Neuron, Vol. 14, 317–327, February, 1995, Copyright © 1995 by Cell Press

Myoblast Fusion and Innervation with Rat Motor Nerve Alter Distribution of Acetylcholinesterase and Its mRNA in Cultures of Human Muscle

Zoran Grubič,* Rado Komel,† Winsome F. Walker,‡ and Armand F. Miranda§
*The Institute of Pathophysiology
†The Institute of Biochemistry
Faculty of Medicine
University of Ljubljana
61105 Ljubljana
Slovenia
‡Department of Neurology
§Department of Pathology
College of Physicians and Surgeons
Columbia University
New York, New York 10032

Summary

To elucidate the mechanisms underlying acetylcholinesterase (AChE) localization, we analyzed the distribution of AChE and *Ache* mRNA during myogenesis in cocultures of human muscle and fetal rat spinal cord. We observed a temporal coincidence in alterations of AChE localization and nuclei expressing the message, suggesting developmental regulation at the mRNA level. Nonuniform mRNA staining among nuclei suggests asynchronous regulation, also supporting an earlier proposal that transcription proceeds intermittently. Asynchrony seems to be overridden by generally acting factors during myoblast fusion, when message is up-regulated, and at the onset of muscle contractions, when it becomes restricted to some nuclei in the junctional region and focal patches of AChE appear near nerve contacts. Coincidence of mRNA down-regulation and synthesis of stable basal lamina-bound AChE suggests coordinated adaptation, so that sufficient enzyme may be derived from low message levels.

Introduction

Hydrolysis of acetylcholine, catalyzed by acetylcholinesterase (AChE; EC 3.1.1.7), is essential for the termination of neurotransmission at the neuromuscular junction (NMJ). In the mature muscle fiber, AChE is concentrated at the NMJ; however, during the differentiation of the skeletal muscle and synaptogenesis, the localization of AChE activity shows different patterns (see reviews by Burden, 1993; Hall and Sanes, 1993; Navarette and Vrbova, 1993). The functional importance and the molecular mechanisms that modulate AChE distribution during myogenesis are not known. Conceivably, changes in the localization of AChE activity could be brought about by developmentally regulated factors that differentially control enzyme synthesis in distinct muscle fiber domains. This could imply that at different developmental stages, expression of AChE is modulated segmentally in multinucleate muscle fibers and that the enzyme is targeted to the surface membrane in the immediate vicinity of myonuclei that are actively transcribing *Ache* mRNA. Evidence that AChE transcripts remain close to the nucleus of origin and are not shared by different nuclear domains has already been provided in studies of avian muscle cultures (Rotundo and Gomez, 1990; Rossi and Rotundo, 1992; Tsim et al., 1992). It has also been shown that a variety of other developmentally regulated muscle proteins are transcribed, translated, and assembled within distinct nuclear territories (see Pavlath et al., 1989; Ralston and Hall, 1989). However, these investigations were not designed to systematically analyze transcription and translation during different stages of muscle development and maturation. Combined localization studies of *Ache* mRNA and AChE at distinct developmental stages of myogenesis and synaptogenesis could help establish conclusively whether or not the changes of AChE localization during muscle development are controlled at the mRNA level. Systematic studies of the development of human NMJ are facilitated by the introduction of a new tissue culture model, in which the progeny of human muscle satellite cells are cultured in monolayer and innervated de novo by fetal rat spinal cord neurons (Kobayashi and Askanas, 1985). In this culture system, the innervation process can be divided into developmental stages that morphologically resemble the development of the NMJs during ontogeny in mouse and rat (Askanas et al., 1987; Kobayashi et al., 1987).

In this study, we followed and compared the localization of *Ache* mRNA and AChE activity during differentiation and formation of the NMJ in human muscle cultures innervated by rat motor neurons. We observed significant changes in the patterns of *Ache* mRNA localization during muscle development, which coincided temporally with altered patterns of AChE localization, suggesting that developmental alterations of AChE localization are mostly regulated at the mRNA level. Based on our observations, we hypothesize that during muscle differentiation, *Ache* mRNA transcription proceeds asynchronously and independently in different nuclei, so that at any given moment, myonuclei are at different phases of their transcription cycles. Conceivably, this asynchrony is overridden by generally acting factors: first, during and immediately following myoblast fusion when *Ache* mRNA transcripts are increased at all nuclei, and second, during synaptogenesis, at onset of muscle contraction when the message disappears or is undetectable at virtually all extrajunctional nuclei. At the nuclei in the junctional region, this down-regulation of *Ache* mRNA expression could be prevented by some nerve-derived factors, possibly those reported to control expression of other synaptic components (reviewed by Laufer and Changeux, 1989; Hall and Sanes, 1993), so that the nuclei in this region resume their intermittent expression of *Ache* mRNA. The reduction of extrajunctional *Ache* mRNA synthesis coincides with innervation-induced contractions, turning on at the same time the synthesis of molecular component(s) that enable AChE binding to the basal lamina where it becomes stable and

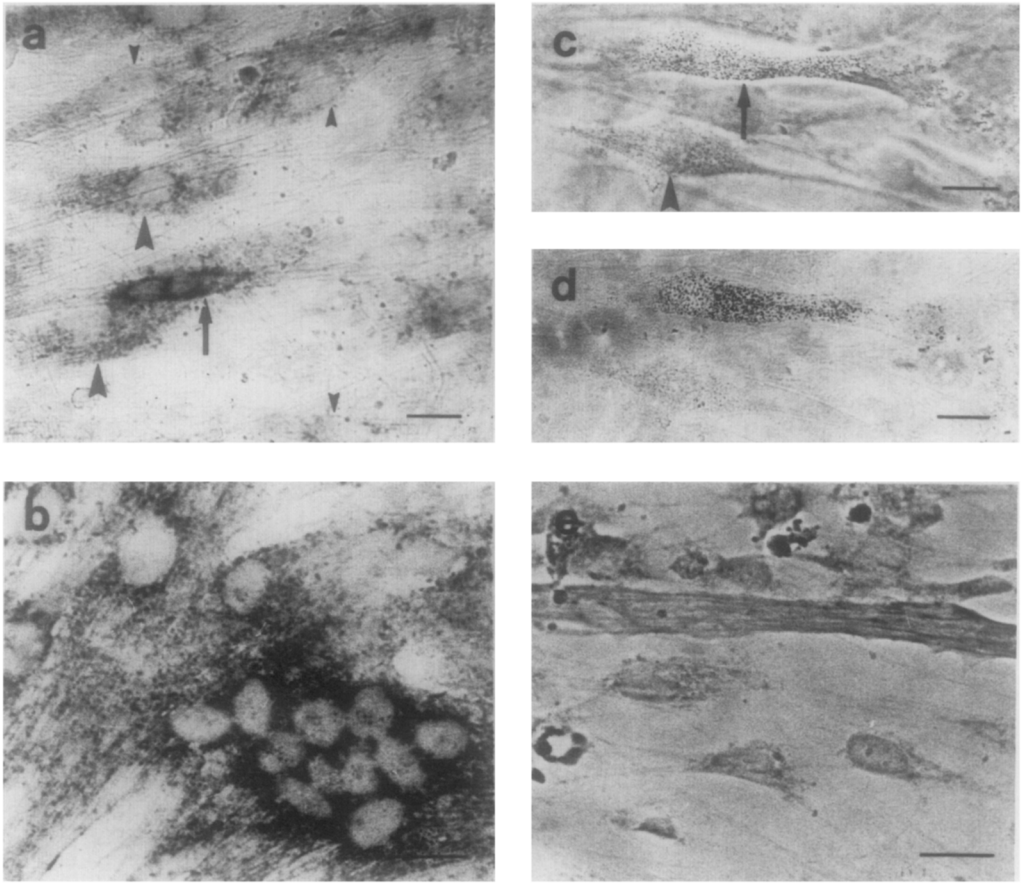

Figure 1. Localization of *Ache* mRNA and AChE Activity in Noninnervated Human Muscle Cultures

Studies were performed two days after plating (t_0), immediately after addition of the rat spinal cord explants. Figure shows *Ache* mRNA localization (a and b) and control (e); (c and d) show AChE staining.

