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Detection of the nicotinic acetylcholine receptor α-subunit mRNA by *in situ* hybridization at neuromuscular junctions of 15-day-old chick striated muscles

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In adult vertebrate striated muscle, the nicotinic acetylcholine receptor (AChR) is almost exclusively localized in the postsynaptic membrane of the neuromuscular junction. Using *in situ* hybridization, we show that, in two different chicken muscles [the slow multi-innervated anterior latissimus dorsi (ALD) and the fast singly innervated posterior latissimus dorsi (PLD)], the AChR α-subunit mRNA is detected at discrete regions on myofibres and that these regions co-localize (80% correspondence) with neuromuscular junctions identified by histochemical staining for acetylcholinesterase. Moreover, autoradiographic grains densely accumulate on and around subsynaptic nuclei. In contrast, hybridization with an actin probe results in a strong signal distributed over the entire length of the myofibres. Denervation increases the level of AChR α-subunit mRNA both in the PLD and to a lesser extent in the ALD. By *in situ* hybridization we observe that, although a perinuclear pattern is maintained, the labelled nuclei appear randomly distributed among ~10% of the nuclei. These results are discussed in a model of AChR gene expression in vertebrate striated muscle fibres.

Key words: latissimus dorsi/denervation/nuclei/acetylcholinesterase

Introduction

The nicotinic acetylcholine receptor (AChR) is a membrane-bound allosteric protein made up of four homologous subunits which form a heterologous $\alpha_2\beta\gamma\delta$ pentamer [reviews in Changeux *et al.* (1984) and Hucho (1986)] and is involved in the transmission of chemical signals at the vertebrate neuromuscular junction [reviews in Changeux *et al.* (1984) and Hucho (1986)]. In adult muscle, the AChR has a surface density ~1000 times higher under the motor nerve endings than outside the junctions [review in Salpeter and Loring (1985)]. The mechanisms underlying the development of this highly localized subsynaptic distribution of the AChR remain obscure. They may intervene in any of the following ways: (i) the interaction of the AChR with components of the basal lamina, such as agrin (Reist *et al.*, 1987) and/or with cytoskeletal proteins, like the 43 K protein [references in Kordeli *et al.* (1986)]; (ii) the covalent processing and conformational maturation of the AChR subunits, their assembly into the $\alpha_2\beta\gamma\delta$ pentamer and their transport and targeting to defined areas of the cell surface via the Golgi apparatus [review in Merlie and Smith (1986)]; and (iii) the differential expression of the AChR genes themselves in muscle nuclei according to whether they are located under the nerve ending or outside the endplate (Merlie and Sanes, 1985; Changeux *et al.*, 1987). In the past several years, the AChR subunit genes from vertebrate skeletal muscle 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; Klarsfeld *et al.*, 1987) now allowing an analysis of these processes at the nucleic acid level. In this context, Merlie and Sanes (1985) have compared steady-state levels of α and δ AChR subunit mRNAs in dissected endplate-rich portions of mouse diaphragm to those in non-endplate regions. They observed that the levels of α- and δ-subunit mRNAs were 3- and 9- to 14-fold higher respectively in the synaptic regions. They therefore suggested that this distribution of AChR mRNAs could arise as a result of: (i) a directed transport of the mRNA from the extra-synaptic regions of the muscle fibres to the synaptic regions; (ii) an increased stability of AChR mRNAs near synapses; or (iii) an increased transcription of AChR subunit genes in nuclei near the synapses compared with that in nuclei elsewhere in the fibre.

In this paper we used *in situ* hybridization with a genomic probe to examine the distribution of the AChR α-subunit mRNA at the subcellular level in chicken muscle fibres. For this purpose, we examined the avian latissimi dorsi which comprises two muscles which have two different patterns of endplates: the slow anterior latissimus dorsi (ALD) possesses multiple 'en grappe' endplates distributed all along the muscle fibres while the fast posterior latissimus dorsi (PLD) receives, like most skeletal muscles in higher vertebrates, a focal innervation with an 'en plaque' endplate (Ginsborg, 1960; Ginsborg and MacKay, 1961). ALD and PLD muscles were examined on day 15 post-hatching, a 'young adult' stage at which neuromuscular junctions are clearly differentiated but when the AchR still displays a rapid metabolic turnover (Burden, 1977; Betz *et al.*, 1980). The staining of the endplates by the Koelle reaction for acetylcholinesterase (Koelle and Friedenwald, 1949) was compared with the distribution of AChR α-subunit mRNA by *in situ* hybridization on the same tissue sections. It has been reported that denervation of skeletal muscle results in an increase in the density of extrajunctional surface AChR and of the corresponding mRNAs coding the different subunits [Merlie *et al.* (1984), Goldman *et al.* (1985), Klarsfeld and Changeux (1985), Evans *et al.* (1987) and Shieh *et al.* (1987)]. Thus, we also examined the PLD and the ALD 4 days following denervation using *in situ* hybridization.

