Localization of Nicotinic Acetylcholine Receptor α-Subunit Transcripts during Myogenesis and Motor Endplate Development in the Chick

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Abstract. In 15-d-old chick latissimi dorsi muscles, the nicotinic acetylcholine receptor (AChR) α-subunit mRNA is densely accumulated at the level of subsynaptic nuclei of the motor endplate (Fontaine et al., 1988). In this paper, using in situ hybridization with genomic probes, we further show that the expression of the AChR α-subunit gene in the embryo, revealed by the accumulation of mature mRNAs, starts in myotomal cells and persists during the first stages of muscle development in a majority of muscle nuclei. Subsequently, the distribution of AChR α-subunit mRNAs becomes restricted to the newly formed motor endplates as neuromuscular junctions develop. To assess the transcriptional activity of individual nuclei in developing muscles, a strictly intronic fragment of the AChR α-subunit gene was used to probe in situ the level of unspliced transcripts. AChR α-subunit unspliced transcripts accumulate around a large number of sarcoplasmic nuclei at embryonic day 11, but can no longer be detected at their level after embryonic day 16 in the embryo. A similar decrease in the accumulation of AChR α-subunit transcripts is observed between day 4 and day 6 in primary cultures of muscle cells. On the other hand, in vivo denervation and in vitro blocking of muscle electrical activity by the sodium channel blocker tetrodotoxin results in an increase in the labeling of muscle nuclei. Yet, only 6% of the muscle nuclei appear labeled by the strictly intronic probes after denervation. The possible significance of such heterogeneity of muscle nuclei during motor endplate formation in AChR gene expression is discussed.

The nicotinic acetylcholine receptor (AChR) is a membrane-bound allosteric protein made up of four homologous subunits assembled into a heterologous α2βγδ pentamer, which transduces the acetylcholine signal at the vertebrate neuromuscular junction (reviews in Changeux et al., 1987a; Lindström et al., 1987). In adult innervated muscle, the surface density of the AChR is ~1000 times higher under the nerve ending than in extrasynaptic areas (review in Salketer and Loring, 1985). The mechanisms underlying the development of this highly localized distribution of AChR are still poorly understood. Yet, in recent years, the genes coding for muscular AChR subunits have been cloned and sequenced (Ballivet et al., 1983; Noda et al., 1983, La Polla et al., 1984; Nef et al., 1984; Boulter et al., 1985; Klarsfeld and Changeux, 1985; Moss et al., 1987), now allowing an analysis of these mechanisms at the nucleic acid level. In this context, Merlie and Sanes (1985) have shown that the steady-state levels of the α- and δ-subunit mRNAs are higher in the endplate-rich part of the mouse diaphragm than outside this region. We recently demonstrated, using in situ hybridization with a genomic coding probe, that the AChR α-subunit mRNAs accumulate under the nerve endings in 15-d-old chick muscles (Fontaine et al., 1988). To investigate the mechanisms involved in this differential accumulation, we examined the distribution of the mature and unspliced AChR α-subunit transcripts using in situ hybridization with genomic coding or strictly intronic probes. Experiments were conducted in vivo, on whole chick embryos during muscle differentiation and on the developing latissimus dorsi muscles: the anterior (ALD) with multiple "en grappe" endplates distributed all along the muscle fibers and the posterior (PLD) with a focal "en plaque" innervation (Ginsborg, 1960; Ginsborg and MacKay, 1961). Parallel experiments were performed in vitro on myoblasts and developing myotubes in primary cultures. Moreover, to investigate the role of muscle activity in the accumulation of AChR α-subunit mature and unspliced transcripts, in situ analysis was also carried out on a 4-d denervated PLD of a 15-d-old chick and on cultured myotubes chronically paralyzed by tetrodotoxin (TTX), a blocker of sodium channels.

Materials and Methods

Tissue Preparation and Cell Cultures

Chick embryos from day 2 to day 19 of incubation were carefully removed...
from fertilized eggs (Centre Avicole de 11e de France, Arpajon, France) incubated at 37°C. Their exact stage was determined according to Hamburger and Hamilton (1951). Each embryo was fixed overnight at 4°C in 4% paraformaldehyde buffered by PBS (140 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, 1.5 mM KH2PO4, pH 7.4) after having removed the dorsal skin for the older embryos. The PLD from day 11 to day 19 embryos and from day 2- and day 15-posthatching chicks were carefully dissected out under a microscope. A similar procedure was followed for 4-d differentiated PLD and controlateral innervated PLD of a 15-d-old chick (as described in Fontaine et al., 1988). A 20-wk-old hen was killed by decapitation and the ALD removed. The samples were fixed overnight at 4°C in 4% paraformaldehyde buffered by PBS (pH 7.4). Whole fixed embryos from day 2 to day 8, fixed PLD muscles from embryonic day 11 to day 15 posthatching and fixed ALD from a 20-wk-old hen were then paraffin (Paraplast) embedded. Sections of 7.5 µm were collected on 0.125% gelatin-coated "subbed" slides (Gall and Pardue, 1977) and stored at 4°C until hybridization.

