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Differential expression of acetylcholine receptor mRNA in nuclei of cultured muscle cells

(myotube/neuromuscular junction/*in situ* hybridization/transcriptional control)

S. BURSZTAJN*†, S. A. BERMAN‡, AND W. GILBERT§

Departments of *Neurology and †Cell Biology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030; ‡Department of Neurology, University of Chicago, 5841 South Maryland Avenue, Chicago, IL 60637; and §Department of Cellular and Developmental Biology, Harvard University, 16 Divinity Avenue, Cambridge, MA 02138

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ABSTRACT **Muscle cells *in vitro* and *in vivo* are multinucleated and express acetylcholine receptors (AcChoRs). On innervated cells, the AcChoRs form clusters which lie under the nerve terminals. However, noninnervated cells in culture also express clusters of AcChoR. Both *in vivo* and *in vitro* the AcChoR clusters appear to be associated with clusters of nuclei. We have used *in situ* hybridization to determine whether all the nuclei in cultured chicken embryo myotubes are equally active in expressing the AcChoR α subunit message. Cells were hybridized with ^{35}S-labeled probes that contained either both an exon and an intron region or only exon sequences. Control cultures were hybridized with a labeled actin DNA probe or poly(U). The hybrids were detected by emulsion autoradiography; simultaneously, the nuclei were visualized with bisbenzamide. Cells hybridized with the intron/exon probe showed a striking preferential silver grain localization in and around some of the myotube nuclei, whereas those hybridized with the exon probe gave a rather homogeneous grain distribution in the cytoplasm. These results show that myotube nuclei possess differential activation capacities for the expression of AcChoR α subunit mRNA and that this difference is due to differential rates of transcription.**

Skeletal muscle cells are multinucleated, and regions of high acetylcholine receptor (AcChoR) concentration (AcChoR clusters) form in response to innervation (1, 2). Such clusters are also present in noninnervated myotubes (1, 3, 4). In culture as well as *in vivo* some nuclei of a multinucleated myotube are associated with AcChoR clusters (5, 6). Are all the nuclei in a single cell equally active in expressing the AcChoR message? To answer this question, we have used *in situ* hybridization with AcChoR α subunit sequences to localize the position of mRNA in intact cells. By hybridizing with probes that carried either both exon and intron or only exon sequences, we show that the activity of nuclei in a myotube varies greatly and that this difference is due to differential rates of transcription. The intron/exon probe shows that individual nuclei differ dramatically in their hybridization. However, the exon probe shows that the mature message is not localized. Thus the hybridization with the intron/exon probe shows primarily intron sequences and hence reflects some property of premessengers. Fontaine *et al.* (7) showed that an intron/exon probe for AcChoR hybridized *in vivo* to nuclei clustered under the neuromuscular junction. Since they could not observe any hybridization to an intron probe, they interpreted their result as hybridization to exons and thus as showing a local accumulation of mRNA under the endplate. Here we show that in myotubes, fused *in vitro* from primary myoblasts which have never been innervated, the nuclei differ in their AcChoR synthetic capacity, while the mature message is not localized near the nuclei but is scattered throughout the cytoplasm.

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MATERIALS AND METHODS

Cell Culture. Pectoral muscle cells were obtained from 11-day-old chicken embryos and plated on collagen-coated coverslips as previously described (4, 5). Cells were maintained in Eagle's minimum essential medium made in Earle's balanced salt solution and supplemented with horse serum (10%, vol/vol) and embryo extracts. Cells were fed every other day and fixed in 4% (wt/vol) paraformaldehyde 4 days after plating.

