Transcripts for the Acetylcholine Receptor and Acetylcholine Esterase Show Distribution Differences in Cultured Chick Muscle Cells

K. W. K. Tsim,* I. Greenberg,‡ M. Rimer,§ W. R. Randall,§ and M. M. Salpeter‡

*Department of Neurobiology, Stanford University School of Medicine, Stanford, California 94305;
‡Department of Neurobiology and Behavior, Cornell University, Ithaca, New York 14853; and
§Department of Pharmacology and Experimental Therapeutics and Medical Biotechnology Center, University of Maryland, Baltimore, Maryland 21201

Abstract. In situ hybridization of chick cultured muscle cells using exonic DNA probes for both AChR α-subunit and the catalytic subunit of AChE, revealed major differences in the distribution of label both over nuclei and in their surrounding cytoplasm, although some overlap in these distributions exists. For the AChR α-subunit there is a highly skewed distribution of labeled nuclei, with 35% of the nuclei being relatively inactive (<0.25 times the mean label) and ~10% being very heavily labeled (>2.5 times the mean label). In contrast the nuclei labeled with the exonic probe for the AChE transcripts had a more Gaussian distribution, yet with some slight skewness in the direction of a few heavily labeled nuclei. There was also a difference in the cytoplasmic distribution of the label. The AChR α-subunit mRNA was mainly within 4 μm of labeled nuclei while the AChE mRNA was more widely distributed throughout the cytoplasm, possibly within a 10 μm rim around labeled nuclei. An intronic probe for the AChE gave the identical distribution of nuclear label to that of the exonic probe (but without any cytoplasmic label). In addition, calibration of the technique indicated that per myotube the AChE transcript is about sixfold more abundant than the AChR α-subunit transcript.

NUCLEI in multinucleated muscle cells in culture are not equally active in expressing muscle-specific molecules (for example see Pavlath et al., 1989; Hall and Ralston, 1989). A dramatic example is the fact that only a subset of nuclei are heavily involved in expressing mRNA for the acetylcholine receptor (AChR)[1] α-subunit (Harris et al., 1989; Bursztajn et al., 1989). In addition, mRNA for the AChR α-subunit is closely associated with the active nuclei, having a restricted localization in the adjacent cytoplasm (Harris et al., 1989; Horovitz et al., 1989; see, however, Bursztajn et al., 1989). In contrast, mRNAs for structural muscle proteins, such as actin, are more widely distributed over the muscle cytoplasm (for example see Fontaine et al., 1988; Harris et al., 1989). such a differential distribution suggests a compartmentalization of different mRNAs as was also shown for the mRNA of different proteins in fibroblasts (Sundell and Singer, 1991) and in neurons (Kleiman et al., 1990; Bruckenstein et al., 1990). It remains to be established whether groups of proteins with a final common location may be processed via the same subset of cellular compartments.

In the present study we examine the relative distribution of the mRNAs of AChRs α subunit and acetylcholine esterases (AChEs) in chick cultured myotubes since these two muscle molecules are encoded by myotube nuclei, and both are destined for the muscle surface. The AChEs in the vertebrate muscle exist in two broad structural classes; globular and asymmetric (Massoulié and Bon, 1982; Massoulié et al., 1984). The catalytic subunits of both classes are now known to be encoded by a single gene (Schumacher et al., 1988; Sikorav et al., 1988; Rotundo et al., 1988; Maulet et al., 1990). AChR and AChE can colocalize at high density clusters on the surface of muscle (Wallace, 1986), yet little is known about the corresponding localization of their respective mRNAs. Consistent with the reported perinuclear localization of AChR mRNA (Harris et al., 1989; Horovitz et al., 1989), the translation and assembly of AChE molecules have also been reported to be restricted to the vicinity of the selected nuclei which are expressing their transcripts in cultured myotubes (Rotundo, 1990). However, this latter study examined only the expression of chimeric AChE subunits from allelic heterokaryons by a genetic analysis of segregation, and did not determine the cytological localization of the mRNA encoding AChE nor the extent of the underlying perinuclear compartment. The present study used in situ hybridization to determine whether AChR α-subunit and AChE expression involves identical subpopulations of nuclei and if their mRNAs are distributed similarly in the

K. W. K. Tsim's present address is Department of Biology, Hong Kong University and Technology, Clear Water Bay Road, Hong Kong.