Myoblasts were obtained from the hind limbs of 11-d-old chick embryos by mechanical dissociation and cultured as described (Betz and Changeux, 1979) on tissue culture chamber slides (Miles Laboratories, Inc., Naperville, IL) coated with 1% collagen or 35-mm plastic dishes (Coming Glass Works, Coming, NY) coated with 0.2% gelatin. From 48 to 96 h of culture, the cells were treated with 10⁻⁵ M cytosine arabinoside (Sigma Chemical Co.). In the plastic dishes, the number of surface AChR was determined according to Koelle and Friedenwald (Koelle and Friedenwald, 1949). Appearance of the reaction positive regions were photographed (the precipitate was washed out by the autoradiography procedure). Slides were then processed for in situ hybridization. In situ hybridization was conducted as recommended by the membrane suppliers (Hybond N; Amersham Corp.) for 5.0 (the younger stages and the cell cultures) or 7.5 min at 37°C in 0.4 M NaCl, 10 rain Tris-HCl (pH 7.4), 0.005 M EDTA, washed 15 min at 0.75 M Na3 citrate, 10 mM Tris-HCl (pH 7.4), 0.005 M EDTA, washed 15 min at 37°C in 0.4 M NaCl, 10 mM Tris-HCl (pH 7.4), 0.005 M EDTA, washed 15 min at 37°C in 0.4 M NaCl, 0.1 mM EDTA, washed 15 min at 37°C in 0.1 M Na3 citrate, 0.1 mM EDTA, washed 15 min at 37°C in 0.1 M Na3 citrate, and 15 min at 37°C in 0.1 SSC (0.015 M NaCl, 0.015 M Na3 citrate). Slides were subsequently dehydrated and air-dried.

For autoradiography, the slides were dipped once in NTB2 emulsion (Eastman Kodak Co.) warmed at 54°C, air-dried, and conserved in a dry, light-tight box with desiccant at 4°C. Slides were developed at 16°C (D19; Eastman Kodak Co.) for 3.5 min, rinsed in a water bath, fixed 4 min in rapid fix (AL4; Kodak) subsequently rinsed, stained with hematoxylin or toluidine blue, and mounted for standard light microscopy (Zeiss Axioshot 2 or Olympus BHS). The number of autoradiographic grains was counted on micrographs.

**Hybridization of the Sections**

Tissue sections pretreatment and hybridization were performed according to Wilkinson et al. (1987) with several modifications (Fontaine et al., 1988). Tissue-sections were deparaffinized in Xylene, rehydrated, washed in 1× PBS (pH 7.4). Tissue sections and culture slides were then fixed with protease K (20 µg/ml; Boehringer Mannheim Biochemicals, Indianapolis, IN) for 5.0 (the younger stages and the cell cultures) or 7.5 min (the older stages) at room temperature, washed in 1× PBS (pH 7.4), acetylated, dehydrated, and air-dried. A low background level and a high specific signal were obtained with an incubation time of 12-16 h in a humid chamber at 50°C and a probe concentration of ~0.5000 cpm/µl. Immediately before hybridization, probes were denatured at 80°C for 2 min and ~5 µl of the hybridization solution was applied either to each section, or to cultured cells and coverslips with a siliconized coverslip. For washing, the coverslips were allowed to float off in a solution of 5× SSC (0.75 M NaCl, 0.75 µM Na3 citrate), 10 mM EDTA at 42°C for 30 min. Slides were then washed in 50% deionized formamide, 2× SSC (0.3 M NaCl, 0.3 M Na3 citrate), 0.1 M EDTA during 20 min at 60°C, and twice in 0.4 M NaCl, 10 mM Tris-HCl (pH 7.4), 0.005 M EDTA, washed 15 min at 0.75 M Na3 citrate, 10 mM Tris-HCl (pH 7.4), 0.005 M EDTA, washed 15 min at 37°C in 0.4 M NaCl, 0.1 mM EDTA, washed 15 min at 37°C in 0.1 M Na3 citrate, 0.1 mM EDTA, washed 15 min at 37°C in 0.1 SSC (0.015 M NaCl, 0.015 M Na3 citrate), and 15 min at 37°C in 0.1 SSC (0.015 M NaCl, 0.015 M Na3 citrate). Slides were subsequently dehydrated and air-dried.