Preparation of Single-Stranded DNA Probe. The single-stranded DNA was synthesized by previously described methods (8, 9) with modification. The 17-mer sequencing primer (3.7 pmol) was annealed with 0.78 pmol of the M13 template on the 3′ end of the polycloning region containing the AcChoR insert. For reactions involving ^{35}S label, 56 pmol of each of two α-[^{35}S]thio-dNTPs was used as well as 0.8 pmol of a [^{32}P]dNTP of one of the same species as a tracer for gel autoradiography. Five nanomoles of each of the remaining two dNTPs was included in a final reaction volume of 45 μl in a buffer containing bovine serum albumin at 0.1 mg/ml, 6.66 mM dithiothreitol, 44.4 mM NaCl, 10 mM $MgCl_2$, 10 mM Tris·HCl at pH 7.4, and 5 units of the Klenow fragment of DNA polymerase. For ^{3}H-labeled probes 200 pmol of each of the four tritiated dNTPs was used. After allowing the reaction to proceed at 37°C for 15 min to 4 hr, we added 2.5 nmol of all four dNTPs at 37°C for 15 min to extend all strands to full length. We inactivated the enzyme by heating at 68°C for 15 min and cut the double-stranded DNA product by incubation with *Eco*RI or *Bgl* I at 37°C for 2 hr as described by BRL. After precipitation with 0.25 vol of 20% (vol/vol) polyethylene glycol (PEG) and 2.5 M NaCl, the product was washed with 70% (vol/vol) ethanol, dried, and then dissolved in formamide loading buffer, denatured by heating at 95°C for 10 min, and electrophoresed on a 6% polyacrylamide/8 M urea strand-separating gel for 2 hr at 250 V. The appropriate single-stranded DNA band was located and cut from the gel, using an autoradiograph as a template. The probe was eluted overnight in 2 ml of 0.2 M NaCl/10 mM Tris·HCl, pH 7.5/1 mM EDTA at 55°C, and then precipitated with ethanol prior to use.

***In Situ* Hybridization and Detection of AcChoR Message.** Dissociated myoblasts from pectoral muscle of 11-day-old chicken embryos were plated on collagen-coated coverslips as previously described. Cells 4 days after plating were fixed in 4% paraformaldehyde, using a modified hybridization protocol of Lawrence and Singer (10). Cells were washed at room temperature with phosphate-buffered saline (PBS, Sigma) or stored in 70% ethanol and rehydrated for 10 min in

Abbreviation: AcChoR, acetylcholine receptor.

PBS plus 5 mM $MgCl_2$ followed by 0.1 M glycine/0.2 M Tris·HCl, pH 7.4. Cells were transferred to 0.1 M acetic anhydride for 10 min and prior to hybridization they were placed in 50% (vol/vol) formamide plus 2× SSC (1× SSC = 0.15 M sodium chloride/0.015 M sodium citrate) for 10 min at 65°C. For each coverslip, 4×10^5 cpm of probe was lyophilized with 40 mg of *Escherichia coli* tRNA and 40 mg of sheared salmon sperm DNA and then resuspended in 10 ml of deionized formamide and combined with 10 ml of 4× SSC/0.4% bovine serum albumin/20 mM vanadyl ribonucleoside inhibitor/20% dextran sulfate/1 mg of heparin per ml/20 mM dithiothreitol buffer. After being heated to 90°C for 10 min, the 20-ml hybridization mixture was placed between Parafilm and the cells on a coverslip and incubated for 4 hr at 37°C. After hybridization, coverslips were washed at 37°C in 10-ml Coplin jars (Thomas Scientific) for 30 min each in 2× SSC/50% formamide, 1× SSC/50% formamide, and, finally, at room temperature in 1× SSC. The coverslips were washed until almost no radioactivity was detected by a minimonitor, dehydrated through 70%, 95%, and 100% ethanol, and then attached to slides with Permount and allowed to air dry. The slides were dipped in Kodak NTB-3 emulsion and stored in light-tight boxes at 4°C for 7–12 days. After developing in Kodak D19, rinsing in water, and fixing in Kodak fixer, they were rinsed twice in water and stained with a DNA dye, bisbenzamide (1 mg/ml in PBS) (11). The cells were then mounted under a coverslip in a PBS/glycerol mixture. Cells were photographed on a Nikon microscope equipped with fluorescence optics and a dark-field condenser.