1. *Abbreviations used in this paper*: AChE, acetylcholine esterase; AChR, acetylcholine receptor; ScEM, scanning EM.

surrounding cytoplasm. We found that the number of active nuclei and the extent of label per nucleus, as well as the distribution of the mRNA away from the nuclei, were very different for these two mRNA species. The possibility of using in situ hybridization for absolute quantitation is also examined.

Materials and Methods

Probes Used for Hybridization

The AChE exon probe is a 790-bp cDNA (about 1/6 total transcript size; Fig. 1 *A*). It was originally isolated from a λgt10 library derived from 14-day-old chick embryo muscle RNA and subsequently subcloned into the EcoRI site of pTZ19R (Gough, N.R., M. Rimer and W. R. Randall, unpublished observations). The cDNA encodes ~260 amino acid sequence ending ~100 residues from the carboxyl terminus of the enzyme. The 3′ end of the clone is homologous and overlaps with amino acid sequence between leu_{320} and phe_{448} of *Torpedo californica* AChE (Schumacher et al., 1986). The 5′ end encodes an additional 132 amino acids distinctive of chick AChE and not found in *Torpedo* or mammalian AChEs so far cloned (Schumacher et al., 1986; Sikorav et al., 1987; Rachinsky et al., 1990; Soreq et al., 1990). The encoding amino acid region is common to all known forms of AChE and thus the cDNA will hybridize to all of its transcripts. The expression of catalytically active AChE from a cDNA containing the sequence used as the exonic probe will be presented elsewhere (Gough, N. R., M. Rimer, and W. R. Randall, manuscript in preparation).

The AChE intron probe was derived by exonuclease III treatment of a 2.18-kb BamHI fragment of the chick AChE gene subcloned in pGEM7Z+. This 2.18-kb BamHI fragment initially contained 2 kb of intron plus 183 bp of exon including the translation initiation ATG codon at its 3′-end (Fig. 1 *B*). The exonuclease treatment deleted ~900 bp of the 3′-end of the BamHI fragment. The resulting 1.3-kb intronic fragment gave no signal when used as a probe in Northern blots of 14-day-old chick embryo muscle or brain poly (A^+) RNA. There is at least one exon mapping upstream of the 1.3-kb probe in different genomic clones (Rimer, M., and W. R. Randall, manuscript in preparation). A cDNA from this exon as well as from the exon immediately downstream from the intron probes hybridizes to AChE transcripts on the same Northern blots described above attesting to the specificity of the intron probe for AChE.

The AChR exonic probe consists of the full-length coding region of chick AChR α-subunit cDNA (1.8 kb), subcloned into the EcoRI site of pTZ19R (Jackson et al., 1986). To prepare the probes, the DNAs (AChR and AChE; exon and intron) were excised from the vectors using restriction enzymes and separated and purified from low-melting-point agarose gels. About 100 ng of DNA was labeled with ^{35}S-dCTP (Amersham Corp., Arlington Heights, IL) by the random hexamer primer method (Feinberg and Vogelstein, 1983) and was separated from unincorporated nucleotides using a 1 ml G-25 Sephadex spun column. The specific activity of the labeled DNA for the different experiments varied from 3×10^8 to 9×10^8 dpm/μg of DNA.

Chick Myotube Cultures

Myotube cultures were prepared from hind limb muscles of 11–12-day-old White Leghorn chick embryos by the method of Fischbach (1972) with minor modifications (Godfrey et al., 1984). In brief, the minced muscles were dissociated for 15 min in 0.02% trypsin at 37°C, the cell suspension was preplated to reduce the number of nonmuscle cells, and $\sim 3 \times 10^5$ cells were plated on 35-mm plastic tissue culture dishes coated with calf skin collagen (Calbiochem-Behring Corp., La Jolla, CA). Culture medium (1.5 ml) consisted of MEM (Gibco Laboratories, Grand Island, NY) supplemented with 10% horse serum (Gibco Laboratories), 2% chick embryo extract (prepared from 11-day-old chick embryos with an equal volume of Puck's saline G), 100 U/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml Fungizone (Gibco Laboratories). Medium was replaced every 2 d; 10^{-5} M cytosine arabinoside was added from days 3–4 to reduce the number of rapidly dividing cells. Cultures were used on day 5 after plating.

