# Denervation Supersensitivity in Skeletal Muscle: Analysis with a Cloned cDNA Probe

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ABSTRACT Motor neurons regulate the acetylcholine sensitivity of the muscles they innervate: denervated muscle fiber become "supersensitive" to acetylcholine, due to insertion of newly synthesized acetylcholine receptors (AChRs) in the plasma membrane. We used hybridization analysis with a cloned cDNA specific for AChR $\alpha$-subunit to compare the abundance of AChR mRNA in innervated and denervated adult mouse muscles. Within 3 d of denervation, levels of AChR mRNA increased 100-fold; levels of actin mRNA changed little. The increase in AChR mRNA level was sufficiently large and rapid to account for denervation supersensitivity.

In adult skeletal muscles, acetylcholine receptors (AChRs)[1] are highly concentrated in the postsynaptic membrane, but virtually absent from the rest of the muscle's plasma membrane. After denervation, however, AChRs appear over the entire muscle fiber surface (1, 2). This phenomenon, called denervation supersensitivity, has been studied extensively, with the aim of learning how nerves cause long-term changes in their targets (reviewed in reference 3). Earlier studies have shown that denervation supersensitivity is due to an increased number of functional receptors rather than to redistribution of synaptic receptors (4–6), and that the receptors that appear after denervation are newly synthesized rather than newly activated (7–10). However, the currently available data do not distinguish among a number of alternative mechanisms by which denervation might induce accumulation of AChRs. Functional AChR is a pentamer of structure $\alpha_2\beta\gamma\delta$, in which the $\alpha$-subunits bear the acetylcholine binding sites (15). AChRs are inserted in the muscle plasma membrane as the final step in a long synthetic pathway that includes transcription of AChR genes, processing of nuclear RNA, transport of mRNA to the cytoplasm, translation of mRNA, co-translational modifications within the rough endoplasmic reticulum, posttranslational modification within the Golgi apparatus, and assembly of AChR subunits (reviewed in references 12 and 13). Any of these processes could be affected by denervation. Thus, while experiments with inhibitors suggest that denervation supersensitivity requires RNA synthesis (3), AChR synthesis is regulated posttranslationally in a mouse muscle cell line (13–15) and in primary cultures of embryonic rat muscle (B. Carlin, J. Lawrence, and J. Merlie, unpublished observations).

To continue the molecular analysis of denervation supersensitivity, it is important to determine the site(s) at which AChR synthesis is regulated. Recently, cDNAs encoding all four subunits of the AChR from *Torpedo* have been cloned (16–20), and we have prepared a cDNA clone that hybridizes specifically to AChR $\alpha$-subunit mRNA from a mouse muscle cell line (21). The availability of this cDNA permits more direct analysis of the effects of nerves on gene expression in their targets than has hitherto been possible. We show here that denervation supersensitivity is preceded by, and presumably largely due to, an approximately 100-fold increase in the level of mRNA encoding AChR.

## MATERIALS AND METHODS

Female Swiss mice were anaesthetized with ether, and the sciatic nerve cut bilaterally in mid-thigh to denervate the hind limbs. 1–15 d later, mice were killed, and the plantar extensor group of lower hind limb muscles was dissected and weighed.

*RNA Preparation:* RNA was extracted from groups of six to eight limbs by homogenization in 7.5 M guanidine hydrochloride, 0.025 M sodium citrate, pH 7.0, using a Polytron homogenizer at a setting of 6 for 1 min (22, 23). The homogenate was acidified with 0.025 vol 1 M acetic acid and precipitated by addition of 0.75 vol ethanol at −20°C. After 12 h, the precipitate was collected by centrifugation and re-extracted twice as described above, except that 0.5 vol ethanol was used for precipitation. The final precipitate was dissolved in 3.75 M guanidine hydrochloride and extracted with phenol: chloroform (1:1). The upper aqueous phase was precipitated, washed twice with ethanol, and dissolved in a small volume of $H_2O$. The yield of RNA, determined spectrophotometrically, was ~250 $\mu$g RNA/g muscle, for all samples. Poly A-containing RNA was enriched by chromatography of total RNA on columns of oligo dT as described previously (24), except that the columns were washed sequentially with 5 vol of 0.5 M NaCl, 10 mM Tris-Cl, pH 7.4, 1% SDS and 5 vol of 0.1 M NaCl, 10 mM Tris-Cl, pH 7.4, 1% SDS before elution with 3 vol 1 mM Tris, 1% SDS. The yield of poly A+ species was ~2% of the total RNA.

*RNA Hybridization:* RNA samples were denatured with formaldehyde and formamide and fractionated by electrophoresis in 1.5% agarose gels containing 2.2 M formaldehyde and 20 mM morpholine propane sulfonic acid

[1] *Abbreviations used in this paper:* AChR, acetylcholine receptor; SSC, standard saline-citrate buffer.



