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Myoblast and Myotube Nuclei Display Similar Patterns of Heterogeneous Acetylcholine Receptor Subunit mRNA Expression

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Abstract Muscle progenitor cells differentiate to myoblasts, and subsequently myotubes, upon expression of muscle specific genes. We and others have previously shown that myotube nuclei, even in the absence of nerve, express AChR α subunit RNA at varying levels, with a small subset (about ten percent) of the nuclei expressing at high levels. These findings raised two important questions: 1) is the observed heterogeneity a unique property of the α subunits, and 2) when does the heterogeneity begin? In particular, is it induced only at or after the time of fusion, or does it exist at the myoblast stage? We have, therefore, extended our observations to the γ and δ subunits and we also have examined the distributions of AChR α, γ, and δ subunit RNAs in both myoblasts and myotubes. We used intron and intron-exon probes to detect prespliced transcripts or mature mRNAs in the cells. Because intron-containing transcripts are not transported out of the nuclei, the distributions of these transcripts can indicate their expression patterns among nuclei in the same myotubes. Our results show that both myotubes and myoblasts have distributions of the AChR α, γ, and δ subunit RNAs which differ sharply from that of the U1 RNA or Myo D. Thus, the heterogeneous expression of AChR genes is not only an intrinsic property of muscle cell nuclei (in the sense that it does not require the presence of nerves), but it also exists prior to fusion. Our results suggest that muscle nuclei attain individualized capacities for AChR subunit mRNA production early in their development. Conceptual models consistent with such individuality imply an additional level of regulation beyond the known diffusible transcriptional factors.

Key words: in situ hybridization, desmin, nuclear diversity, MyoD, cell fusion

The muscle nicotinic acetylcholine receptor (AChR[1]), a membrane-bound oligomeric protein, is comprised of five subunits ($\alpha_2\beta\gamma\delta$ or $\alpha_2\beta\epsilon\delta$) encoded by four separate genes [Mishina et al., 1986; Changeux, 1989]. The receptor is a ligand gated ion channel responding to environmental cues and motor neuron innervation. During muscle development, the receptor undergoes physiological, biochemical, and molecular alterations, including changes in channel open time and turnover rate, an increase in the receptor number, and a switch from γ subunit to ε subunit in the adult [Changeux, 1989; Hall and Sanes, 1993; Mishina et al., 1986; Gu and Hall, 1988; Fontaine et al., 1989; Fontaine and Changeux, 1989; Goldman and Staple, 1989; Brenner et al., 1990]. In the adult neuromuscular junction, the density of AChR is about 1,000-fold higher than in the extrajunctional regions [Salpeter and Loring, 1985]. Other components that have been shown to concentrate at the postsynaptic sites of neuromuscular junctions include N-CAM in the plasma membrane, s-laminin, agrin, acetylcholinesterase in the basal lamina, the 43-KDa rapsin proteins, and dystrophinlike proteins in the cytoskeleton [Frail et al., 1988; Phillips et al., 1991; Froehner et al., 1990; Froehner, 1991; Ferns and Hall, 1992; McMahon and Wallace, 1989; Sanes, 1989].

This accumulation of AChR beneath the nerve terminals is not limited to the increase in protein. It has been shown that the mRNA levels for the α and δ subunits are much higher at the junctional regions than in areas outside them [Merlie and Sanes, 1985] and, indeed, in mature

Abbreviations used: AChE, acetylcholinesterase; AChR, acetylcholine receptor; BSA, bovine serum albumin; DTT, dithiothreitol; PBS, phosphate buffered saline; PCR, polymerase chain reaction; mAb: monoclonal antibody; SDS, sodium dodecyl sulfate.

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innervated muscle higher level expression of the AChR subunit RNAs appears to be restricted to a certain population of postsynaptic nuclei [Fontaine et al., 1989; Fontaine and Changeux, 1989; Brenner et al., 1990; Goldman and Staple, 1989; Sanes et al., 1991; Simon et al., 1992]. Our previous studies and those of others have shown that the diversity among nuclei in AChR α subunit expression is not limited to the subsynaptic nuclei: in situ hybridization studies with cultured, noninnervated myotubes showed that a certain population of nuclei are highly active in the expression of the α subunit RNA and others are inactive or express AChR RNAs in low abundance [Bursztajn et al., 1989; Harris et al., 1989; Horovitz et al., 1989; Fontaine and Changeux, 1989]. Furthermore, muscle mRNAs and their corresponding proteins appear to have relatively confined distributions around the nuclei from which they originated [Ralston and Hall, 1989, 1992; Hall and Ralston, 1989]. The AChE mRNA appears to be transported further away from its origins than the AChR α subunit mRNA [Tsim et al., 1992].

Because AChR subunits are products of different genes, it is important to determine how the synthesis of the subunits is coordinated among the nuclei of multinucleated muscle cells. It has been shown in a number of species that the gene expression at steady-state is not the same for all the subunits during embryonic development or after denervation [Moss et al., 1987, 1989; Shieh et al., 1987; Goldman et al., 1988; Buonanno and Merlie, 1986; Baldwin et al., 1988]. The mechanism responsible for the differential expression of subunit RNAs has not been elucidated. Trophic factors such as acetylcholine receptor-inducing activity (ARIA) [Harris et al., 1988] or the calcitonin gene related peptide (CGRP) induce a larger increase of the α mRNA than of the γ or δ subunit mRNAs [Osterlund et al., 1989; Kirilovsky et al., 1989]. Muscle activities and neuronal factors play major roles in the AChR regulation [Shieh et al., 1987; Goldman et al., 1988; Kirilovsky et al., 1989; Bursztajn et al., 1990; Witzemann and Sakmann, 1991]. In addition, AChR subunit genes, like many other muscle specific genes, appear to be activated by a family of muscle-specific transcription factors such as MyoD [Weintraub et al., 1991; Li and Olson, 1992; Piette et al., 1990]. Because there is a close association of myotube nuclei with AChR clusters [Bruner and Bursztajn, 1986] and acetylcholinesterase [Rossi and Rotundo, 1992], the expression of AChR and muscle specific genes may also be regulated by the subcellular environments of multinucleated myotubes.

Despite the large amount of work regarding AChR subunit RNA distribution and the factors affecting their regulation, many questions remain unanswered. For example, when during myotube differentiation is the diversity among nuclei initiated? Does it occur in prefusion myoblasts? Do intrinsically different nuclei become incorporated into a single multinucleated myotube? How does the expression of the myogenic factors, such as MyoD, change in prefusion myoblasts and myotubes? And is the MyoD mRNA distributed in equal amounts among the myoblast and myotube nuclei? To answer these questions, we have examined the expression of three AChR subunit genes (α, γ, and δ), as well as the CMD1 (chicken MyoD) gene [Lin et al., 1989] in cultured chicken myoblasts and myotubes by in situ hybridization. Our studies indicate that the diversity among nuclei in AChR subunits expression is controlled by a program already active prior to fusion.

MATERIALS AND METHODS

Cell Culture

Myoblasts were isolated from 11-day-old White Leghorn chick embryos and were plated on collagenized coverslips in 35-mm petri dishes (1.5×10^5 cells/plate) as described previously [Bursztajn, 1984]. At this stage of development, fetal myoblasts but no adult satellite cells are present [Feldman and Stockdale, 1992]. Cells were grown in Eagle's minimum essential media made in Earle's balanced salt solution (Sigma, St. Louis, MO), supplemented with 10% horse serum, 100 μ/ml of penicillin/streptomycin, and 2% of chick embryo extract [Bursztajn, 1984]. Media were changed on the second day after plating and every other day afterwards. To delay cell fusion, low calcium media (Cellgro/Mediatech, Herndon, VA) were used for initial plating and the cells were grown for two days before changing to regular media.

Immunocytochemistry

The cultured cells were fixed in 4% paraformaldehyde prepared in 10 mM $MgCl_2$ and PBS (10 mM phosphate buffered salts plus 120 mM NaCl and 2.7 mM KCl), washed with PBS, and preserved in 70% ethanol at 4°C. Before immunostaining, fixed cells were rehydrated in PBS, permeabilized with 0.1% Triton X-100 and incu-

bated with 0.1% BSA in PBS (PBS-BSA) for 30 min. A primary antibody (anti-desmin, Developmental Studies, Hybridoma Bank, or anti-fast myosin isoform, a gift from F.E. Stockdale, University of Iowa) was diluted (1:20) in PBS-BSA and cells were incubated for 1 h at 37°C. After washing with PBS-BSA, biotinylated anti-mouse IgG was used to bind to the primary antibody and, subsequently, Texas Red-conjugated streptavidin (1:150) was applied to the samples for 1 h at 37°C, followed by washing with PBS. The stained cells were examined with fluorescent microscopy and kept in 70% ethanol at 4°C until ready for use in the in situ hybridization studies.