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Results

Localization of AChR α-subunit mRNA by in situ hybridization in chick innervated muscles

As a template for the AChR α-subunit RNA probe, we used a fragment of the chicken genomic DNA delimited by the restriction sites *Hin*dIII and *Sal*I. The restriction site *Sal*I is located ~40 bp from the beginning of exon 2 in this allelic variant of the chicken AChR α-subunit gene (Klarsfeld and Changeux, 1985; A.Klarsfeld personal communication). This 2.3-kb fragment includes exons 2–6 (~800 bp of coding sequence) (see Figure 1). The antisense ^{32}P-labelled transcript hybridized with a single band of ~2.8 kb on a Northern blot analysis of chicken hind-limb denervated-muscle RNA (gift of A.Klarsfeld), a size reported for the AChR α-subunit mRNA by Klarsfeld and Changeux (1985) (Figure 1). In contrast, the sense ^{32}P-labelled transcript did not give any visible signal under the same conditions (Figure 1).

In situ hybridization on ALD and PLD sections with the antisense ^{35}S-labelled transcript revealed highly localized grain clusters with both innervated muscles (Figures 2–4). (Note that the same results were obtained both in operated and non-operated innervated chick muscles.) After 8 days exposure, the number of grains per section area in these regions was at least 10-fold the background level in PLD sections and 50-fold in ALD sections (Figures 2b, 3a and 4b).

To test for the specificity of this signal, we hybridized some sections with a sense ^{35}S-labelled transcript. No signal was detected over the background level (data not shown). A treatment with RNase A before the hybridization with the antisense probe also abolished any detectable signal (data not shown).

In contrast, the actin probe under the same conditions resulted in a uniformly distributed signal throughout both muscles (Figures 3c and 4e). The same controls were also performed for the actin probe.

Comparison of the distribution of AChR α-subunit mRNA-rich regions and motor endplates revealed by the Koelle reaction in ALD and PLD

ALD and PLD neuromuscular junctions were stained by the reaction of Koelle (Koelle and Friedenwald, 1949) for acetylcholinesterase, a protein highly concentrated at the level of the basal lamina on the postsynaptic side of the endplate [reviews in Couteaux (1978) and MacMahan *et al.* (1978)]. Since the precipitate of the Koelle reaction was subsequently lost by the *in situ* hybridization procedures, the sections were photographed before hybridization. Photographs were also taken after the *in situ* hybridization and were used to establish a correlation between positive regions for the acetylcholinesterase staining and regions with a high density of grains on the autoradiograph as shown in Figures 2a,b,c and 4a,b. In four different experiments with the PLD (focally innervated muscle), 32 acetylcholinesterase-stained sites and 28 clusters of grains for the AChR α-subunit mRNA were counted and co-localization observed 27 times [mean = 85.7 ± 11.7% (SD); n = 4]. An example of this is illustrated in Figure 2 showing the co-localization of acetylcholinesterase (Figure 2a, arrows) and AChR mRNA-rich clusters (Figure 2b,c arrows). In the case of the ALD muscle which possesses distributed endplates, a similar co-localization was observed, as shown in Figure 4a,b. For example, arrows 1–3 show a correlation between Koelle-positive regions and AChR α-subunit mRNA-rich regions. Arrow 3 shows AChR α-subunit mRNA-rich regions that were detected under the postsynaptic membrane of neuromuscular junctions, suggesting a specialized contribution by these regions to the high levels of AChR mRNA. In five different experiments, we counted 136 Koelle-positive regions and 145 spots of grains. A co-localization was found in 118 cases [mean = 73.5 ± 6.4% (SD); n = 5]. The minor discrepancy (~20%) between Koelle-positive and AChR α-subunit-positive regions likely results from sections containing acetylcholinesterase-positive regions and lacking subsynaptic nuclei or vice versa. Additional control experiments showed that grain accumulation did not differ significantly in cholinesterase reacted and unreacted muscles (data not shown).