Quantitation of Autoradiographic Grain Distribution. Muscle cells were viewed with fluorescence optics and a dark-field condenser, allowing the simultaneous observation of the position of the silver grains with respect to the nuclei. All counts were done with a ×60 objective, focusing up and down to locate all the grains. In cases where the nuclei were in close proximity to one another the total grains were counted in all nuclei and divided by the number of these nuclei. A total of 468 nuclei in 65 muscle cells from three different experiments were counted.

RESULTS

We have hybridized cultured chicken embryonic muscle cells with radiolabeled probes of the AcChoR message and counterstained the nuclei with bisbenzamide (which binds specifically to DNA) to visualize the RNA and the nuclei simultaneously. Myoblasts were obtained from 11-day-old chicken embryo pectoral muscle and allowed to fuse and form multinucleated myotubes in culture. The myotubes, 4 days after plating, were hybridized with short, highly radioactive single-strand antisense DNA obtained from an M13mp8 subclone of a λ phage clone that had been isolated from a chicken genomic library by cross-hybridization with *Torpedo* AcChoR cDNA. [The subclone was a gift from A. Klarsfeld and J.-P. Changeux (12, 13).] The subclone consists of intron and exon sequences from the α subunit, comprising 465 nucleotides, of which the exon portion is 125 nucleotides (Fig. 1; A. Klarsfeld and J.-P. Changeux, personal communication). We made radiolabeled single-stranded DNA probes by the primer extension method (see *Materials and Methods*). As Fig. 1 shows, by cutting the newly synthesized DNA with *Eco*RI, we obtained a probe comprising both the exon and intron sequences, while by cutting with *Bgl* I, we obtained an exon-specific probe. Most of the experiments used ^{35}S-labeled probes rather than tritium label, because the development time was much less: 7–12 days versus 3 months. The ^{35}S-labeled probes gave results identical to those obtained with tritium.

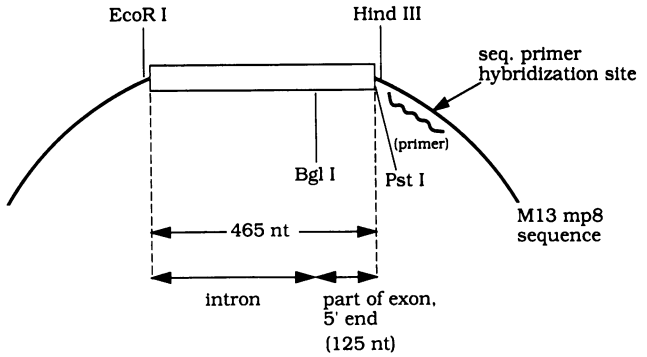

FIG. 1. Diagram of chicken AcChoR α subunit genomic subclone (A. Klarsfeld and J.-P. Changeux, personal communication). The clone is composed of intron and exon sequences. nt, Nucleotides. Single-stranded DNA probes were synthesized by primer extension.

When the coverslips bearing the myotubes and the developed emulsion were examined, we observed that the intron/exon probe showed a distribution of AcChoR mRNA that was strikingly inhomogeneous. Only some of the nuclei in a myotube are covered with grains. Fig. 2 *A* and *B* show this phenomenon. Fig. 2*A* is a tracing of the positions of the nuclei and the outlines of the muscle cells corresponding to the photograph in Fig. 2*B*, which shows the fluorescent nuclei and the dark-field illuminated silver grains produced by the radioactive decays. The labeling appears to be perinuclear and nuclei associated with both low and high grain densities are found in the same muscle cells. In general, high densities of AcChoR α subunit mRNA appear both around nuclei that are in close proximity to one another and around single nuclei, independently of whether the nuclei are at the center or periphery of the cells. Fig. 2*C* shows a control hybridization with an actin probe. [The subclone was a gift from Robert Schwartz (14).] The silver grains are uniformly distributed throughout the cytoplasm of all cells. Furthermore, hybridization with ^3H-labeled poly(U) (New England Nuclear), which should bind to all poly(A)$^+$ RNA, resulted in uniform grain densities over all cells, as would be expected from an even distribution of poly(A)$^+$ RNA in the cytoplasm. The control in Fig. 2*D* shows that essentially no grains appear when cells are hybridized with a probe from an M13 bacteriophage lacking the AcChoR α subunit insert. Both cells hybridized at an early stage of development (3 days after plating) and more mature cells (8 days after plating) reveal a heterogeneous pattern of low and high grain distribution around certain nuclei.