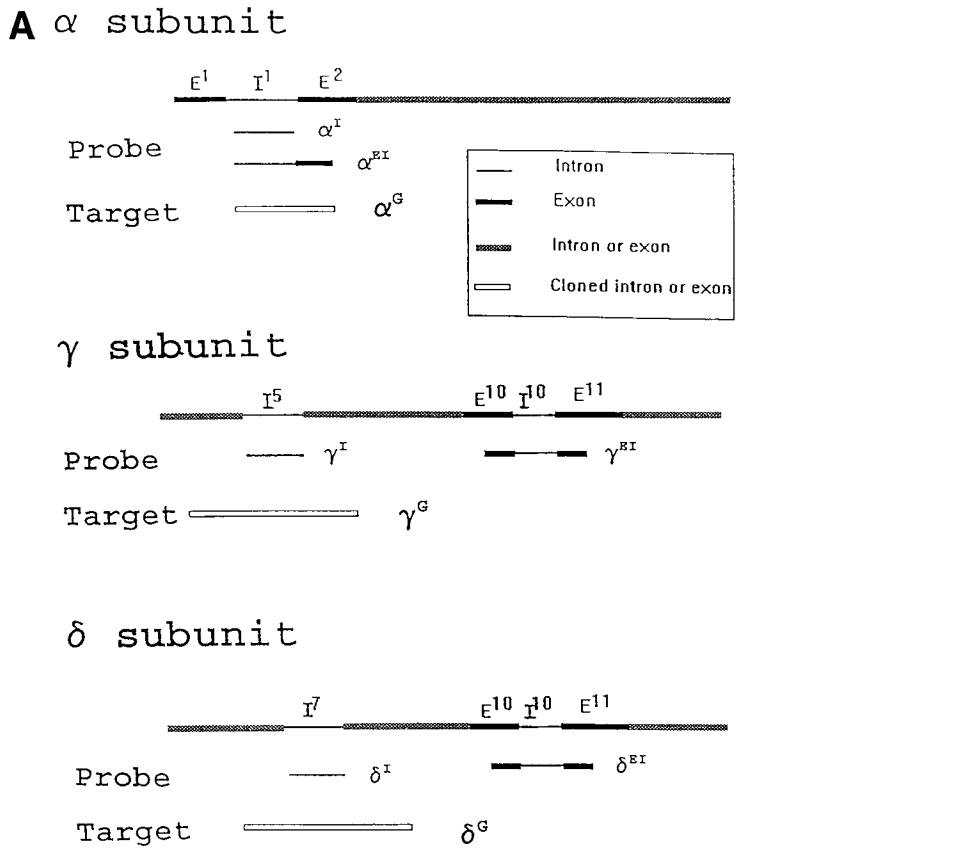
DNA Cloning

Origins and constructs for the cloned DNA fragments used in this study are diagramed in Figure 1.

Intron-exon DNA (465 bp) of AChR α subunit was in M13mp8 vector [Klarsfeld and Changuex, 1985; Bursztajn et al., 1989] or re-cloned in pBluescript SK(+) plasmid (pBS, Stratagene, La Jolla, CA). The 340 bp intron portion of the DNA was subcloned into pBS KS(−) [Berman et al., 1990]. A near full-length α cDNA clone, which did not contain the complete second exon sequence, was a gift of Dr. K.M. Rosen of Harvard Medical School. The probe DNAs of the γ and δ subunits were cloned by the PCR-aided method [Su and Bogorad, 1991] from nonhomologous regions according to published sequences [Nef et al., 1984; Genbank sequences: K02903 and K02904]. Chick embryonic tissues were homogenized with a mortar and pestle in a solution containing 10 mM Tris-HCl, 1 mM EDTA, and 2% SDS. After phenol/chloroform extraction, the DNA was purified with Geneclean (Bio101, La Jolla, CA). About 1 μg of the genomic DNA was used for PCR with specific primers under conditions similar to those suggested by Perkin-Elmer-Cetus. PCR product sizes and PCR primers used were γ intron (288 bp), 5′ primer—GTGAGCACAGCTTGG; 3′ primer—GGGAGGTTGGCAATC; γ intron-exon (199 bp), 5′ primer—AGGGATGGACTGATG; 3′ primer—GTTGCAGGCCTCAAC; δ intron (250 bp), 5′ primer—AGTGAGTGGCACCTG, 3′ primer—ACCTGGAGAGCAGAG; δ intron-exon (186 bp), 5′ primer—GCACGGGCTGGCCAG; 3′ primer—GTTGGCCTCATCCAG. About 10 ng each of the amplified and purified DNA was ligated to 10 ng of pBS SK(+) plasmid linearized with *Eco*RV. The ligated DNA was subjected to PCR with the universal primer (GTAAAACGACGGCCAGT) and the 5′ primer specific to a subunit DNA. The DNA product was blunt-ended by treatment with T4 DNA polymerase in the presence of 200 μM dNTP for 10 min and then digested with *Hind*III. The DNA was then cloned into the same vector which had been digested with *Eco*RV and *Hind*III. The cloned DNA was sequenced by the dideoxy nucleotide method [Sanger et al., 1977]. The full-length cDNA clones for γ and δ and the partial genomic DNAs p9 (γ) and p1.6 (δ) were gifts of Dr. M. Ballivet [Nef et al., 1984].

Probe DNA for the CMD1 was a 609 bp fragment of the 5′ region of the CMD1 cDNA (153 bp 5′ noncoding region and 456 bp of the 5′ coding region) prepared by *Pst*I digestion of the cDNA clone in pBS KS(+) [Lin et al., 1989]. After removal of the 3′ region, the digested plasmid was relegated. The 297 bp of the chicken U1 gene [Earley et al., 1984; Berman et al., 1990] was cloned in pBS SK(+). A 224 bp sequence between the reverse primer (AACAGCTATGACCATG) site and the universal primer

Fig. 1. Schematic diagrams of probe DNAs (not to scale). **A:** Origins of subunit DNA clones. Locations of relevant introns (I) and exons (E) in each subunit gene are shown and labeled by superscripted numbers in the upper line. The DNA clones are identified with the Greek letters (α, γ, and δ) for the subunits and superscripted letters for intron (I) and exon-intron (EI) or genomic DNA clone (G). The cloned DNAs are aligned directly under the corresponding regions of the genes and overlapped DNAs share common sequences. Probe: DNAs used for hybridization in this study. Target: DNAs used for probe specificity test (see text), which include α^G (genomic DNA of α subunit cloned in M13mp8 vector, see Bursztajn et al. [1989]), γ^G and δ^G (same as p9 and p1.6, respectively, in Nef et al. [1984]). The boundaries of the DNA clones in the genes can be found from corresponding primers listed in the text and cited references. **B:** Probe DNA constructs. DNAs used for probe preparation were cloned in pBluescript (pBS) KS (±) or SK (±). The names of the inserts are identified in the diagrams, except that "Other intron/exon" includes exon-intron DNA of α, γ, and δ, as well as intron DNA of δ and γ (see A for more details). The restriction enzyme sites are the places where the DNAs were inserted. All enzyme sites are intact except the *Eco*RV sites were altered after ligation. Arrow heads in the insert DNAs indicate the relative orientations of the insert DNAs in the vectors (5′ to 3′). Letters R and U represent positions of the reverse and the universal primers. KS and SK are also two primer sites. All primers are orientated from 5′ to 3′ on these sites as indicated by the arrows. T3 and T7 are promoters in orientations as indicated by the arrows (5′ to 3′). The plasmids containing inserts were either digested by *Pvu*II or amplified by PCR with the reverse and the universal primers. cRNAs were then produced using the promoters labeled with stars. cDNA probes were generated by reverse transcription from these cRNAs using the primer sites labeled with stars.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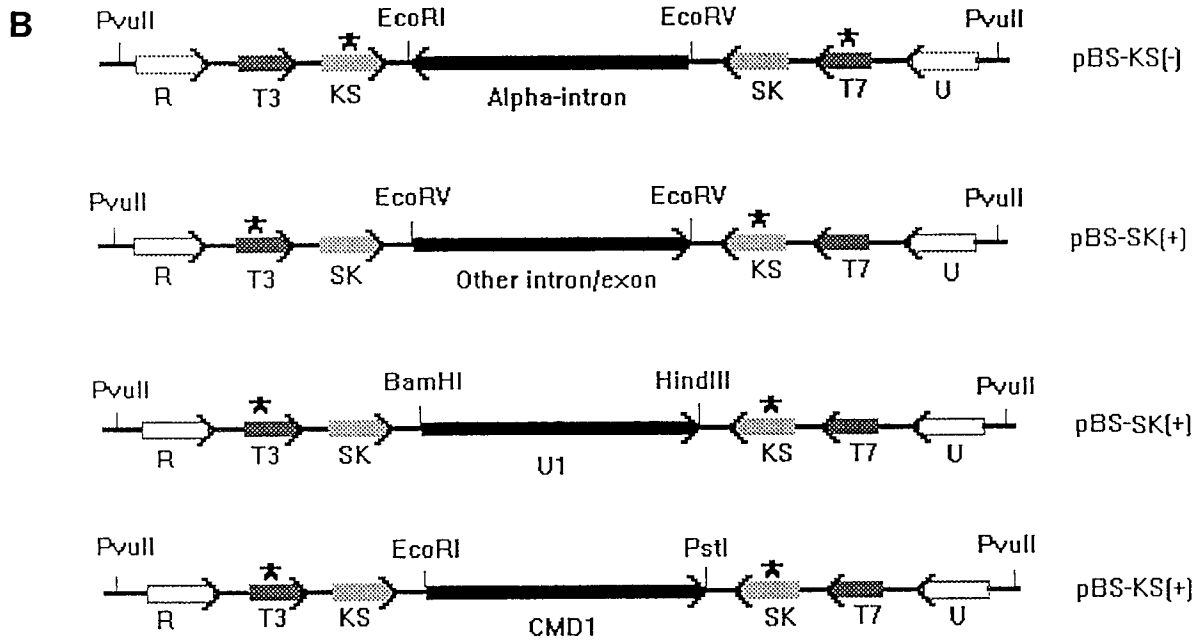
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BamHI
HindIII
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site of pBS vector was used as a probe DNA for a negative control.