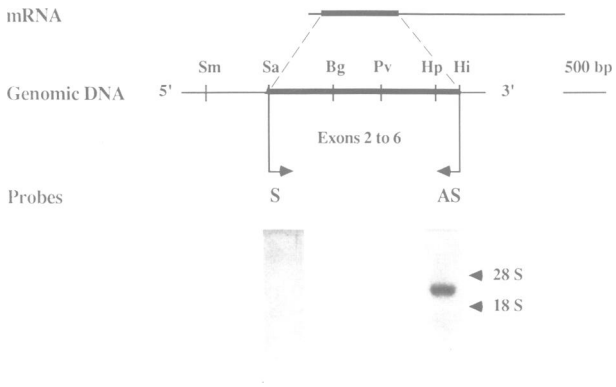

Fig. 1. Characterization of ^{32}P-labelled transcripts from a fragment of chick nicotinic acetylcholine receptor α-subunit gene: A. Restriction map of chick genomic DNA (Sm = *Sma*I, Sa = *Sal*I, Bg = *Bgl*II, Pv = *Pvu*I; Hp = *Hpa*I, Hi = *Hin*dIII). The *Sal*I cutting site is an allelic variation of the chicken AChR α-subunit gene (Klarsfeld and Changeux, 1985) and is located 40 nucleotides from the beginning of exon 2 (A.Klarsfeld, personal communication). Boxes indicate the length of genomic DNA transcribed *in vitro* and the corresponding coding region in the mature mRNA. The sense (S) and the antisense (AS) transcripts are hybridized to chicken denervated hind-limb muscle RNA as described in Materials and methods.

Effect of denervation on ALD and PLD

A Northern blot analysis of AChR α-subunit mRNA steady-state levels in both denervated or innervated ALD and PLD was performed. After 4-day denervation, these levels increased in both muscles for the same quantity (10 μg) of total RNA analysed, although to a lesser extent in ALD (Figure 5). Note that in the innervated muscles the AChR α-subunit mRNA level in PLD is lower than in ALD (Figure 5). The levels of muscular α-actin mRNA slightly decreased after 4-day denervation (Figure 5).

PLD is a focally innervated muscle and accordingly only one cluster of grains for the AChR α-subunit probe per muscle fibre was detected in longitudinal sections (Figure 3a). After 4-day denervation, clusters of grains appeared all along the myofibre with an apparently random distribution (Figure 3b). The same observation was made for the ALD (Figure 4c arrows, d). However, due to the greater abundance of synapses in the innervated ALD muscle, the effect of denervation appears as a smaller change in overall levels.

After hybridization of the sections with the actin probe, grains appeared uniformly distributed on the myotubes (Figures 3c and 4e) and no significant difference was noticed in either ALD or PLD after denervation (data not shown).

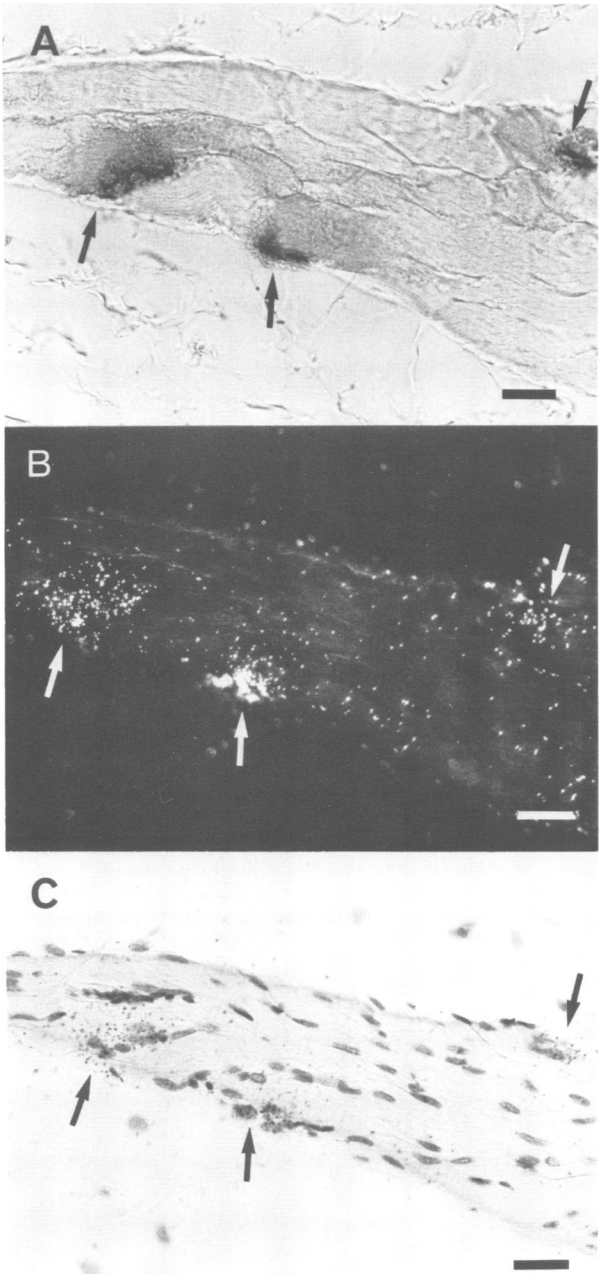

Fig. 2. Detection of acetylcholinesterase-stained regions by the Koelle reaction and AChR α-subunit mRNA revealed by *in situ* hybridization in a section of 15-day-old chick innervated PLD. (**A**) Section stained by the Koelle reaction. (**B**) and (**C**), respectively dark-field and light-field pictures of the same section as in (A) hybridized with an AChR α-subunit genomic probe. Arrows indicate the corresponding sites between Koelle and *in situ* positive regions. Slides were exposed for 8 days. Bar = 10 μm.