Fig. 3 shows a frequency distribution of the grain densities per nucleus for the α-subunit intron/exon probe; 468 nuclei were counted, and the distribution was compared to that for a Poisson distribution of the same mean. The grain counts do not follow the Poisson distribution, showing that the variation in the number of grains associated with different nuclei is not random. In fact, the 14% most active nuclei account for 50% of the grains.

Does this differential hybridization reflect a difference in the amount of messenger or a difference in the rate of synthesis? Since the probe we used contained both intron and exon sequences, its distribution does not necessarily reflect the distribution of mature message. Fig. 2 *E* and *F* show that cells hybridized with an α subunit exon probe made with *Bgl* I display a homogeneous distribution of grains throughout the cytoplasm, in contrast to the intron/exon probe. The excess of grains at the nuclei seen with the intron/exon *Eco*RI probe must be due to hybridization to intron sequences. Since we expect the intron RNA to be unstable, this hybridization should follow the instantaneous rate of synthesis. Thus the transcription rate for α subunit AcChoR mRNA varies

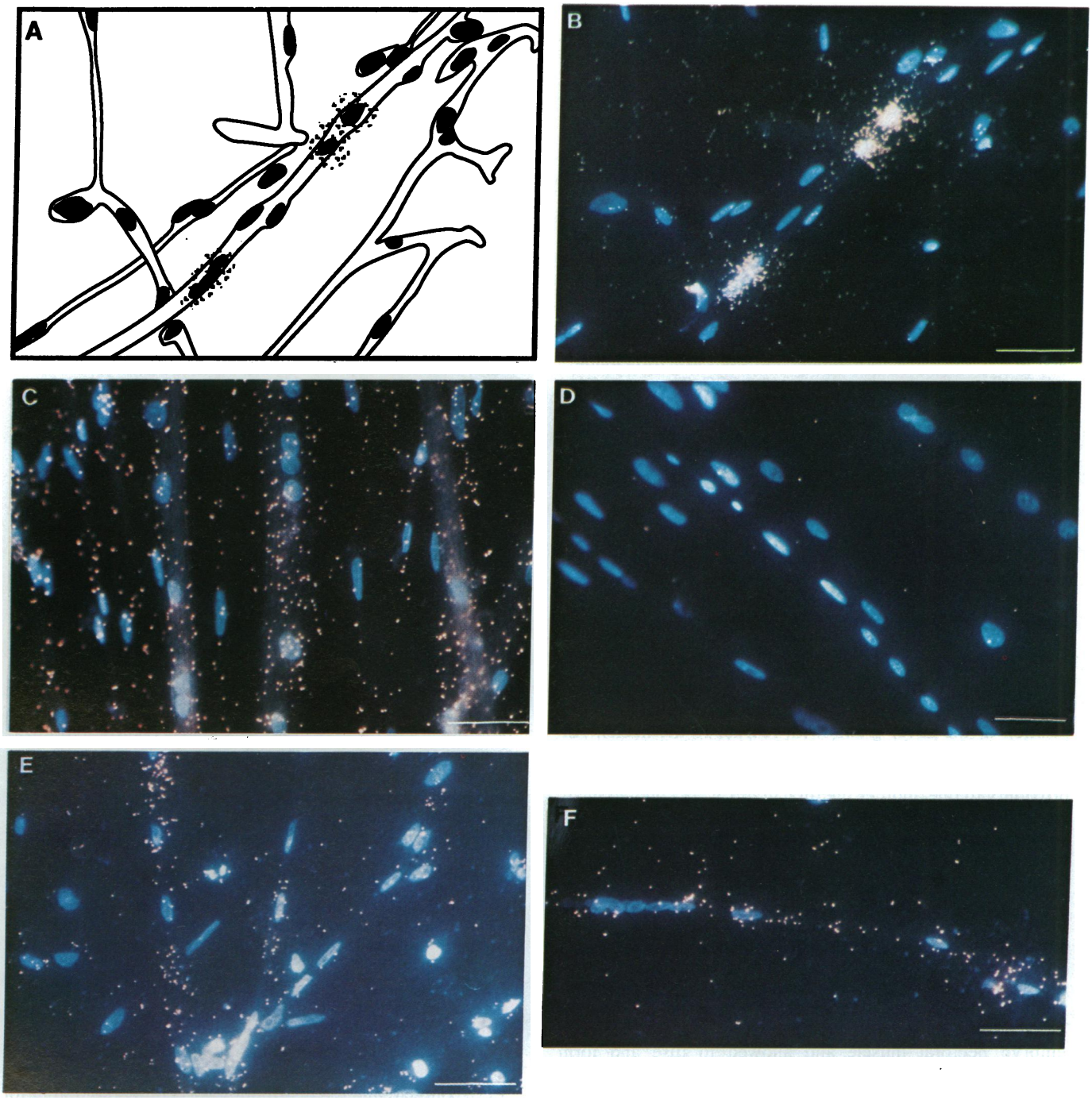

FIG. 2. Autoradiographs of muscle cell RNA *in situ* hybridizations. (*A* and *B*) Hybridization with ^{35}S-labeled AcChoR α subunit intron/exon probe. (*A*) Tracing of an image obtained by projecting the original slide of the fluorescent and dark-field image. (*B*) The autoradiograph whose tracing is shown in *A*. The tracing reveals the muscle cell boundaries, which are not clearly seen in *B*. The fluorescent nuclei, stained