R.Zak*+. Departments of Medicine, Organismal Biology and Anatomy*, and The Pharmacological and Physiological Sciences+, The University of Chicago, Chicago, IL 60637

Myosin heavy chain (MHC) is a major component of the muscle contractile machinery. It is coded by a multigene family and expression of each isoform is regulated in a developmental and tissue specific manner as well as in response to a variety of epigenetic factors. Immunochemical analysis of chicken slow anterior latissimus dorsi (ALD) muscle during regeneration after cold injury showed reactivity to adult ventricle-specific monoclonal antibody (MnAb) CCM18. To determine if this putative primordial MHC is identical to the ventricular one, we have constructed cDNA expression libraries primed with either Not I adaptor, random hexanucleotides or oligo $d(T)_{18}$. cDNA was prepared from 7-wk-old injured ALD muscle after 4 days of regeneration. Not I-primed library was first screened with MnAb CCM52 which reacts with all sarcomeric MHC; positive plaques were screened again with MnAb CCM18. Cloned DNAs were analyzed by Southern blot using a 0.9 kb DNA fragment (from chicken ventricular MHC LMM region) as a probe. Under moderately stringent conditions, inserts (0.8-2.0 kb) from six clones showed cross-hybridization. No bands were observed when rat embryonic-MHC 3'-untranslated (UT) probe was used. One of these clones (pCP29) reacted weakly to rat alpha-MHC 3'-UT probe, and comparison of its partial sequence revealed 69% and 97% homology with embryonic and ventricular chicken cDNAs, respectively. Although putative primordial and ventricular MHCs were immunologically indistinguishable, analysis of libraries from two different chicken strains suggests that these proteins are encoded by non-identical genes.

STUDY OF A 5 kb REGION CONTAINING THE 3 PROMOTERS OF THE HUMAN ALDOLASE A GENE, Jean-Paul Concordet, Pascal Maire, Marjo Salminen, Clara Moch, Axel Kahn and Dominique Daegelen, Inserm U129, I.C.G.M., 75014 Paris, France.

We have undertaken the study of the 5' region of the gene coding for aldolase A. This gene is under the control of 3 promoters : two of these, "N" and "H", are active in all tissues ; the third, "M", is specific to adult skeletal muscle. In rat muscle, M is specific to fast-twitch fibers whereas H activity is restricted to slow-twitch fibers.

Two lines of mice containing an aldolase A/ß globin hybrid transgene with all 3 promoters, were analyzed. Both lines exhibited expression of the transgene in a pattern reproducing that known in humans. The M promoter was active only in adult skeletal muscle. In contrast, activity of the H promoter was observed in fetal muscle ; in adult muscle and heart, it was more than 10-fold higher than in other tissues. Parallel studies by transient transfections showed, that the M promoter, by itself, is active in H9 rat myotubes, and, that an ubiquitous enhancer is located in the 400 bp proximal to the H promoter. Muscle and heart specificity of the H promoter was reproduced in one line of mice with a transgene driven only by these 400 bp and the H promoter.

L-14, A DEVELOPMENTALLY SECRETED LECTIN, BINDS TO AND CO-LOCALIZES WITH LAMININ IN MUSCLE BASEMENT MEMBRANE. Douglas N.W. Cooper, Steven M. Massa, and Samuel H. Barondes, University of California, San Francisco, CA 94143-0984

A soluble lactose-binding lectin with subunit Mr of 14,500 (L-14) is prominently localized in extracellular matrix of many vertebrate tissues, including muscle, where it is presumed to function by interaction with complementary glycoconjugates. However, the nature of this function has been unclear. To begin investigation of L-14 function, we have indentified and localized cognate glycoconjugate ligands in cultured C2C12 muscle cells. When these cells are induced to differentiate to form myotubes, they secrete L-14 which co-localizes with laminin in the basement membrane. Staining myotubes with fluorescein-conjugated L-14 reveals that L-14 ligands also co-localize with laminin. L-14 has particularly high affinity for polylactosaminoglycans and has been shown to bind to the polylactosamine chains of purified laminin. Using L-14 affinity chromatography of metabolically labeled C2C12 extracts, we demonstrate that laminin is the predominant glycoprotein ligand for L-14 in these cells. Since laminin has been implicated in developmental regulation of muscle proliferation, migration, differentiation, and synaptogenesis, we are now investigating possible functional participation of L-14 in these processes.