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**Colocalization of Acetylcholinesterase and AChR α-Subunit mRNA-rich Regions in the ALD of a 20-wk-old Hen**

After being dissected out, connective tissue was carefully removed from the ALD muscle under a microscope. The muscle was then fixed overnight at 4°C at its resting length in 2% paraformaldehyde buffered by PBS (pH 7.4) (Betz et al., 1980) and stained for acetylcholinesterase activity by the reaction of Koelle and Friedenwald (Koelle and Friedenwald, 1949). Appearance of the reaction precipitate was monitored under the dissection microscope. The muscle was then fixed in 4% paraformaldehyde buffered by PBS (pH 7.4) overnight at 4°C, and subsequently paraffin (paraplast) embedded. Sections (7.5 µm) were deparaffinized in xylene, rehydrated, mounted in glycerol, and Koelle-positive regions were photographed (the precipitate was washed out by the autoradiography procedure). Slides were then processed for in situ hybridization. In the case of the adult ALD, pretreatment required successive methanol dehydrations followed by a treatment of the slides by 0.2 N HCl during 20 min and a digestion by protease K (40 µg/ml; Boehringer Mannheim) for 10 min at room temperature. For the autoradiography, slides were exposed 10 d. Further details are given in Fontaine et al., 1988.

**Results**

**Localization of AChR α-Subunit Unspliced and Mature Transcripts by In Situ Hybridization**

The mature AChR α-subunit mRNA was detected in situ with a 2.3-kb genomic probe that includes coding sequences.
from exon two to six as described in Fontaine et al. (1988). The antisense 32P-labeled transcript of this 2.3-kb fragment hybridized on Northern blots of RNA from chick hind limb denervated muscle with a single band of ~2.8 kb assigned to the mature AChR α-subunit mRNA (Klarsfeld and Changeux, 1985). In situ hybridization with the antisense 35S-labeled transcript of the 2.3-kb fragment revealed signals on muscle tissues (for details see Fontaine et al., 1988), while sense 35S-labeled transcripts did not (data not shown). Treatment with RNase A before hybridization with the antisense probe abolished any detectable signal over background levels (data not shown). The background level was estimated as the number of grains found on nonmuscular tissues (for example blood vessels or connective tissue). The size of the AChR α-subunit mRNA did not significantly differ in developing, adult, or denervated chick muscles (Moss et al., 1987) and the AChR α-subunit gene was shown to be present as a single copy in chick genome (Ballivet et al., 1983; Klarsfeld and Changeux, 1985). The specific signal we observed thus most likely reflected the accumulation of transcripts from the same AChR α-subunit gene at the different stages of development.

To detect unspliced "primary" transcripts of the AChR α-subunit gene, we used two different intronic probes. A restriction map of a part of the AChR α-subunit gene is shown in Fig. 1 (Klarsfeld, A., personal communication; Shieh et al., 1987). Northern blot hybridization of antisense 32P-labeled transcripts of the intron-VII probe and the intron-VIII probe with RNA from chick hind limb denervated muscle did not reveal repetitive sequences (data not shown). The antisense 35S-labeled transcripts of the intron-VII and the intron-VIII probe were used for the in situ studies and similar control experiments as for the 2.3 kb probe (see above) were performed.

Actin transcripts were detected in situ by a similar procedure with a 1,150-bp mouse cDNA (see Materials and Methods). After 4 d of exposure, a signal of 15 ± 1 grains/10^3 μm^2 (mean ± SD, n = 4 countings) was detected all over muscle cells, which most likely resulted from hybridization with muscle specific α-actin mRNA (Schwartz and Roth-
Figure 3. AChR α-subunit mRNAs in myotomal cells and presumptive muscle precursor cells. (A) Transverse sections (TM) and longitudinal sections (LM) of the myotomes in a stage 24 chick embryo. (B) Muscle cells gathered around the developing humerus of the wing bud of a stage 24 chick embryo. (C) Presumptive muscle precursor cells bridging the myotome and the hind limb of a stage 24 embryo. Sections were hybridized with the 2.3-kb probe, exposed 6 d, and stained with hematoxyline. All these pictures are superpositions of dark-field and bright-field micrographs (SC, spinal cord; M, myotome; H, presumptive humerus; W, Wing bud; L, Hind-limb bud). Bars: (A) 200 µm; (B and C) 100 µm.

The cells from most skeletal muscles, especially those from limb and trunk, originate from the somites (Chevallier et al., 1977; Christ et al., 1977; review in Bennett, 1983). Since the torsion of the embryo prevents the observation of all somites in a single section (Patten, 1950), we examined serial sections of the same embryo starting from the caudal part (where the youngest somites are located) towards the more differentiated proximal somites (Patten, 1950). Different stages were studied with the 2.3-kb probe: stage 13 (19 somites), stage 17-18 (30 somites), stage 20 (42 somites), and stage 24. In the unsegmented primitive mesoderm and at the very early stages of somite differentiation, we did not detect any accumulation of AChR α-subunit mRNAs (Fig. 2 A in a stage 13 chick embryo). As somite differentiation proceeds, some cells become spindle shaped with an elongated nuclei and form the myotomal part of the somite (Holtzer et al., 1957). In our preparations, these cells which were most likely mononucleated (Holtzer et al., 1957) were intensively labeled by the 2.3-kb probe (Fig. 2 B in stage 20 chick embryo but a similar result was also obtained in stage 17-18 chick embryos). Clusters of grains (up to the saturation of the emulsion with a background level <1 grain/10^3 µm^2 after a 6-d exposure) accumulated on and around these elongated nuclei and grains were also seen over the cytoplasm of these cells (Fig. 2 B). Distinct populations of cells form the dermatomyotome and the sclerotome (Holtzer, 1957): Fig. 2 C shows that only the cells composing the myotome in a stage 24 chick embryo were labeled by the 2.3-kb probe with an intensity similar to that found at the level of the mononucleated muscle cells in the somite of a stage 20 chick embryo.