with bisbenzamide, are blue. The silver grains from the autoradiography are golden. Control hybridizations are to a cardiac actin probe (*C*) and bacteriophage M13mp19 lacking any insert (*D*). (*E* and *F*) Cells hybridized with the AcChoR α subunit exon probe show a more homogeneous distribution of grains than those hybridized with the intron/exon probe. The grains are predominantly located in the cytoplasm. Specific activities of probes are approximately 1×10^8 cpm/mg. Exposure times are 7–12 days. Some grains are out of the plane of focus. (Bar = 50 μm.)

markedly between nuclei in the same myotube. However, we cannot rule out the possibility that the differential appearance is due to some nuclei destroying the intron sequences unusually rapidly.

DISCUSSION

Prior investigations using RNA from *in vivo* dissection (15) and *in situ* hybridization of sections from innervated vertebrate skeletal muscle (7) have found high levels of AcChoR RNA sequences near the neuromuscular junction. In the *in situ* studies of Fontaine *et al.* (7) these RNA accumulations had a high (80%) correlation to the staining for acetylcholinesterase activity associated with neuromuscular junctions, and they possessed a generally perinuclear localization, similar to our own results. Although these authors used a mixed intron/exon probe, they inferred that their probe showed differential AcChoR mRNA *localization* rather than the differential rate of transcription that we find. Because all the muscle cells were innervated, their results implied that

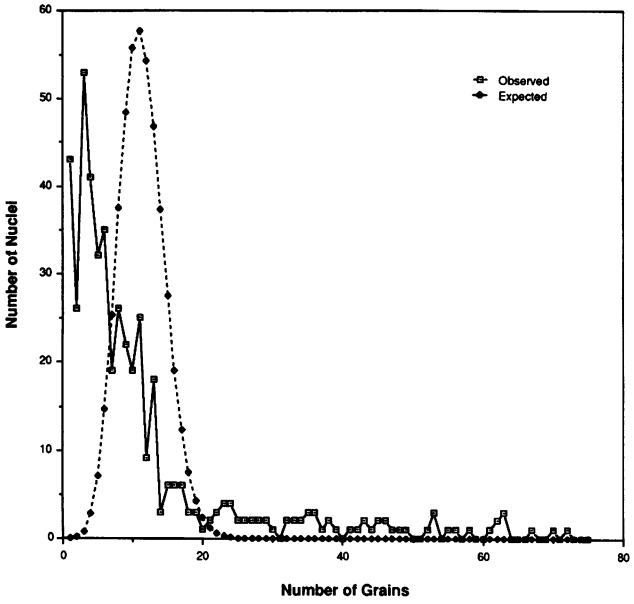

FIG. 3. Comparison of grain distribution in and around nuclei with calculated Poisson frequencies.