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buffer (25). RNA was transferred to Gene Screen (New England Nuclear, Boston, MA) according to the manufacturer's instructions. Hybridization was to the nick-translated (26) probe ($2 \times 10^8$ dpm/$\mu$g) in 50% formamide, 5 × standard saline-citrate buffer (SSC) (0.15 M NaCl, 0.015 M Na-citrate), 1 × Denhardt's solution (27), 1% SDS, 100 $\mu$g/ml salmon sperm DNA at 42°C for 72 h. Membranes were washed twice each in SSC at 22°C, SSC at 60°C, and 0.1 × SSC at 22°C, then exposed to pre-fogged x-ray film with an intensifying screen. The probes used for hybridization were either a 700 base pairs (bp) Pst I insert, purified by electrophoresis from the AChR $\alpha$-subunit-specific clone, pA59 (see reference 21), used at 4 ng/ml, or total plasmid DNA of the skeletal muscle actin specific subclone pAM 91-1, generously provided by M. Buckingham (28), and used at 40 ng/ml.

*AChR Assay:* The AChR content of innervated and denervated limb muscles was measured as described by Brockes and Hall (29). Muscles were homogenized in 50 mM NaCl, 50 mM Tris, 1 mM EDTA, 1 mM EGTA, pH 7.2, and centrifuged at 20,000 *g* for 1 h. The pellet was resuspended in 50 mM NaCl, 50 mM Tris, 1% Triton X-100, incubated for 1 h at 4°C, and recentrifuged. Aliquots of the supernatant were incubated with 4 nM $^{125}$I-$\alpha$-bungarotoxin for 2 h at 37°C. Bungarotoxin-AChR complexes were collected and separated from free toxin by filtration through Whatman DE-80 filters; filter-associated radioactivity was determined in a gamma counter. Values presented have been corrected for nonspecific binding, determined in the presence of 500 nM nonradioactive toxin, and represent moles of $^{125}$I-bungarotoxin-AChR complex per limb.

## RESULTS AND DISCUSSION

Our analysis of AChR mRNA made use of a cDNA probe, called A59, which was prepared by reverse transcription of mRNA from a mouse muscle cell line and cloned by conventional methods. We showed previously that this 700 bp cDNA hybridizes specifically to an mRNA that encodes the skeletal muscle AChR $\alpha$-subunit (21). Determination of the A59 sequence has allowed us to verify its identity by comparison with published sequences of other AChR subunits. The amino acid sequence predicted from A59 is very similar to the sequences predicted for $\alpha$-subunits of human and *Torpedo* AChR; homology is significantly lower with *Torpedo* $\beta$-, $\gamma$-, and $\delta$-subunits (16, 17) (Fig. 1). Thus, A59 is a specific probe for mRNA encoding the ACh-binding $\alpha$-subunit of the mouse AChR.

To compare AChR mRNA levels of innervated and denervated mouse muscles, we isolated mRNA-rich (poly A+) fractions, fractionated them by gel electrophoresis, and transferred them to nitrocellulose membrane. The membrane was then incubated with $^{32}$P-labeled DNA under hybridization conditions, washed, and submitted to autoradiography. A59-DNA bound to a ~2,000 base RNA species in denervated muscle (Fig. 2*a*, lane *4*), which was identified as AChR $\alpha$-subunit mRNA because it co-electrophoresed with previously characterized $\alpha$-subunit mRNA from cultured muscle cells (Fig. 2*a*, lane *1*), was absent from AChR-poor tissues such as liver (Fig. 2*a*, lane *2*), and was not detected by other $^{32}$P-DNA probes (e.g., see Fig. 2*b*). AChR $\alpha$-subunit mRNA was also present in innervated muscle, but in far lower abundance. Thus, while $\alpha$-subunit mRNA from denervated muscle was easily detectable on autoradiographs after 4-h exposure, the corresponding species from normal muscle was only barely detectable after 40-h exposure (Fig. 2*a*, lanes *3* and *3'*). Densitometry of appropriately exposed autoradiographs showed that the concentration of AChR $\alpha$-subunit mRNA was approximately 100-fold higher in denervated than in innervated muscle.