Probe Labeling

Dot blot hybridization was used to determine the specificity of probes. Probe DNAs were labeled by the random primer labeling method [Feinberg and Vogelstein, 1983, 1984] with a nonradioactive digoxigenin labeling kit obtained from Boehringer Mannheim (Germany). The signals were detected by the chemiluminescent procedure specified by the same manufacturer.

Probes used for in situ hybridization were prepared as follows. The insert DNAs (Fig. 1B) were isolated after PCR with the universal and reverse primers or, in some cases, *Pvu*II digestion. Sense RNAs were prepared by in vitro transcription with T7 or T3 RNA polymerase (Stratagene). After treatment with RNase-free DNase, the cRNAs were preserved in 70% ethanol without salt. About 100 ng of each RNA was dried and used for cDNA (antisense) labeling with the SuperScript Preamplification system (*Gibco-BRL*, Gaithersburg, MD) and 10 ng of specific primer (KS primer, CGAGGTCGACGGTATCG or SK primer, TCTAGAACTAGTGGATC, Stratagene). Besides the cRNA and primer, a labeling reaction (10 μl) includes 10 mM DTT, 100 μM dGTP and dTTP, 20 μCi of each ^{35}S-α-dATP and ^{35}S-α-dCTP, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 1 μg BSA, and 100 units reverse transcriptase. After 40 min at 42°C, 100 μM of each dATP and dCTP were added and the reaction continued for another 20 min. At the end of the reaction, 1 μg RNase A and 0.5 units of RNase H were added to digest the RNA at 42°C for 30 min. The reaction was heated by boiling, then cooled on ice-water and diluted with 60 μl of 10 mM DTT. The diluted reaction was incubated for 42°C for another 30 min, then boiled and cooled on ice-water. Labeled cDNAs were purified with a G-50 mini column (Stratagene) or Geneclean (Bio101). Alternatively, single stranded DNA probes were prepared by a primer extension method [Bursztajn et al., 1993]. Labeled probes with specific activities of about 1×10^8 cpm/μg were used for in situ hybridization.

In Situ Hybridization

Cells grown on coverslips were fixed, stained, and treated sequentially in each of the following solutions for 10 min: 1) 1 × PBS; 2) 0.25% acetic anhydride in 0.1 Methanolamine, pH 8.0; 3) 0.2 M Tris-HCl (pH 7.5) and 0.1 M glycine; 4) 2 × SSC in 50% formamide (1 × SSC = 0.3 M NaCl and 0.03 M NaCitrate). Hybridization buffer (50 μl/sample) contained 2 × SSC, 1 × Denhardt's solution (0.02% BSA, 0.02% ficoll, and 0.02% polyvinylpyrrolidone), 5% dextran sulfate, 0.5 mg/ml salmon sperm DNA, 0.25 mg/ml yeast tRNA, 20 mM vanadyl ribonucleoside complex, 20 mM DTT, 0.5 mg/ml heparin, 50% formamide, and probe of 4×10^5 cpm. Hybridization was carried out at 37°C for 4–6 h. The samples were washed for 20–30 min at 37°C in each of the following solutions containing 20 mM DTT: 2 × SSC, 0.5 × SSC, 0.1 × SSC (twice). After dehydration and mounting on glass slides, the samples were coated in a dark room with NTB-3 emulsion (1:1 diluted, Eastman Kodak, Rochester, NY) at 42°C. The length of exposure time depended on the probes used (from 1 day to 1 month). After development of the slides, the nuclei of cells were stained with bisbenzimide (Hoescht 33258, Sigma) (37 μg/ml in 56% glycerol and 6 mM Tris-HCl, pH 8).

Microscopic and Statistical Analyses

After immunocytochemistry and in situ hybridization, the cells were examined with combined dark field and fluorescent microscopy [Bursztajn et al., 1993]. Triple exposures of color photographs were taken with a Nikon microscope using Kodak Ektachrome ASA-400 films (Eastman Kodak): dark-field for grain photography (auto setting −1, about 2 s), fluorescence photography for Texas-Red with a DM580/G-1B filter (auto setting −1, about 30 s), and fluorescence photography for bisbenzimide with a DM400/UV2A filter (setting −2, about 5 s).

We quantified hybridization signals by counting autoradiographic grains over 200 nuclei for each molecular probe and constructed frequency distributions to show the number of nuclei which contained a given number or range of grains. Grain counts were done with dark-field and fluorescence microscopy using through focus, thus allowing us to count grains above and below nuclei. The combination of these optics allowed us simultaneously to count grains and see the bisbenzimide labeled nuclei. This procedure allowed us to readily identify mononucleated cells and their distribution of grains as well as multinucleated myotubes and their grain distribution. We normalized the grain counts for each slide by the mean number of counts per nucleus similar to methods described by others [Tsim et

al., 1992] to facilitate comparisons between different experiments and between different probes. We initially evaluated the resultant distributions by visual inspection, also smoothing the data using a locally weighted least mean square procedure, the Cleveland-Devlin algorithm [Cleveland and Devlin, 1988] as implemented in the Axum Technical Graphics and Data Analysis software [1993] to facilitate such comparisons. We then compared one distribution in several different ways. To compare the distributions in general, without arbitrary subdivisions, we used the nonparametric Kolmogorov-Smirnov two sample test. We also divided the nuclei into two groups (>2.0 times the mean vs. all others), using the Fisher exact test to detect significant differences between probes, and into three groups (≤ 0.5, >0.5 and ≤ 1.5, >1.5 times the mean), using Chi-square analysis to test for significant differences [Sokal and Rohlf, 1981; Blackford, 1992].

RESULTS

Probe Specificity

To study AChR mRNA distributions, we obtained intron and intron-exon probes for mRNAs of the AChR subunits α, γ, and δ and an exon probe for MyoD mRNA. To check metabolic activity of cells, we used U1 small nuclear RNA (snRNA) which served as a positive control and the multi-cloning site region of the pBS vector served as a negative control. Figure 1A shows the origins of relevant AChR subunit probes used in this study and Figure 1B shows the DNA constructs and their restriction sites. To test the specificity of these probes, the DNAs were labeled with digoxigenin and hybridized to cDNAs or genomic DNAs fixed on membranes. Figure 2 shows that all the intron probes hybridized to the corresponding genomic DNAs. The intron-exon probes hybridized selectively to the corresponding cDNA and genomic clones but not to genomic clones that lacked the corresponding sequences.

AChR Subunit mRNA Expression After Fusion

Our previous studies showed that RNA for the α subunit of the AChR was heterogeneously expressed among nuclei in myotubes [Bursztajn et al., 1989]. To extend this study, we prepared intron probes for the γ and δ subunit mRNAs. Because our cultures contain fibroblasts, we used an antibody to desmin in combination with defined morphological criteria [Feldman and Stockdale, 1992] that allowed us to distinguish myoblasts from fibroblasts. When the probes were used to hybridize to myotubes in two-day old culture, we consistently observed heterogeneous localization of the subunit RNAs among the myotube nuclei (Figs. 3A, 4C). Some nuclei were associated with few grains while others were heavily labeled. To rule out the possibility that some of the nuclei were metabolically inactive in the cultures, we used the U1 probe to hybridize to nuclei of the myotubes. Our results show that all the nuclei were active in producing U1 RNA (Fig. 3D). The negative control probe did not produce hybridization signals (data not shown).