(a) There is moderate perinuclear staining in some mononuclear myoblasts (large arrowheads) and virtually no staining in others (small arrowheads). There is intense perinuclear *Ache* mRNA staining in a small myotube containing two compact nuclei (arrow).

(b) In areas of the monolayer where myoblasts appear to be actively fusing, there is *Ache* mRNA staining around all nuclei.

(c and d) AChE localization. The same culture was studied with phase (c) and bright field (d) optics. AChE activity in a small myotube (arrow in [c]) is much higher in comparison with a mononuclear myoblast (arrowhead in [c]).

(e) To evaluate *Ache* mRNA staining specificity, some cultures were incubated with linearized pBR328 plasmid DNA. There was only very mild nonspecific staining, even after prolonged incubation (the culture in [b] was stained simultaneously with *Ache* probe).

Bars, 20 μm.

long lived (McMahan et al., 1978). This scheme could represent an energy-saving adaptation in the mature muscle fiber, whereby sufficient quantities of functional AChE can be derived from low levels of message synthesized, as needed, in short pulses by myonuclei near the NMJ.

Results

We studied the expression of *Ache* mRNA and AChE activity during myogenesis and synaptogenesis in human muscle cocultured with fetal rat spinal cord complex. Four stages of muscle development were studied, spanning the period from the first day in coculture until the formation of nearly mature NMJs (6 weeks in coculture). To localize *Ache* mRNA, we performed nonradioactive, colorimetric in situ hybridization. We used an exonic probe that spans a 339 bp sequence of the constant-catalytic core region of the rat AChE gene, upstream from the sequence coding for amino acids around the active site serine. It encodes a region common to all known forms of the enzyme (Taylor, 1992) and, therefore, should be able to detect AChE transcripts indiscriminately. To localize AChE activity, we used the thiocholine method, modified to visualize high junctional, as well as low extrajunctional, enzyme activities.

Localization of *Ache* mRNA and AChE Activity in Mononuclear Myoblasts and Developing Myotubes

The spinal cord explants were positioned on human muscle culture monolayers 2 days after plating (t_0), when only a few small myotubes were present (Figure 1; Table 1). About 87% of the mononuclear cells expressed *Ache* mRNA at this stage (Figure 2), but staining intensity was highly variable, and a concentration gradient of *Ache*

Table 1. Fusion Index

	t_0	t_{1w}	t_{3w}	t_{6w}
Innervation region	—	61.8 ± 3.6	82.0 ± 2.1	83.9 ± 2.8
Noninnervated region	9.6 ± 2.9	29.2 ± 2.4	58.7 ± 2.1	63.9 ± 2.5

The percentage of total nuclei incorporated into myotubes (fusion index; mean ± SE) was determined within a 3 mm zone surrounding the cord explants where innervation generally occurs (innervation region) and in random fields outside these zones where nerve processes are absent (noninnervated region). Counts were performed in 2-day-old muscle cultures, when the spinal cord explants were added (t_0), at 1 week of coculture (t_{1w}), when myotubes did not pulsate, and at 3 weeks (t_{3w}) and 6 weeks (t_{6w}), when continuous pulsations indicative of innervation were evident.

mRNA from the nuclear membrane toward the periphery was observed in all mononuclear cells expressing the transcripts (Figures 1a and 1b). At the time of fusion, the myonuclei appeared smaller and more compact, and *Ache* mRNA staining became intense in the newly formed myotubes (Figures 1a and 1b). At this stage, some cytoplasmic AChE activity was detectable in 83% of the mononuclear cells (Figure 2). In myotubes, enzyme activity appeared more abundant than in mononuclear cells and was localized along their entire length (Figures 1c and 1d).

Approximately 1 week after coculture (t_{1w}), a substantial difference was observed between the myotubes located within 2–3 mm of the spinal cord explant (region of innervation) and myotubes located more distally from it. The latter resembled the flattened, scattered muscle syncytia also commonly observed in aneurally grown human muscle cultures. Typical for the regions of innervation, there were elaborate bundles or networks of myotubes near the cord explants. These were not normally observed more than 3 mm away from the spinal cord, the region not invaded by neuronal processes. The formation of such myotube aggregates appeared to result from continued, intensive fusion of many mononuclear cells with myotubes that had already formed, resulting in their growth in girth and length (Figure 3a). The fusion index within the 3 mm zone around the cord explant was also much higher (61.8%) than in the area outside this region (29.2%; Table 1). In areas of active fusion, *Ache* mRNA staining was significantly more intense (Figures 3a and 3b) than in the more mature regions of the myotubes (Figures 3a and 3c). In the more mature regions, transcripts were detectable around most myotube nuclei (Figure 3c and see Figure 2), but staining intensity was variable (Figures 3a, 3c,and 3f). Unfused mononuclear cells located in the area of fusion also stained more intensely for *Ache* mRNA (Figures 3a and 3b) than mononuclear cells outside that area. The myotubes were not yet contracting at this stage; however, long neurites had already emerged from the spinal cord and were branching in the region of the myotube network. No differentiated neuromuscular contacts could be observed at the newly formed segments of the myotubes (Figure 3b), but immature bouton-like structures were already present at nerve–muscle contacts at the more mature region of the myotube network (Figure 3c). At this stage, no AChE patches or focal staining could be observed at such contacts or at any other segments of the myotubes, but AChE activity was detectable virtually along their entire length (Figures 3d and 3e). This was also the case in myotubes, where perinuclear *Ache* mRNA staining was nonuniform (compare Figures 3g and 3f). Even though AChE staining intensity was greater in the areas of active fusion where *Ache* mRNA levels were also high (Figures 3b, 3d, and 3e), most nuclei in the more mature area of the myotube network were *Ache* mRNA positive (97%) and AChE positive (98%; see Figure 2).

Localization of *Ache* mRNA and AChE Activity in Muscle Fibers after Functional Innervation

Occasional intermittent contractions were already noted in a few myotubes of some cultures after 6–7 days in coculture, but continually pulsating muscle fibers were usually not noted until 3 to 4 days later. It should be emphasized that in the culture system under study, contractions were always indicative of neuromuscular transmission; contractions ceased after addition of d-tubocurarine to the me-

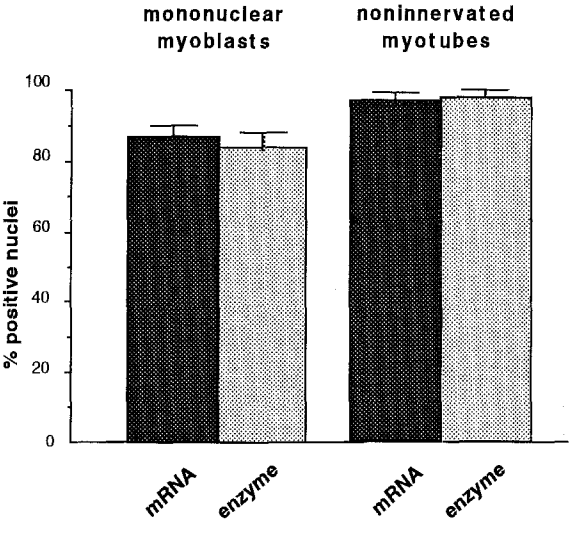

Figure 2. The Percentage of Myoblast and Myotube Nuclei Showing *Ache* mRNA and AChE Staining in Their Domains

Ache mRNA (mRNA) and AChE staining (enzyme) in myoblasts were evaluated at the time of coculture. In myotubes, the percentage of nuclei surrounded by *Ache* mRNA and AChE reaction products were determined 1 week after coculture. Percentages (± SE) were determined as described in Experimental Procedures. A statistically significant difference (unpaired Student t test, $p < .05$) was noted between *Ache* mRNA-positive nuclei in mononuclear cells (87 ± 2.7) and in myotubes (97 ± 1.2). For AChE, the data were also statistically significant (83 ± 3.0 and 98 ± 1.1, respectively; unpaired t test, $p < .05$). The difference between *Ache* mRNA and AChE staining was not significant, neither in myoblasts, nor in myotubes.