A ROLE FOR EXON SEQUENCE IN ALTERNATIVE SPLICING, Thomas A. Cooper and Ruishi Xu, Department of Pathology, Baylor College of Medicine, Houston, TX 77030.

For many muscle specific genes, alternative splice site selection is a determinative regulatory event for the expression of divergent protein isoforms. To investigate the mechanism of alternative splicing, we are using the avian cardiac troponin T (cTNT) gene which produces two mRNAs via inclusion or exclusion of a single exon, exon 5. Alternative splicing of the cTNT pre-mRNA is developmentally regulated such that exon 5 inclusion predominates in the early embryo and exon 5 exclusion predominates in the adult. Using transfection of cTNT minigenes into both muscle and nonmuscle cultures, we have determined that multiple distinct pre-mRNA cis elements are required for alternative splicing of exon 5. Of particular interest is the finding that as few as four nucleotide substitutions within the alternative exon, distal from known splicing cis elements, prevent its inclusion into processed mRNA in both muscle and nonmuscle cells. The fact that sequence within the alternative exon is required for exon inclusion suggests the possibility that exon inclusion requires direct interaction between the exon and the splicing machinery. This may be a distinguishing feature of alternative splicing since the cis-elements known to be required for splicing of constitutive exons are essentially confined to introns. To analyze the interactions between the splicing machinery and the cTNT pre-mRNA, we have established in vitro splicing of cTNT precursors. cTNT precursors containing two exons, either exons 4 and 5 or exons 5 and 6, accurately splice the two exons together. Interestingly, the mutations within exon 5 that prevent its inclusion during splicing in vivo block in vitro splicing of exons 4 and 5 but have no effect on in vitro splicing of exons 5 and 6. These results indicate that sequence within exon 5 is specifically required for joining of exons 4 and 5. RNA binding studies using mutant and wild type exon 5 sequences are in progress to define the specific interactions disrupted by the mutation and to characterize the trans acting components involved.

DIFFERENTIAL EXPRESSION OF THE PROTO-ONCOGENE C-SRC IN CHICKEN SKELETAL MUSCLE. Thambi Dorai and Lu-Hai Wang. Department of Microbiology, Mount Sinai School of Medicine, New York, NY 10029.

We have investigated the expression of the proto-oncogene c-src in developing chicken skeletal muscle. While the 4 kb mRNA coding for $pp60^{c\text{-}src}$ is expressed in embryonic muscle, it is permanently switched off at the prehatching stage and novel alternatively spliced, smaller sized c-src mRNAs of about 3 kb are produced thenafter. We have analyzed the primary structures of both these c-src mRNAs. The 3 kb mRNA encoded a 24 KDa protein rich in cysteine and proline residues. Antibodies to the 24 KDa protein detected in vivo a muscle specific 24 KDa protein which was developmentally regulated and corresponded to the switch from the 4kb to the 3 kb c-src mRNA. Sequence and structural mapping of the 5′ non-coding exons of the 4 kb mRNA and of the 3 kb mRNA indicated that the transcription unit for the 3 kb mRNA is interdigitated in the c-src locus. Generation of the 4 kb and the 3 kb mRNA from the c-src gene may involve multiple initiations, multiple poly A sites as well as differential splicing.

