Nucleus-specific Translation and Assembly of Acetylcholinesterase in Multinucleated Muscle Cells

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Abstract. Multinucleated skeletal muscle fibers synthesize cell surface and secreted oligomeric forms of acetylcholinesterase (AChE) that accumulate at specialized locations on the cell surface, such as sites of nerve-muscle contact. Using allelic variants of the AChE polypeptide chains as genetic markers, we show that nuclei homozygous for either the α or β alleles residing in chimeric myotubes preferentially translate their AChE mRNAs on their respective ERs. These results indicate that the events of transcription, translation, and assembly of this membrane protein are compartmentalized into nuclear domains in multinucleated cells, and provide the structural basis for the possible localized expression and regulation of synaptic components at the neuromuscular junctions of vertebrate skeletal muscle fibers.

SKELETAL muscle fibers are long, multinucleated cells containing many hundreds of nuclei dispersed at regular intervals along their length. Upon fusion of myoblasts to form myotubes, each myoblast nucleus retains its own complement of intracellular organelles, including the RER, the Golgi apparatus, and mitochondria. At sites of nerve-muscle contact, clusters of nuclei accumulate within the sarcoplasm to form the soleplate or fundamental nuclei (Ranvier, 1878; Kuhne, 1886; cited in Couteaux, 1973). Because of their unique location beneath the neuromuscular junction, these nuclei have been postulated to be the principal sites of expression and regulation of synaptic proteins such as acetylcholine receptors (AChRs)[1] and acetylcholinesterases (AChEs) (Couteaux, 1981). In support of this hypothesis, Merlie and Sanes (1985) demonstrated a severalfold higher concentration of AChR alpha-subunit mRNA in regions of skeletal muscle fibers enriched in neuromuscular junctions. More recently Fontaine et al. (1988), using higher resolution in situ hybridization techniques, showed that these higher levels of AChR alpha-subunit mRNA were confined to the cytoplasmic regions surrounding the subsynaptic nuclei. These observations suggest the interesting possibility that individual nuclei within skeletal muscle fibers are compartmentalized and are autonomous with respect to the transcription and translation of at least a specific subset of membrane proteins.

AChE is an essential component of all cholinergic synapses, including the neuromuscular junction. Vertebrate AChE occurs as a complex family of membrane-bound and secreted oligomeric forms (for reviews see Brimijoin, 1983; Rosenberry, 1985; Rotundo, 1987; Toutant and Massoulié, 1987) encoded by a single gene (Rotundo et al., 1988; Schumacher et al., 1988; Sikorav et al., 1988). We have recently described two allelic variants of quail AChE catalytic subunit with apparent molecular masses of 110 (α) and 100 (β) kD (Rotundo et al., 1988; Fernandez-Valle and Rotundo, 1989). These allelic polypeptide chains are codominantly expressed, are functionally identical, and can assemble randomly into the multiple oligomeric forms of the enzyme found in nerves and muscle. AChE is a glycoprotein and, after synthesis on the RER, assembles into disulfide-bonded dimeric and tetrameric forms. A subset of these oligomers is subsequently transported through the Golgi apparatus where they become associated with a collagen-like tail giving rise to the 20S asymmetric form of the enzyme (Rotundo, 1984; Brockman et al., 1986; Rotundo, 1988).

In the present studies we have used these allelic variants to test the hypothesis that mRNAs encoding an identified synaptic component, AChE, are preferentially translated on the RER surrounding the nucleus of transcription. Chimeric myotubes were grown in culture from myoblasts obtained from quail embryos homozygous for either the α or β AChE alleles. The resulting chimeric myotubes expressed predominantly homodimers of the two AChE allelic variants in contrast to the random assembly of heterodimers expressed in myotubes made from heterozygous myoblasts. Our results indicate that the mRNAs encoding exportable proteins in multinucleated skeletal muscle fibers are not freely diffusible, but rather are restricted to the vicinity of the nucleus of transcription. These findings support the hypothesis that individual nuclei within multinucleated cells are functionally compartmentalized with respect to the synthesis and assembly of membrane proteins and provide a physical basis for the possible localized translation, transport, and insertion of synaptic components at the neuromuscular junction.