AChR α-Subunit Transcripts in Muscle Cells during Migration and First Events of Fusion

Migration of the myoblasts from the somite to their definitive location proceeds along a craniocaudal gradient and begins...
as early as stage 13 at the level of brachial somites (Chevallier, 1978; Jacob et al., 1978). Fig. 3 A shows myotomes in a stage 24 embryo. Because of the torsion of the embryo, longitudinal sections (LM) of the myotomes were seen together with transverse sections (TM) in the same preparation (Fig. 3 A). Fig. 3 C illustrates the continuous and homogeneous labeling by the 2.3-kb probe of putative muscle cells (arrow) bridging the myotome (M) and the hind limb bud (L). In the limb buds, the myoblasts regroup into ventral and dorsal cell masses (references in Bennett, 1983). Fig. 3 B shows...
Figure 5. AChR α-subunit mRNAs in developing PLD. (A) Longitudinal section of a 11-embryonic-day-old PLD. (B) Longitudinal section of a 14-embryonic-day-old PLD. (C) Longitudinal section of a 16-embryonic-day-old PLD. (D) Longitudinal section of a 19-embryonic-day-old PLD. A, B, C, and D are dark-field pictures. PLD were fixed in situ after the dorsal skin of the embryos had been removed. Sections were processed for in situ hybridization with the 2.3-kb probe as described in Material and Methods and exposed 9 d. Sections were stained with hematoxylin. Photographs were focused on the autoradiographic grains. The arrows indicate regions with a high level of autoradiographic grains. Bar, 120 μm.
two cell masses gathered around the presumptive humerus (H) in the developing wing (W). At this stage, nearly all cells were labeled by the 2.3-kb probe with the same intensity as the myotomal cells. Note that our results were obtained in a stage 24 embryo and that migration of myoblasts has been found to start as early as at stage 13 in chick embryo (Chevallier, 1978; Jacob et al., 1978). Therefore, we can not exclude the possibility that early migrating myoblasts would not express the AChR α-subunit.

First events of fusion can be detected at stage 24 (Holtzer et al., 1957). Myoblasts begin to leave the division cycle around stage 21–22 (Janners and Searls, 1970; Buckley and Konigsberg, 1977; Marchok and Hermann, 1967) and fuse to form primary myotubes in the proximal limb compartment at stage 25 (Bennett, 1983). Fig. 4 A shows that a majority of cells were labeled by the 2.3-kb probe in the proximal muscles of the hind-limb of a stage 29 embryo. A similar result is shown for the paravertebral muscles of a stage 34 embryo (Fig. 4 B). Fig. 4 C illustrates that in the paravertebral muscles of a stage 34 embryo, autoradiographic grains were found on and around the majority of muscle nuclei (59.5 ± 3.3%, mean ± SD, n = 4 different countings for a total number of 466 nuclei examined). These nuclei gave a signal that saturated the emulsion after a 6-d exposure.

**AChR α-Subunit Gene Expression in Developing PLD and in Adult ALD**

In the Latissimus dorsi, the first electrophysiological signs of nerve–muscle interaction have been recorded at 9 d of incubation (Bennett and Pettigrew, 1974). Around embryonic day 10 (stage 36), the formation of secondary myotubes also begins (Mac Lennan, 1983). Fig 5 A shows that at day 11, autoradiographic grains for the 2.3-kb probe were, in majority, diffusely distributed all over the developing muscle fibers with a density of 5.9 ± 1.5 grains/103 μm2 (mean ± SD, n = 4 countings). Some regions with higher levels of grains (14.3 ± 1.5 grains/103 μm2, mean ± SD, n = 4 countings) were however noticed (Fig. 5 A). At stage 40 (day 14) (Fig. 5 B) and at stage 42 (day 16) (Fig. 5 C) the total number of grains distributed all over the myofibers decreased, but several clusters of grains persisted along the developing muscle fibers. At stage 45 (day 19) (Fig 5 D) only one cluster of grains (68 ± 1.7 grains/103 μm2, mean ± SD, n = 4 countings) was observed per muscle fiber in longitudinal sections of PLD and the grains distributed over the rest of the muscle fiber did not exceed background level (0.5 ± 0.1 grain/103 μm2, mean ± SD, n = 4 countings). The same results held for 2- and 15-d posthatching chick PLD (data not shown; see also Fontaine et al., 1988). In contrast, hybridization studies with the actin probe disclosed that muscle cells were uniformly labeled along their entire length in the developing PLD (data not shown).

