

# Quantitation of an Alpha Subunit Splicing Intermediate: Evidence for Transcriptional Activation in the Control of Acetylcholine Receptor Expression in Denervated Chick Skeletal Muscle

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**Abstract.** We have investigated the mechanisms responsible for the increase in acetylcholine receptor subunit mRNAs during the induction of denervation supersensitivity in skeletal muscle. Using a cRNA probe specific for exon 7 (224 nucleotides; with flanking intron sequences of 105 nucleotides on the 3' end, and of 70 nucleotides on the 5' end) of the alpha subunit of the chicken muscle acetylcholine receptor gene, we were able to quantitate the concentration of mature alpha subunit mRNA and its precursor. In 3-wk-old chicks, the concentration of alpha subunit message in leg muscle was found to be 4.0 attomoles per  $\mu\text{g}$  total

RNA, and to increase 40-fold within 1 wk after section of the sciatic nerve. The molar ratio of precursor/mature mRNA, which was  $\sim 0.023$  in innervated as well as denervated muscle, transiently rose to 0.047 at the beginning of the second postoperative day when mature message content increased 20-fold; the rise in precursor level preceded the increase in mature message content. These findings suggest that an accelerated rate of transcription of the message coding for the alpha subunit causes increased message content and the stimulation of receptor synthesis characteristic of denervated muscle.

UPON denervation of skeletal muscle a number of physiological and biochemical changes occur; among these an increased sensitivity to the neuromuscular transmitter acetylcholine is particularly conspicuous. The mechanism by which this "denervation supersensitivity" arises has been under intensive investigation for some time. It is now known to be the result of increased acetylcholine receptor (AChR)<sup>1</sup> synthesis (for review see Fambrough, 1979; Pumplin and Fambrough, 1982; Salpeter and Loring, 1985). In addition, it has become apparent that the increase in receptor expression is preceded by a rise in the level of mRNAs coding for receptor subunits (Merlie et al., 1984; Klarsfeld and Changeux, 1985; Goldman et al., 1985) suggesting that the denervation signal activates the genes that code for the four receptor subunits. A change in mRNA level could also arise posttranscriptionally, from a change in mRNA half-life. To distinguish between these two possibilities, mRNA transcription and/or turnover rates must be measured; neither of these measurements, however, are readily conducted in vivo. Skeletal muscle in particular defies the subcellular fractionation techniques required for such metabolic analysis. Tetrodotoxin treatment of cultured myotubes is an in vitro model of denervation, but differs from it in the rather modest effect on message levels (Shieh, B. H., M. Bal-

livet, and J. Schmidt, manuscript submitted for publication) and in that direct neural ("trophic") influences elude analysis. Cell culture is better suited for developmental analysis; transcriptional activation has recently been shown to account for much of the increase in AChR alpha and delta subunit mRNA during differentiation of the mouse muscle cell line C2 (Buonanno and Merlie, 1986).

Here we report the quantitative analysis of alpha subunit mRNA and a putative precursor of it in vivo. The results strongly support the notion that in adult skeletal muscle the denervation signal prompts enhanced transcription of receptor messages.

## Materials and Methods

### Denervation

Chickens were operated by crush or section of the left sciatic nerve as described (Shieh, B. H., M. Ballivet, and J. Schmidt, manuscript submitted for publication). At the desired time after operation, animals were sacrificed by ether overdose or CO<sub>2</sub> asphyxiation, and the calf muscles of the denervated and control legs removed and either processed immediately or frozen in liquid nitrogen for storage at  $-70^{\circ}\text{C}$  before use.

### Preparation of <sup>125</sup>I-Alpha-Bungarotoxin

Alpha-bungarotoxin was isolated from *Bungarus multicinctus* venom (Miami Serpentarium, Miami, FL) and radioiodinated as described previously

1. Abbreviation used in this paper: AChR, acetylcholine receptor.

(Wang and Schmidt, 1980). Specific activity ranged from 3.5 to  $1.0 \times 10^7$  GBq/mol, depending on the age of the preparation.