1. *Abbreviations used in this paper*: AChE, acetylcholinesterase; AChR, acetylcholine receptor.

Materials and Methods

Tissue Culture

Quail muscle cultures were prepared as previously described using myoblasts isolated from individual embryos (Rotundo et al., 1988). The genotype of each embryo was determined by immunoprecipitating the AChE polypeptides isolated from brain and analysis by SDS-PAGE (Rotundo, 1988). Sufficient AChE protein is precipitated under these conditions to be readily visualized by Coomassie blue staining of the gel. Myoblasts isolated from embryos homozygous for either the α or β alleles were counted, mixed together in the appropriate ratios, and plated in 100-mm collagen-coated culture dishes. Pure cultures made from α/α, β/β, or α/β myoblasts served as controls.

Metabolic Labeling and Analysis of AChE Polypeptides

After fusion and differentiation, the multinucleated myotubes were labeled with 100 μCi/ml [^{35}S]methionine for 4 h and the AChE was extracted and immunoprecipitated as previously described (Rotundo, 1988). The AChE polypeptides were resuspended in 30–50 μl SDS sample buffer without reducing agents and analyzed by electrophoresis on 7.5% polyacrylamide gels to resolve the dimeric molecules. Alternatively, aliquots of the samples were reduced with 100 mM DTT and analyzed on 10% polyacrylamide gels to determine total radioactivity incorporated into each allelic polypeptide chain. After fluorography to localize the AChE polypeptides, the bands were excised from the dried gels, and the labeled proteins solubilized with 0.5 M NaOH and counted in a liquid scintillation counter after neutralization with acetic acid.

Autoradiography of Muscle Cultures

Mononucleated myoblasts isolated from homozygous α or β quail embryos were initially plated in separate 100-mm culture dishes at low densities to prevent fusion. The β/β myoblasts were labeled overnight with 1 μCi [^{3}H]thymidine (New England Nuclear, Boston, MA; 84.0 Ci/mmol sp act) per ml of medium. The α/α and β/β myoblasts were detached by gentle trypsinization, counted in a hemocytometer, and replated on 35-mm collagen-coated culture dishes, as pure homozygous populations or as 1:1 mixtures, at a density of 4×10^4 cells/dish. After fusion to form multinucleated myotubes the cells were fixed with PBS-buffered 4% paraformaldehyde, dehydrated with 95% ethanol, air dried, covered with Kodak nuclear emulsion (NTB3; Eastman Kodak Co., Rochester, NY), and exposed for 2 wk at room temperature. The cultures were developed in Kodak D76 (Eastman Kodak Co.), fixed with Rapid Fix, rinsed, stained with HOESCHT 33258, and viewed with a microscope (Carl Zeiss, Inc., Thornwood, NY) using a 40× water immersion objective and epifluorescence illumination. Under these conditions the background autofluorescence permits visualization of the cells; the labeled nuclei appear black and the unlabeled nuclei appear bluish white. To determine labeling efficiency a total of 587 myotube nuclei were counted in 10–13 randomly chosen myotubes from each of three culture dishes of labeled β/β myotubes alone. In these experiments we found that 78.7 $\pm$ 2.3% of the nuclei showed heavy or intermediate labeling.

Statistical Analyses

The predicted distribution of dimeric AChE molecules is calculated from the binomial $\alpha^2 + 2\alpha\beta + \beta^2 = 1$, where the frequency of the β allele is determined directly by counting the number of labeled nuclei and correcting for the labeling efficiency of 79%. In this experiment 35.5% of the nuclei in myotubes were labeled; therefore after correcting for efficiency the frequency of the α allele is 0.55, the β allele 0.45, and the frequency of each dimer class is 0.30 (α/α), 0.50 (α/β), and 0.20 (β/β).