The grains associated with AChR α-subunit mRNA probe are distributed at the level of nuclei

Figure 2c (arrows) and Figure 4b (arrow 3) show that, in both PLD and ALD muscles, the grains associated with the AChR α-subunit probe are localized over the nuclei located under the postsynaptic membrane of the endplate. In contrast, in denervated PLD, the grains due to the AChR α-subunit probe were also distributed along the length of the fibre, although they remained perinuclear in their distribution (Figures 3b and 6a). Interestingly, we note in this situation that only ~10% of the nuclei were labelled by the AChR α-subunit probe (in Figure 6a, arrow 1 points to a positive nucleus and arrow 2 to a negative one).

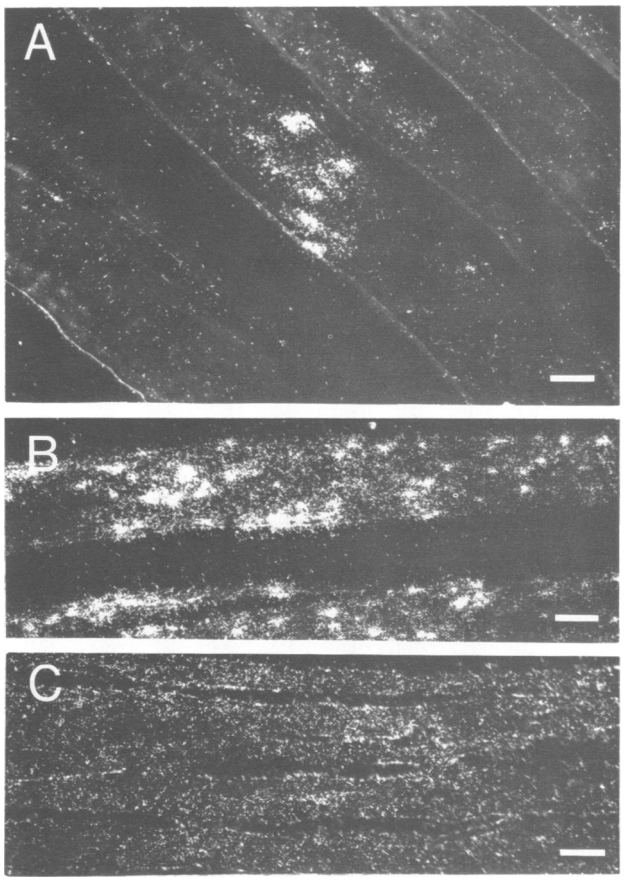

Fig. 3. Dark-field photographs of longitudinal PLD sections. (**A**) Longitudinal section of 15-day-old chick innervated PLD hybridized with an AChR α-subunit probe. (**B**) Longitudinal section of 15-day-old chick denervated PLD hybridized with an AChR α-subunit probe. In (A) and (B) slides were exposed for 8 days. (**C**) Longitudinal section of 15-day-old chick innervated PLD hybridized with the actin probe (24 h exposure). Bar = 20 μm.

In contrast, Figures 3c, 4e and 6b show that, with the actin probe, grains appeared diffusely distributed (all along the myofibre) without a preferential nuclear localization all along the myofibre.

Discussion

In vertebrate striated fast muscle, motor endplates are highly specialized structures which represent only ~0.1% of total muscle fibre surface. The subsynaptic domain of the endplate is characterized by a high density of AChR, a granulated sarcoplasm and four to eight specialized myonuclei referred to as 'fundamental' (Kühne, 1862; Ranvier, 1888; Couteaux, 1960; Roberts, 1986). These 'fundamental' myonuclei, which represent only a small fraction of the myonuclei within the entire muscle fibre, have a distinct morphology and are associated with AChR clusters in rat myotubes (Bruner and Bursztajn, 1986). The role of these 'fundamental' myonuclei in the expression of synapse-specific proteins has long been proposed [review in Couteaux (1960)]. In this context, using Northern blot analysis, Merlie and Sanes (1985) have shown that AChR α- and δ-subunit