Northern Blot Analysis

The exonic AChR α-subunit and AChE probes were compared using Northern blot analyses. Poly (A)+ RNA was isolated from 5-day-old cultured chick muscle cells by Fast Track mRNA isolation kit (Invitrogen, San Diego, CA). 5 μg of mRNA per lane were electrophoresed in a 1% formaldehyde-agarose gel and blotted onto a BA-S nitrocellulose membrane (Schleicher & Schuell, Inc., Keene, NH). Prehybridization was done for 4 h at 42°C in a buffer containing 50% formamide, 5× SSC, 5× Denhardt's reagent, 0.5% SDS, and 0.1 mg/ml denatured salmon sperm DNA. Hybridization was overnight at 42°C in prehybridization buffer plus 10% dextran sulfate, 0.1 mg/ml tRNA, and 5×10^6 cpm/ml of ^{32}P-DNA probe labeled as for in situ hybridization. After overnight hybridization at 42°C the blot was washed twice in 1× SSC, 0.1% SDS for 30 min at room temperature. The final washes were for 30 min in 0.1× SSC, 0.1% SDS at 65°C. A RNA ladder (Bethesda Research Laboratories, Bethesda, MD) was used to standardize the RNA sizes. The density of each hybridized band, after autoradiography, was measured using a densitometer (Pharmacia-LKB Biotechnology Inc., Piscataway, NJ).

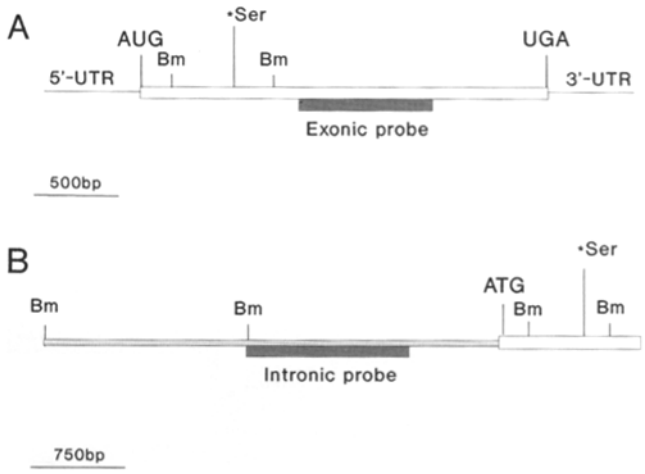

Figure 1. DNA exon and intron fragments of chick AChE used as probes for in situ hybridization. (*A*) The transcript encoding AChE showing translation start (*AUG*) and stop (*UGA*) codons, untranslated regions (*UTR*), the active site serine (**Ser*), and the BamHI restriction sites (*Bm*) from the corresponding cDNA. The exonic probe (▩), located in its position relative to the coding region of the transcript is a 790-bp cDNA cloned into the EcoRI site of pTZ19R and encodes an amino acid sequence common to all known forms of AChE polypeptides. (*B*) Partial restriction map of the chick AChE gene showing the location of the 1.3-kb intron probe (▩) in relation to the exon (□) containing the translation start codon (*ATG*). An additional exon is located upstream of the 5′ end of this map.