ENTACTIN PROMOTES ADHESION AND MATURATION OF CULTURED REGENERATED SKELETAL MYOTUBES, Vicky L. Funanage and Susan M. Smith, Research Department, Alfred I. duPont Institute, Wilmington, Delaware 19899

The basal lamina protein, laminin, has been shown to promote migration and proliferation of cultured skeletal myoblasts, resulting in increased myotube formation. However, skeletal myotubes adhere poorly to a laminin substrate, and long-term cultures of skeletal myotubes on laminin have not been achieved. We have found that cultured satellite cells from bupivacaine-damaged rat skeletal muscle actively proliferate and differentiate on a diluted Matrigel substrate composed of laminin, type IV collagen, heparan sulfate proteoglycan, and entactin. Myotubes cultured on diluted Matrigel are contractile and have never been observed to detach from the culture dish; rather, myotubes generally atrophy after 2-3 weeks in culture. Antibodies directed against the various components of Matrigel were used to determine the role of each component in enhancing muscle differentiation. Anti-laminin impaired satellite cell proliferation, whereas antibodies against either type IV collagen or heparan sulfate proteoglycan had no effect. Anti-entactin did not affect proliferation or fusion of cultured satellite cells; however, myotubes exposed to anti-entactin failed to adhere to the culture dish after spontaneous myotube contractions began. We conclude that entactin is responsible for long-term maintenance and maturation of contractile skeletal myotubes on a diluted Matrigel substrate. This is the first study to assign a biological function for entactin in myogenesis.

IDENTIFICATION OF A NOVEL ZINC-FINGER PROTEIN THAT BINDS E-BOXES WITHIN THE MCK AND Ig ENHANCERS, Tom Genetta, Diane Ruesinsky and Tom Kadesch, HHMI and Dept. of Human Genetics, University of Pennsylvania School of Medicine, Philadelphia, PA, 19104-6145.

The E-box, a DNA sequence with the consensus CANNTG, binds an extensive family of proteins, all of which contain a basic-helix-loop-helix (bHLH) domain. These factors have been shown to play key roles in a variety of developmental and cell type-specific programs, from *drosophila* (e.g. *daughterless*) to human (e.g. MyoD). Using an E-box-containing oligonucleotide (the μE5 site of the immunoglobulin heavy chain enhancer), we screened a HeLa cDNA library for DNA binding proteins (bHLH or otherwise) that may play a role in transcriptional regulation. We have isolated a cDNA encoding a novel E-box-binding protein with three zinc fingers - designated ZEB (Zinc finger E-box-Binding protein). ZEB specifically binds E-box elements from a variety of genes, including the MEF1 site of the muscle creatine kinase enhancer. A general role for ZEB in the regulation of E-box-containing genes will be discussed.

MULTIPLE CONSERVED CBAR PROMOTER ELEMENTS INTERACT SELECTIVELY AND DIFFERENTIALLY WITH TWO DISTINCT NUCLEAR FACTORS:IMPLICATIONS FOR A POSITIVE REGULATORY ROLE OF SERUM RESPONSE FACTOR IN THE SKELETAL α-ACTIN GENE EXPRESSION, Te-Chung Lee and Robert J. Schwartz, Department of Cell Biology, Baylor College of Medicine, Houston, TX 77030

The avian skeletal α-actin promoter contains three copies of a CCAAT-box-associated repeat (CBAR) centered at -85 (DBAR), -130 (UBAR) and -175 (UTA) that are required for directing myogenic cell type restricted expression. Although CBARs contain the motif $CC(A/T)_6GG$, which is also the core binding site for serum response factor (SRF), this study indicates the nonequivalence nature of the three CBAR elements for binding SRF. The SRF-CBARs interactions are complex in that the DBAR and UTA sites are first cooperatively occupied by SRF, thus facilitating subsequent binding of SRF to the middle CBAR which exhibits a poor SRF affinity. In addition, the DBAR is uniquely recognized by another nuclear factor, F-ACT1, which can be clearly distinguished from SRF. F-ACT1, present at higher levels in nonmyogenic cells and replicating myoblasts, contacts the GG doublet of the core sequence on the coding strand and therefore is likely to compete for DBAR-binding with SRF, which symmetrically contacts the GG doublet on each strand. Our results suggest that skeletal α-actin promoter activity may be facilitated by SRF forming a stable multiprotein-promoter complex through the three CBAR elements.