To determine whether the distribution of [^{3}H]thymidine-labeled β/β and unlabeled α/α nuclei in chimeric multinucleated myotubes was random, a "nearest neighbor" analysis was performed. The linear arrangement of labeled and unlabeled nuclei from 30 randomly selected $\alpha/\alpha + \beta/\beta$ myotubes (10 myotubes/culture dish; three cultures) was determined. Of a total of 747 nuclei, 242 (32.4%) were labeled and 505 (67.6%) were unlabeled. The end nuclei from each myotube were not counted since they did not have two neighbors. Total numbers of labeled and unlabeled nuclei with either one or two like or unlike neighbors were determined by direct observation. The predicted distribution of this nearest neighbor analysis is given by the polynominal $a^3 + 3a^2b + 3ab^2 + b^3 = 1.0$ where a is the frequency of labeled nuclei and b is the frequency of unlabeled nuclei in the sample population. Thus, for example, the frequency of labeled nuclei having two labeled nuclei as neighbors is 0.324^3 or 3.4% of the total sample.

Results

AChE Homodimers Are Preferentially Assembled in Chimeric Myotubes

Muscle cultures made from quail embryos heterozygous for

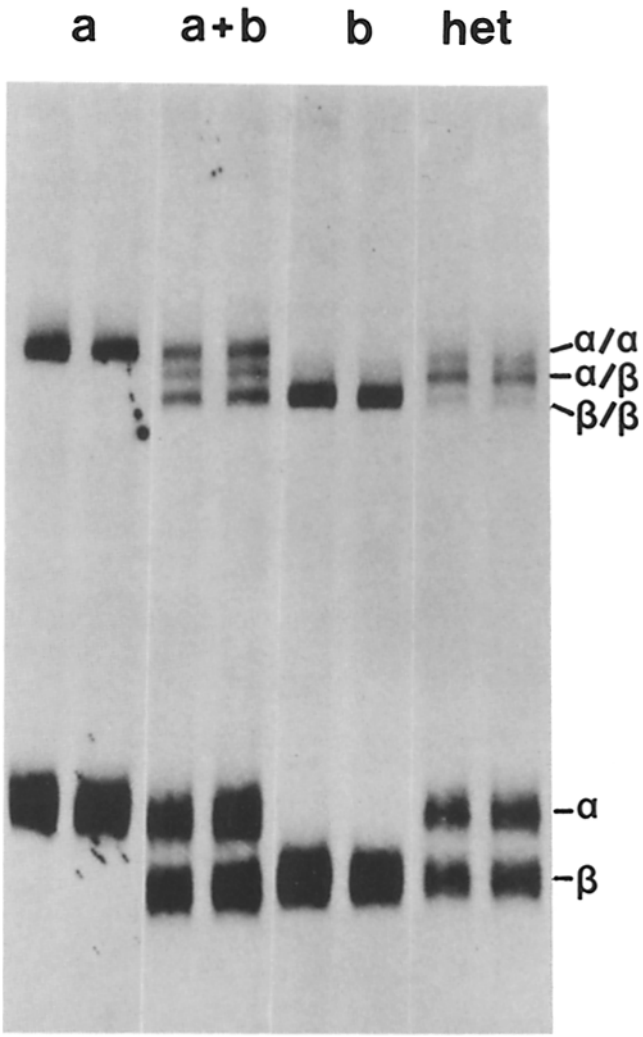

Figure 1. Expression of dimeric AChE molecules in homozygous, heterozygous, or chimeric homozygous multinucleated myotubes. Muscle cultures from homozygous α, homozygous β, or heterozygous quail myoblasts were prepared and labeled as described in Materials and Methods. The isotopically labeled AChE polypeptides were immunoprecipitated and analyzed by SDS-PAGE under nonreducing conditions and fluorographed. (Lane *a*) Monomeric α and dimeric α/α AChE polypeptides from homozygous α/α myotubes; (lane *a + b*) AChE polypeptides immunoprecipitated from myotubes expressing equal levels of the α and β polypeptide chains showing the unequal distribution of the dimeric AChE molecules; (lane *b*) AChE from homozygous β/β myotubes; and (lane *het*) distribution of AChE dimers in heterozygous α/β myotubes. The heterozygous myotubes randomly assemble disulfide-bonded AChE dimers, whereas chimeric myotubes made by mixing homozygous myoblasts expressing both AChE alleles assemble the polypeptides in a clearly nonrandom manner. These distributions are representative of a total of 41 cultures made from 22 embryos.