Hybridization studies of similar sections of the developing PLD with the intron-VII and the intron-VIII probes revealed that at embryonic day 11, grains accumulated over muscle nuclei (results are shown for the intron-VII probes in Fig. 6 A). At embryonic day 16, the number of muscle nuclei labeled by the probe decreased (Fig. 6 B, arrows). At em-
Figure 7. AChR α-subunit and actin mRNAs in the ALD of a 20-wk-old hen. (A) Longitudinal section of ALD stained by the acetylcholinesterase reaction of Koelle. (B) Same section as in A, hybridized with the 2.3-kb probe. After being photographed for the acetylcholinesterase staining, slides were processed for the in situ reaction with the 2.3-kb probe. (C) Longitudinal section of ALD hybridized with the 2.3-kb fragment. Autoradiograms were exposed 10 d. Sections were stained with toluidine blue (C) and hematoxylin (B). Photographs are bright-field pictures. (D) Longitudinal section of ALD hybridized with the actin probe. Slides were exposed 4 d and stained by hematoxylin. The photograph is a dark-field picture focused on the autoradiographic grains. Bars: (A and B) 30 μm; (C) 60 μm; (D) 70 μm.
bryonic day 19, as well as at posthatching day 2 or 15, no specific labeling was found with both intronic probes (data are shown for the innervated PLD of a 15-d-old chick in Fig. 6 C), even at neuromuscular junctions of 15-d-old chick PLD revealed by the Koelle reaction (data not shown). In contrast, in 4-d-denervated PLD of a 15-d-old chick, clusters of grains (3.3 ± 0.5 grains/10³ μm², mean ± SD, n = 4 countings for a background level of 1.2 ± 0.3 grains/10³ μm², mean ± SD, n = 3 different countings) were found to accumulate over 6 ± 2% (mean ± SD, n = 7 different countings for a total of 788 nuclei examined) of muscle nuclei with both intron-VII and intron-VIII probes (results are shown for the intron-VII probe in Fig. 6 D).

During the first weeks after hatching, neuromuscular junctions grow in length and the metabolic degradation rate of the AChR slows down (Burden, 1977a; Betz et al., 1977; Betz et al., 1980). In the ALD from a 20-wk-old hen ALD, autoradiographic grains from the 2.3-kb probe were found distributed exclusively within discrete clusters (Fig. 7 C). Fig. 7 A shows a neuromuscular junction revealed by the Koelle reaction (dotted line). After being photographed, the same section was hybridized with the 2.3-kb probe and autoradiographic grains were found to colocalize with this particular junction (Fig. 7 B, arrows) as reported for 80% of the cases examined in our study of the PLD and ALD in 15-d-old chicks (Fontaine et al., 1988). Note, however, that the number of autoradiographic grains was low (5 ± 1 grains/10³ μm², mean ± SD, n = 4 countings) even after a 10-d exposure but that autoradiographic grains accumulated on and around subsynaptic muscle nuclei as described in ALD and PLD from 15-d-old chicks (Fontaine et al., 1988). In contrast, hybridization with the actin probe of ALD sections disclosed that muscle cells were uniformly labeled along their entire length (Fig. 7 D). These results are consistent with those reported for 15-d-old PLD and ALD (Fontaine et al., 1988).

Expression of the AChR α-Subunit Gene in Primary Cultures of Chick Muscle Cells

Primary cultures of chick muscle cells were made from heterogeneous suspensions of cells composed mainly of myoblasts but containing also fibroblasts. Myoblasts divided during the first 24 h and then left the division cycle to fuse into multinucleated myotubes. 24 h after plating, the majority of the cells were mononucleated but only ∼17 ± 3% (mean ± SD, n = 5 different countings) for a total number of 707 nuclei examined were intensely labeled by the 2.3-kb probe (Fig. 8 A, arrows 1). A few small myotubes were also noticed. Arrows 2 in Fig. 8 A show that within these myotubes some of the muscle nuclei were labeled by the 2.3-kb probe while other were not (arrows 3). Fig. 8 C further illustrates that such heterogeneous labeling of muscle nuclei by the 2.3-kb probe also occurs in 4-d-old cultures. Autoradiographic grains densely accumulated over some muscle nuclei designed by arrow 1. Other muscle nuclei designed by arrow 2 displayed a significantly lower label, and muscle nuclei designed by arrow 3 were not labeled at all by the 2.3-kb probe (Fig. 8 C). Hybridization of cultured muscle cells with the intron-VII probe revealed that unspliced precursors of the AChR α-subunit mRNA also accumulated differentially at the level of some nuclei within the same myotube in a 4-d-old culture. Arrows 1 and 2 in Fig. 8 D designate muscle nuclei which were, respectively, not labeled and labeled by the intron-VII probe. In contrast, autoradiographic grains for the actin probe were distributed over the entire length of the myotubes in culture (results are shown for a 5-d-old culture in Fig. 8 B).