BOVINE GENE FOR THE HEART/MUSCLE ISOFORM OF CYTOCHROME c OXIDASE SUBUNIT VIA
Margaret I. Lomax*, Ellen O. Smith+, Dawn M. BeMent*, and Edna Gamliel*,
*Department of Anatomy and Cell Biology, University of Michigan Medical School, Ann Arbor, MI 48109, and +Department of Biology, Madonna College, Livonia, MI 48150.
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 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 and may modulate cytochrome oxidase activity in response to different physiological signals or metabolic environments. Three nuclear COX subunits have isoforms found only in contractile muscle. To understand the transcriptional regulation of genes for these muscle-specific subunits, we have isolated the bovine gene (*COX6AH*) for the heart isoform of COX subunit VIa. Genomic Southern blots indicated that COX6AH is a single-copy gene located within a 6.2-kb HindIII fragment. The gene is composed of four exons; the three coding exons are contained within a 2.3 kb EcoRI fragment. Exons three and four are separated by a small 97 bp intron. The promoter region and intron 1 are being sequenced to determine whether previously described muscle-specific transcription factors also regulate expression of this important class of muscle-specific genes.

INTERACTIONS OF MYO D, MYOGENIN, MRF 4 AND MYF 5 WITH THE CHICKEN CARDIAC AND SKELETAL α-ACTIN PROMOTERS, Jennifer Barnett Moss, Brent A. French and Robert J. Schwartz, Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77030

Four members of the helix-loop-helix family of proteins: Myo D, myogenin, MRF 4 and Myf 5 are capable of initiating a myogenic pathway when transfected into 10T1/2 fibroblasts. Promoter/enhancer sequences of target genes which bind these proteins contain a consensus sequence CANNTG; the "E box" element. The avian cardiac α-actin promoter has two E box elements one of which clearly binds myogenin as well as Myo D (French, B.A. et.al., submitted, MCB). We now demonstrate binding of all four myogenic factors to the cardiac promoter. In addition, transfection experiments indicate differential activation of the cardiac as well as skeletal α-actin promoters. Binding but not transactivation by MRF 4 suggests a differential interaction between protein domains.

TWO FORMS OF MOUSE MUSCLE NCAM: CLONING MUSCLE GENES USING EPISOMAL SHUTTLE VECTORS. Lydia C. Pan, William A. Mohler and Helen M. Blau, Department of Pharmacology, Stanford University School of Medicine, Stanford, CA 94305.

Differentiation of myoblasts into myotubes is accompanied by the induction of transcripts that encode phosphoinositol (PI)-linked forms of neural cell adhesion molecule (NCAM). Using PCR, we have shown that the marked increase in NCAM at the onset of myogenesis is due to the induction of two PI-linked isoforms which are alternatively spliced within the extracellular "hinge" region of the molecule. Full-length clones encoding the smaller isoform were obtained by screening a mouse cDNA expression library constructed in the episomal shuttle vector EBO-pcD which can replicate extrachromosomally in human cell hosts. Following transfection with the EBO-pcD library, cells expressing mouse NCAM were identified by immunofluorescence and enriched using the fluorescence-activated cell sorter. Expressible NCAM clones recovered in Hirt supernatants from the sorted cells encoded a PI-linked NCAM with an insert of 18 nucleotides between exons 12 and 13: this isoform also occurs in brain (Santoni et al., 1989, EMBO J. 8, 385). We cloned a second NCAM that is the predominant form expressed by myotubes. It differs from the smaller form by the inclusion of a 90-nucleotide segment which is 80% homologous to the muscle-specific human MSD1b and MSD1c exons (Thompson et al., 1988, Genes and Development 3, 348). None of the NCAM clones from our muscle library utilize the pi (Π) exon found in a subset of brain NCAMs (Santoni, ibid). These results suggest that PI-linked NCAMs play a central role at the onset of myogenesis. Moreover, they demonstrate the efficiency of cloning strategies based on the transfection of episomal cDNA expression libraries. (Supported by NIH, Damon Runyon)

EXPRESSION OF α-CARDIAC MHC IN HUMAN MASSETER AND EXTRAOCULAR MUSCLES, Fatima Pedrosa[1], Per-Olof Eriksson[2], Gillian S. Butler-Browne[3] and Lars-Eric Thornell[1], [1]Dept of Anatomy and [2]Dept of Clinical Oral Physiology, University of Umeå, S-901 87 Umeå; [3]INSERM U262, Clinique Universitaire Baudelocque, 123 Port Royale, 75674 Paris Cedex 14, France