Table I. Distribution of Dimeric AChE Molecules in Mixed Muscle Cultures

Approximate nuclear ratio (α/β)	Frequencies of dimer classes (O/E) α/α	α/β	β/β	Significance	(df = 2)
3.35:1	80.8	11.4	7.8	$X^2 = 24.6$	$P < 0.0005$
	60.0	34.9	5.0		
1.34:1	59.1	21.9	19.0	$X^2 = 25.4$	$P < 0.0005$
	39.1	46.9	14.0		
1:1.34	37.6	19.5	42.9	$X^2 = 55.5$	$P < 0.0005$
	14.1	46.9	39.1		
Heterozygotes	28.4	49.9	21.8	$X^2 = 0.67$	$P < 0.70$
	25.0	50.0	25.0		

Quail muscle cultures were prepared by mixing different proportions of α/α and β/β myoblasts and the resulting myotubes isotopically labeled with [^{35}S]methionine as described in Fig. 1. After immunoprecipitation and separation of the dimeric and monomeric AChE polypeptide chains by SDS gel electrophoresis, the bands were localized by fluorography, excised from the gel, and counted in a liquid scintillation counter. To determine the expected ratios of the three possible dimeric AChE polypeptide combinations, aliquots of the samples were reduced with 100 mM DTT, analyzed on 10% SDS-polyacrylamide gels, and the bands localized and counted as above. The deviations of the observed (O) from the expected (E) values were statistically analyzed using the chi-square test of binomial distributions.

the α and β AChE alleles express both polypeptides in equal amounts (Fig. 1, *het*). Analysis and quantitation of the dimeric AChE forms synthesized by these cells shows that they are assembled in a ratio of ~0.25:0.50:0.25 (α/α:α/β:β/β), indicating random pairing of the catalytic subunits in the RER before disulfide bond formation (Fig. 1 and Table I). These events, in which individual subunits of two "genotypes" combine within the lumen of the RER, are quantitatively analogous to "randomly mating" populations and hence can be analyzed statistically using binomial distribution equations. Myotubes made from individual embryos homozygous for either the α or β allele express only homodimers of the respective allele (Fig. 1, lanes *a* and *b*). In contrast, chimeric myotubes, produced by mixing approximately equal numbers of myoblasts derived from embryos homozygous for the α and β alleles, assembled AChE dimers in a nonrandom 0.40:0.20:0.40 (α/α:α/β:β/β) ratio (Fig. 1, *a+b*). Furthermore, in a similar experiment, mixing of homozygous α/α and β/β myoblasts in unequal ratios yielded similar deviations from that predicted from the binomial distribution equation (Table I). As assembly of the disulfide-bonded AChE dimers occurs in the lumen of the RER before transport to the Golgi apparatus (Rotundo, 1984, 1987; Toutant and Massoulié, 1987), these results will reflect the relative distributions of the different AChE mRNAs being translated on each RER at any given time.

Preferential Assembly of AChE Homodimers Is Not Due to Clonal Origins of Individual Myotubes

Because AChE subunits expressed in heterozygous muscle cells assemble randomly, the preferential assembly of homodimers in mixed cultures could arise through three possible means: (*a*) individual myotubes could arise clonally thus selecting for individual nuclei expressing one or the other allelic polypeptide chain; (*b*) a significant fraction of the AChE could be synthesized by mononucleated cells expressing either the α or β AChE alleles such that mixing of the mRNAs and polypeptides could not occur; or (*c*) individual mRNAs encoding AChE could be translated preferentially on the RER surrounding the nucleus of origin. To distinguish between these alternatives, muscle cultures were made by plating approximately equal numbers of α/α and β/β myoblasts in which one group of myoblasts was prelabeled with [^{3}H]-thymidine (see Materials and Methods for details). Pure cultures of [^{3}H]thymidine-labeled myoblasts were made to determine the percent labeling of nuclei and to ensure that the label itself had no effect on the development and differentiation of these cells. After myoblast fusion and differentiation on day 5 after plating, the cultures were fixed and autora-