Around day 3 after plating, myotubes start to contract as a consequence of their spontaneous firing. This spontaneous electrical activity has been shown to repress AChR biosynthesis in vitro and chronic blocking of electrical activity by TTX results in an increase of AChR protein levels (Shainberg et al., 1976; Betz and Changeux, 1979; Fambrough, 1979) and of the amount of AChR α-subunit mRNA (Klarfeld and Changeux, 1985). After a 48-h treatment of 4-d-old cultures by tetrodotoxin, we observed, as described by these authors, an increase of ∼100% in the number of surface AChR measured by the binding of 125I-α-bungarotoxin. After 6 d in culture, clusters of grains for the 2.3-kb probe were rare in nontreated cultures and the number of autoradiographic grains that accumulated over the myotubes rather small (Fig. 9 A). In TTX-treated cultures (Fig. 9 B), the labeling of the myotubes by the 2.3-kb probe became very significant, yet, in a nonuniform manner. Some myotubes remained unlabeled, while others displayed a strong hybridization with the 2.3-kb probe. Fig. 9, C and D further shows that a similar effect of TTX was noticed with the intron-VII probe, after 48 h treatment in 6-d-old myotube cultures. Again, in some myotubes, some nuclei were found significantly labeled and others not.

Discussion

Early Accumulation of AChR α-Subunit Transcripts During Myogenesis

Meiniel and Bourgeois (1982) have detected high amounts of α-bungarotoxin binding sites in myotomal cells from 4-d-old chick embryo somites (stages 24–25). In the present study, we report that AChR α-subunit mRNAs accumulate in the mononucleated myotomal cells of the somites at least from stages 17 to 18 and 20 in chick embryos. Our observations are consistent with those of Baldwin et al. (1988) who recently showed that AChR subunit mRNAs accumulate in the somites of Xenopus. In mouse, an accumulation of transcripts coding for other muscle specific proteins (α-cardiac and α-skeletal actins) has also been observed in the myotome of developing somites as early as day 9 post coitum (15 somites) (Sassoon et al., 1988). We further show that high levels of AChR α-subunit transcripts persist during the first stages of myotube formation. Our results thus suggest that the accumulation of AChR α-subunit mRNA might be considered as an early sign of muscle cell differentiation in vivo.

Differential Distribution of AChR α-Subunit Transcripts during Synaptogenesis

At the early stage of development of a focally innervated muscle such as PLD, the AChR, revealed by both electrophysiological methods and α-bungarotoxin binding, appears distributed all along the developing myofiber (Burden, 1977b; Betz et al., 1980; Bennett, 1983). At the moment AChR accumulates under the motor nerve ending, the level of the
diffusely distributed surface AChR starts to decline (around day 11) to reach the background level (around day 19) (Burden, 1977b; Betz et al., 1977, 1980). We obtained parallel results by following the distribution of α-subunit mRNA, in contrast with actin transcripts that we found diffusely distributed throughout development. At embryonic day 19, a few regions of high labeling persisted but the number of grains counted in these clusters appeared less important than at embryonic day 14 or 16. We tentatively assigned them to developing motor endplates because we recently showed that in 15-d-old chick muscles, these clusters of grains detected with the AChR α-subunit coding probe colocalized with neuromuscular junctions (Fontaine et al., 1988). We further demonstrate that this result also holds for the multiply innervated ALD in the adult hen. Yet, the number of grains detected at the level of the adult endplate appeared significantly lower than that found at junctions of younger animals. These results are consistent with those of Pestronk (1985) who followed the distribution of intracellular and surface AChR protein during development of the motor endplate in the rat. This author found that intracellular AChR colocalized with neuromuscular junctions and that its level decreased throughout development while the level of surface AChR present in the subsynaptic domain remained relatively constant (Pestronk, 1985). Taken together, our results indicate that a differential accumulation of AChR transcripts in junctional and extra-junctional areas of the developing neuromuscular junction correlates with the differentiation of the motor endplate (Merlie and Sanes, 1985; Changeux et al., 1987b; Fontaine et al., 1988).

**Cellular and Nuclear Heterogeneity in the Accumulation of AChR α-Subunit Transcripts by Developing Muscle Cells**

In a previous study (Fontaine et al., 1988), we noticed that both at the level of the young adult motor endplate and in denervated muscle, AChR α-subunit mRNAs clustered on and around sarcoplasmic nuclei. It was further shown that 4 d after muscle denervation in a 15-d-old chick, only a
minority of the muscle nuclei (~10%) accumulated AChR α-subunit transcripts. We observed a similar heterogeneity at intermediate stages of motor endplate formation (between embryonic day 11 and 19) and in primary muscle cultures consistently with a previous autoradiographic study of surface AChR distribution in cell cultures using 125I-α-bungarotoxin (Entwistle et al., 1988). The heterogeneity in the accumulation of AChR α-subunit mRNAs in muscle cells thus seems a general feature at both the cellular and the nuclear levels and appears particularly striking when the spontaneous activity of cells in primary culture is blocked by TTX. To account for our observations, we suggest that, among other possibilities, discrete switches in gene expression regulate in an all-or-none manner the accumulation of AChR α-subunit mRNA by a given nucleus. Such switches between these several different nuclear states would, themselves, be under the control of intracellular messengers, as discussed in the next paragraph.