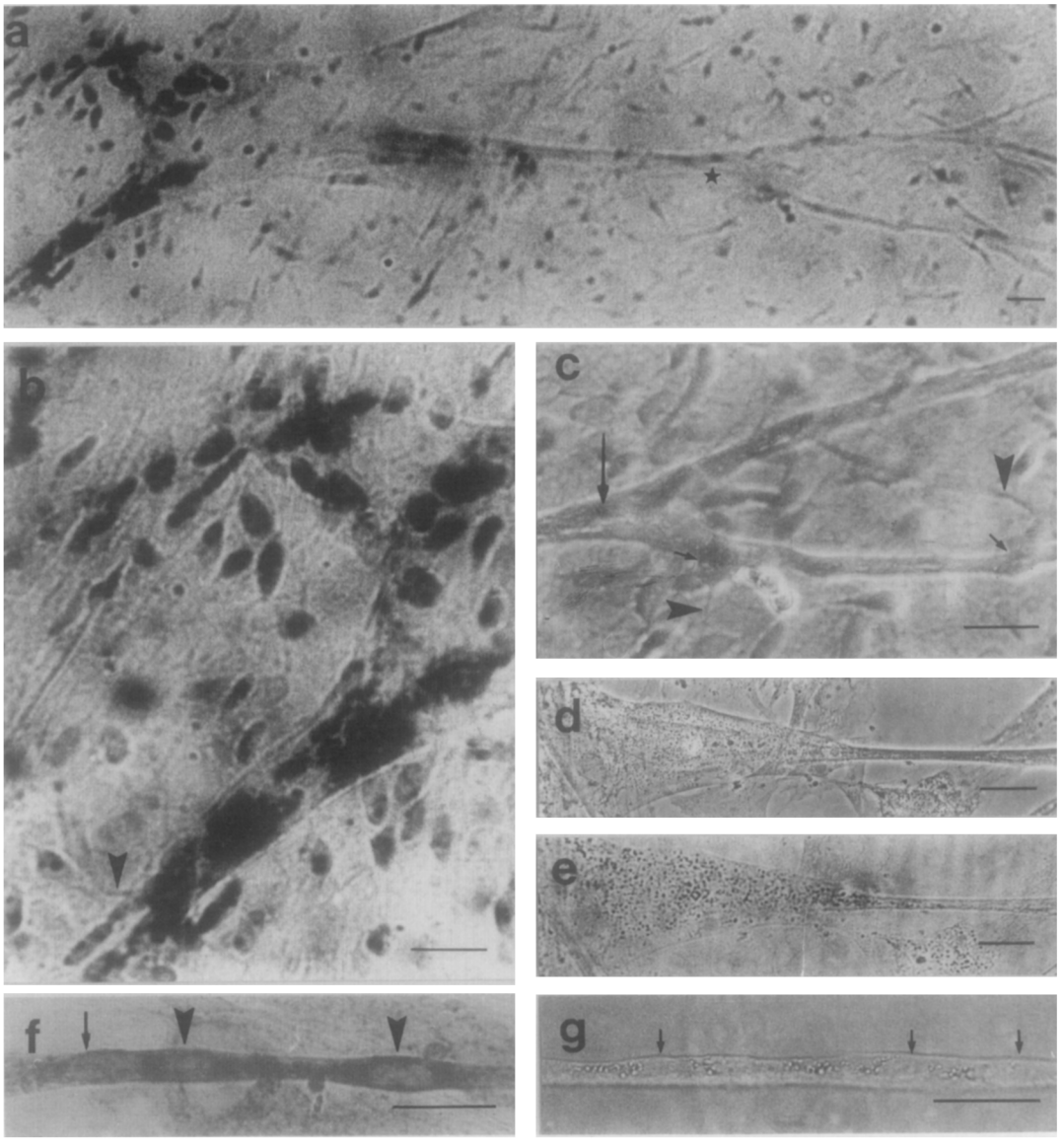

Figure 3. Localization of *Ache* mRNA and AChE Activity in Fusing Human Muscle Cultures

Studies were performed 1 week after addition of the rat spinal cord explants (t_{1w}).

(a) Formation of a branched myotube network, typically seen in regions of innervation, located 2 to 3 mm from the spinal cord explants. Branching myotubes to the right appear to be growing further through continued fusion of myoblasts to the left.

(b and c) Higher magnification micrographs of (a) reveal intensely stained clusters of myotube nuclei and adjacent unfused myoblasts (b) apparently becoming incorporated into the bifurcated myotube (c). Branching nerve fibers (one marked in [b] by arrowhead) are seen near the developing myotube. No differentiated neuromuscular contacts were observed in this myotube segment. Higher magnification phase microscopy of the more mature myotube segment in (c), representing the right side of (a) near the bifurcation marked with a star, reveals branches of nerve fibers (arrowheads in [c]) making bouton-like contacts (small arrows in [c]) with the myotubes. Dark spots, somewhat out of the focal plane (one marked by large arrow in [c]), represent nuclei expressing *Ache* mRNA.

(d and e) In the fusion region of a typical myotube (phase [d]; bright field [e]), there is high AChE activity.

(f) *Ache* mRNA localization studied with bright-field optics. A myotube located in the innervation region shows characteristic intense staining around some nuclei. Compare intense staining at two nuclei (large arrowheads) with faint staining at a nucleus marked with an arrow.

(g) AChE localization of a myotube in a region of innervation studied with phase optics. White cytoplasmic reaction product, showing up as refractile globules, is uniformly distributed among nuclei (small arrows) along the myotube.

Bars, 20 μm.

dium. As also reported previously (Askanas et al., 1987), aneurally grown human muscle does not contract spontaneously, does not possess well aligned sarcomeres, and does not have a well developed basal lamina and T system. This is in contrast with avian and rodent muscle cultures, which do not require innervation for advanced morphologic development and contractile activity. At 2–3 weeks after coculture, the number of contracting muscle fibers had increased. Most of these were entirely cross-striated, with well aligned sarcomeres (Figures 4a–4c). At