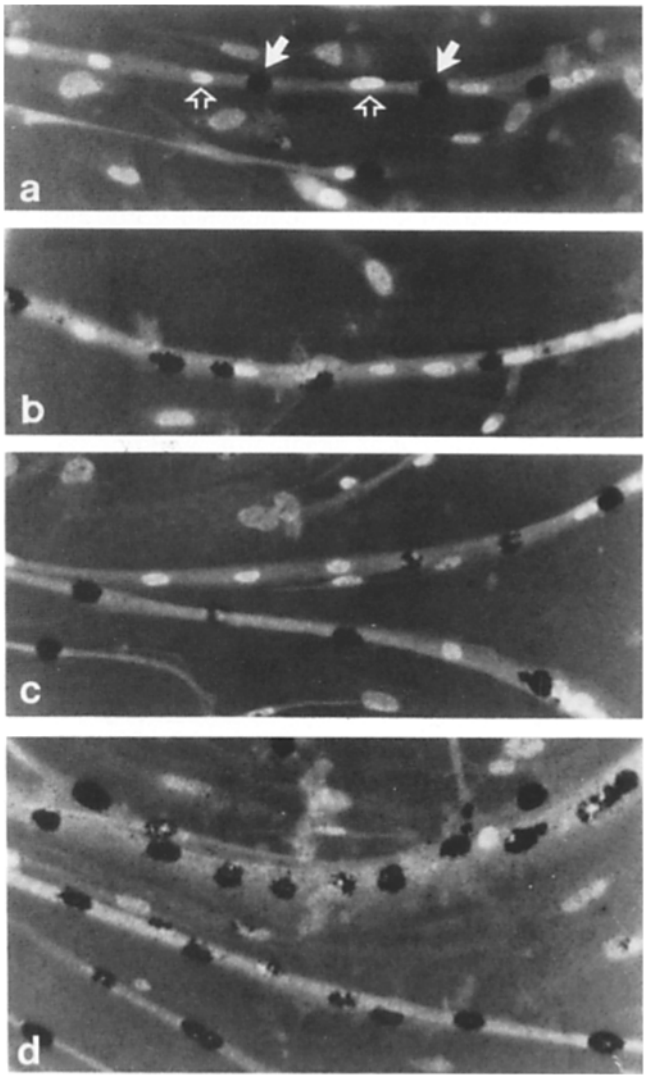

Figure 2. Distribution of α/α and β/β nuclei in chimeric myotubes. Unlabeled α/α myoblasts and [^{3}H]thymidine-labeled β/β myoblasts were mixed and allowed to differentiate into multinucleated myotubes. The cultures were then fixed, autoradiographed, and stained as described. (*a–c*) Myotubes formed by mixing approximately equal numbers of unlabeled α/α myoblasts and [^{3}H]thymidine-labeled β/β myoblasts. (*Open arrows*) Unlabeled nuclei; (*solid arrows*) labeled nuclei. (*d*) Multinucleated myotubes form pure [^{3}H]thymidine-labeled β/β myoblasts showing that the labeling procedure did not affect their ability to develop and differentiate into normal myotubes. The labeling efficiency in these experiments was 79% (see Materials and Methods).

diographed. After developing the emulsion cultures were stained with the DNA-binding fluorochrome HOESCHT 33258 to visualize individual nuclei (Fig. 2). In this manner one can readily quantitate nuclei in multinucleated myotubes and mononucleated cells as well as distinguish between labeled and unlabeled nuclei.

When unlabeled α/α and labeled β/β myoblasts were mixed in an ~1:1 ratio, 35.5 ± 5.1% of the nuclei in multinucleated myotubes were labeled. In this group a total of 807 nuclei were counted in 10 myotubes from each of three culture dishes (n = 30). Corrected for labeling efficiency (79%; see legend to Fig. 2, and Materials and Methods), these values indicate that an average of 45.1 ± 5.1% of the nuclei in the mixed myotubes were expressing the β AChE allele. If the mRNAs encoding each allele were being randomly translated on the RER, then the predicted distribution of dimers would be 0.30:0.50:0.20 (α/α:α/β:β/β) (see Materials and Methods), which is significantly different from the distribution of AChE dimers observed in these experiments (Fig. 1 and Table I).