Because cells were grown on many different coverslips and were hybridized with several different probes, we compared results after normalizing the data by dividing the number of grains per nucleus by the mean number of grains per nucleus obtained for the same probe and the same slide (Fig. 4). All nuclei expressed U1. No nucleus lacked grains and none had as many as 3 times the mean number of grains per nucleus (Fig. 4A–C). When we categorized our normalized counts for U1 into three levels, about 75% of the nuclei had normalized grain count values between 0.5 and 1.5, with 7% below 0.5 and 18% above 1.5. When probes for AChR subunits were used, there were clearly many nuclei which lacked grains and some with more than three times the mean value (Fig. 4A–D). About 50% of the nuclei had normalized values between 0.5 and 1.5, about 25% had values larger than 1.5, and 25% had less than 0.5. These differences in distribution among the three levels of intensity were highly statistically significant ($P < 0.001$) whenever U1 was compared to an AChR subunit probe. There were, however, no significant differences ($P > 0.1$) between the results obtained from one subunit probe compared to another (Chi square [Sokal and Rohlf, 1981; Blackford, 1992]). We also divided the grain levels into two groups: those ≥ 2.0, and all the rest. In this case, almost none of the nuclei probed with U1 showed a normalized grain count ≥ 2.0, whereas 8 to 12% of the nuclei probed with the subunit probes yielded normalized counts ≥ 2.0. This difference between U1 and the subunit probes was significant with $P < 0.001$ and there was no difference ($P > 0.1$) between one subunit and another (Fischer exact test). The overall normalized frequency distributions for the 3 subunits (Fig.

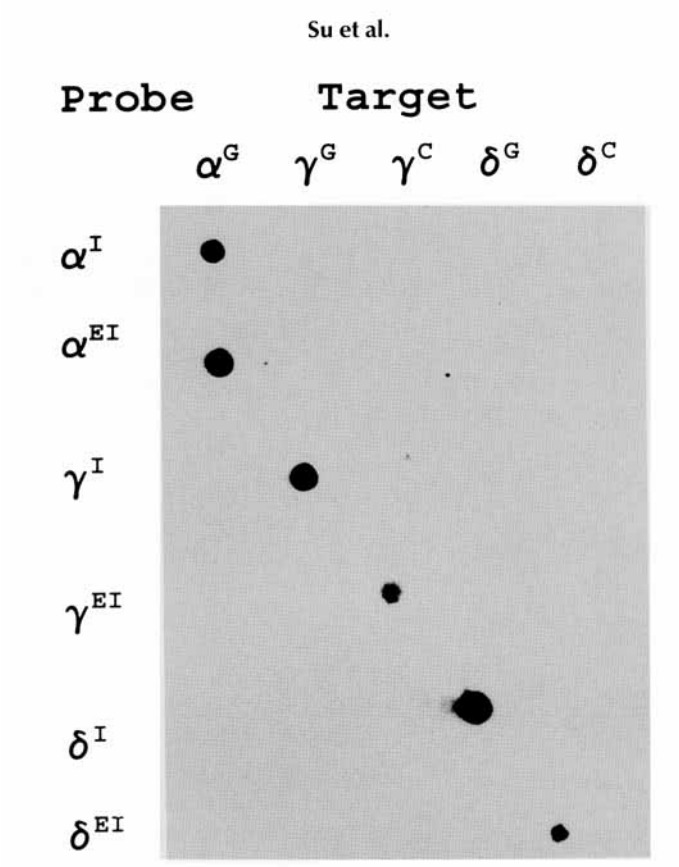

Fig. 2. Specificity test of the AChR subunit probes. The relation between the probe DNAs and the target genomic DNAs (α^G, γ^G, and δ^G) are described in Figure 1, except that the target α^G was in M13mp8 vector. γ^C and δ^C are full-length cDNA clones for γ and δ (gift of Dr. M. Ballivet) cloned in pBS. A full-length α cDNA clone containing the second exon region was not available. The γ cDNA clone was digested by *Pst*I and *Hind*III and the δ cDNA clone by *Eco*RI to separate the inserts from the vector which was the same as that used for probe DNAs (pBS). The gel-purified DNAs and other target DNAs were denatured in 0.4 N NaOH. Aliquots (1 ng DNA in 2 μl) of each denatured DNA were spotted on a nylon membrane in rows. The membrane was trimmed to columns, each of them containing all the target DNAs. The probe DNAs were isolated after PCR with the reverse and the universal primers. They were then labeled with digoxigenin-11-dUTP and hybridized separately to the trimmed membranes. Black dots are positive chemiluminescent signals of hybridization recorded on a X-ray film.

4A–C) were essentially identical to each other and appeared very similar to those of previous studies on α subunits [Bursztajn et al., 1989; Tsim et al., 1992]. The differences of the RNA distribution patterns between U1 and the subunits were statistically significant ($P < 0.001$), but there were no significant differences between the subunits themselves (Kolmogorov-Smirnov two sample test [Sokal and Rohlf, 1981]).

We also examined the distribution of MyoD mRNA which appeared visually similar, though not identical, to that of U1 (Fig. 4D). Like U1, the MyoD distribution differed significantly ($P < 0.001$) from each subunit distribution (Komologorov-Smirnov two sample test, Chi-square, Fisher exact test [Sokal and Rohlf, 1981; Blackford, 1992]). Though both the Myo D and U1 distributions differed markedly from those of the subunits, they were not completely identical to each other. When divided into low, medium, and high expression (≤ 0.5, > 0.5 and ≤ 1.5, > 1.5 times the mean) groups, myo D and U1 differed significantly ($P < 0.01$, Chi-square) though when divided into two groups (> 2.0 times the mean vs. all others), they did not ($P = 0.13$, Fisher exact test). Goodness of fit analysis of the entire distributions shows no difference between U1 and Myo D ($P > 0.1$, Komolgorov-Smirnov two sample test [Sokal and Rohlf, 1981; Blackford, 1992]). Although the myo D and U1 distributions are not completely identical, they are not as strikingly different from each other as they are from the subunit distributions.

AChR Subunit RNA Expression in Myoblasts

In order to learn when the heterogeneity of AChR gene expression occurs during muscle development, we carried out in situ hybridiza-