### Receptor Quantitation

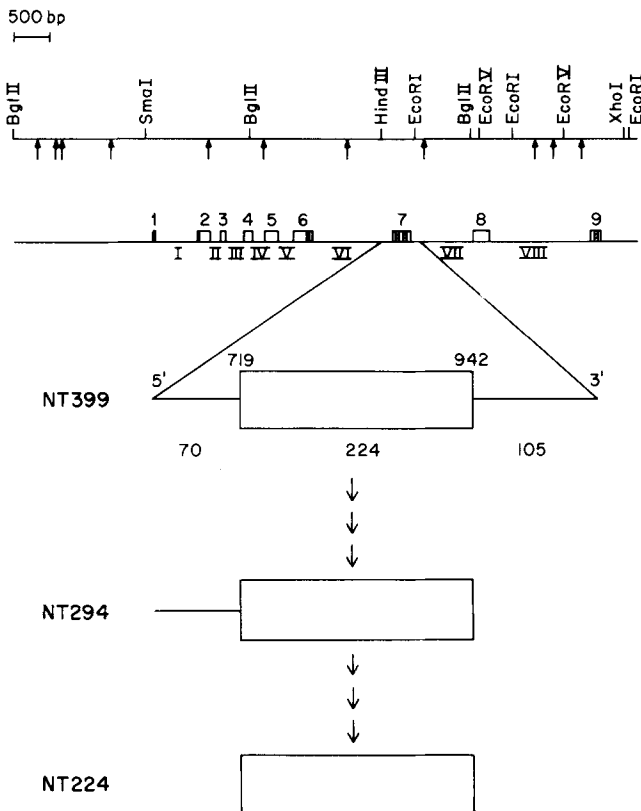
Muscle samples were assayed for AChR content by measuring  $^{125}\text{I}$ -alpha-bungarotoxin-binding activity in detergent extracts. Briefly, tissue was homogenized in 10 mM Na phosphate (pH 7.4) containing protease inhibitors (5 mM iodoacetamide, 1  $\mu\text{g}/\text{ml}$  aprotinin, 10 mM phenylmethylsulfonyl fluoride), and a crude membrane fraction was prepared and extracted with 1% Triton X-100. The detergent extract was then incubated with  $^{125}\text{I}$ -alpha-bungarotoxin and receptor-bound ligand determined by the DEAE-cellulose disk technique (Schmidt and Raftery, 1973). No attempt was made to separate the tissue into individual muscles of specific fiber type.

### Isolation and Quantitation of RNA

Total RNA was extracted by the guanidinium isothiocyanate/hot phenol method (Feramisco et al., 1982) and quantitated spectrophotometrically. One  $A_{260}$  unit equals 40  $\mu\text{g}$  RNA.

### Solution Hybridization

A portion of the chick muscle AChR alpha subunit gene, comprising the seventh exon (224 nucleotides from position 719 up to and including nucleotide 942) and flanking sequences of 70 nucleotides of the sixth intron up-