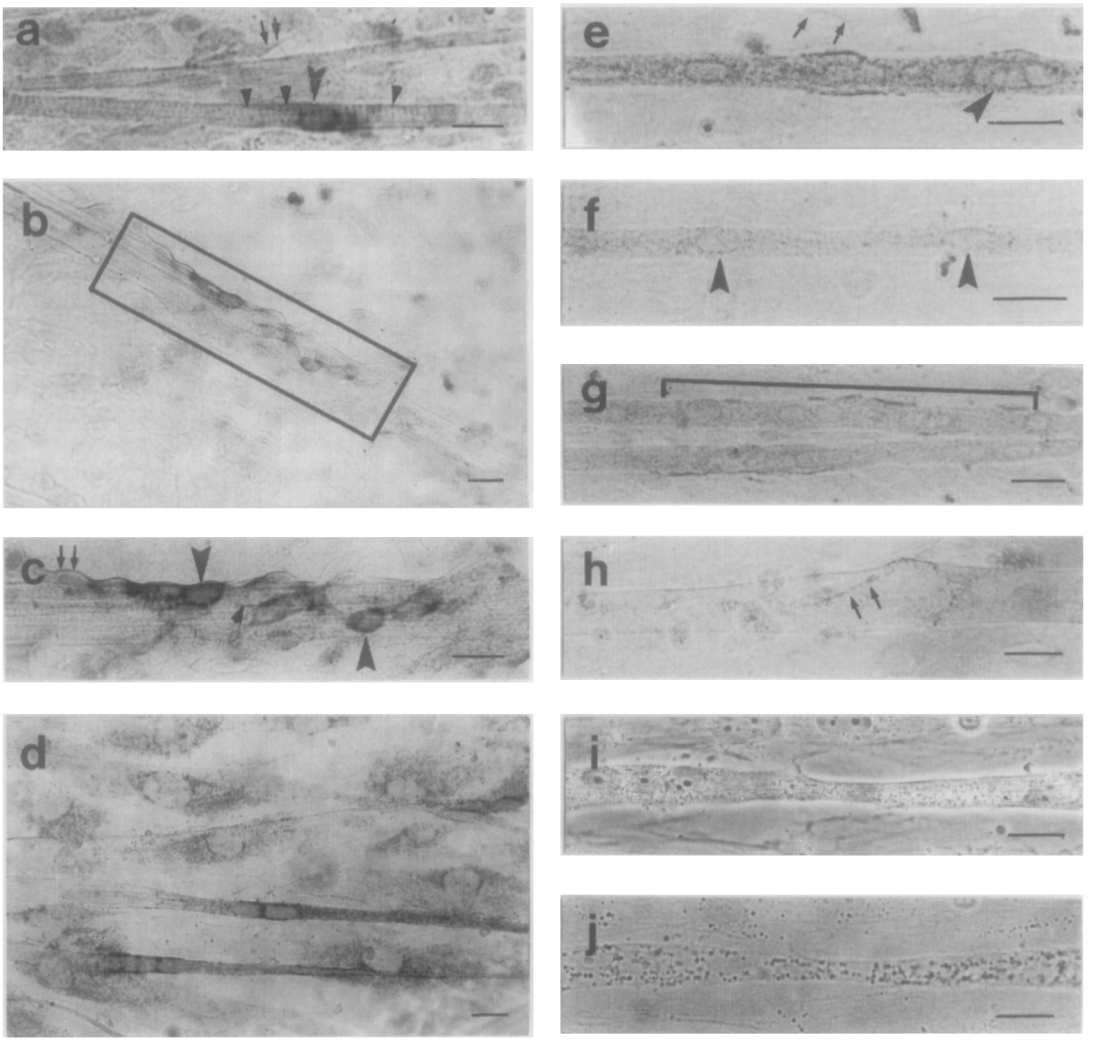

Figure 4. Localization of *Ache* mRNA and AChE Activity in Innervated Myofibers

The cultures were studied at three weeks (t_{3w}) after addition of the spinal cord explants (mRNA [a–d]; AChE [e–j]).

(a–c) In cross-striated myofibers that had been contracting (phase, [a–c]), *Ache* mRNA staining was only detectable near myonuclei located at loci that were contacted by nerve processes (small arrows). (a), as well as (c), which is an enlargement of the boxed junctional region in (b), show nuclei exhibiting *Ache* mRNA, some indicated by large arrowheads. Adjacent unstained nuclei are indicated by small arrowheads. At this stage, nuclei outside the junctional region usually did not stain.

(d) In noninnervated regions more than 3 mm away from the spinal cord, myotubes remained immature, and intense *Ache* mRNA staining was present at most or all nuclei. Moderate staining was also present in unfused mononuclear cells.

(e, g, and h) At the same stage of development, multiple focal patches exhibiting high AChE activity were noted at junctional regions of most innervated myofibers. The patches were generally superimposed on clusters of nuclei exhibiting perinuclear AChE staining (one cluster indicated in [e] with large arrowhead; a junctional region is marked by a bracket in [g]). AChE patches always formed at loci contacted by nerve. Segments of nerve processes traversing in the same focal plane as the myofibers are indicated (small arrows in [e] and [h]). The nerve processes also showed some staining.

(f) AChE staining was also present extrajunctionally in some innervated myofibers, often delineating the nuclei (large arrowheads). However, staining was generally less intense than at clustered nuclei in junctional areas.

(i and j) In noninnervated nonpulsating myotubes, AChE staining remained intense along the length of the myotubes (phase [i]; bright field [j]). This was similar to the staining pattern noted for *Ache* mRNA (see [d]). No densely stained focal patches of AChE activity were ever noted in noninnervated myotubes (i and j), and *Ache* mRNA staining remained intense around most nuclei (see [d]).

Bars, 20 μm.

this stage of development, the fusion index in the innervation regions was 82%, much higher than in noninnervated regions (58.7%; Table 1). In situ hybridization performed 3 weeks after coculture (t_{3w}) revealed that virtually all mRNA-positive nuclei were now located at the loci invaded by nerve processes. Even in these regions, only a small, highly variable proportion, not exceeding one-third of the total number of nuclei in the junctional region, expressed *Ache* mRNA (Figure 5). Apparently silent nuclei were often immediately adjacent to active ones (Figures 4a and 4c). Virtually no message was detectable by our technique around nuclei outside this region (Figures 4a and 4b; Fig-

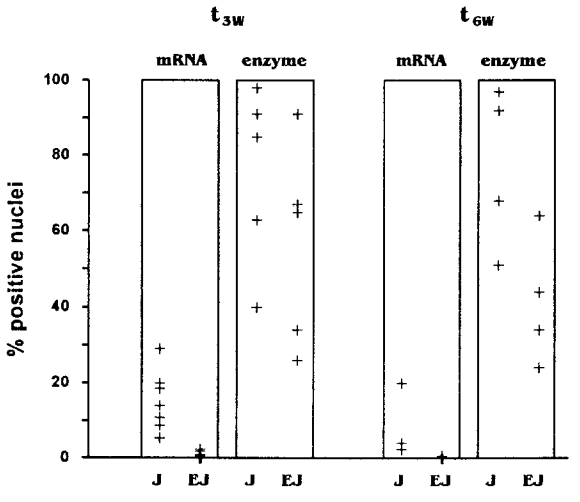

Figure 5. The Percentage of *Ache* mRNA and AChE Stained Nuclei in Functionally Innervated Fibers

The measurements were performed after 3 weeks (t_{3w}) and 6 weeks (t_{6w}) of coculture (mean ± SE), determined as described in Experimental Procedures. J denotes the junctional region; EJ, the extrajunctional region. Crosses are individual determinations. In many fibers, the number of nuclei was difficult to determine accurately because they were tightly clustered and overlapping. In fibers in which counts were feasible, the data are presented here. The average length of J, defined as the segment of the fiber bundle invaded by nerve and containing an entire cluster of AChE patches, was 131 ± 18 μm ($n = 5$) at t_{3w} and 104 ± 21 μm ($n = 4$) at t_{6w}. The difference between t_{3w} and t_{6w} was not statistically significant at our number of determinations ($p > .05$; unpaired t test). In some instances, J corresponded well with the length of the segment in the fiber bundles invaded by nerve and containing clustered *Ache* mRNA-stained nuclei (compare Figures 4b and 4g). However, this segment was frequently shorter, showing only two, or even one *Ache* mRNA-stained nucleus (see Figure 4a). EJ, defined as a 150 μm segment of the muscle bundle located on either side of J, contained virtually no *Ache* mRNA–stained nuclei at t_{3w} or t_{6w}, but AChE-stained nuclei were present. A significant difference was observed between *Ache* mRNA–stained nuclei in J and EJ at t_{3w} ($p < .05$; paired t test). At t_{6w}, the nuclei in J were almost always too tightly clustered to analyze, and the number of determinations that were feasible (three) was too low for comparative statistical analysis. However, in three determinations, we found no mRNA staining at nuclei in adjacent EJ regions. No significant difference was observed between AChE-stained nuclei in EJ and J at t_{3w} ($p > .05$; paired t test), but at t_{6w}, this difference was significant ($p < .05$; paired t test). There was no significant difference between t_{3w} and t_{6w} mRNA or AChE–stained nuclei in J ($p > .05$; unpaired t test). Staining intensity among individual nuclei was not taken into account. However, as determined by visual inspection, AChE staining was always much less intense in EJ than in J, and the total number of nuclei per 100 μm fiber length was also less in EJ (4.7 ± 2.3; $n = 12$) than in J (9.3 ± 1.1; $n = 12$).

ure 5). AChE patches were generally located above a single nucleus or clusters of nuclei (Figures 4e and 4g) and were only found at areas of nerve contacts (Figures 4e and 4h). In addition to AChE patches at the cell surface, some diffuse, rather uniform AChE activity could be observed in the myofibers, especially prominent around most nuclei in the junctional regions (Figure 4e). However, diffuse AChE staining, albeit more faint, was also seen in extrajunctional areas of innervated, contracting fibers (Figure 4f). The number of AChE-stained nuclei was significantly higher than the number of mRNA-stained nuclei, not only in the junctional region, but also in the extrajunctional region (Figure 5). Myotubes in aneural control cultures grown for the same length of time remained immature (Figures 4d, 4i, and 4j) in comparison with innervated myotubes in cocultures of the same age (Figures 4a–4c). In these noninnervated myotubes, *Ache* mRNA and AChE staining remained about the same as at the earlier stages. Unlike innervated cultures expressing *Ache* mRNA only in the region of innervation, noninnervated myotubes continued to show intense mRNA staining around most myonuclei (Figure 4d). High transcript levels in the myotubes were generally accompanied by high AChE activity distributed uniformly among the nuclei along their length (Figures 4i and 4j). Patches of AChE staining were never observed at the sarcolemma of noninnervated myotubes.