nuclei active and inactive for AcChoR mRNA production must coexist within the same myotube, though the presence of connective tissue cells as well as the use of sectioned material obscured the direct demonstration of such nuclei. With denervated material, the number of active nuclei rose to 10% of the total (7). In this case as well, it is likely that active and inactive nuclei occupy the same myotube, but one cannot infer such coexistence conclusively; the radioactive nuclei might all have been in the same cells. Furthermore, the role of the nerve was left problematic. Clearly it must influence the process of nuclear activity, but the *in vivo* experiments do not indicate whether or not the nerve is necessary to establish the phenomenon itself.

Our experiments show directly that nuclei with markedly different AcChoR mRNA synthetic abilities coexist within the same myotubes. Since these cells have never been innervated, the variation must be an intrinsic property of the myotube, a capacity that may be modulated by the nerve but does not require nerve to initiate. Furthermore, the differential nuclear expression is retained in innervated cultures (preliminarily reported in ref. 16), although in this case, the general level of AcChoR mRNA rises, as it does in denervated muscle (17–20). Recent studies have shown that, in contrast to previous thinking, avian muscle fiber-type differentiation may be intrinsically programmed (21, 22). Miller and Stockdale's (23) studies on myogenic cell lineages, using monoclonal antibodies specific to the fast and slow classes of myosin heavy chain (MHC), have shown that when myoblasts were taken from embryonic day 5 and 6 they fused to form three types of myotubes (myotubes containing the fast class of MHC, myotubes containing both fast and slow classes of MHC, and myotubes containing only the slow class of MHC isoforms). Myoblasts from older embryos (day 8–12) formed a single type of myotube that contained only a fast class of MHC. Just as they concluded that the early myoblasts had the intrinsic (i.e., innervation-free) capacity to produce all three fiber types and that their late myoblasts had the intrinsic capacity to produce one fiber type, so do we conclude that our myoblasts (which are the same age as their late group) have the intrinsic capacity of differential nuclear expression of AcChoR mRNA. This conclusion cannot be reached from the *in vivo* studies of Fontaine *et al.* (7), which examine cells that have been innervated.

We cannot rule out that myoblasts may have experienced different environments prior to plating. However, it is universally accepted that the myoblasts obtained to produce primary cultures are not innervated nor do they form synapses with motor neurons. Many factors may contribute towards the activation of nuclei in muscle cells. As we have shown, physical contact of motor neurons with muscle cells or factors released by the neuron are not necessary for the basic phenomenon of differential activation. Perhaps the positioning of the nuclei or the muscle activity (12, 20) may dictate which nuclei actively express AcChoR mRNA. Such a possibility would require the involvement of the cytoskeleton for the alignment of nuclei and to make them receptive to surrounding structures and environmental cues. Direct contact with neurons, or factors released by them, could modulate the fundamental differential activation process in the context of the formation of the neuromuscular junction.

We conjecture that the transcription of the mRNA of the other AcChoR subunits will also be localized in the same nuclei and that this differential message synthesis will also exist for other proteins needed at the synapse.

Note Added in Proof. After this paper was submitted, another report showing differential activation of myotube nuclei appeared (24). Whereas we find that exon sequences are scattered throughout the cytoplasm and the probe containing intron sequences shows differential nuclear function, Harris *et al.* demonstrate that a probe corresponding to anti-message is localized around certain nuclei, particularly in cells stimulated with a brain-derived factor. These interesting differences may be partially explained by the fact they are studying mainly a short-term induction of message synthesis, whereas we are studying the basal state. They detect no intronic signal in unstimulated cells, but in stimulated cells they detect intronic sequences over certain nuclei. Further hybridizations in our laboratories with a purely intronic probe show clear signals over some nuclei of unstimulated cells. This difference may be explained by an intrinsically higher transcription rate in our cells or, perhaps, by differences in detection sensitivities with the intron probe.

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