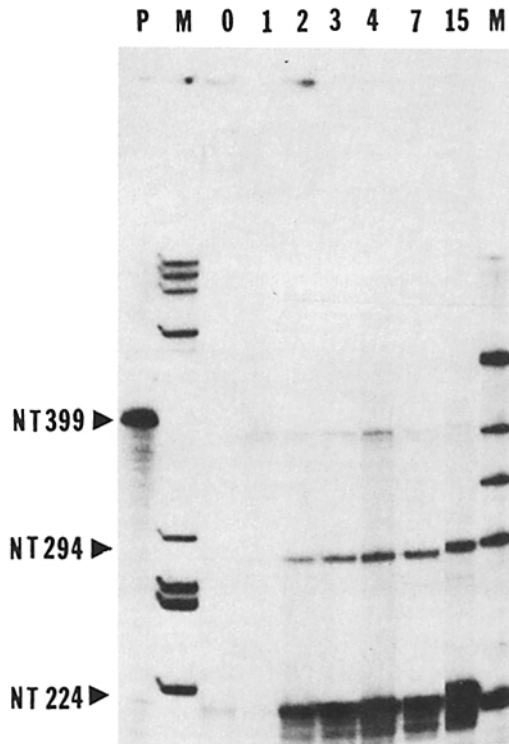


**Figure 1.** Summary of splicing pathway as determined by RNase mapping using cRNA derived from a genomic sequence. The top diagram shows the restriction map of the gene coding for alpha subunit, with arrows indicating Pst I sites. Underneath it the gene structure is displayed with the nine exons indicated as boxes. The signal peptide and the four conserved transmembrane regions are shaded and hatched, respectively. The gene fragment used for construction of the riboprobe and the two splicing derivatives occupy the bottom half. Exons and introns are labeled in sequence of transcription in arabic and Roman numerals, respectively. The numbering of nucleotides is based on the protein-coding portion of the mRNA.

stream and 105 nucleotides of the seventh intron downstream, was cloned into the Eco RI/Hind III site of pT7-1 (United States Biochemical Corp., Cleveland, OH). Single-stranded cRNA was prepared by in vitro transcription using T7 RNA polymerase (United States Biochemical Corp.) on the linearized template in the presence of alpha- $^{32}\text{P}$ -UTP (ICN K&K Laboratories Inc., Irvine, CA) as described (Shieh B. H., M. Ballivet, and J. Schmidt, manuscript submitted for publication). An excess of transcripts, comprising at least 90% of the full-length probe, were annealed with total cellular RNA in the presence of 750 mM NaCl, 75 mM Na citrate (pH 7.0), and 50% formamide for 24 h at 55°C. The hybridization reaction was terminated by addition of 10 vol of RNase buffer (270 mM NaCl, 5 mM EDTA, 10 mM Tris-HCl, pH 7.5) containing RNase I "A" (80  $\mu\text{g}/\text{ml}$ , Pharmacia Fine Chemicals, Piscataway, NJ) and RNase T<sub>1</sub> (4 U/ml; Calbiochem-Behring Corp., San Diego, CA), followed by incubation at 37°C for 30 min. Total RNase-resistant fragments were precipitated with 10% TCA, collected on glass fiber filters, and counted. Probe excess and linearity of the assay were ascertained in each case by using three sample aliquots differing in the amount of total RNA. The specific activity of the probe was calculated from the specific activity of the alpha- $^{32}\text{P}$ -UTP (ICN K&K Laboratories, Inc.) used for probe synthesis (600 Ci/mmol) and the number of uridine moieties in the probe (86). Samples containing  $^{32}\text{P}$  were quantitated by measuring Cerenkov radiation in a liquid scintillation spectrometer, at a counting efficiency of 32%.

### Densitometric Quantitation

At the end of the RNase digestion (see above), proteinase K (50  $\mu\text{g}$ ; Bethesda Research Laboratories, Gaithersburg, MD) was added, and the incubation continued for 15 min. After phenol/chloroform extraction, the RNase-resistant fragments were precipitated with ethanol in the presence of yeast tRNA as a carrier and fractionated on a denaturing 6% urea/poly-