Further studies compared the denervation-induced increases in AChR $\alpha$-subunit mRNA and AChRs. $\alpha$-Subunit mRNA levels increased significantly within 1–2 d after denervation, and reached a constant value 2–3 d later (Figs. 2*b* and 3*a*). Levels of AChR, determined by $^{125}$I-$\alpha$-bungarotoxin binding, began to increase 2–3 d after denervation, and reached a constant value 3–4 d later (Fig. 3*b*). Thus, the rise in AChR mRNA precedes the rise in AChR itself. The AChR content of denervated limbs rises to four- to fivefold that of controls; others have reported similar values (4–6, 30–32). Since extrasynaptic AChR is degraded with a 10–20-fold higher rate constant than synaptic AChR (10, 31, 32), the rate of AChR production must increase 40–100-fold (4–5 × 10–20) after denervation. This is consistent with our determination of a 100-fold increase in mRNA levels. Thus, the denervation-induced increase in AChR mRNA is sufficiently early and large to account for denervation supersensitivity.

Several observations indicate that the increase in AChR $\alpha$-subunit mRNA level we observed is a specific consequence of denervation. First, the level of adult skeletal muscle actin mRNA, determined with a specific cDNA probe (28), changed little after denervation (Fig. 2*b*). Second, AChR mRNA levels increased greatly during the first several days of denervation, before denervation atrophy was marked, but changed little during the second week, when atrophy was rapid (compare Fig. 3, *a* and *c*). Finally, the amount and yield of muscle RNA changed little after denervation as evidenced by similar recoveries from innervated and denervated muscles of total RNA and of a $^3$H-RNA standard added at the first step of tissue extraction (see also reference 33). Together, these results argue that neither selective recovery of RNA from denervated muscle, nor late consequences of denervation atrophy accounted for our detection of high levels of AChR mRNA in denervated muscle.

While we have shown that AChR $\alpha$-subunit mRNA levels increase after denervation, our results do not rule out the possibility that the rate of AChR assembly also changes after

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 A59              GTG TGT ... CTC ATC GGG ACG CTG GCT GTG TTT ... GCA GGT CGG CTC ATT GAG TTA CAT CAA ... ... ... CAA GGA TGA
Mouse    α        Val Cys --- Leu Ile Gly Thr Leu Ala Val Phe --- Ala Gly Arg Leu Ile Glu Leu His Gln --- --- --- Gln Gly ***
                                        520                                     530
Human    α        Val Cys --- Ile Ile Gly Thr Leu Ala Val Phe --- Ala Gly Arg Leu Ile Glu Leu Asn Gln --- --- --- Gln Gly ***
Torpedo  α        Ile Cys --- Ile Ile Gly Thr Val Ser Val Phe --- Ala Gly Arg Leu Ile Glu Leu Ser Gln --- --- --- Glu Gly ***
         β        Ile Cys --- Ser Ile Gly Thr Phe Ser Ile Phe Leu Asp Ala Ser His Asp Val Pro Pro Asp Asn Pro Phe --- Ala ***
         γ        Leu Leu Phe Ser Ile Gly Thr Leu Ala Ile Phe Leu Thr Gly His Phe Asp Gln Val Pro Glu Phe Pro Phe Pro Gly Asp
         δ        Val Met --- Val Leu Gly Thr Ile Phe Ile Phe Val Met Gly Asn Phe Asn His Pro Pro Ala Lys Pro Phe Glu Gly Asp
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FIGURE 1 Partial sequence of the cloned cDNA, A59, showing its homology with human and *Torpedo* AChR subunits. The nucleotide sequence of A59 was determined by the method of Maxam and Gilbert (36) (K. Isenberg, S. Russell, and J. P. Merlie, unpublished observations). The first line shows a portion of this sequence derived from a HinfI fragment that spanned the C terminus of the amino acid coding region. A single open reading frame allowed prediction of the amino acid sequence, which is shown in the second line. Subsequent lines show predicted sequences of human (17) and *Torpedo* (16) AChR subunits, numbered as in reference 16. Gaps are inserted in the amino acid sequences to maximize homology among subunits (16), and translation stop codons are indicated by ***. In the region shown here, homology of the A59 sequence is 90% with the human $\alpha$-subunit, 75% with the *Torpedo* $\alpha$-subunit, and 25–40% with *Torpedo* $\beta$-, $\gamma$-, and $\delta$-subunits. These homology relationships are maintained throughout the amino acid coding region of A59.