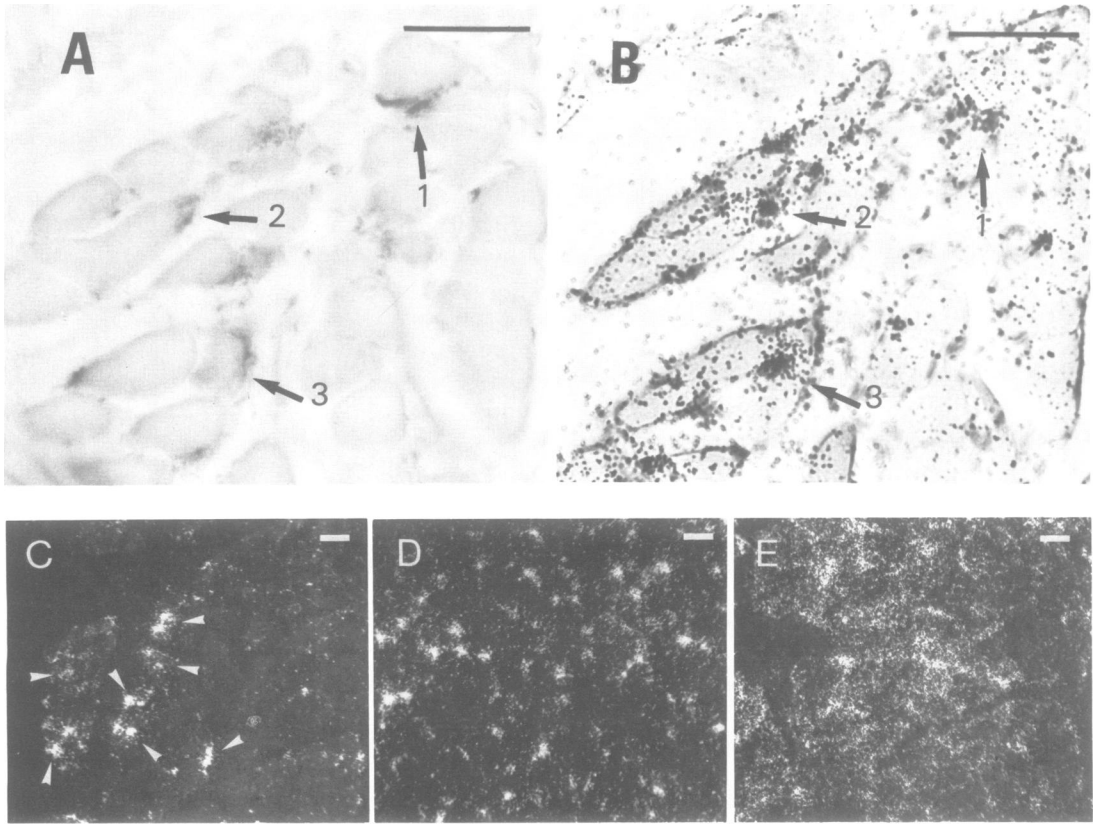

Fig. 4. Transversal sections of 15-day-old chick ALD. (**A**) Innervated ALD stained for the acetylcholinesterase by the Koelle reaction. (**B**) Same section as in (A) hybridized with an AChR α-subunit probe. Arrows 1–3 indicate three corresponding regions between the acetylcholinesterase and the *in situ* stainings. Arrow 3 particularly shows that the *in situ* positive region is under the AChR acetylcholinesterase-rich postsynaptic membrane. (**C**) and (**D**), respectively innervated ALD, denervated ALD hybridized with the AChR α-subunit probe. In (C) arrows indicate *in situ* positive regions. (A)–(D) are 8-day exposures. (**E**) Innervated ALD hybridized with the actin probe (24 h exposure). Bar = 20 μm.

mRNAs preferentially accumulate in synaptic regions of the mouse diaphragm. Such an uneven distribution can either be due to transport of AChR transcripts to subsynaptic regions and/or stabilization of messenger at the subsynaptic region. An alternative possibility is that the subsynaptic nuclei are the major sites of AChR mRNA synthesis. In this paper, we report, using *in situ* hybridization, that, in innervated 15-day-old chick muscle, only subsynaptic regions of the muscle fibres contain a high level of AChR α-subunit mRNA. Moreover, in transverse sections of ALD, we observe a high density of AChR α-subunit mRNA at the level of the nuclei localized under the postsynaptic membrane. The present data are not consistent with the hypothesis of a transport and collection of AChR mRNAs from wide areas of the muscle fibres, since, in such a case, a gradient of messengers toward the endplate would have been expected. The localization of the AChR α-subunit mRNA is markedly discrete and autoradiographic grains are distributed on and around the subsynaptic nuclei only. Moreover, no signal is observed between these clusters of grains. Therefore, the most plausible interpretation of our data is that, in the innervated muscle fibre, the AChR mRNAs detected by *in situ* hybridization are synthesized by the subsynaptic myonuclei, although we cannot exclude that AChR mRNA stability is not also a factor.