Six weeks (t_{6w}) after coculture, the fusion indices were about the same as observed at t_{3w} (Table 1). AChE transcripts remained confined to the few nuclei located in the vicinity of the innervation site. At this stage, the number of nuclei expressing *Ache* mRNA remained variable, and the percentage appeared to be even smaller. In three junctional regions that were analyzed, the percentages of positive nuclei were 2.6%, 3.5%, and 20.8%, respectively (Figure 5). However, at our number of determinations, no statistically significant difference was observed between the percentage of junctional nuclei expressing *Ache* mRNA at t_{3w} and t_{6w}. At some locations, AChE patches on individual myotubes became less numerous at the junctional regions.

Localization of *Ache* mRNA and AChE Activity in the Spinal Cord

Ache mRNA staining of high intensity could be observed in the developing fetal rat spinal cord at 12 or 13 days of gestation (Figure 6a). However, as was also observed in the nuclear domains of myotubes, the intensity of staining at individual neuronal cells was nonuniform (Figure 6b). This pattern remained unchanged throughout our study. High levels of transcript were accompanied by intense and uniform AChE staining in the spinal cord cells, which also remained unchanged throughout the study (Figures 6c–6e). After 6 weeks of coculture, the nerve cell cytoplasm was more abundant (Figure 6e), but there was no detectable change in the intensity of AChE staining. AChE activity was also noted in axons (Figure 6e), with no apparent difference between those growing freely and those establishing contacts with myotubes.

Discussion

Ache mRNA and AChE activity were already detectable in mononuclear myogenic cells derived from human muscle satellite cells. This is in agreement with earlier observations demonstrating AChE expression in myoblasts of embryonic chick (Mumenthaler and Engel, 1961) and embryonic rabbit (Tennyson et al., 1971). The perinuclear localization of *Ache* mRNA, with a gradient toward the periphery, presumably corresponds to the rough endoplasmic reticulum, where the catalytic subunits of AChE are translated and glycosylated (Rotundo et al., 1991).

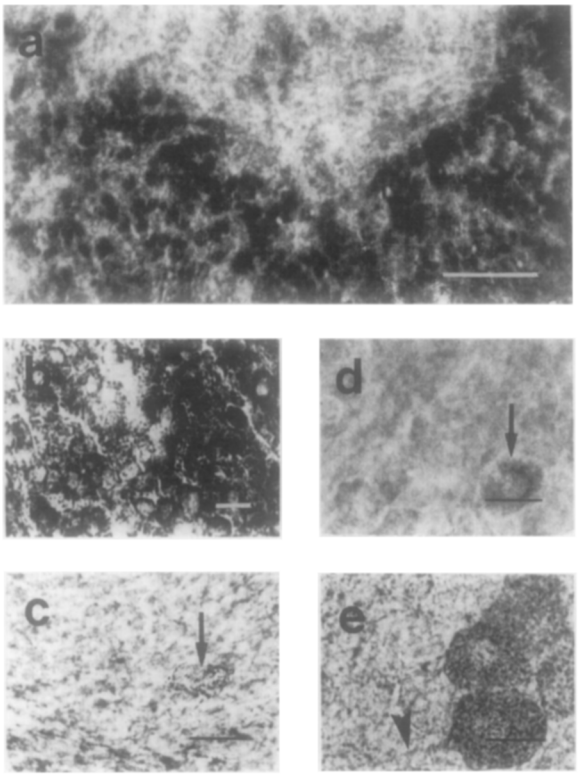

Figure 6. Localization of *Ache* mRNA and AChE Activity in the Rat Spinal Cord Explants

At the first day of coculture, there is intense mRNA staining in some neuronal cells in the presumptive ventral horns of the cord (a), at 1 week (b) and throughout the time of experimentation. AChE in neuronal cell bodies (arrows) and along axons (arrowhead) is also prominent and unchanged after 1 day (c), 1 week (d), and 6 weeks (e) in coculture. However, at 6 weeks, the neuronal cell bodies were significantly larger (e). Owing to the thickness of the explants, only a few cells in the same focal plane could be visualized simultaneously. Bars, 20 μm.

On the other hand, the perinuclear localization of AChE activity most probably corresponds to the Golgi area (Pavlath et al., 1989). The fusion of myoblasts into multinucleate myotubes and the formation of the NMJ are two developmental stages of muscle differentiation that are associated with distinct alterations in AChE expression. Rieger et al. (1980) and Inestrosa et al. (1983) observed that AChE activity is increased following fusion of cultured rodent myoblasts. More recently, Fuentes and Taylor (1993) demonstrated by quantitative RNAse protection assay that *Ache* mRNA is significantly higher in myotubes when compared with mononuclear myoblasts. In agreement with these data, we observed a statistically significant increase in the number of *Ache* mRNA-positive nuclei and AChE staining after fusion (see Figure 2). *Ache* mRNA and AChE staining were particularly intense in areas where active fusion appeared to occur. The pattern of staining that we observed at the fusion stage seems to indicate that the increase of AChE activity in early myotubes results from the presence of a large pool of *Ache* mRNA that is contributed by most or all myonuclei. Tsim et al. (1992) had previously demonstrated in aneurally grown avian muscle cultures that the number of *Ache* mRNA-positive nuclei in myotubes was more than 90%. Fuentes and Taylor (1993) showed that the increase of *Ache* mRNA in developing myotubes results from the stabilization of preexisting message rather than an increase in the rate of transcription. These investigators also noted superinduction of *Ache* mRNA in cultures of fusing myoblasts and differentiating myotubes exposed to cycloheximide, an inhibitor of protein synthesis. More mature myotubes became resistant to this treatment. It was hypothesized that cycloheximide inhibited the synthesis of some yet unknown *Ache* mRNA destabilizing factor.

At the onset of muscle contractions, which in the culture system we used is indicative of functional innervation, *Ache* mRNA staining became restricted to some nuclei in the junctional region. At the same time, patches of AChE activity appeared at the sites contacted by nerve, usually superimposed on tightly clustered nuclei. The noted decrease of extrajunctional *Ache* mRNA staining appears to be contraction mediated rather than the result of nerve-derived trophic factors, because Rotundo et al. (1991) found a significant decrease of total *Ache* mRNA in aneurally cultured quail muscle following the onset of spontaneous pulsations. On the other hand, prevention of *Ache* mRNA downregulation at the junctional region might result from the action of nerve-derived trophic factors. It has been shown previously that in the human muscle–rodent nerve cocultures, muscle contractions are always indicative of functional neuromuscular transmission, and remarkably mature motor endplates were found on the myofibers by electron microscopy (see Askanas et al., 1987; Kobayashi et al., 1987; Miranda et al., 1988). In agreement with Kobayashi et al. (1987), we did not observe focal patches of AChE activity at loci not contacted by nerve in human muscle–rat spinal cord cocultures. The importance of innervation for the formation of focal AChE patches has also been noted previously in other nerve–muscle culture systems (Koenig and Vigny, 1978; Rubin et al., 1980; De la Porte et al., 1993). However, nerve contact is not an absolute prerequisite for their formation in different vertebrate muscle cultures, because in the C2 mouse muscle cell line (Inestrosa et al., 1982) and in cultures of myotomal cells from Xenopus (Moody-Corbett and Cohen, 1981) grown aneurally, AChE patches were also found on the myotubes. The appearance of AChE patches indicates that the asymmetric, collagen-tailed A12 form of AChE and the basal lamina with its specialized proteoglycans (Inestrosa et al., 1992) are present at this stage, because they arise by attachment of the collagen tail of A12 to the basal lamina (Hall and Kelly, 1971; Hall, 1973; Inestrosa et al., 1982). There is evidence that muscle contraction is needed for the synthesis of the asymmetric A12 molecular form of AChE in muscle cultures (Rieger et al., 1980; Rubin et al., 1980; Brockman et al., 1984; Toutant and Massoulie, 1987). Its synthesis is probably triggered by expression of the structural tail subunit (Massoulie et al., 1993). However, AChE expression at the NMJ may also be regulated by the nerve through other mechanisms. Asymmetric A12 forms of AChE were shown to move along the motor nerve by fast axonal transport (Di Giamberardino and Couraud,

1978), and the possibility that nerve contributes at least some of the enzyme at the NMJ was suggested by Anglister (1991). In our experiments, intense staining for *Ache* mRNA and AChE was detected in neural cells of the spinal cord explants throughout the period of our study. Additionally, axons approaching the myotubes and forming contacts with them always stained first when our innervated cultures were processed for AChE staining. It has also been shown that agrin, a nerve derived factor, has the ability to induce accumulation of muscle-derived AChE at nerve contacts (Wallace et al., 1985). This mechanism might be especially important for the neural control of synaptic AChE after the onset of muscle contractions when *Ache* mRNA is down-regulated in the rest of the muscle fiber.