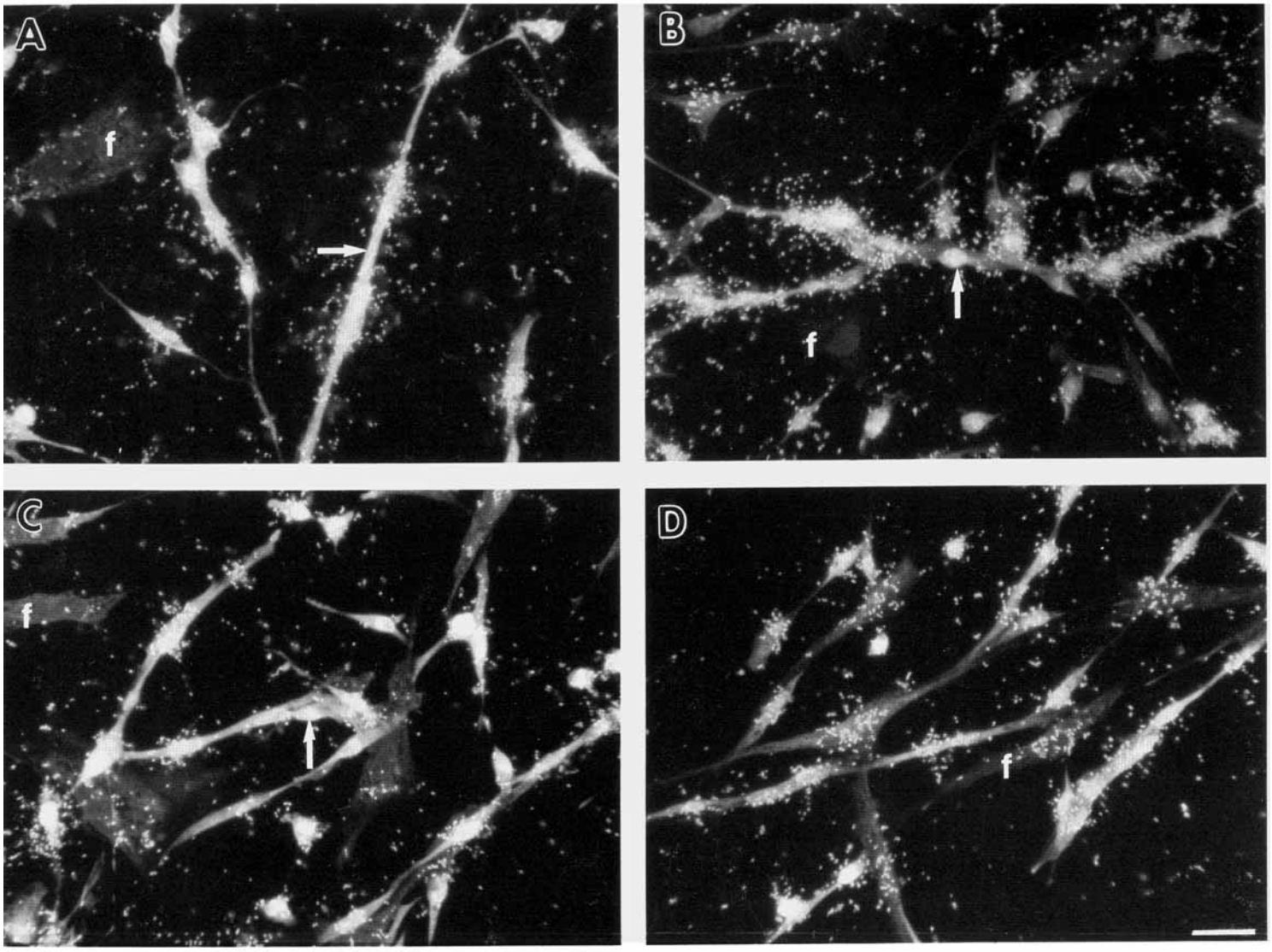

Fig. 3. In situ hybridization of newly formed myotubes with intron probes. Two-day-old muscle cells were hybridized with ^{35}S-dATP labeled intron probes for α (**A**), γ (**B**), and δ (**C**) subunit mRNAs and a probe for U1 (**D**) RNA. Hybridization signals were grains (which appear gold in color by dark field illumination) generated in the emulsion due to radiation from ^{35}S-labeled probes. Arrows point to the nuclei which express few or no AChR subunit RNAs. The cells (red) were stained with an anti-desmin antibody and Texas Red before hybridization. Fibroblasts (f) show background staining and were not hybridized by the subunit intron probes. The U1 probe hybridized to every nucleus including fibroblast nuclei. The nuclei were stained with bisbenzimide and appeared blue in UV light. Bar, 20 μm.

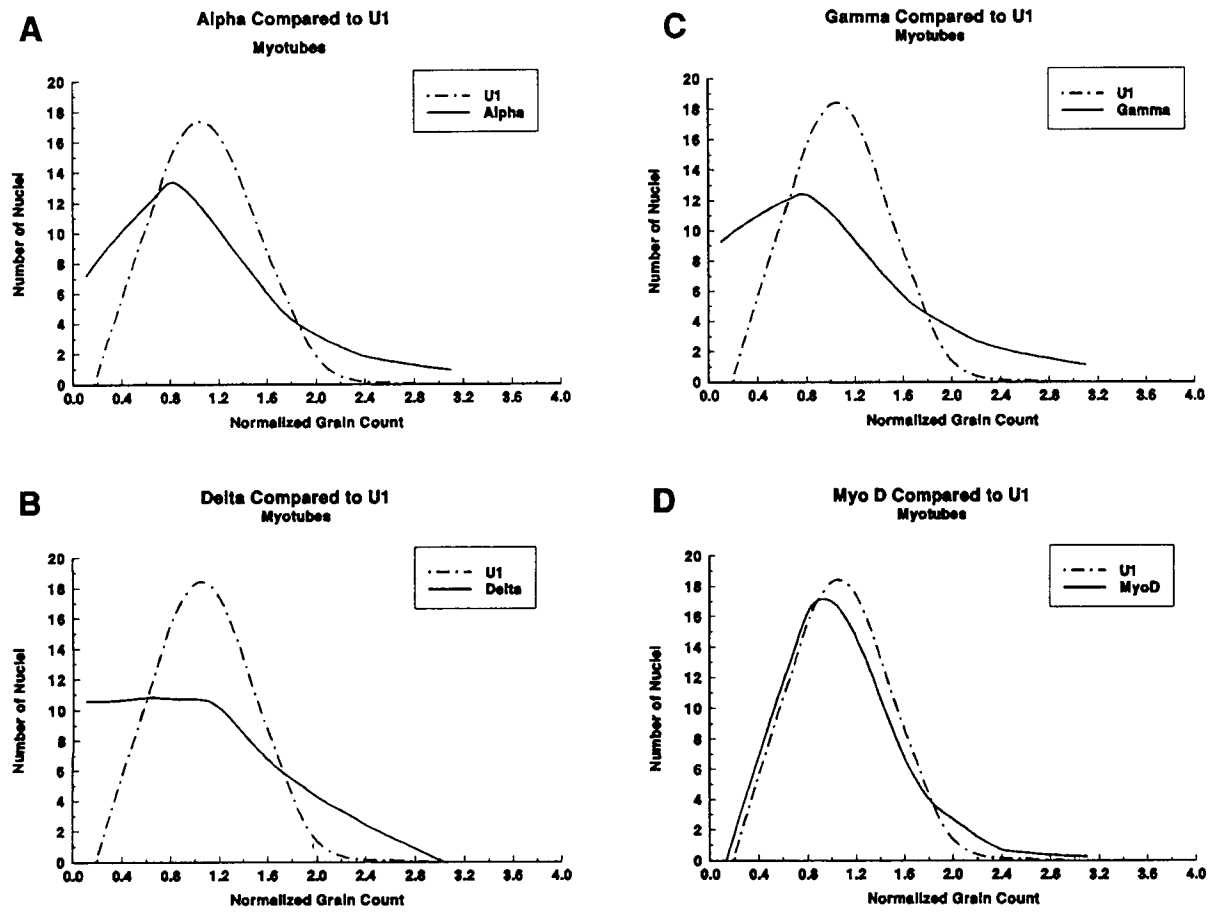

Fig. 4. Grain distribution in myotubes. Silver grains generated after in situ hybridization were counted manually in 200 nuclei for each probe. The mean value of grains per nucleus was calculated from the data. The ratio of actual grain count of a nucleus to the corresponding mean value is defined as the normalized grain count. We plotted the frequency distribution of the number of nuclei at each level of normalized grains counts using a bin width of 0.1. The smooth curve shown resulted from applying a locally weight least mean square procedure to the frequency distribution [Cleveland and Devlin, 1988; Axum Manual, 1993]. Visually, U1 appears markedly different from alpha (**A**), delta (**B**), or gamma (**C**). Analysis of the unsoothed distributions, as described in Materials and Methods, showed that U1 differs from each subunit ($P < 0.001$) but that the subunit distributions do not differ from one another ($P > 0.1$) (Kolmogorov-Smirnov two sample test, Fisher exact test, Chi-square) [Sokal and Rohlf, 1981; Blackford, 1992]. The U1 and MyoD distributions (**D**) in myotubes and myoblasts are similar to each other, but not identical. The distributions as a whole show no significant differences ($P > 0.1$, Kolmogorov-Smirnov two sample test) [Sokal and Rohlf, 1981; Blackford, 1992]. When stratified into three groups they do show a difference ($P < 0.01$, Chi-square test) [Sokal and Rohlf, 1981; Blackford, 1992], but not when divided into two groups (Fisher exact test) [Sokal and Rohlf, 1981; Blackford, 1992].

tion experiments on myoblasts before and during fusion. Because primary myoblasts might have different birthdays and exist in different developmental stages, we used low calcium media to synchronize the cells. In the low calcium media, myoblast fusion was inhibited, but cells divided and were competent to fuse when changed to calcium containing medium or in extended cultures.

In situ hybridization of myoblasts with the α, γ, and δ subunit gene probes showed that only certain populations of cells were heavily hybridized with these probes. Many myoblasts were devoid of grains or have very few grains (Fig. 5A–C). In contrast, in situ hybridization with the U1 probe show that all the cells, including fibroblasts, actively produced the U1 RNA (Figs. 3D and 5D), meaning that all the cells were metabolically active. Hybridization with the MyoD probe produced signals in the desmin-positive cells (myoblasts) but not in desmin-negative cells (fibroblasts) (Fig. 5E). This confirms that all the myoblasts expressed the MyoD RNA.