We have recently shown that the bag fibres in rat hindlimb muscle spindles specifically express an α-cardiac like MHC, which is detected by a monoclonal antibody (anti-α MHC) raised against human α-cardiac MHC. Here we report that, in humans, in addition to the spindle bag fibres, a small subset of the slow fibres in the extraocular muscles and a subpopulation of the type 2 extrafusal fibres in the masseter are moderately stained with anti-α MHC. In fetuses from 14 to 22 weeks of gestation, staining with this antibody is only seen in spindle bag fibres in the masseter and is absent in the extraocular muscles. Seven to 9 weeks after birth a large number of fast fibres in the masseter display moderate reactivity to anti-α MHC. The staining pattern obtained with this antibody is distinct, at all stages, from those seen with antibodies against slow tonic, slow twitch, fast twitch, embryonic or neonatal MHC. Cat masseter extrafusal fibres containing superfast MHC and rat diaphragm fibres containing fast 2X/2C MHC are unreactive to anti-α MHC, whereas the bag fibres in both cat and rat spindles are stained.

These preliminary data show that α-cardiac MHC or a closely related isoform is expressed in some extrafusal fibres of the human masseter and extraocular muscles, as well as in spindle bag fibres. Furthermore, the initiation of expression of this isomyosin is a late event in development and its expression in the masseter seems to be species specific. These findings further stress the complexity of MHC expression in specialized muscles.

SEQUENCE AND SECONDARY STRUCTURE OF A NOVEL TRANSLATION INHIBITORY RNA OF CHICK EMBRYONIC MUSCLE, Z-C.Zheng, G-J.Cao, Y.Du, P.McCartin and S.Sarkar Dept. of Anatomy & Cell Biology, Tufts University, Health Sc. Campus, Boston, MA 02111

Previous work by us has shown that the translation inhibitory RNA (iRNA; 60-150 nucleotides size range) isolated from the 12S RNP (iRNP) particles of chick embryonic muscle inhibits mRNA translation in vitro by blocking mRNA binding to the 43S preinitiation complex. The iRNA has no effect on the double-stranded RNA sensitive protein kinase activity. The iRNA has now been resolved into multiple biologically active subspecies which inhibit mRNA translation in a discriminatory manner (muscle poly A⁺ mRNA being inhibited more strongly than non-muscle mRNA). A cDNA library for iRNA was prepared in pUC18 vector using random priming of total iRNA and the positive clones were identified by screening with radio labeled iRNA. The 114 nucleotide insert of one clone was subcloned in the Bluescript vector. The in vitro transcript of this insert (only the 5'-3' and not the reverse orientation) showed inhibitory activity of mRNA translation in vitro. The sequence of the iRNA derived from DNA sequencing of the insert is:5'AUCCAUUUUCAGGGCUAGUUGAUUCGGCAGGUGAGUUGUUCACACUCCUUAGCGGGUUCCGACUUCCAU GGCCACGUCCUGCUGUCUAGAUCAACCAGCCCUGAAAAUGGAU 3'. Sequence analysis with the data base indicates that it is a new sequence in which 27 nucleotides underlined at the 3'and 5' ends can form base paired stem, the remaining of the molecule having a loop structure. These results suggest that iRNA(s) are true cellular entities that may play a role in the regulation of translation during myogenesis. Studies on structure-function analysis of cDNA clones for other iRNA subspecies are now in progress.