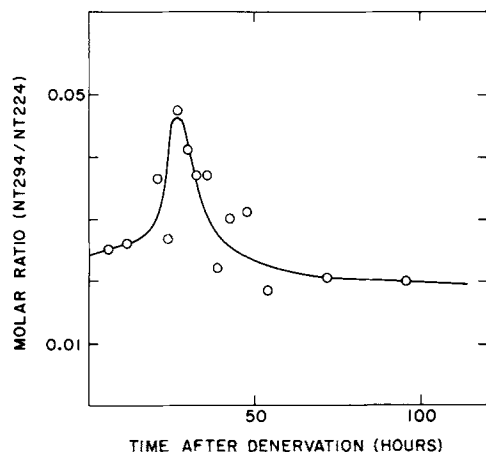
**Figure 2.** Appearance of alpha subunit mRNA precursors after denervation. White Mountain Cross chickens ( $\text{f}$ ,  $\sim 2$  mo old; Nevin's Poultry Farm, Farmingville, NY) were denervated, and total cellular RNA (10–50  $\mu\text{g}$ ) was analyzed as described in Materials and Methods. Lane P shows the intact riboprobe. The numbers above the lanes indicate how many days after the operation the tissue was analyzed. Lanes M contain size markers ( $\text{OXI}74$ , Hae II fragments, end labeled; pBR322, Hinf I fragments, end filled). Note that on the first day, NT399 and NT224 occur at comparable levels.

acrylamide gel. The abundance of different sizes of the protected RNA species was quantitated by densitometric tracing of the resulting autoradiograph. Autoradiographic exposure time was adjusted so as to attain maximal sensitivity without generating artifacts due to the saturation of the film. Molar ratios were determined assuming that the specific radioactivities of the observed fragments are proportional to their uridine content (NT294 and NT224: 64 and 46 uridine moieties, respectively).

## Results and Discussion

When RNA from denervated skeletal muscle is hybridized with the RNA probe complementary to a portion of the genomic sequence coding for alpha subunit of AChR, and protected sequences are analyzed by gel electrophoresis, three bands are discernible: the major one (NT224) representing the completely processed exon 7, and thus including all mature mRNA; and two fainter ones, one which is 400 nucleotides long (NT399), corresponding to the original transcript and all intermediates with unspliced introns VI and VII, and one which is 300 nucleotides long (NT294), corresponding to all intermediates with intron VI intact and intron VII excised (Fig. 1). The presence of NT399 results neither from incomplete digestion, as it disappears in control incubations with nonhomologous RNA, nor from contamination with genomic DNA, since double-stranded DNA is not accessible to the single-stranded probe under the experimental conditions used (data not shown).

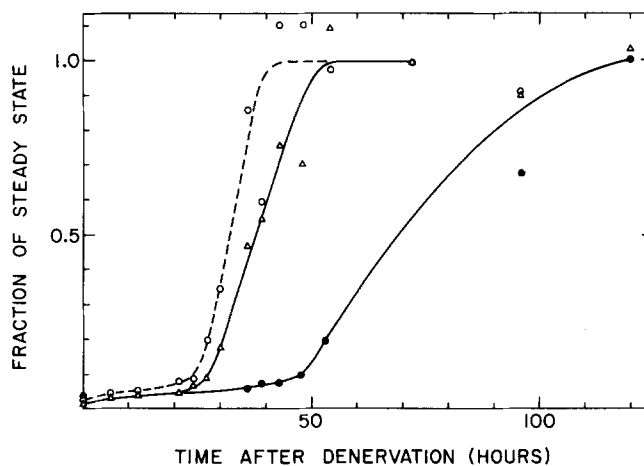
It is not known whether the eight intervening sequences of the original transcript are removed in an ordered or random fashion. Since within the limits of the sensitivity of the methods used it appears that intron VII is always excised before intron VI, a specific splicing pathway may exist, although it obviously does not operate in a sequential 5' to 3' fashion. This agrees with observations on the nonrandom splicing order of introns in vitellogenin gene transcripts (Ryffel et al., 1980). Fig. 2 shows that, as a consequence of denervation, the concentration not only of mature alpha subunit mRNA, but also of the precursors NT399 and NT294 increases significantly.



**Figure 3.** The molar ratio of fragments NT294 and NT224 as a function of time after denervation. Leghorn chicks (3 wk old; SPAFAS, Norwich, CT) were used in this experiment. Absolute quantities of alpha subunit-related sequences were measured by solution hybridization at the indicated times after denervation, and the molar ratio of NT294 and NT224 was determined as described in Materials and Methods.