In addition, we determined the potential contribution of clonally derived myotubes to the overall distribution of AChE dimers. Of 40 randomly chosen myotubes from three individual cultures only 2 (5%) had only unlabeled nuclei. These two myotubes were unusually small, containing 6–8 nuclei compared to a mean of 27 nuclei/myotube, and could also have contained unlabeled nuclei of the β/β genotype (Fig. 2). Thus nuclei in clonally derived myotubes would only contribute a very small percentage (1–2%) of the total AChE synthesized in these cultures.

The relative contributions of mononucleated myoblasts to the total AChE expressed in these cultures would also be very small. Excluding fibroblasts, which are large flattened cells with prominent nuclei that do not express AChE (Fig. 2), 91 ± 2% of the nuclei were found in multinucleated myotubes. Because myoblasts synthesize AChE at less than 1/10 the rate of differentiated myotubes (our unpublished observations), and assuming that the mononucleated cell population consists of equal numbers of α/α and β/β cells, their relative contributions to total AChE would be ~0.5–1.0%.

The Distribution of Homozygous Nuclei within Individual Myotubes Is Random

As a final consideration we examined the distribution of nuclei of each genotype within a series of 30 randomly selected myotubes having a total of 747 nuclei. Although on the average each myotube contained approximately equal proportions of nuclei encoding the α and β alleles, a nonrandom distribution of nuclei within each myotube could favor assembly of the homodimers. For this nearest neighbor analysis the linear order of labeled and unlabeled nuclei was determined for the 30 myotubes analyzed with an average of 27 nuclei per myotube. Nuclei at either end of the myotubes were eliminated from the analysis since they did not have two neighbors. Of 747 nuclei analyzed, 37.0% had a nucleus of the same genotype on either side. This value is very close to the predicted value based upon the frequency of labeled and unlabeled nuclei in the sample population (Fig. 3). Furthermore, the observed distribution of nearest neighbor labeled and unlabeled nuclei corresponds closely to that expected for a random distribution (Fig. 3). Thus, local statistical fluctuations in the distribution of α/α and β/β nuclei cannot account for the observed distribution of AChE dimers.

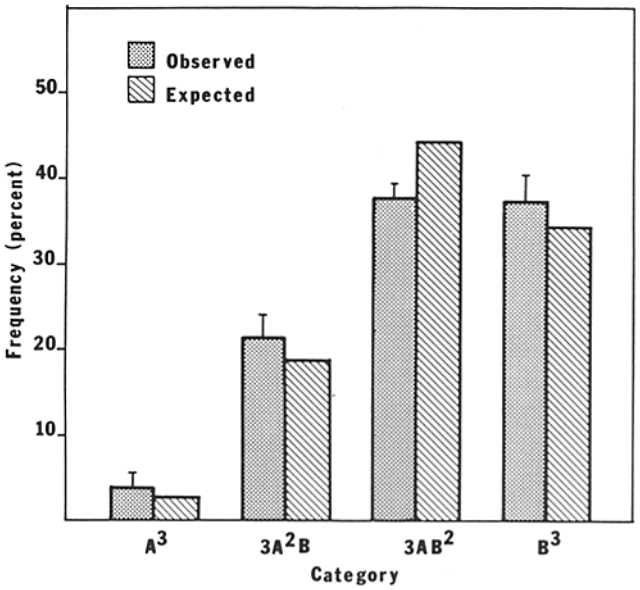

Figure 3. Frequency distribution of homozygous nuclei in chimeric myotubes. The distribution of labeled (A) and unlabeled (B) nuclei in 30 randomly selected myotubes was analyzed statistically using a nearest neighbor analysis (see Materials and Methods for details) and compared to the predicted distribution based upon the total numbers of labeled (32.4%) and unlabeled (67.6%) nuclei in the sample (n = 747 nuclei). The observed distribution of labeled and unlabeled nuclei closely approximates the predicted distribution (X^2 = 1.65; df = 3; P = 0.5), indicating random distribution of nuclei throughout the myotubes.