GENERATION OF MYONUCLEI BY SATELLITE CELLS DURING SKELETAL MUSCLE GROWTH, Edward Schultz and Jill Heckman-Jones, Department of Anatomy, University of Wisconsin, Madison, WI 53706

Since the report of Moss and Leblond ('71) demonstrated that satellite cells are the source of new myonuclei during postnatal growth, it has been generally accepted that the entire population is mitotically active and participates equally in the production of myonuclei. The pattern of satellite cell proliferation and fusion was examined in 30 day old rats during a specific interval of the postnatal growth period to determine if, in fact, the entire population is dividing and sharing equally in the production of myonuclei. Mitotic activity of satellite cells in the soleus and extensor digitorum longus (EDL) muscles was monitored by continuous infusion of bromodeoxyuridine (BrdU) using mini-osmotic pumps for periods of up to 14 days followed by electron microscopic immunocytochemistry. At each time point studied, the percentage of labeled satellite cells was greater in the soleus than EDL, although the pattern of labeling in both muscles was the same. Labeling in the satellite cell population increased linearly at a constant rate during the initial 5 days of continuous infusion at which time approximately 73% of the satellite cells in the EDL and 80% in the soleus were labeled. During this same infusion period there was a linear increase in the percent of labeled myonuclei and a constant percentage of satellite cells in both muscles suggesting that the concentrations of BrdU used did not inhibit terminal differentiation of the cells. After 5 days the rate of increase in labeled satellite cells was reduced suggesting that the remaining unlabeled cells constituted a slowly dividing population. Unlabeled satellite cells were still present after 14 days of infusion. These results suggest that satellite cell populations in growing muscle are not homogeneous and that a subpopulation of slowly dividing satellite cells may function as progenitor cells for more rapidly dividing, fusion competent cells. The size of this slowly dividing population is not the same in all muscles and may be regulated according to individual muscle growth characteristics. (Supported by NIH AR38033 & MDA)

COMPARISON OF THE ACCUMULATION OF VASCULAR SMOOTH AND SKELETAL MUSCLE ACTIN mRNAS IN C2C12 AND BC3H1 CELLS DURING GROWTH AND DIFFERENTIATION,

Sandra B. Sharp, Shamshad Khan, Dinithi Silva, Moises Theimer and Abel Vazquez, Department of Biology, California State University, Los Angeles, CA 90032

BC3H1 cells are myogenic and express many characteristics of skeletal muscle cells, but do not fuse or undergo terminal differentiation. It is known that these cells express vascular smooth (vsm) and skeletal (skm) muscle actin mRNAs during differentiation. In the present work we have examined the accumulation of these two actin mRNAs in both growing and differentiating BC3H1 cells under a number of different culture conditions. In addition, we have analyzed the accumulation of these two mRNAs in C2C12 cells, which do fuse and terminally differentiate. Our results show that vsm, but not skm, actin mRNA is expressed in both BC3H1 and C2C12 cells which are dividing. When BC3H1 cells are induced to differentiate, whether by serum depletion or by switch from high serum medium to medium supplemented with only 2% fetal bovine or 2% horse serum, the amount of vsm actin mRNA stays the same or increases over time, and skeletal actin mRNA appears for the first time and increases in abundance. In differentiating C2C12 cells, skm actin mRNA is similarly induced, but vsm actin mRNA clearly declines in abundance. These observations are consistent with at least two models: 1) a mechanism for down-regulating vsm actin mRNA accumulation is operative in C2C12 cells, but not in BC3H1 cells; or 2) a mechanism for up-regulating vsm actin mRNA accumulation is operative in BC3H1 cells but not in C2C12 cells. Investigation of the expression of vsm and skm actin mRNAs in myogenic lines derived by transfer of myogenesis determination genes present in C2C12 cells but absent in BC3H1 cells may help to elucidate these observations. We thank B.H. Min, D. Foster, and A. Strauch for the vsm probe. Supported by grants from NIH-MBRS and NSF-RIMI.