Denervation of ALD and PLD has been reported to increase acetylcholine sensitivity (Benett *et al.*, 1973) as in other vertebrate skeletal muscles [review in Salpeter and Lor-

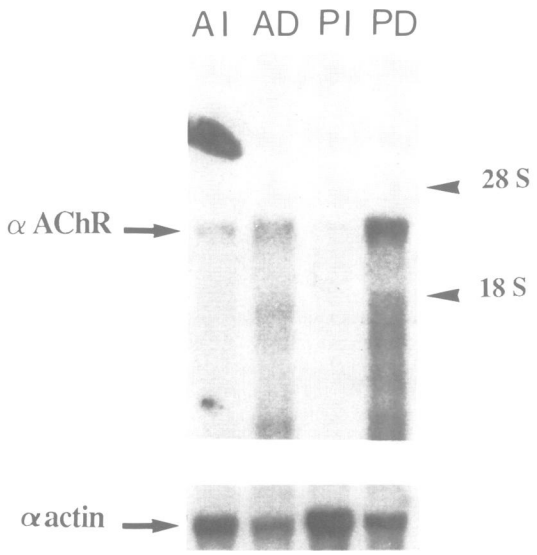

Fig. 5. Northern blot analysis of 15-day-old chick innervated and denervated ALD and PLD. After 4 days of denervation, total RNA was extracted from samples of 10 muscles and Northern blot analysis was performed as described in Materials and methods. Size markers were chicken rRNAs. Each track contained 10 μg of total RNA (AI = innervated ALD, AD = denervated ALD, PI = innervated PLD, PD = denervated PLD). Note that the same blot was used successfully with the AChR α-subunit and the actin probes.

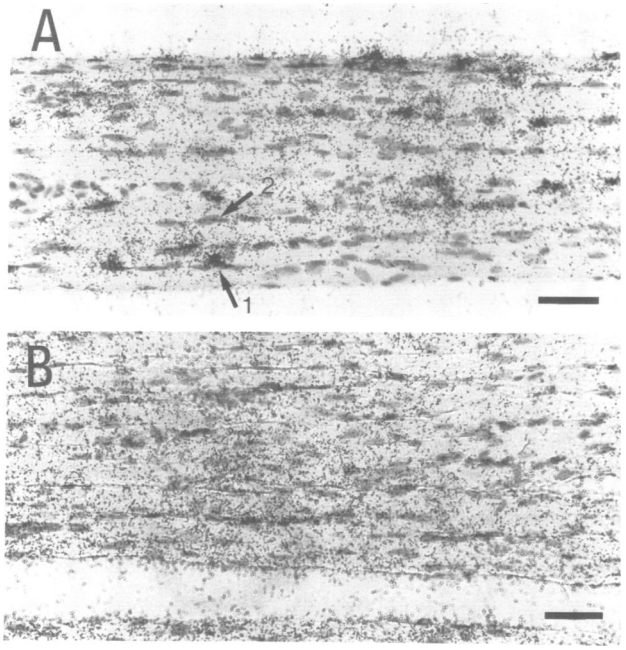

Fig. 6. Light-field pictures of longitudinal sections of PLD. (**A**) Four days denervated PLD hybridization with an AChR α-subunit probe. Arrow 1 shows a spot of autoradiographic grains over a nucleus and arrow 2 a non-labelled nucleus (8-day exposure). (**B**) Hybridization of innervated PLD with the actin probe (24-h exposure). Bar = 15 μm.

ing (1985)]. Moreover, after chronic injection *in ovo* of the curare-like agent flaxedil, which blocks the neurally evoked muscle activity, the total content of AChR increases by 1.15-fold in ALD and by 2-fold in PLD by the 18th day of incubation (a stage at which neuromuscular junctions are clearly individualized) (Betz *et al.*, 1980). These results correlate well with our observation that the AChR α-subunit mRNA content of PLD and ALD increases 4 days after denervation and that this relative increase is larger in the PLD than in the ALD. Moreover, the steady-state level of the AChR α-subunit mRNA is lower in the normally innervated PLD than in the ALD, a finding consistent with the different modes of innervation of the PLD and the ALD since more subsynaptic nuclei are expected to be 'activated' in the multiply innervated ALD. In contrast, after denervation, the proportion of myonuclei 'activated' in the singly innervated PLD might be more important than in the ALD. In agreement with this interpretation, we observe, using *in situ* hybridization, that the increase of AChR α-subunit mRNA-rich regions is more marked in the PLD than in the ALD. In the innervated state, only one cluster of grains is visible in a longitudinal section of the PLD. After denervation, numerous clusters of grains appear all along the PLD myofibre, while in the multiply innervated ALD, clusters already present reduce the magnitude of the relative effect caused by denervation.

After 4 days of denervation, only 10% of nuclei appear labelled by the AChR α-subunit RNA probe in a random fashion. This result correlates well with the observation that, early after denervation, some of the non-endplate myonuclei become enlarged and resemble the end plate 'fundamental' nuclei (Tower, 1939; Levitt-Gilmour and Salpeter, 1986). However, at this point, we cannot exclude a contribution of connective tissue nuclei to the unlabelled nuclei. Further studies are needed to quantify the number of myonuclei labelled after denervation.