Discussion

In conclusion, these data support the hypothesis that transcripts encoding membrane-bound and secreted proteins in multinucleated skeletal muscle fibers are preferentially translated and assembled in the vicinity of the nucleus of origin. The small percentage of heterodimers expressed in mixed α/α + β/β cultures (Fig. 1 and Table I) could reflect very limited diffusion of AChE mRNAs, as has been recently shown by Ralston and Hall (1989) for cytoplasmically expressed β-galactosidase with a nuclear localization signal. Alternatively, the fact that in tissue culture myotube nuclei will spontaneously form occasional clusters could also favor translation of mRNAs expressed in neighboring nuclei. Thus, closely apposed nuclei of opposite genotypes in mixed cultures would translate each other's mRNAs and allow for some heterodimer assembly. At present we cannot distinguish between these possibilities; however, we favor the interpretation of limited diffusion of the mRNAs encoding proteins translated on the RER. Despite these limitations, the vast majority of AChE mRNAs are translated on the RER surrounding their nucleus of origin. These results are in striking contrast to earlier studies on expression of cytoplasmic and mitochondrial proteins in multinucleated muscle cells, which are translated on free ribosomes, where either the mRNAs or proteins were shown to be free to diffuse before translation or assembly (Mintz and Baker, 1967; Frair et al., 1979; Frair and Peterson, 1983).

At the morphological level, precise in situ hybridization studies indicate that mRNAs encoding the α-subunit of the AChR may be localized to the vicinity of individual nuclei either in vivo (Fontaine et al., 1988) or in tissue-cultured myotubes (Harris et al., 1989; Fontaine and Changeux, 1989; Horovitz et al., 1989). Although these latter studies have been questioned (Bursztajn et al., 1989), a likely explanation for the differences in localization reported by these investigators is that they used 3-d-old cultures which have only just begun to differentiate whereas Harris et al. (1989) and Fontaine and Changeux (1989) used older, more mature myotubes in their studies. Since myoblast nuclei are expressing AChR α-subunit mRNA at the time of fusion (Fontaine and Changeux, 1989), the younger cultures would be expected to show a less restricted distribution of AChR α-subunit mRNA.

Recent experiments using chimeric marker proteins capable of translocating into the nucleus either constitutively or upon induction (Ralston and Hall, 1989), and chimeric mouse-human myotubes expressing locally assembled contractile proteins or a cell surface marker (Pavlath et al., 1989), have shown that nucleus-specific domains may indeed form in multinucleated cells. In the former, Ralston and Hall (1989) used a β-galactosidase–glucocorticoid receptor nuclear localization signal chimeric protein, which was constitutively transported into the nucleus of myotubes to show that the mRNAs and/or proteins expressed in the vicinity of a particular nucleus diffused but a short distance within the multinucleated myotube. However, when the same β-galactosidase was coupled to a nuclear localization signal under glucocorticoid induction, the chimeric protein was able to diffuse long distances in the absence of activated nuclear localization signal. When glucocorticoids were added to the medium the reporter gene was then capable of translocating into the nuclei. These studies provide direct evidence that cytoplasmically translated proteins can diffuse long distances in the absence of a specific means of anchoring them, but that functional nuclear domains can be established by the relative rates of translation, diffusion, and localization of specific proteins. In the second set of studies, the expression of myosin heavy chains and of proteins retained in the Golgi apparatus associated with a particular myoblast nucleus remained in the vicinity of that nucleus after fusion to form the muscle fibers (Pavlath et al., 1989).

Thus, a picture emerges whereby the majority of mRNAs and/or proteins translated on free ribosomes of skeletal muscle fibers are free to diffuse throughout the sarcoplasm, whereas mRNAs encoding proteins destined for export to the cell surface, or translocation into the nucleus, are restricted in mobility and hence are preferentially translated in the vicinity of the nucleus of origin. These results have important implications for the regulation of neuromuscular junction components in muscle in that they provide a physical basis for the preferential expression and possible local regulation of these membrane proteins in a very narrow compartment of the muscle fiber.

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