Under steady-state conditions the ratio of these three fragments is determined by processing and turnover rates (see Appendix). To gain a better understanding of the kinetics of alpha subunit mRNA processing, a denervation experiment was carried out focusing on the second day after the operation when the concentration of the alpha subunit message rises sharply. The absolute amount of hybridizing RNA was measured by probe excess solution hybridization, and the relative abundance of precursor NT294 and "mature" mRNA determined by densitometry. It was found that the precursor-product ratio was 0.020 to 0.025 in innervated as well as denervated muscle (Fig. 3). For a half-life of mRNA of several hours (see below) this would indicate a transcript processing time of several minutes, in agreement with what is known about the kinetics of processing in other systems (Darnell et al., 1986). It is to be kept in mind that the calculated interval reflects the time elapsed between the two splicing events that generate and eliminate NT294. Whether these two events are immediately consecutive or the first and last steps in the splicing pathway is not known (i.e., the time estimate deduced from the precursor-product ratio represents a lower limit of the complete processing time). NT399 could not be quantitated accurately, but occurred, under steady-state conditions, at concentrations  $\sim 5$ –10 times lower than NT294. This may indicate that the splicing between nucleotides 942 and 943 occurs soon (perhaps within a minute) after transcription.

Since after the first postoperative day alpha subunit mRNA production is stimulated approximately 20-fold (see Fig. 4), one might expect the NT294/NT224 ratio to rise sharply and transiently by a comparable factor. This is not the case. Instead a fairly slow increase to about twice the steady-state value was observed, presumably because the response of the musculature representing each time point is not synchronized to within the time constant of RNA processing (i.e., less than an hour). Such synchronization is unlikely in vivo



**Figure 4.** Time course of AChR, alpha subunit mRNA, and precursor NT294 after denervation. AChR levels were determined by alpha-bungarotoxin binding, total alpha subunit mRNA by solution hybridization, and molar concentrations of NT294 by multiplying the latter values with the ratios in Fig. 3. Values are normalized to steady-state levels reached within several days after denervation ( $\bullet$ , 200 pmol of alpha-bungarotoxin-binding sites per g of muscle for AChR;  $\Delta$ , 340 fmol per g of tissue for alpha subunit mRNA; and  $\circ$ , 9.5 fmol per g of tissue for NT294).

of the presence of different fiber types and nerve stump lengths in individual muscles; in addition it is not obvious that all fibers within a muscle, or even all nuclei within a fiber, should be responding in unison. In Fig. 4, the time course of alpha subunit mRNA precursor, alpha subunit mRNA, and AChR after denervation is compared. The half-time of approach to steady state of the three species is a reflection not only of their turnover rate, but, as pointed out above, also of a lack of synchronization. For instance, the apparent  $t_{1/2}$  of NT294 is  $\sim 6$  h, while its true half-life should be of the order of a fraction of an hour. We therefore estimate the half-life of mature alpha mRNA in denervated muscle to be considerably shorter than the 12 h suggested by the time course, perhaps  $\sim 6$  h.

Both the alpha subunit mRNA and its precursor rise only slightly (about twofold) during the first postoperative day. Why it should take about a day before the major response is seen remains a challenge for future research. Are we perhaps witnessing the depletion of a repressor molecule below a critical concentration? The delay in the appearance of receptor molecules long after the increase in alpha subunit messages requires an explanation. Perhaps it is caused by the belated elaboration of other subunits. We have noted that mRNAs for the gamma and delta subunits rise significantly 2 d after denervation (Shieh, B. H., M. Ballivet, and J. Schmidt, manuscript submitted for publication).