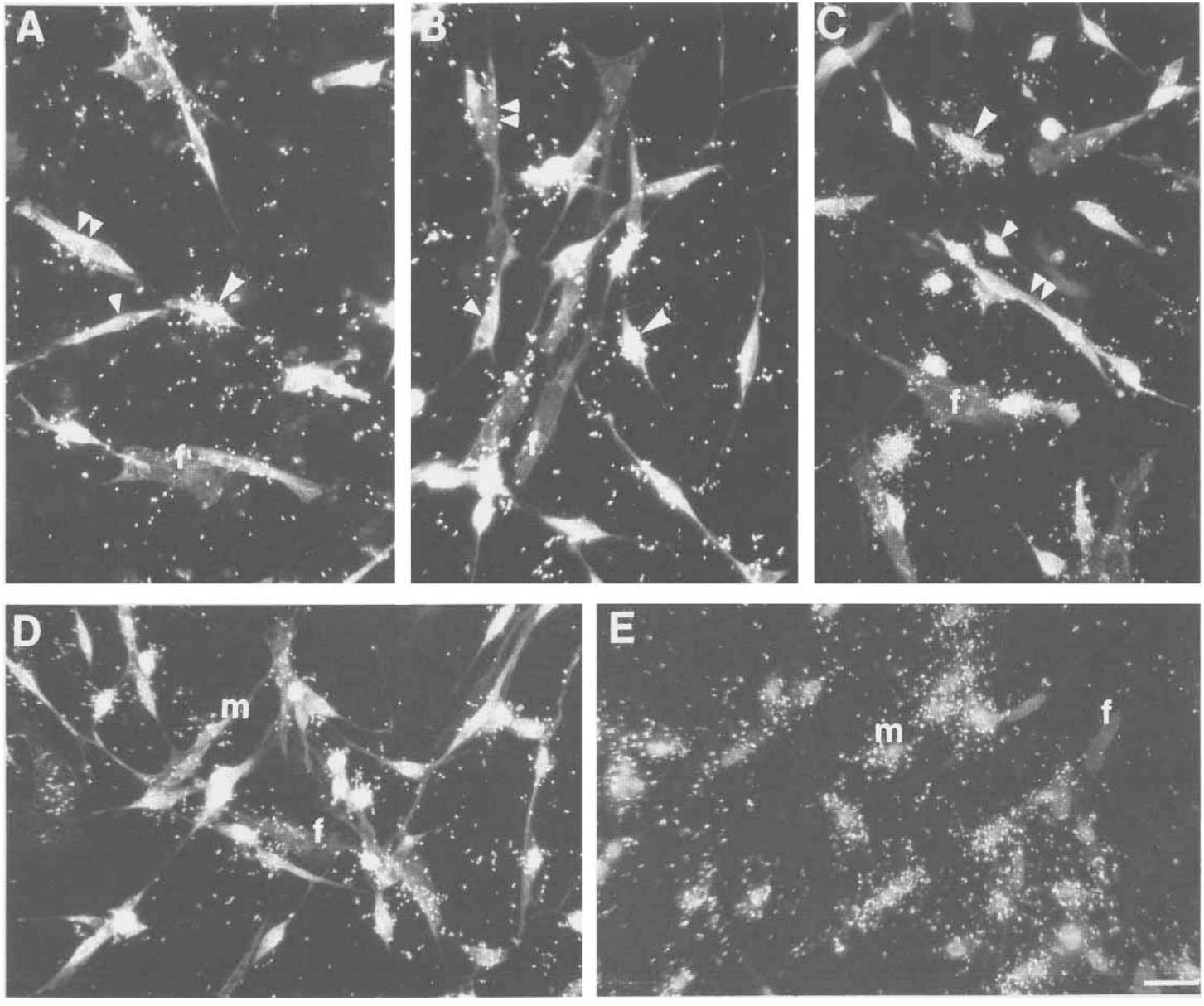

Fig. 5. In situ hybridization of myoblasts with intron-exon probes. Myoblasts were cultured in low calcium media for 2 days. After immunocytochemistry staining with the anti-desmin antibody and Texas-Red, the cells were hybridized with intron-exon probes for AChR subunits α (**A**), γ (**B**), and δ (**C**), as well as probes for U1 (**D**) and myoD (**E**). The letter f marks fibroblasts and m myoblasts. Large arrowheads point to cells which were heavily labeled, small arrowheads point to cells which were associated with few grains or are devoid of grains, double arrowheads indicate cells which were undergoing alignment and possibly fusion. Bar, 20 μm.

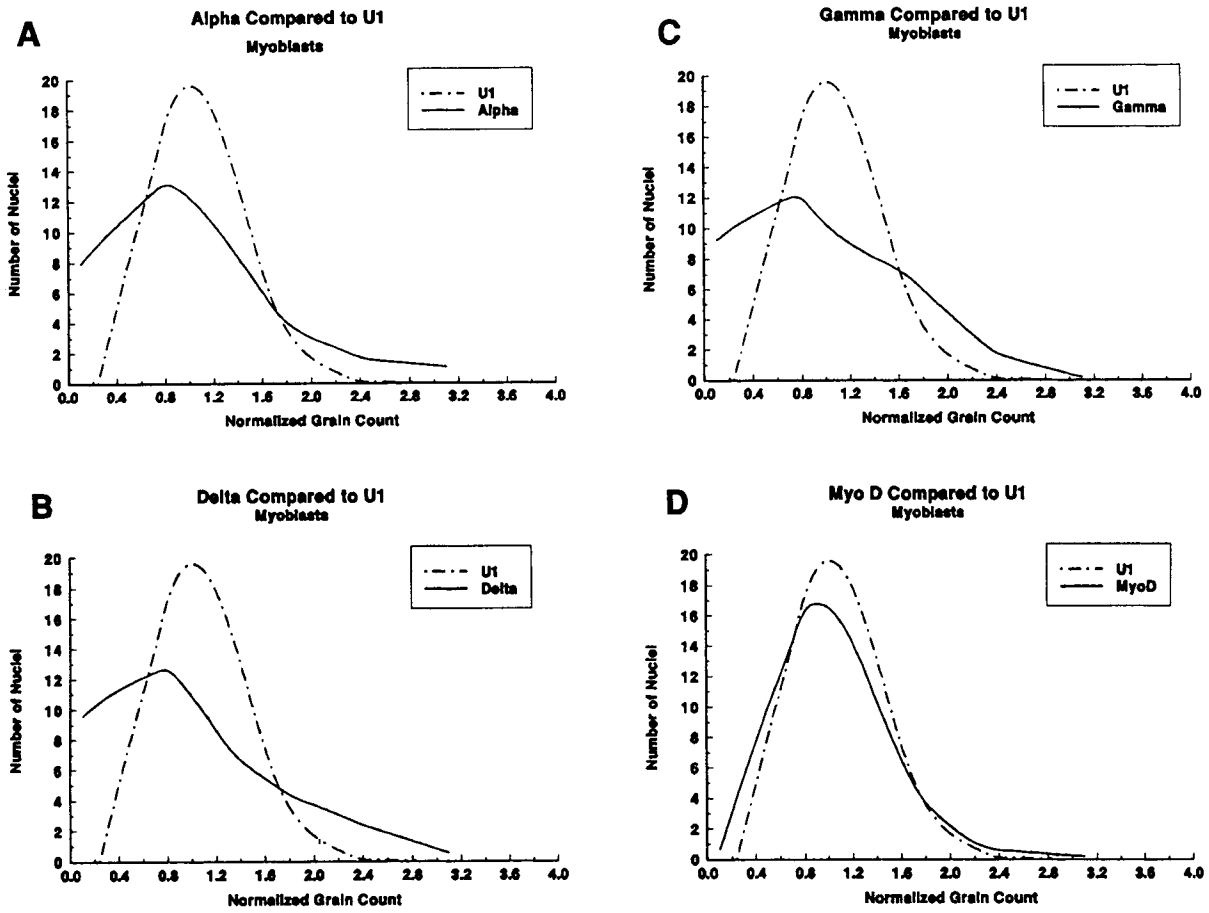

Fig. 6. Grain distribution in myoblasts. Grain distributions for myoblasts were analyzed by the same methods employed for myotubes. Again, U1 appears much different from alpha (**A**), delta (**B**), or gamma (**C**). Analysis of the unsoothed distributions, as described in Materials and Methods, showed that U1 differs from each subunit ($P < 0.001$) but that the subunit distributions do not differ from one another ($P > 0.1$) (Kolmogorov-Smirnov two sample test, Fisher exact test, Chi-square) [Sokal and Rohlf, 1981; Blackford, 1992]. Grain distributions for myoD and U1 in myoblasts were compared in **D**. They were not significantly different at the 0.01 level but a difference was detectable at the less stringent 0.05 level (Kolmogorov-Smirnov test) [Sokal and Rohlf, 1981; Blackford, 1992]. There was also a difference at the 0.01 when they were stratified into three levels (Chi-square test) [Sokal and Rohlf, 1981; Blackford, 1992], but not when they were divided into two levels (Fisher exact test) [Sokal and Rohlf, 1981; Blackford, 1992].