In Situ Hybridization

The procedure followed was as previously described in Horovitz et al. (1989) with minor modifications. Chick myotubes grown on 35-mm dishes were fixed with a freshly prepared 4% paraformaldehyde in PBS, pH 7.4, for 30 min at 4°C. Cultures were acetylated for 10 min at room temperature using a solution of 0.15 ml acetic anhydride in 50 ml of 0.1 M triethanolamine, pH 8.0. Cells were rinsed 3 times with 2× SSC, and were prehybridized at 55°C for 4 h in a buffer containing 50% formamide (deionized), 10% dextran sulfate, 1% SDS, 0.1 M DTT, 1 M NaCl, and 0.1 mg/ml of denatured salmon sperm DNA. The prehybridization buffer was removed, and replaced by 20 μl hybridization buffer, containing everything in the prehybridization buffer plus 2 or 4 ng of ^{35}S-labeled cDNA probe, which had been denatured by boiling for 5 min and rapidly chilled. The hybridized area was covered with an 18 × 18-mm coverslip. Culture dishes were placed in a moist closed plastic box and were hybridized at 55°C for 16–20 h. After hybridization, coverslips were washed off with 2× SSC, 50% formamide. Plates were washed twice (30 min each) while shaking with 2× SSC, 50% formamide at room temperature, then with 1× SSC , 50% formamide at 55°C, and finally with 1× SSC at room temperature. Cells were dehydrated rapidly through 50, 70, 95, and 100% ethanol, and air dried. Duplicate plates for each condition were treated with 0.1 mg/ml of RNase A (United States Biochemical Corp., Cleveland, Ohio) for 30 min at 37°C before the

acetylation step, and were used as controls for nonspecific binding of the probe.

Preparing Autoradiograms

Autoradiograms were prepared as previously described (Horovitz et al., 1989). Briefly, the air-dried cells were covered with a layer of carbon by evaporation to prevent chemography, and then coated with either a monolayer or a double layer of Ilford L4 emulsion (Ilford, Knutsford, Cheshire, England) which had been prepared as a stripping film on collodion-coated slides (Salpeter, 1981). The thickness of the preformed emulsion layers was judged by its interference color (purple for single layer, deep red for double layer; Harris and Salpeter, 1980). Double layers were often used since the energy of ^{35}S is high enough to penetrate a double layer of Ilford L4 emulsion, giving almost twice the grain yield (Harris and Salpeter, 1980). The use of the stripping film prevents emulsion from piling up around the edges of the myotubes and eliminates the well known edge artifact in which developed grains accumulate along the edges of the tissue. The stripping film also insures a uniform layer over all the tissue, allowing the emulsion to be calibrated for sensitivity, and provides for quantitative analysis. The emulsion-coated tissue culture dishes were stored in black slide boxes containing packets of "dryrite," exposed at 4°C for 2–5 wk and developed in D19, for 2 min at room temperature.

Autoradiograms were analyzed using primarily light microscopy (Reichert Diapan with anoptral negative contrast oil immersion objectives). However, to get large areas of autoradiograms in focus simultaneously, scanning EM (ScEM) autoradiography was also used. For the ScEM procedure, the bottom of the tissue culture dish was cut out and attached to the ScEM stubs. Subsequently, the procedure was as previously described (Salpeter et al., 1988) except that a thin layer of gold rather than carbon was evaporated over the developed emulsion before viewing.

Analysis of Autoradiograms

Three conditions were compared: mature AChE mRNA and AChR α-subunit mRNA using exonic probes and AChE intronic RNA using a genomic probe. Three experiments were involved and 6–8 dishes were included in the final analysis for each condition. Using oil immersion light optics, 5–10 muscle fibers were selected randomly for each dish. To get a broad sampling of nuclei, every second or third nucleus was analyzed until 10–20 were tabulated per fiber, for a final number of 100–200 nuclei per dish. The nuclei included in the tabulation were either single or were part of nuclear clusters.

Developed grains were counted either over the entire nucleus, or only over a unit area (39 μm^2, which was the size of one square of a measuring grid in the ocular), chosen to encompass the most heavily labeled area of the nucleus. Most nuclei had an ~80–100-μm^2 top surface area. When analyzing the mRNA distribution, grains were also counted in consecutive grid squares up to ~18 μm from the edge of a nucleus to determine the gradient of mRNA around active nuclei. To minimize overlap of grain distributions, nuclei were included in this latter analysis only if they were >40 μm from an adjacent nucleus in the direction that the gradient was being measured. This analysis of the perinuclear cytoplasmic gradient therefore included only single nuclei or nuclei at the edge of a cluster.