Ever since Fambrough (1970) showed that the development of denervation supersensitivity can be blocked by the RNA synthesis inhibitor actinomycin D, the expectation has been that receptor synthesis in denervated or paralyzed muscle is prompted as a result of transcriptional activation. Since then evidence for other modes of regulation, posttranscriptional and even posttranslational, has been reported (Pezementi and Schmidt, 1981; Olson et al., 1983, 1984; Carlin et al., 1986). Thus in the interpretation of findings the possibility of multiple regulatory mechanisms must be kept in mind.

The precursor-product ratio increases transiently during the time of rapid accumulation of alpha subunit mRNA. This observation is most simply explained by transcriptional activation, with processing of transcripts and message turnover continuing at fixed rates. The most plausible alternative to transcriptional activation is message stabilization. A 20-fold increase in half-life would lead to the observed 20-fold increase in the steady-state level of alpha subunit mRNA. However, precursor concentration should then remain constant (and precursor-product ratio should fall) which is clearly not the case. A transient block of mRNA maturation (or increase in processing time) would mimic the transient increase in precursor-product ratio that is observed; however such a temporary inhibition would not result in increased mRNA levels.

The converse situation is also conceivable; namely, the operation, in normal muscle, of a "discard" pathway (Darnell, 1982) that branches off the splicing route distally to the formation of NT294. If upon denervation this discard pathway were to be blocked, more precursor would be channeled into mature mRNA, and mRNA levels would accordingly rise. However, pre-mRNA would presumably not change in concentration, and the precursor-product ratio would temporarily decline rather than increase. If, on the other hand, the discard pathway is postulated as branching off the main

route before the first splicing event, the distinction between transcriptional activation and the shutdown of such a discard pathway is not possible any longer. Since there is no direct evidence for a discard pathway in other systems, it is not very likely to play an important role in denervation-induced supersensitivity either.

Another mechanism can be envisaged in which NT294 is not a precursor of NT224, namely alternate splicing of the primary transcript of the alpha subunit gene to give rise to two different messages one of which retains 70 or more nucleotides of the 3' end of intron VI. To test this possibility we analyzed cytoplasmic RNA for the presence of NT294. We found that the proportion of NT294 drops by a factor of 6 when RNA from a crude postnuclear fraction is compared to total RNA from chick embryo muscle (data not shown). Thus NT294 does not normally occur outside of the nucleus, strongly suggesting that it represents a short-lived intermediate in the synthesis of alpha subunit mRNA. The conclusion that denervation activates the alpha subunit gene is difficult to avoid.

## Appendix

Let  $S$  = rate of transcription,  $\tau$  = time required to process transcript to mature mRNA, and  $t_{1/2}$  = half-life of mature mRNA.

Assuming that all transcripts yield mature mRNA (and that therefore in the steady state the rate of transcript synthesis equals the rate of mRNA production) the concentration of mRNA precursors is given by the term  $S \cdot \tau$ , and the concentration of mature mRNA by the expression  $S \cdot t_{1/2} / \ln 2$ . Under steady-state conditions, the ratio of the two pools is  $\tau \cdot \ln 2 / t_{1/2}$ ; i.e., is a function of processing and turnover rates, but independent of the synthesis rate  $S$ . Similarly, if processing is divided into several steps, the ratio of any particular intermediate to another intermediate or to mature mRNA will be determined by the times required for the respective conversions and by the mRNA turnover rate. Thus the ratio NT294/NT224 can be written as  $\tau_1 / (t_{1/2} / \ln 2 + \tau_2)$ , where  $\tau_1$  is the time elapsed between the splicing event between nucleotides 942 and 943 that generates NT294 and the splicing event between nucleotides 718 and 719 that converts NT294 to NT224, and  $\tau_2$  is the time required for complete maturation after the latter splicing step. This interval  $\tau_2$  is here presumed to be negligible compared to  $t_{1/2}$  ( $\sim 6$  h; see Fig. 4). Consequently, the experimentally determined ratio NT294/NT224 ( $\sim 0.022$ ) simplifies to  $\tau_1 \ln 2 / t_{1/2}$ , yielding an estimate of 11.4 min for  $\tau_1$ .