Similar to the myotube analysis, the U1 distribution differed significantly ($P < 0.0001$) from those seen with the AChR subunit probes (Fig. 6A–C), as did the MyoD distribution ($P < 0.001$), but the subunit patterns did not differ among themselves (Komolgorov-Smirnov two sample test, Chi square test and Fisher's exact test [Sokal and Rohlf, 1981; Blackford, 1992]). Again, the distribution of MyoD mRNA appeared similar, though not identical, to that of U1 (Fig. 6D).

Comparison of Expression Patterns in Myotubes vs. Myoblasts

We compared the patterns obtained for the myotubes with those obtained for the myoblasts (Fig. 7A,B). There was no significant difference between any grain distribution produced by a subunit probe applied to the myotubes and any grain distribution produced by a subunit probe applied to the myoblasts (Fig. 7A). There were also no differences between the distribution for the U1 probe applied to the myotubes vs. the U1 distribution obtained from the myoblasts (Fig. 7B). As expected, the U1 distributions for the myoblasts differed significantly ($P < 0.001$) from the subunit distributions for the myotubes and the U1 distributions for the myotubes differed significantly ($P < 0.001$) from the subunit distributions for the myoblasts.

Comparison of AChR Expression Levels in Myotubes vs. Myoblasts

In contrast to the cells grown in low calcium medium, 2-day-old cultures grown in a calcium

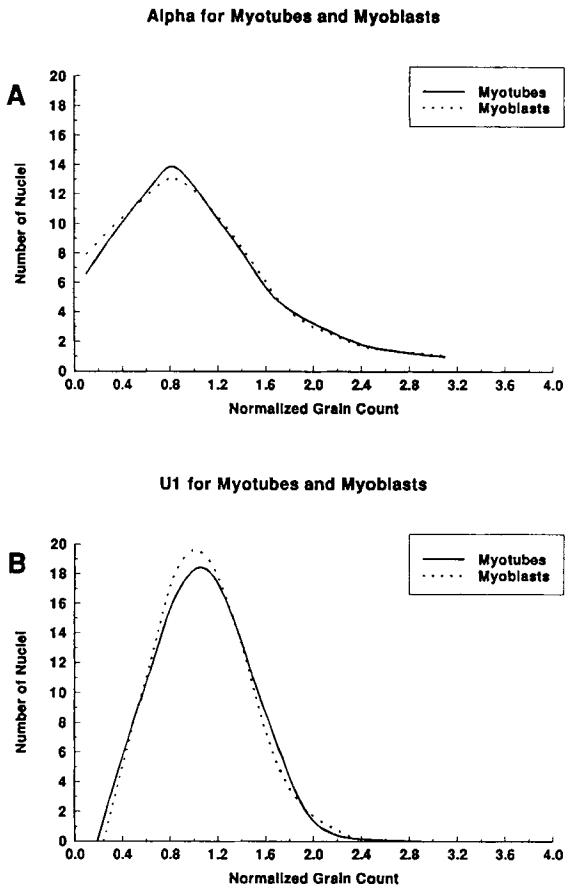

Fig. 7. Grain distributions in myoblasts and myotubes. Data were obtained from experiments as described in Figures 4 and 6 and analyzed as described in Materials and Methods and Figure 5. The grain distribution for the α subunit in myotubes vs. myoblasts is shown in **A**, indicating nearly identical patterns. The same results were obtained for γ and δ subunit (not shown). In agreement with the visual appearance, we found no statistically significant differences between the subunit distributions in the myotubes vs. the myoblasts by either of the three methods of analysis described in Materials and Methods (Kolmogorov-Smirnov test, Fisher exact test, Chi-square test) [Sokal and Rohlf, 1981; Blackford, 1992]. Likewise, grain distributions for U1 RNA were compared in **B** and no difference was found between the two types of cells.

containing medium contains both myoblasts and myotubes. After in situ hybridization, we counted separately the grains on nuclei of myoblasts and the adjacent myotubes on the same slides and the mean values are compared in Figure 8. For the AChR subunits, there clearly were, on average, almost twice as many grains associated with myotube nuclei as with myoblast nuclei and the differences between the myotube and myoblast grain numbers were highly significant ($P < 0.0001$, Mann-Whitney test and sign test). MyoD RNA levels decreased ($P < 0.001$, Mann-Whitney and sign tests), and U1 levels remained about the same ($P > 0.4$, Mann-Whitney, and $P > 0.3$, sign test) [Sokal and Rohlf, 1981; Blackford, 1992] after cell fusion. This result demonstrates that fusion increases the level of AChR subunit gene expression.

DISCUSSION

The AChR, a membrane-bound oligomeric protein, comprises 5 subunits encoded by 4 separate genes [Mishina et al., 1986; Changeux, 1989]. Previous studies focused on the expression of the AChR subunits during development, denervation, muscle activity, and addition of receptor blockers or trophic factors [Goldman et al., 1988; Klarsfeld and Changeux, 1985; Shieh et al., 1987; Fontaine and Changeux, 1989; Falls et al., 1993; Goldman and Staple, 1989; Klarsfeld et al., 1989; Witzemann and Sakmann, 1991]. Many of these studies have shown that the AChR α subunit RNA is highly concentrated at the postsynaptic region of the neuromuscular junction.

We have previously shown that myotube nuclei, even in the absence of nerve, express AChR α subunit RNA at varying levels, with a small subset (about ten percent) of the nuclei expressing much more than the others [Bursztajn et al., 1989; Berman et al., 1990]. Similar findings have been made in other laboratories [Fontaine and Changeux, 1989; Harris et al., 1989; Piette et al., 1993]. These findings raised two important questions: 1) is the observed heterogeneity a unique property of the α subunits, and 2) when does the heterogeneity begin? In particular, is it induced at the time of fusion or does it exist at the myoblast stage? We have, therefore, not only extended these observations to the γ and δ subunits, but we have also probed the foundation for this diversification by examining the distributions of AChR α, γ, and δ subunit RNAs in pre-fusion myoblasts as well as postfusion myotubes. As in our previous work, we used intron and intron-exon probes to detect prespliced transcripts or mature mRNAs in the cells [Bursztajn et al., 1989, 1990; Berman et al., 1990]. Because intron-containing transcripts are not transported out of the nuclei, the distributions of these transcripts can indicate their expression patterns among nuclei in the same myotubes. Our results show that both myotubes and myoblasts have distributions of the AChR α, γ, and δ subunit RNAs which differ sharply from that of the U1 RNA as well as from Myo D. Thus, the heterogeneous expression of AChR genes is not only an intrinsic property of muscle

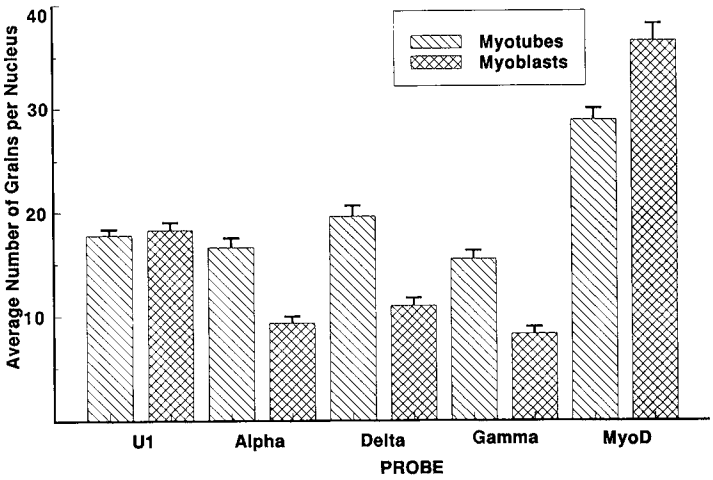

Fig. 8. Transcript levels in myotubes and myoblasts. After in situ hybridization of two-day-old cultures with a probe (intron-exon), grains were counted in 100 nuclei of myoblasts and 100 nuclei of adjacent myotubes. The mean grain value for 100 nuclei for each probe was determined. For U1, there is no significant difference in numbers of grains per nucleus between myotubes and myoblasts ($P > 0.4$ Mann-Whitney test, $P > 0.3$ sign test). For the subunits, the myotubes showed about twice the grain number per nucleus ($P < 0.0001$, Mann-Whitney test and sign test) [Sokal and Rohlf, 1981; Blackford, 1992]. For MyoD the average grain count per nucleus of the myoblasts exceeded that of the myotubes by a modest amount ($P < 0.001$, Mann-Whitney test and sign test) [Sokal and Rohlf, 1981; Blackford, 1992].

cell nuclei (in the sense that it does not require the presence of nerves), but it also exists prior to fusion.