We noted considerable variability of mRNA staining around individual myonuclei, even between adjacent ones. It is highly unlikely that this was due to two distinct active and inactive pools of myogenic precursor cells, because at the fusion stage, intense *Ache* mRNA staining was found at almost all of the myoblast nuclei. The number of *Ache* mRNA–stained nuclei was particularly variable in the junctional region, as was the amount of transcripts in their domains. This finding is in agreement with the results of Jasmin et al. (1993), who showed by quantitative reverse transcription–polymerase chain reaction (RT–PCR) that *Ache* mRNA expression in homogenates of single isolated junctional regions of adult quail skeletal muscle was highly variable and undetectable in more than half of the samples. Based on this finding, the authors proposed that AChE transcription proceeds intermittently rather than constitutively. Our morphologic data also support the quantitative PCR data, showing that *Ache* mRNA is only found at junctional regions of mature muscle fibers and is virtually undetectable extrajunctionally.

In the junctional regions of pulsating fibers, we noted that the percentage of nuclei surrounded by AChE stain was significantly higher than the percentage of mRNA-stained nuclei. This is best explained by the higher stability of the catalytically active enzyme than its message, so that some nuclei ceased transcribing *Ache* mRNA while the enzyme persisted in their vicinity. This is in agreement with available data demonstrating that the half-life of *Ache* mRNA in cultured mouse C2–C12 myotubes is approximately 4–5 hr (Fuentes and Taylor, 1993), whereas the half-life of AChE in chick embryo muscle cultures is about 10 times longer (Rotundo and Fambrough, 1980). It is interesting that Landwehrmeyer et al. (1993) also showed in human striatum that relatively low levels of *Ache* mRNA accompany high AChE activity. Probably relevant to these data, we also observed nonuniform *Ache* mRNA staining in the neural cells of the spinal cord, suggesting intermittent transcription in the CNS as well.

We also observed faint AChE staining at the extrajunctional nuclei of innervated fibers, even though *Ache* mRNA staining in this region was virtually absent. Conceivably, transcription in this region is very low and not detectable by our technique. Supporting this assumption is an earlier observation showing that low AChE levels are found extrajunctionally in mature muscle fibers of different species. In human muscle, extrajunctional-specific AChE activity is particularly high, as is the relative proportion of the asymmetric forms (Carson et al., 1979).

In conclusion, although *Ache* mRNA expression appears to be up-regulated by generally acting regulatory factors at the time of fusion and down-regulated extrajunctionally at the onset of muscle contractions, AChE transcription also seems to be controlled at the level of individual nuclei within developing myofibers, because we frequently observed nuclei that appeared to be transcriptionally silent immediately adjacent to very active ones. As also proposed earlier by Jasmin et al. (1993), transcription seems to occur intermittently and at low levels at the NMJs as needed. Such tight regulation would be an efficient way to reduce the metabolic expenditure needed for the synthesis of AChE. AChE transcription also seems to occur intermittently in neural cells of the spinal cord, because the intensity of *Ache* mRNA staining of individual cells was also nonuniform. However, the phasic nature of transcription can only be hypothesized at present. Kinetic analysis of the expression of *Ache* mRNA around individual nuclei would be necessary before this question can be answered conclusively.

Experimental Procedures

Muscle-Spinal Cord Cocultures

Human muscle cultures were prepared from diagnostic muscle biopsies of 11 patients considered free of intrinsic muscle disease after all diagnostic studies were performed. The muscle was cleaned of adhering connective tissue, cut into 0.5–1 mm pieces, and trypsinized to release muscle satellite cells. The cells were initially grown at clonal density (300–400 cells) in 100 mm petri dishes at saturation humidity in 5% CO_2 and air. After 2–3 weeks, the muscle cell clones were trypsinized and pooled for further growth in 75 cm^2 flasks, as previously described (Miranda et al., 1986). As the cell layer approached confluency, prior to myoblast fusion, the cultures were again trypsinized and plated in 35 mm cluster dishes (5×10^4 cells per dish) with coverslip inserts that were previously coated with a 2:1 mixture of 1.5% gelatin and human plasma. The cells were initially cultured in Ham's F14 nutrient mixture with 10% fetal bovine serum and supplemented with 50 ng/ml basic fibroblast growth factor, 10 ng/ml epidermal growth factor, and 10 μg/ml insulin (Kobayashi and Askanas, 1985; Askanas et al., 1987). Two days after plating, 1 mm thick spinal cord sections from Sprague-Dawley rat fetuses (12–13 days gestation), with dorsal root ganglia attached and meninges intact, were applied onto each monolayer (4–5 explants per dish). To promote further differentiation, the fetal bovine serum in the growth medium was reduced to 5%, and fibroblast growth factor and epidermal growth factor were no longer added after 1 week in coculture (Kobayashi and Askanas, 1985; Askanas et al., 1987). To assure that no contaminating rat myoblasts were present, some cord explants were cultured without muscle. The human nature of the muscle in the cocultures was further confirmed by staining the developing myotubes in some cultures with a monoclonal antibody that specifically recognizes human neural-cell adhesion molecule (Walsh et al., 1989; Tanji et al., 1994). The cultures were fed twice a week and examined microscopically at least once a day. The research protocol for these studies was reviewed and approved by the Review Board of Columbia University.

AChE Probe for In Situ Hybridization

We used a rat AChE probe to detect human muscle and rat spinal cord *Ache* mRNAs by in situ hybridization in muscle–nerve cocultures. This probe was prepared by RT–PCR (Riboclone cDNA Synthesis System; Promega, Madison, WI) of poly(A)-selected (PolyA Tract System; Promega) rat brain RNA (Chomczynski and Sacchi, 1987), using forward and reverse PCR primers based on the published mouse AChE cDNA sequence (Rachinsky et al., 1990). Forward (A1) and

reverse (A2) PCR primers were selected from highly conservative regions, corresponding to nucleotides 370–393 and 691–708, respectively. The resulting 339 bp fragment (from the deduced Leu-92 to Gly-205 mouse sequence) was subcloned into the BamHI site of pUC19, using primers analogous to A1 and A2, but which contained 12 nt extentions harboring BamHI sequences. This region showed 83% nucleotide identity to the same region of the human AChE gene (Soreq et al., 1990).