We report here that in chicken denervated muscle, the AChR α-subunit mRNAs are localized on or around nuclei. In contrast, the distribution of the grains with the actin probe is uniform all along both denervated or innervated myofibre. The simplest explanation for these observations is that the AChR is a transmembrane protein whose mRNAs are, as shown with the BC_3 H1 muscle cell line, translated on ribosomes bound to the endoplasmic reticulum membrane and then transported to the cell surface via the Golgi apparatus (Merlie and Smith, 1986). In contrast, the actin mRNA is probably translated on free ribosomes and dispersed throughout the sarcoplasm of the muscle fibre. The presence of an intronic sequence in the AChR probe we used is probably not responsible for the nuclear association on hybridization, since with other AChR intronic probes alone, we did not detect a significant signal under these conditions.

An important question concerns the causes of the local accumulation of AChR α-subunit mRNA around the nuclei. Such high local levels might result from an enhanced transcription of the AChR α-subunit gene or from a protection of the transcribed mRNA from degradation. The available data about the effect of denervation in vertebrate muscle show that an enhanced rate of transcription (Shieh *et al.*, 1987; A.Klarsfeld, personal communication) accounts, at least partly, for the observed steady-state increase of mRNA content (Merlie *et al.*, 1984; Goldman *et al.*, 1985; Klarsfeld and Changeux, 1985; Evans *et al.*, 1987; Shieh *et al.*, 1987). The contribution of an enhanced transcription rate to the labelling of the nuclei observed by *in situ* hybridization can, in theory, be tested by using strictly intronic probes, as successfully demonstrated in the case of the pro-opio melanocortin gene (Fremeau *et al.*, 1986). This method should also allow a comparison of the mechanisms responsible for the 'activity' of the nuclei at the endplate under normal conditions and for that occurring outside the endplate after denervation.

Based on these results, we would suggest that at least in 15-day-old chicks, the potential capacity of individual nuclei within the same cytoplasm for expression of the AChR α-subunit gene (and possibly for other genes coding for synaptic proteins) is different. It has been recently shown that experimentally induced ectopic innervation by fast motor neurons can induce fast myosin which is restricted to the ectopic endplate region in a slow muscle (Salviati *et al.*, 1986). Tentatively, one may think that the ectopic endplate myonuclei and the other myonuclei are in distinct 'activated' states.

To account for such a locally differential 'activation' of subsynaptic and extrasynaptic nuclei, Changeux *et al.* (1987) have previously proposed that: (i) different 'first messengers' released by the nerve ending [review in Salpeter and Loring (1985); see also Fontaine *et al.* (1986)] might transcriptionally or post-transcriptionally increase AChR biosynthesis in junctional nuclei while muscle electrical activity would repress AChR biosynthesis in the extrajunctional nuclei [see also Fambrough (1979)], (ii) distinct 'second messenger' systems might be involved in such a differential regulation: for instance, the cyclic AMP pathway would have a positive [see also Betz and Changeux (1979), Blosser and Appel

(1980) and Laufer and Changeux (1987)] and the Ca^{2+}/diacylglycerol system a negative effect on AChR biosynthesis [see also MacManaman *et al.* (1982), Vergara (1985) and Fontaine *et al.* (1987)].

An important issue thus becomes the identification of regulatory mechanisms involved in the expression of the different AChR subunits during embryonic development, and the maintenance of this expression beyond the 15th day post-hatching in the adult and aged chick.

Materials and methods

Tissue preparation

ALD and PLD muscles of 11-day-old chicks were unilaterally denervated under anaesthesia by surgical excision of ~5 mm of nervis latissimus dorsi. The controlateral muscles were used as controls for innervated ALD and PLD muscles. Four days following denervation, animals were killed by decapitation and fixed after evisceration in 2% PBS-buffered (pH 7.4) paraformaldehyde overnight at 4°C. The PLD and ALD muscles were then dissected out and processed for acetylcholinesterase staining on whole tissue (Koelle and Friedenwald, 1949). They were then fixed overnight at 4°C in 4% PBS-buffered (pH 7.4) paraformaldehyde, and paraffin (Paraplast) embedded. Sections of 7.5 μm were collected on 0.125% gelatin-coated 'subbed' slides (Gall and Pardue, 1971) and stored at 4°C until hybridization.