**Regulation of the Expression of the AChR α-Subunit Gene during Myogenesis and Synaptogenesis Studied Using Strictly Intronic Probes**

As discussed in our previous study (Fontaine et al., 1988), the accumulation of AChR α-subunit mRNAs on and around muscle nuclei might reflect a high transcriptional activity of these individual nuclei, a local protection of the mRNA from the action of nucleases or a differential transport of the mRNAs to given muscle nuclei. To get further insight into this question and into the mechanisms regulating the expression of the AChR α-subunit gene, we took advantage of strictly intronic probes already successfully used to detect unspliced transcripts in situ in the case of the proopiomelanocortin gene (Fremeau et al., 1986). At variance with mature mRNAs, the unspliced transcripts exhibit a rapid turnover (half-life in the range of a few seconds) and are not transported out of the nucleus (Darnell et al., 1986). Even though some examples of precursor stabilization have been documented (Leys et al., 1984; Narayan and Towle, 1985) their accumulation is generally assumed to reflect the transcriptional activity of the nuclei. In the case of the AChR, Buonanno and Merlie (1986) have reported an increase of the transcriptional rate for the α- and δ-subunit revealed by run-on experiments during myotube differentiation in a mouse cell line. In this context, the most likely interpretation of our in situ data obtained with strictly intronic probes is that the labeling of nuclei parallels the state of transcription of the AChR α-subunit gene.

We observed that the accumulation of unspliced tran-
scripts for the AChR α-subunit decreased throughout development to reach undetectable level at embryonic day 19 PLD and at the following stages, as well as after 6 d for muscle cells in culture. Denervation in vivo (Mertie et al., 1984; Kolarsfeld and Changeux, 1985; Goldman et al., 1985; Evans et al., 1987; Moss et al., 1987; Shieh et al., 1987; Fontaine et al., 1988), chronic paralysis in ovo (Burden, 1977b; Bourgeois et al., 1978), and blocking the spontaneous electrical activity in vitro (Kolarsfeld and Changeux, 1985; Shieh et al., 1988) is known to increase AChR mRNA levels. Blocking muscle activity in vivo and in vitro resulted in an increase in the accumulation of unspliced transcripts but only in some of the muscle nuclei (6% in the denervated muscle). Our observations are consistent with that of Shieh et al. (1987) who reported an increase of AChR α-subunit unspliced transcripts after denervation, revealed by a RNase protection assay. Taken together, our results, obtained using the strictly intronic probes, strengthen the notion that muscle activity inhibits the AChR biosynthetic pathway, in the case of the α-subunit at the transcriptional level, and suggest that the level of muscle nuclei transcriptional activity for the AChR α-subunit gene might play a role in the formation of the neuromuscular junction. The local accumulation of AChR and AChR α-subunit mRNA (Fontaine et al., 1988) at the level of the subnuclear “fundamental” nuclei (Ranvier, 1888) and their disappearance from extrajunctional regions would thus result from a differential transcription of AChR subunit genes in distinct nuclei present in different regions of the same sarcoplasm (Changeux et al., 1987b). In support to this idea, muscle nuclei within a same muscle fiber were indeed found to be labeled in an all-or-none manner using strictly intronic probes revealing the AChR α-subunit unspliced transcripts. The previous report that synthesis of fast myosin induced by fast ectopic innervation of rat soleus muscle is restricted to the ectopic endplate region is also consistent with this notion (Salviati et al., 1986).

Yet, the observation that trans-acting factors may diffuse from one nucleus to another in heterokaryons (Blau et al., 1985) raises a problem about the mechanism involved. Two possibilities may be considered. (a) Different “first messengers” are involved in junctional and extra-junctional domains; electrical activity outside the synapse and “trophic” factors released by the nerve ending (such as calcitonin gene-related peptide [Fontaine et al., 1986, 1987; New and Baldwin, 1985] or Myo D1 (Davis et al., 1987). Small variations of intracellular regulatory signals may then suffice to switch the nuclei between these discrete states.

These speculations point to the importance of the identification of the factors which actually contribute to the in vivo development of the neuromuscular junction and the regulatory mechanisms involved, at the gene level, in the expression of the different AChR subunits during embryonic development and motor endplate formation.