In Situ Hybridization

Cultures were washed with phosphate buffered saline (PBS; pH 7.4) and fixed for 30 min at room temperature with 4% formaldehyde (freshly prepared from paraformaldehyde), containing 0.3% Triton X-100. After rinsing in PBS, the coverslips were mounted on microscope slides for easier handling. Samples were prehybridized in humid chamber with prehybridization solution (5× SSC, 5× Denhardt's solution, 50% deionized formamide, 250 μg/ml yeast tRNA, 250 μg/ml freshly denatured herring sperm DNA, 4 mM EDTA) at 44°C for 4 hr. After prehybridization, the coverslips were dehydrated in ethanol of increasing concentrations (70%–100%) and air dried briefly. Cultures were hybridized for 15 hr at 44°C in a hybridization mixture having the same composition as the prehybridization solution, except that herring sperm DNA was replaced by freshly denaturated AChE DNA probe labeled with digoxigenin using a Genius DNA Labeling and Detection Kit (Boehringer Mannheim, Germany). The concentration of the DNA probe was between 0.5 and 2 ng per 10 μl, as determined by dot blot analysis. The coverslips were rinsed two times for 15 min each at 42°C with 2× SSC buffer, one time for 15 min with 0.2× SSC, and 8–12 hr in several changes of 0.1× SSC. Hybridized probe was detected with the alkaline phosphatase–coupled anti-digoxigenin antibody, according to the Genius kit protocol, except that incubation with anti-digoxigenin antibody was prolonged to 1 hr. The color was developed with alkaline phosphatase substrate (10–12 hr for air-dried and frozen samples; 2 to 3 hr for samples processed immediately; see below). Cultures were mounted in PBS and examined in a Zeiss microscope equipped with phase optics. Control experiments included preincubation with RNAse prior to hybridization, hybridization with labeled linearized pBR328 plasmid DNA instead of AChE probe (see Figure 1e), and detection without the application of antibodies. In some experiments, the fixed cultures were air dried at 37°C overnight and placed in a sealed black box containing dessicant. These cultures were stored at −80°C for up to 2 months. If formaldehyde-fixed cultures were air dried and frozen prior to prehybridization, *Ache* mRNA was also detected within the nuclei (see Figures 3a and 3b and Figure 6a), most likely because the nuclear envelope became more permeable to the probe. Without drying and freezing, intranuclear localization of *Ache* mRNA could be detected only after prolonged alkaline phosphatase staining (see Figure 1b). However, perinuclear mRNA staining was more intense, suggesting better preservation of the message when mild conditions were applied prior to prehybridization. Dried and frozen cultures were rehydrated in a graded series of ethanol, rinsed in PBS, and processed for prehybridization as described.

Localization of AChE Activity

We used the thiocholine procedure of Koelle and Friedenwald (1949) modified according to Brzin and Pucihar (1976). The iodide ion in the substrate mixture was replaced by a cyanide ion, which gave a fine amorphous copper salt precipitate at the sites of AChE activity, instead of fast growing needle-like crystals generated with iodide copper salt. Cultures grown on coverslips were fixed as described above. They were preincubated for 20 min in the freshly prepared medium containing 10 mM $CuSO_4$, 20 mM glycine, and 5 mM KCN in PBS (pH 7.0), then incubated for up to 40 min in the same medium containing 3 mM acetylthiocholine chloride. Staining was monitored in a Zeiss microscope. Iso-OMPA (10 μM) was present in the preincubation and incubation medium to inhibit butyrylcholinesterase activity. Incubation was stopped by rinsing in PBS; the cultures were then examined and photographed. Depending on the microscope technique used (bright field, phase, or a combination of both) AChE reaction product appeared either as white, often refractile globules or as a black precipitate. Sometimes the same field was represented, both in phase and in bright field, to reveal both morphological details and AChE localization. In control experiments, Triton X-100 was omitted during fixation, with no detectable difference in AChE localization.

Quantitative Procedures

Myoblast fusion was evaluated by determining the percentage of total nuclei incorporated into myotubes (fusion index) at times specified in the legend to Table 1. For each determination, at least 1000 nuclei were counted in each of 2–4 different cultures, using phase optics, a calibrated reticle, and an ocular grid. The human myoblasts, which were elongate or fusiform in shape, were easily distinguishable from rat mononuclear cells, which at t_{3w} and t_{6w} formed a continuous sheet of polygonal epitheloid cells within 2 mm of the cord explants.

Ache mRNA and AChE measurements were performed in an Image Analyzer (Imaging Research, Brock University, Ontario, Canada) equipped with MCID (Micro Computer Imaging Device). At the myoblast stage (t_0), 2 days after culture when the spinal cord explants were added, the percentage of *Ache* mRNA and AChE-positive nuclei was determined in randomly chosen 680 × 500 μm fields containing 50–100 mononuclear cells. A total of 687 nuclei in 10 fields were counted for mRNA and 593 nuclei in 10 fields for the enzyme. The percentages of *Ache* mRNA and AChE-stained nuclei were also determined in 200 μm myotube segments (one in each myotube; ten segments in each of three different cultures) at the noninnervated early myotube stage (t_{1w}). No myotube segments were selected in areas where active myoblast fusion still appeared to be going on (see Figure 3b). A total of 372 nuclei were evaluated for mRNA and 329 nuclei for the enzyme. Intensity and size of the mRNA staining area, which varied substantially, was not taken into account. AChE reaction product per cell was also measured regardless of quantity. In myotubes, the precise nuclear domains for AChE synthesis was impossible to assess at this stage of development because staining was diffuse without nuclear membrane localization. Therefore, the myotube segments were divided into stained and unstained areas. All nuclei in stained areas were considered positive for AChE.

At three (t_{3w})and six weeks (t_{6w}) after coculture, *Ache* mRNA and AChE-positive nuclei were counted in junctional segments (J) and extrajunctional segments (EJ) of myotubes that had been pulsating. J and EJ were determined as described in the legend to Figure 5.

To correct for mRNA background staining, baselines were determined for each of the determinations using control cultures processed with heterologous pBR328 plasmid DNA instead of the probe. No baseline correction was necessary for AChE, since no staining was observed in control cultures treated with 1 nM methyl pinacolyl phosphonofluoridate.

Acknowledgments

This work was supported by grants from the National Institutes of Health (NINCDS, NS 11766) and the Muscular Dystrophy Association. Dr. Grubič was a Visiting Research Scientist at Columbia University, Department of Neurology. During his tenure, he was supported by the Ministry of Science of Slovenia. The authors are grateful to Drs. H. Soreq and P. Taylor for their support during the preparation of the probe. The authors also acknowledge Drs. H. W. Chang, C. T. Moraes, E. A. Schon, C. Sobreira, and K. Tanji for advice and comments, Drs. V. Askanas and J. McFerrin for their invaluable advice in growing the cultures, and M. Kadunc for his help in preparation of the photographs.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *"advertisement"* in accordance with 18 USC Section 1734 solely to indicate this fact.

Received April 13, 1994; revised October 15, 1994.

References

Anglister, L. (1991). Acetylcholinesterase from the motor nerve terminal accumulates on the synaptic basal lamina of the myofiber. J. Cell Biol. *115*, 755–764.

Askanas, V., Kwan, H., Alvarez, R. B., King Engel, W., Kobayashi, T., Martinuzzi, A., and Hawkins, E. F. (1987). *De novo* neuromuscular junction formation on human muscle fibres cultured in monolayer and

innervated by fetal rat spinal cord: ultrastructural and ultrastructural cytochemical studies. J. Neurocytol. *16*, 523–537.

Brockman, S. K., Younkin, L. H., and Younkin, S. G. (1984). The effect of spontaneous electromechanical activity on the metabolism of acetylcholinesterase in cultured embryonic rat myotubes. J. Neurosci. *4*, 131–140.

Brzin, M., and Pucihar, S. (1976). Iodide, thiocyanate and cyanide ions as capturing reagents in one-step copper-thiocholine method for cytochemical localization of cholinesterase activity. Histochemistry *48*, 283–292.

Burden, S. J. (1993). Synapse-specific gene expression. Trends Genet. *9*, 12–16.

Carson, S., Bon, S., Vigny, M., Massoulie, J., and Fardeau, M. (1979). Distribution of acetylcholinesterase molecular forms in neural and non-neural sections of human muscle. FEBS Lett. *96*, 348–352.

Chomczynski, P., and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. *162*, 156–159.

De la Porte, S., Ragueh, F., Eymard, B., Courbin, P., Chapron, J., and Koenig, J. (1993). Effect of sera from myasthenia gravis patients and of alpha-bungarotoxin on acetylcholinesterase during in vitro neuromuscular synaptogenesis. J. Neurol. Sci. *117*, 92–102.

Di Giamberardino, L., and Couraud, J. Y. (1978). Rapid accumulation of high molecular weight acetylcholinesterase in transected sciatic nerve. Nature *271*, 170–172.

Fuentes, M. E., and Taylor, P. (1993). Control of acetylcholinesterase gene expression during myogenesis. Neuron *10*, 679–687.

Hall, Z. W. (1973). Multiple forms of acetylcholinesterase and their distribution in endplate and non-endplate regions of rat diaphragm muscle. J. Neurobiol. *4*, 343–361.

Hall, Z. W., and Kelly, R. B. (1971). Enzymatic detachment of endplate acetylcholinesterase from muscle. Nature New Biol. *232*, 62–63.