Multiple Diffusable Transactivating Factors Can Influence Subunit Expression

What can account for the nuclear heterogeneity on AChR subunit RNA expression? Though MyoD is a basic prerequisite for cellular expression of muscle characteristics [Weintraub et al., 1991], its expression does not correlate in detail with that of the AChR subunit genes in each individual nucleus. This finding probably reflects a complex and only partially understood control system for acetylcholine receptor subunits' expression levels involving MyoD and several other myogenic factors. The α subunit, for example, has a 842 bp to 850 bp promoter region whose activity correlates highly with expression patterns seen by in situ hybridization [Klarsfeld et al., 1991; Salmon and Changeux, 1992]. Within that region there are two major MyoD binding sites [Piette et al., 1990] which activate α AChR expression. However, nearby MRF4 binding sites also increase α AChR expression [Prody and Merlie, 1991]. Since all muscle must have experienced the influence of myogenic promoters, inhibitors of α subunit transcription may help to explain heterogeneity. Phorbol esters inhibit α subunit promoter-reporter constructs containing the sequence between 110 and −45 (measuring from the start point of the α subunit transcription), perhaps by down regulation of MyoD production [Laufer et al., 1991]. The α enhancer region also contains overlapping binding sites for Ap1 and a G-C monopolymer binding factor (GBF). Sp1 binding enhances activation by MyoD and myogenic, and GBF binding can decrease α subunit expression by blocking Sp1 binding [Bessereau et al., 1993]. Though less work has been done on the other subunits and despite differences in detail between different subunits and different species, the overall pattern suggests multiple myogenic regulatory factor sites at the 5′ flanking regions with a subset of sites displaying relative primacy.

Differing General Models Could Explain the Nuclear Heterogeneity

Several models of the heterogeneity, viewed in a developmental context, lead to differing regulatory expectations but all suggest more long lasting or specific influences than can be accounted for by diffusible transactivators alone. First, the individual nuclei may have permanently fixed capacities to produce AChR subunit RNA. These capacities could allow variation dependent on the intracellular milieu or the extracellular environment. Thus, AChR levels from a given nucleus could change with innervation or myo-

blast fusion. Indeed, our previous [Bursztajn et al., 1989] and present studies suggest that innervation and fusion do alter the subunit RNA production. However, nuclei subject to the same external influences, e.g., nuclei within the same myotube, would still express their innate capacities, relative to each other, and would, thus, display heterogeneity. This model is somewhat conceptually similar to the model of preprogrammed ATPase fiber type determination [Gunning and Hardeman, 1991; DiMario et al., 1993] in that there is a commitment as early as the myoblast stage, as well as a capacity for modification via innervation. It differs from that of ATPase commitment in that, presumably, the myotube forms from myoblasts of a single ATPase type, whereas we see heterogenous nuclei within the same cell. Indeed, it is remarkable that nuclei very close together can show completely different levels of AChR RNA, suggesting that at least one component of the molecular control of nuclear AChR expression is not a highly diffusible inducer or promoter molecule such as a phorbol ester [Laufer et al., 1991] or a factor blockading an Sp1 binding site [Bessereau et al., 1993] but, rather, a relatively stable chemical or structural alteration. Changes in the chromatin accessibility and/or conformation may underly such a phenomenon [Crowder and Merlie, 1986, 1988]. Such changes may also correlate with other clonal phenomena, such as time of withdrawal from the cell cycle [Feldman and Stockdale, 1992]. However, the age, size, and BrdU uptake of our myoblasts suggest that they are all or almost all of fetal type which are still dividing.

A second model envisions changes in nuclear AChR production capability occurring slowly over periods of days, or at least many hours, as part of development. In such a model, the nuclei which express the highest levels as myoblast nuclei might even express the lowest levels as myotube nuclei. One significant task for this model is to accommodate our observed data showing that the shapes of the AChR receptor output distributions are the same between myoblasts and myotubes with an equal percentage of nuclei at each output level despite an overall increase in output after fusion. Thus, changes in nuclear output must be coordinated with compensatory increases and decreases and we would need to envision a different category of control mechanism than for the first model. The different expression levels displayed at a given developmental period by two nuclei located close to each other could no longer be explained by a stable chemical determinants in the genome because each would be gradually changing. However, their proximity would still rule out complete control by a diffusible inducer.

Finally, the observed heterogeneity could result from a more rapid cyclic variation over smaller periods, perhaps minutes to an hour. One might argue that this model is not qualitatively different than the previous one; however, the essential difference is that the period of time over which variation in RNA output might occur would be short in comparison to protein stability (though still long in comparison to RNA stability). In this case, the in situ hybridization autoradiographs, which show significant heterogeneity, would be similar to static photographs of blinking lights which are not blinking in phase. Averaged over an appropriate time period, such as 5 or 6 h, the actual AChR RNA output of the nuclei might be the same! Or, at least it could be more similar than apparent from the static photographs.

A recent study of the mouse δ subunit, using cultured cells obtained from transgenic mice with the δ subunit promoter linked to a human growth hormone hGH reporter, showed differing levels of hGH associated with different nuclei in the same myotube [Simon et al., 1992]. Because the hGH is quite stable, this finding favors long lived differences in subunit RNA output among nuclei rather than brief, cyclic variations. However, there were a "substantial number" rather than the small percentage of highly expressing nuclei which had been found in situ hybridization studies. This could reflect a difference in the particular cell type, subunit, or culture conditions employed. It could also suggest a difference between accumulation of a stable protein product and a short lived RNA species and, thus, favor the hypothesis that some of the heterogeneity seen with RNA probes does result from fluctuations in RNA production. Diffusion of either RNA or protein, which, though restricted, has been found to be as large as 100 μm in some cases [Ralston and Hall, 1989, 1992; Hall and Ralston, 1989] could underlie variations in results as well. Also, we do not know how closely the reporter gene mirrors the output of the natural gene. Indeed, it is possible that the two δ promoters in the transgenic cells compete for the same transcription factors and, thus, alter each other's expression. Use of the

transgenic construct simultaneously with in situ hybridization could clarify these possibilities. We believe that temporal heterogeneity remains a possibility, though the complexity of control mechanisms that might be necessary to explain it diminishes its attractiveness. The ultimate regulation of synapse formation, however, may require such a mechanism, and oscillations in RNA output may reflect the early workings of that mechanism. This mechanism may account for the formation of synapses at specific membrane domains. In that case, the development of stable innervation could synchronize the outputs near the motor endplate and lengthen the period such nuclei produce high AChR RNA levels, while decreasing the production by the more remote nuclei.

Early Heterogeneity May Have Implications for Later Development

The three models of variation in nuclear production of AChR subunit RNA are not mutually exclusive, but we believe it is important to think about them individually because they offer some guidance toward integrating the growing mass of detailed regulatory information about the AChR and searching for new mechanisms. In addition, the precise meaning of the apparent differential distribution of all three AChR subunit RNAs in pre-fusion myoblasts and myotubes has significant implications for the selective accumulation of the AchR protein during early stages of synapse formation. Previous studies have shown that AChR clusters are associated with nuclei [Bruner and Bursztajn, 1986; Englander and Rubin, 1987]. On the subcellular level, the AChR is present in coated vesicles and transported to plasma membrane where they have been shown to accumulate beneath the nerve terminal [Bursztajn and Fischbach, 1984; Porter-Jordan et al., 1986]. Recent findings have shown that mRNA for a given protein may have a restricted distribution around myotube nuclei [Ralston and Hall, 1989, 1992; Hall and Ralston, 1989]. In the first two models (fixed capacities for AChR RNA production or slow variation in levels over development) certain nuclei may be potentially more fit to participate in a synapse. Such active nuclei may migrate to regions of muscle where the motor neurons make the initial contacts and their migration may bring other proteins which promote synaptic stabilization [Englander and Rubin, 1987]. The second model, which embodies a combination of variation together with constant relative levels of output, implies a high degree of control of individual nuclei and such a control mechanism may be responsible for suppression of AChR output in other nuclei as the synapse matures. The third model is quite different. Here the heterogeneity occurs over very small units of time. Averaged over a longer period, the nuclei (prior to innervation) may be essentially identical with respect to AChR RNA output. In this case, the clear heterogeneity which exists after innervation is a qualitatively different heterogeneity, though it still could develop from an earlier phasic heterogeneity by an increase in the percentage of time the nuclei at the endplate produced AChR RNA and a decrease in the percentage for other nuclei. Further studies should allow us to distinguish between these possibilities.

In conclusion, we have shown that the nuclear heterogeneity in AChR subunit RNA accumulation in noninnervated cultured myotubes is not a unique property of the α subunit but it also exists for both γ and δ. In addition, the heterogeneity begins well before the period of fusion. Though at least three different developmental models can accommodate the observed heterogeneity, all models appear to require an additional level of regulation beyond the known diffusible transcriptional factors. Further knowledge of the basis of the heterogeneity may contribute to our understanding of the selective AChR accumulation during early synapse formation. Combined, simultaneous studies of receptor RNA and subunit accumulation, presently underway in our laboratory, can help refine our understanding of these phenomena.

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