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References

Alonso, S., A. Minty, Y. Bourlet, and M. Buckingham. 1986. Comparison of three actin-coding sequences in the mouse; evolutionary relationships between the actin genes of warm-blooded vertebrates. J. Mol. Evol. 23:11–22.
Baldwin, T. J., C. M. Yoshitohara, K. Blackmer, C. R. Kintner, and S. J. Burden. 1988. Regulation of acetylcholine receptor transcript expression during development in Xenopus laevis. J. Cell Biol. 106:469–478.
Ballivet, M., P. Nef, R. Stalder, and B. Fulpitsu. 1983. Genomic sequences encoding the alpha-subunit of acetylcholine receptor are conserved in evolution. Cold Spring Harbor Symp. Quant. Biol. 48:83–87.
Bennett, M. R. 1983. Development of neuromuscular synapses. Physiol. Rev. 63:915–1048.
Bennett, M. R., and A. G. Pettigrew. 1974. The formation of synapses in striated muscle during development. J. Physiol. (Lond.) 241:515–545.
Betz, H., and J. P. Changeux. 1979. Regulation of muscle acetylcholine receptor synthesis in vitro by derivatives of cyclic nucleotides. Nature (Lond.). 278:749–752.
Betz, H., J. P. Bourgeois, and J. P. Changeux. 1977. Evidence for degradation of the acetylcholine (nicotinic) receptor in skeletal muscle during the development of the chick embryo. FEBS (Fed. Eur. Biochem. Soc.) Lett. 77:219–224.
Betz, H., J. P. Bourgeois, and J. P. Changeux. 1980. Evolution of cholinergic proteins in developing slow and fast skeletal muscles from chick embryo. J. Physiol. (Lond.) 302:197–218.
Blau, H. M., G. K. Pavlath, E. C. Hardeman, C. P. Chiu, L. Silberstein, S. G. Webster, S. C. Miller, and C. Webster. 1985. Plasticity of the differentiated state. Science (Wash. DC). 230:758–766.
Boulter, J., W. Layten, K. Evans, P. Mason, M. Ballivet, D. Goldman, S. Stengelin, G. Martin, S. Heinemann, and J. Patrick. 1985. Isolation of a clone coding for the α-subunit of a mouse acetylcholine receptor. J. Neurosci. 5:2545–2552.
Bourgeois, J. P., H. Betz, and J. P. Changeux. 1978. Effets de la paralysie chronique de l'embryon de poulet par le flaxedil sur le développement de la jonction neuromusculaire. C. R. Hebld. Séances Acad. Sci. 286:773–776.
Buckley, P. A., and E. R. Konigsen. 1977. Do myoblasts in vivo withdraw from the cell cycle? A reexamination. Proc. Natl. Acad. Sci. USA. 5:2031–2035.
Buonanno, A., and J. P. Merlie. 1986. Transcriptional regulation of nicotinic acetylcholine receptor genes during muscle development. J. Biol. Chem. 261:11442–11455.
Burden, S. 1977a. Acetylcholine receptors at the neuromuscular junction: developmental changes in recombinant myoblasts in vivo. Dev. Biol. 61:79–85.
Burden, S. 1977b. Development of the neuromuscular junction in the chick embryo: the number, distribution and stability of acetylcholine receptors. Dev. Biol. 57:317–329.
Changeux, J. P., J. Giraudat, and M. Dennis. 1987a. The acetylcholine receptor: molecular architecture of a ligand-regulated ion channel. Trends Pharmacol. Sci. 8:459–465.
Changeux, J. P., A. Devilliers-Théry, J. Giraudat, M. Dennis, T. Heidmann, F. Revah, C. Malle, O. Heidmann, A. Kolarsfeld, B. Fontaine, R. Lauger, H. O. Nghia, E. Kordeli, and J. Cartaud. 1987b. The acetylcholine receptor: functional organization and evolution during synapse formation. In Strategy and prospects in Neuroscience. Taniguchi Symposia on Brain Sciences n° 10. O. Hayashi, editor. Japan Scientific Societies Press, Tokyo. 29–76.
Chevallier, A. 1978. Etude de la migration des cellules somatiques dans le mésoderm somatopleural de l'ébauche de l'œuf. Wilhem Roux'Arch. Ent- wicklungsmech. Org. 184:1–73.
Chevallier, A., M. Kiener, and A. Mauger. 1977. Limb-somite relationship:
origin of the limb musculature. J. Embryol. Exp. Morphol. 41:243–258.

Christ, B. H., J. Jacob, and M. Jacob. 1977. Experimental analysis of the origin of the wing musculature in avian embryos. Anat. Embryol. 150:171–186.

Darnell, J., H. Lodish, and D. Baltimore. 1986. Molecular Cell Biology. Scientific American Inc., New York. 1187 pp.

Davis, R. L., H. Weintraub, and A. B. Lassar. 1987. Expression of a single transferr inhibit gene. Nature (Lond.). 329:345–348.

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Darnell, J., H. Lodish, and D. Baltimore. 1986. Molecular Cell Biology. Scientific American Inc., New York. 1187 pp.

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