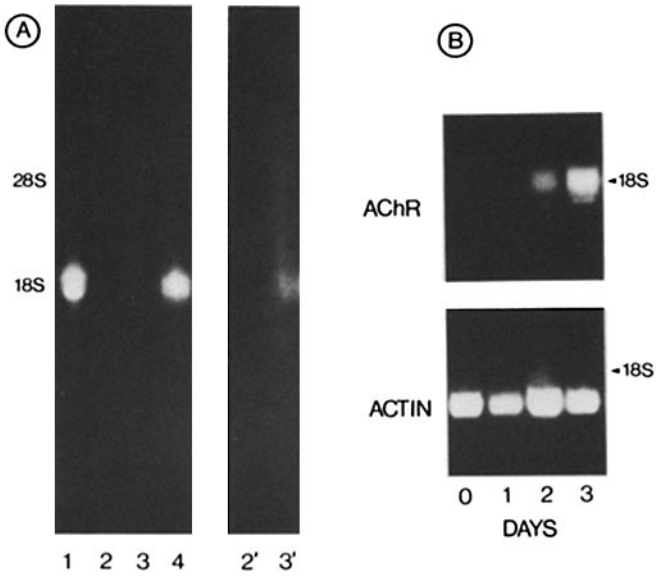


FIGURE 2 AChR α-subunit mRNA detected in mouse skeletal muscle by hybridization of a cDNA probe. (*A*) RNA was fractionated by gel electrophoresis, transferred to nitrocellulose, incubated with $^{32}$P-A59-DNA specific for AChR α-subunit, and exposed to x-ray film. Lane *1*: 10 μg of total RNA from a mouse muscle cell line, BC3H1, which accumulated high levels of AChR (12–14). Lane *2*: 10 μg of total RNA from liver. Lane *3*: 3 μg of poly A+ RNA from innervated skeletal muscle. Lane *4*: 3 μg of poly A+ RNA from denervated skeletal muscle. Lanes *2'* and *3'* show autoradiographs exposed 10-fold longer than lanes *2* and *3* (40 h vs. 4 h). Total liver RNA was used in lane *2* to show that A59 does not hybridize nonspecifically to 18S ribosomal RNA, which migrates near α-subunit mRNA in these gels; negative results were also obtained when poly A+ liver RNA was used. Positions of 18S and 28S ribosomal RNA size markers are indicated. (*B*) Total RNA from innervated muscles, or from muscles denervated for 1, 2, or 3 d, was fractionated and transferred as above, then incubated with $^{32}$P-DNA specific for AChR α-subunit (*top* panel) or for skeletal muscle actin (*bottom* panel). The ~2 kbp region of autoradiographs exposed for 4 d is shown. 10 μg of RNA was applied to each lane.

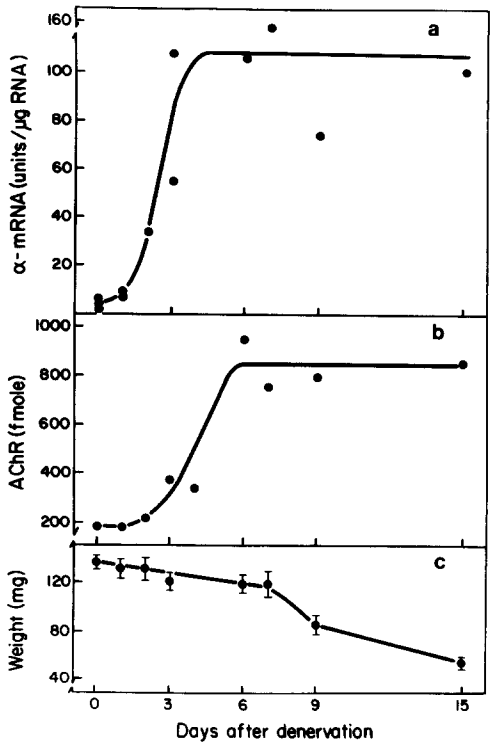


FIGURE 3 Time course of the increase in AChR α-subunit mRNA and AChR after denervation. (*a*) AChR α-subunit mRNA levels, obtained by densitometric scanning of films such as those shown in Fig. 2 b and expressed as arbitrary units of film density per microgram of RNA. Each point represents one pool of six to eight limbs. (*b*) AChR content per limb, as measured by a $^{125}$I-α-bungarotoxin binding assay. Each point shows the mean of triplicate assays performed on two to four separate limbs. (*c*) Wet weight of the plantar extensor muscles from which data in *a* and *b* were obtained. Each point shows mean ± SE of 8–20 limbs.

denervation (see references 13–15). Furthermore, subtle molecular differences exist between the AChRs of normal and denervated muscles (3, 34), and these could arise pre- or posttranslationally. If different transcripts encode the α-subunits of synaptic and extrasynaptic AChRs, our probe (A59) might hybridize differently to them. However, our findings clearly indicate that denervation supersensitivity is mediated in large part by a specific alteration in transcription or post-transcriptional processing of AChR RNA. Because neural control of muscle AChR levels is mediated by electrical and/or contractile activity (3, 9, 10, 35), we conclude that neurons can use conventional processes of synaptic transmission to regulate gene expression in their synaptic targets.

We thank M. Buckingham and A. Minty for providing pAM91-1, and R. Winkle and S. Eads for assistance.

This work was supported by grants from the National Institutes of Health and Muscular Dystrophy Association. J. Sanes is an established Investigator of the American Heart Association.

*Received for publication 15 March 1984, and in revised form 6 April 1984.*

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