Northern blot analysis

Normal and 4-day denervated chick ALD and PLD muscles were dissected free of surrounding tissue on ice and immediately frozen in liquid N_2. Total RNA from 10 muscles was then prepared according to the procedure of Auffray and Rougeon (1980). RNA samples (10 μg) were subjected to electrophoresis in a 1.2% formaldehyde agarose gel and transferred onto a nylon membrane (Hybond N, Amersham Corp.) by standard procedures (Maniatis *et al.*, 1982). Hybridization and final washes of the blots were performed as recommended by the suppliers (Amersham Corp.). The AChR α-subunit single-stranded probe (Biggin *et al.*, 1984) was synthesized from a DNA fragment of 500 nucleotides containing 120 nucleotides of exon 2 of the chicken AChR α-subunit gene (Klarsfeld and Changeux, 1985) and labelled by [α-^{32}P]dCTP (800 Ci/mmol, Amersham Corp.). The actin probe was a 1150-bp mouse cDNA insert in plasmid pAL41 corresponding to most of the coding sequence of cytoskeletal β-actin mRNA (Alonso *et al.*, 1986). This cDNA cross-hybridizes with muscle and non-muscle actin mRNAs derived from chick (Alonso *et al.*, 1986). From Northern blot analysis of chick muscle, only α-actin mRNA is detectable as a single band of ~1.7 kb (Schwartz and Rothblum, 1981; Klarsfeld and Changeux, 1985). The actin probe was labelled according to the Amersham multiprime procedure by [α-^{32}P]dCTP (800 Ci/mmol, Amersham Corp.).

Preparation of SP6, T7-labeled RNA probes

A 2.3-kb fragment of the chicken AChR α-subunit gene (Klarsfeld and Changeux, 1985) was subcloned into the polylinker region of pGEM3 vector (Promega Biotec) by standard methods (Maniatis *et al.*, 1982). The same procedure was followed with a 1150-bp mouse cDNA insert in bluescribe (Promega Biotec) derived from the pAL41 plasmid (courtesy of R.D.Cox).

High specific activity RNA (~1×10^8 c.p.m./μg) was prepared from the sense and the antisense strands of the fragments using 100 μCi [^{32}P]-UTP (800 Ci/mmol, Amersham Corp.) for blot analysis or 100 μCi [α-^{35}S]UTP (>1000 Ci/mmol, Amersham Corp.) for *in situ* analysis. After a limited alkaline hydrolysis of the probes for *in situ* hybridization generating a size range of ~50–200 bp as determined by gel electrophoresis, unincorporated nucleotides were removed by G 50 column filtration. After ethanol precipitation, the RNA pellet was dissolved in the hybridization buffer [50% deionized formamide, 0.3 M NaCl, 20 mM Tris–HCl (pH 7.4), 5 mM EDTA, 10 mM Na H_2PO_4 (pH 8), 10% dextran sulfate, 1 × Denhardt's solution, 0.5 mg/ml total yeast RNA and 10 mM dithiothreitol]. The concentration of the probe was adjusted to 50 000 c.p.m./μl. In this solution, probe was stored for a maximum of 3 weeks at −80°C.

Hybridization of the sections

Section pretreatment and hybridization was performed according to Wilkinson *et al.* (1987) with several modifications (Sassoon *et al.*, 1988; this paper). Sections were deparaffinized in xylene, rehydrated, mounted in glycerol and Koelle-positive regions were photographed. Slides were then washed in 1 × PBS (pH 7.4), treated with proteinase K (20 μg/ml, Boehringer-Mannheim) for 7.5 min at room temperature, washed in 1 × PBS (pH 7.4), acetylated, dehydrated and air-dried. A low background level and a high specific signal was obtained with an incubation-time of 16 h in a humid chamber at 50°C and a probe concentration of 50 000 c.p.m./μl. Immediately prior to hybridization, probes were denatured at 80°C for 2 min and ~10 μl of the hybridization solution was applied to each section, and covered 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 M Na_3 citrate) – 10 mM dithiothreitol at 42°C for 30 min. Slides were then washed in 50% deionized formamide, 2 × SSC (0.3 M NaCl, 0.3 M Na_3 citrate), 0.1 M dithiothreitol for 20 min at 60°C, in 0.4 M NaCl, 10 mM Tris–HCl (pH 7.4), 0.05 M EDTA twice for 10 min at 37°C. The sections were then treated with RNase A (20 μg/ml, Boehringer-Mannheim) for 30 min at 37°C in 0.4 M NaCl, 10 mM Tris–HCl (pH 7.4), 0.05 M EDTA, 15 min at 37°C in 2 × SSC (0.3 M NaCl, 0.3 M Na_3 citrate) and 15 min at 37°C in 0.1 SSC (0.015 M NaCl, 0.015 M Na_3 citrate). Slides were subsequently dehydrated and air-dried.

For autoradiography, the slides were dipped once in Kodak NTB2 emulsion (45°C), air-dried and conserved in a dry, light-tight box with desiccant at 4°C. Slides were exposed for 24 h for the actin probe and 8–9 days for the AChR probe and developed at 16°C (Kodak D19) for 3.5 min. The slides were rinsed in a water bath, fixed for 4 min in Kodak rapid fix AL4, subsequently rinsed, stained with haematoxylin and mounted for standard light microscopy (Zeiss Axiophot).

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