LONGTERM STABLE EXPRESSION OF FOREIGN GENES DIRECTLY TRANSFERRED INTO MOUSE SKELETAL MUSCLE, Jon A. Wolff, Gyula Acsadi, Agnes Jani, Phillip Williams, Wang Chong, Department of Pediatrics and Genetics, Waisman Center, University of Wisconsin, Madison, WI 53706

The direct introduction of genetic information into skeletal muscle cells *in vivo* would be useful for gene therapy purposes. *In situ* cytochemical staining for ß-galactosidase activity was localized to muscle cells following injection of a ß-galactosidase DNA vector (pRSVLac-Z). DNA vectors consisting of genes for chloramphenicol acetyltransferase (pRSVCAT) and luciferase (pRSVL) were *separately injected into mouse skeletal muscle. Protein expression was readily* detected in all cases; no special delivery system was required for these effects. The levels of expression from both constructs were comparable to levels of expression obtained from fibroblasts transiently transfected *in vitro* under optimal conditions. Protein expressions from the pRSVCAT vector and pRSVL vector were stable for at least two and twelve months, respectively. The molecular basis for the stable expression has been investigated and it appears that the stable expression is due to the persistence of the injected DNA in an extrachromosomal, circular form. This has implications for gene therapy and for our understanding of the maintenance of nuclear DNA in non-dividing cells.

FUNCTIONAL ANALYSIS OF THE EMBRYONIC AND ADULT ISOFORMS OF CHICKEN CARDIAC TROPONIN T IN RECONSTITUTED THIN-FILAMENTS

Ted S. Wong, Keyvan J. Nouri, Sarah Shull, Roger A. Cooke and Charles P. Ordahl, Depts. Anatomy, Biochemistry and Biophysics and the Cardiovascular Research Institute, University of California, San Francisco, CA. 94143

Two cardiac isoforms of the thin filament regulatory protein, troponin T (cTnT), are expressed in the developing chicken embryo. $cTnT_{emb}$ is expressed during early embryonic stages in both cardiac and skeletal muscle but is gradually replaced in mature muscle by the adult form of cTnT ($cTnT_{adult}$) in the heart and by skeletal isoforms (skTnT) in skeletal muscle. The two cTnT isoforms are products of a common gene and differ only by the inclusion of a 10 amino acid peptide in $cTnT_{emb}$ that is excluded from $cTnT_{adult}$ by developmentally regulated splicing (Cooper and Ordahl, J. Biol. Chem. 260:11140-11148, 1985). This tissue and developmentally regulated program of switching implies that each isoform offers a distinct function to the cell when expressed. One possibility is that each cTNT isoform differentially affects the contractile properties of the muscle. This hypothesis was examined by comparing the ability of TnT isoforms to activate the calcium-regulated Mg^{2+}-ATPase rate in muscle fibers using a model of *in vitro* thin filment reconstitution with alternative TnT isoforms. Adult chicken cardiac muscle thin filaments substituted with bacterially expressed $cTnT_{emb}$ showed a decreased ability to activate myosin ATPase rate compared to the native isoform ($cTnT_{adult}$) or to filaments substituted with bacterially produced $cTnT_{adult}$. These data suggest that the 10 amino acid embryo-specific region of $cTnT_{emb}$ may affect myofiber assembly or cooperative interactions between thin filament proteins. Supported by grants from the NIH-Program of Excellence in Molecular Biology.

SATELLITE CELLS AS SOURCE OF bFGF AND ROLE IN MUSCLE HYPERTROPHY, Shigeru Yamada, Takashi Kitajima+, Yoriko Atomi and Richard Strohman*, Department of Sports Sciences, University of Tokyo, Tokyo Japan 153, Fuji Rebio Research Laboratory, Tokyo Japan+, Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720*

Evidence will be presented showing that skeletal muscle satellite cells are the source of new fibers seen in hypertrophied skeletal muscle. The mechanism by which these cells are activated in vivo is not understood. bFGF is a known mitogen for these cells in vitro and bFGF is localized together with satellite cells in muscle fiber basal lamina in vivo. We have attempted to identify the source of bFGF found in the basal lamina. Chicken myoblast cells in vitro produce a conditioned medium (CM) which stimulates new cell replication when used in primary cultures of chicken embryonic myoblasts or fibroblasts. The activity of CM can be absorbed by heparin-Sepharose and is eluted by 2M NaCl and resembles bFGF. Myoblasts but not fibroblasts from 12 day chicken embryo muscles in culture react with an antibody to bFGF and we are able to detect bFGF mRNA transcripts in myoblasts using a rat bFGF probe and PCR.