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## References

- Buonanno, A., and J. P. Merlie. 1986. Transcriptional regulation of nicotinic acetylcholine receptor genes during muscle development. *J. Biol. Chem.* 261:11452-11455.
- Carlin, B. E., J. C. Lawrence, J. M. Lindstrom, and J. P. Merlie. 1986. Inhibition of acetylcholine receptor assembly by activity in primary cultures of embryonic rat muscle cells. *J. Biol. Chem.* 261:5180-5186.
- Darnell, J. E. 1982. Variety in the level of gene control in eukaryotic cells. *Nature (Lond.)* 297:365-371.
- Darnell, J. E., H. Lodish, and D. Baltimore. 1986. *Molecular Cell Biology*.

- W. H. Freeman, New York. 1187 pp.
- Fambrough, D. M. 1970. Acetylcholine sensitivity of muscle fiber membranes: mechanism of regulation by motoneurons. *Science (Wash. DC)*. 168:372-373.
- Fambrough, D. M. 1979. Control of acetylcholine receptors in skeletal muscle. *Physiol. Rev.* 59:165-227.
- Feramisco, J. R., J. E. Smart, K. Burrige, D. Helfman, and G. P. Thomas. 1982. Coexistence of vinculin and a vinculin-like protein of higher molecular weight in smooth muscle. *J. Biol. Chem.* 257:11024-11031.
- Goldman, D., J. Boulter, S. Heinemann, and J. Patrick. 1985. Muscle denervation increases the levels of two mRNAs coding for the acetylcholine receptor alpha subunit. *J. Neurosci.* 5:2553-2558.
- Klarsfeld, A., and J.-P. Changeux. 1985. Activity regulates the level of acetylcholine receptor alpha subunit mRNA in cultured chicken myotubes. *Proc. Natl. Acad. Sci. USA.* 82:4558-4562.
- Merlie, J. P., K. E. Isenberg, S. D. Russell, and J. R. Sanes. 1984. Denervation supersensitivity in skeletal muscle: analysis with a cloned cDNA probe. *J. Cell Biol.* 99:332-335.
- Olson, E. N., L. Glaser, J. P. Merlie, and J. Lindstrom. 1984. Expression of acetylcholine receptor alpha subunit mRNA during differentiation of the BC<sub>3</sub>H-1 muscle cell line. *J. Biol. Chem.* 259:3330-3336.
- Olson, E. N., L. Glaser, J. P. Merlie, R. Sebbane, and J. Lindstrom. 1983. Regulation of surface expression of acetylcholine receptors in response to serum and cell growth in the BC<sub>3</sub>H-1 muscle cell line. *J. Biol. Chem.* 258:13946-13953.
- Pezzeменти, L., and J. Schmidt. 1981. Rapid modulation of acetylcholine receptor synthesis. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 135:103-106.
- Pumplin, D. W., and D. M. Fambrough. 1982. Turnover of acetylcholine receptors in skeletal muscle. *Annu. Rev. Physiol.* 44:319-335.
- Ryffel, G. U., T. Wyler, D. B. Muellener, and R. Weber. 1980. Identification, organization and processing intermediates of the putative precursors of *Xenopus* vitellogenin messenger RNA. *Cell.* 19:53-61.
- Salpeter, M. M., and R. H. Loring. 1985. Nicotinic acetylcholine receptors in vertebrate muscle: properties, distribution and neural control. *Prog. Neurobiol. (Oxf.)*. 25:297-325.
- Schmidt, J., and M. A. Raftery. 1973. A simple assay for the study of solubilized acetylcholine receptors. *Anal. Biochem.* 52:349-353.
- Wang, G. K., and J. Schmidt. 1980. Primary structure and binding properties of iodinated derivatives of alpha-bungarotoxin. *J. Biol. Chem.* 255:11156-11162.