Hall, Z. W., and Sanes, J. R. (1993). Synaptic structure and development: the neuromuscular junction. Cell *72*/Neuron *10* (Suppl.), 99–121.

Inestrosa, N. C., Silberstein, L., and Hall, Z. W. (1982). Association of the synaptic form of acetylcholinesterase with extracellular matrix in cultured mouse muscle cells. Cell *29*, 71–79.

Inestrosa, N. C., Miller, J. B., Silberstein, L., Ziskind-Conhaim, L., and Hall, Z. W. (1983). Development and regulation of 16S acetylcholinesterase and acetylcholine receptors in a mouse cell line. Exp. Cell Res. *147*, 393–405.

Inestrosa, N. C., Gordon, H., Esko, J. D., and Hall, Z. W. (1992). Binding of asymmetric (A12) acetylcholinesterase to C2 muscle cells and to CHO mutants defective in glycosaminoglycan synthesis. In Multidisciplinary Approaches to Cholinesterase Function, A. Shafferman and B. Velan, eds. (New York: Plenum Press), pp. 25–32.

Jasmin, B. J., Lee, R. K., and Rotundo, R. L. (1993). Compartmentalization of acetylcholinesterase mRNA and enzyme at the vertebrate neuromuscular junction. Neuron *11*, 467–477.

Kobayashi, T., and Askanas, V. (1985). Acetylcholine receptors and acetylcholinesterase accumulate at the nerve-muscle contacts of *de novo* grown human monolayer muscle co-cultured with fetal rat spinal cord. Exp. Neurol. *88*, 327–335.

Kobayashi, T., Askanas, V., and King Engel, W. (1987). Human muscle cultured in monolayer and cocultured with fetal rat spinal cord: importance of dorsal root ganglia for achieving successful functional innervation. J. Neurosci. *7*, 3131–3141.

Koelle, G. B., and Friedenwald, J. S. (1949). A histochemical method for localization of cholinesterase activity. Proc. Soc. Exp. Biol. NY *70*, 617–622.

Koenig, J., and Vigny, M. (1978). Neural induction of the 16S acetylcholinesterase in muscle cell cultures. Nature *271*, 75–77.

Landwehrmeyer, B., Probst, A., Palacios, J. M., and Mengod, G. (1993). Expression of acetylcholinesterase messenger RNA in human brain: an in situ hybridization study. Neuroscience *57*, 615–634.

Laufer, R., and Changeux, J.-P. (1989). Activity-dependent regulation of gene expression in muscle and neuronal cells. Mol. Neurobiol. *3*, 3–53.

Massoulie, J., Pezzementi, L., Bon, S., Krejci, E., and Vallette, F.-M. (1993). Molecular and cellular biology of cholinesterases. Prog. Neurobiol. *41*, 31–91.

McMahan, U. J., Sanes, J. R., and Marshall, L. M. (1978). Cholinesterase is associated with the basal lamina at the neuromuscular junction. Nature *271*, 172–174.

Miranda, A. F., Babiss, L. E., and Fisher, P. B. (1986). Measurement of the effect of interferons on cellular differentiation in human muscle cultures. Methods Enzymol. *119*, 619–628.

Miranda, A. F., Peterson, E. R., and Masurovsky, E. B. (1988). Differential expression of creatine kinase and phosphoglycerate mutase isozymes during development in aneural and innervated human muscle culture. Tissue Cell *20*, 179–191.

Moody-Corbett, F., and Cohen, M. W. (1981). Localization of cholinesterase at sites of high acetylcholine receptor density on embryonic amphibian muscle cells cultured without nerve. J. Neurosci. *1*, 596–605.

Mumenthaler, M., and Engel, W. K. (1961). Cytological localization of cholinesterase in developing chick embryo skeletal muscle. Acta Anat. (Basel) *47*, 274–284.

Navarette, R., and Vrbova, G. (1993). Activity-dependent interactions between motor neurons and muscles: their role in the development of the motor unit. Prog. Neurobiol. *41*, 93–124.

Pavlath, G. K., Rich, K., Webster, S. G., and Blau, H. M. (1989). Localization of muscle gene products in nuclear domains. Nature *337*, 570–572.

Rachinsky, T. L., Camp, S., Li, Y., Ekstrom, T. J., Newton, M., and Taylor, P. (1990). Molecular cloning of mouse acetylcholinesterase: tissue distribution of alternatively spliced mRNA species. Neuron *5*, 317–327.

Ralston, E., and Hall, Z. W. (1989). Transfer of a protein encoded by a single nucleus to nearby nuclei in multinucleated myotubes. Science *244*, 1066–1069.

Rieger, F., Koenig, J., and Vigny, M. (1980). Spontaneous contractile activity and the presence of the 16S form of acetylcholinesterase in rat muscle cells in culture: reversible suppressive action of tetrodotoxin. Dev. Biol. *76*, 358–365.

Rossi, S. G., and Rotundo, R. L. (1992). Cell surface acetylcholinesterase molecules on multinucleated myotubes are clustered over the nucleus of origin. J. Cell Biol. *119*, 1657–1667.

Rotundo, R. L., and Fambrough, D. M. (1980). Synthesis, transport, and fate of acetylcholinesterase in cultured chick embryo cells. Cell *22*, 583–594.

Rotundo, R. L, with the assistance of Gomez, A. M. (1990). Nucleus-specific translation and assembly of acetylcholinesterase in multinucleated muscle cells. J. Cell Biol. *110*, 715–719.

Rotundo, R. L., Fernandez-Valle, C., Gomez, A. M., Barton, F., and Leff, P. (1991). Regulation of acetylcholinesterase expression in electrically excitable cells. In Cholinesterases: Structure, Function, Mechanism, Genetics and Cell Biology, J. Massoulie, F. Bacou, E. A. Barnard, A. Chatonnet, B. P. Doctor, and D. M. Quinn, eds. (Washington, D. C.: American Chemical Society), pp. 146–151.

Rubin, L. L., Schuetze, S. M., Weill, C. L., and Fischbach, G. D. (1980). Regulation of acetylcholinesterase appearance at neuromuscular junctions *in vitro*. Nature *283*, 264–267.

Soreq, H., Ben-Aziz, R., Prody, C. A., Seidman, S., Gnatt, A., Neville, L., Lieman-Hurwitz, J., Lev-Lehman, E., Ginzberg, D., Lapidot-Lifson, Y., and Zakut, H. (1990). Molecular cloning and construction of the coding region for human acetylcholinesterase reveals a G+C-rich attenuating structure. Proc. Natl. Acad. Sci. USA. *87*, 9688–9692.

Tanji, K., Sancho, S., and Miranda, A. F. (1994). Innervation of MyoD-converted amniocytes and fibroblasts by fetal rodent spinal cord neurons. Neuromuscular Disorders *4*, 317–324.

Taylor, P. (1992). Impact of recombinant DNA technology and protein structure determination on past and future studies on acetylcholines-

terases. In Multidisciplinary Approaches to Cholinesterase Function, A. Shafferman and B. Velan, eds. (New York and London: Plenum Press), pp. 1–15.

Tennyson, V. M., Brzin, M., and Slotwiner, P. (1971). The appearance of acetylcholine esterase in the myotome of the embryonic rabbit: an electron microscope cytochemical and biochemical study. J. Cell Biol. *51*, 703–721.

Toutant, J.-P., and Massoulie, J. (1987). Acetylcholinesterase. In Mammalian Ectoenzymes, A. J. Kenny and A. J. Turner, eds. (Amsterdam: Elsevier), pp. 289–328.

Tsim, K. W. K., Greenberg, I., Rimer, M., Randall, W. R., and Salpeter, M. M. (1992). Transcripts for the acetylcholine receptor and acetylcholine esterase show distribution differences in cultured chick muscle cells. J. Cell Biol. *118*, 1201–1212.

Wallace, B. G., Nitkin, R. M., Reist, N. E., Fallon, J. R., Moayeri, N. N., and McMahan, U. J. (1985). Aggregates of acetylcholinesterase induced by acetylcholine receptor aggregating factor. Nature *315*, 574–577.

Walsh, F. S., Dickson, G., Moore, S. E., and Barton, C. H. (1989). Unmasking N-CAM. Nature *339*, 516.