Grains were expressed either as grains/nucleus, or grains/μm^2. No dish was included in this study which had an average grain density of <6 grains/nucleus. Nuclear label above 20 grains/39 μm^2 was corrected for lack of linearity as previously described (Podleski and Salpeter, 1988) and grains were not counted above 39 grains/39 μm^2. Exposure times were varied to avoid such high label, but on the rare occasion when it did occur (~1% of the nuclei for AChR and essentially none for AChE had such a high label), the nuclear label gave a lower limit to the actual label. Grains were also counted over regions adjacent to the myotubes (excluding mononucleated cells) to obtain an emulsion background value.

Normalization of Data for Easy Comparison

To compare grain densities over nuclei from dishes with different emulsion layers, different exposure times, or which came from experiments with different specific activity probes, the data was normalized to the mean value per dish. This was most important for comparing the results obtained from the AChE with those from the AChR probes. Briefly, for each dish a value for the average grain density per nucleus was first obtained and a frequency histogram of the number of grains per nucleus was constructed so that the mean grain density value was always contained within the 4th of a total of 11 histogram columns, and each histogram column was of equal width (i.e., contained an equal number of integer grains with zero grains included as an integer). Thus, since the mean is in column 4, column 1 was equal to or less than about one quarter of the mean, and above column 10, values were >2.5 times the mean. The histogram columns for all dishes from the same experimental group were then pooled, giving the average number of nuclei ± SEM which had the same label relative to the mean.

Results

Distribution of mRNA for AChE and AChR α-Subunit

Using exonic cDNA probes to localize the mRNA for AChR α-subunit in the cultured chick myotubes, we found that, as previously reported (Harris et al., 1989; Horovitz et al., 1989), the α-subunit mRNA is not uniformly distributed throughout the myotubes, but is concentrated over and around specific nuclei (Fig. 2). We found however, that the AChE mRNA is distributed much more widely throughout the myotubes. Figs. 3 and 4 give sample autoradiograms which illustrate these points qualitatively.

To analyze the distribution of the grains more quantitatively, we initially counted the grains over nuclei. Although such grains are due to hybridization inside the nucleus as well as in the cytoplasm above or below that nucleus, we will refer to the grains overlying nuclei as "nuclear label" and the associated nuclei as the "labeled nuclei." We also determined the distribution of grains away from the nuclei into the cytoplasm and will refer to the labeled nucleus plus the associated labeled cytoplasmic rim as the "nuclear domain."

Nuclear Label

The histograms in Fig. 5 illustrate that for the AChR α-subunit mRNA, the nuclear label has a considerably skewed distribution (i.e., a peak with low label at the origin with a long tail towards the heavily labeled end). We found that ~35% of the nuclei had a label at or below 0.25 the mean grain density per nucleus (and ~14% of the nuclei had essentially no label) while ~10% of the nuclei are labeled at >2.5 times the mean, half of which (i.e., 5%) were greater than four times the mean. (Fig. 4 illustrates the fact that even within a cluster of labeled nuclei one finds unlabeled ones.) In contrast, Fig. 5 shows that for the AChE exonic probe the nuclear label had a more nearly Gaussian distribution, although with a slight skewness towards the high nuclear label range. Most (65%) of the nuclei had a grain density in the mid range between 0.75 and 1.25 times the mean (compared to ~23% of the nuclei hybridized with the probe for the AChR α-subunit that fell within this range). Only ~3% of the nuclei had a label which was below 0.25 times the mean (while none were unlabeled) and ~1% of the nuclei had a nuclear label >2.5 times the mean (all of which were in fact more than four times the mean value). With so few nuclei, the significance of this last observation is difficult to assess. However, it suggests the potential for having a subclass of very active nuclei expressing the AChE similar to that seen for the AChR.

The difference between the AChE and AChR nuclear label can also be illustrated by the ratio of the median to the mean grain density/nucleus, which was ~1 for the AChE and ~0.6 for the AChR nuclear label. Finally we calculate that 50% of the total nuclear label is contained in only 14% of the nuclei expressing the AChR α-subunit mRNA, compared to 32% of the nuclei expressing AChE mRNA.

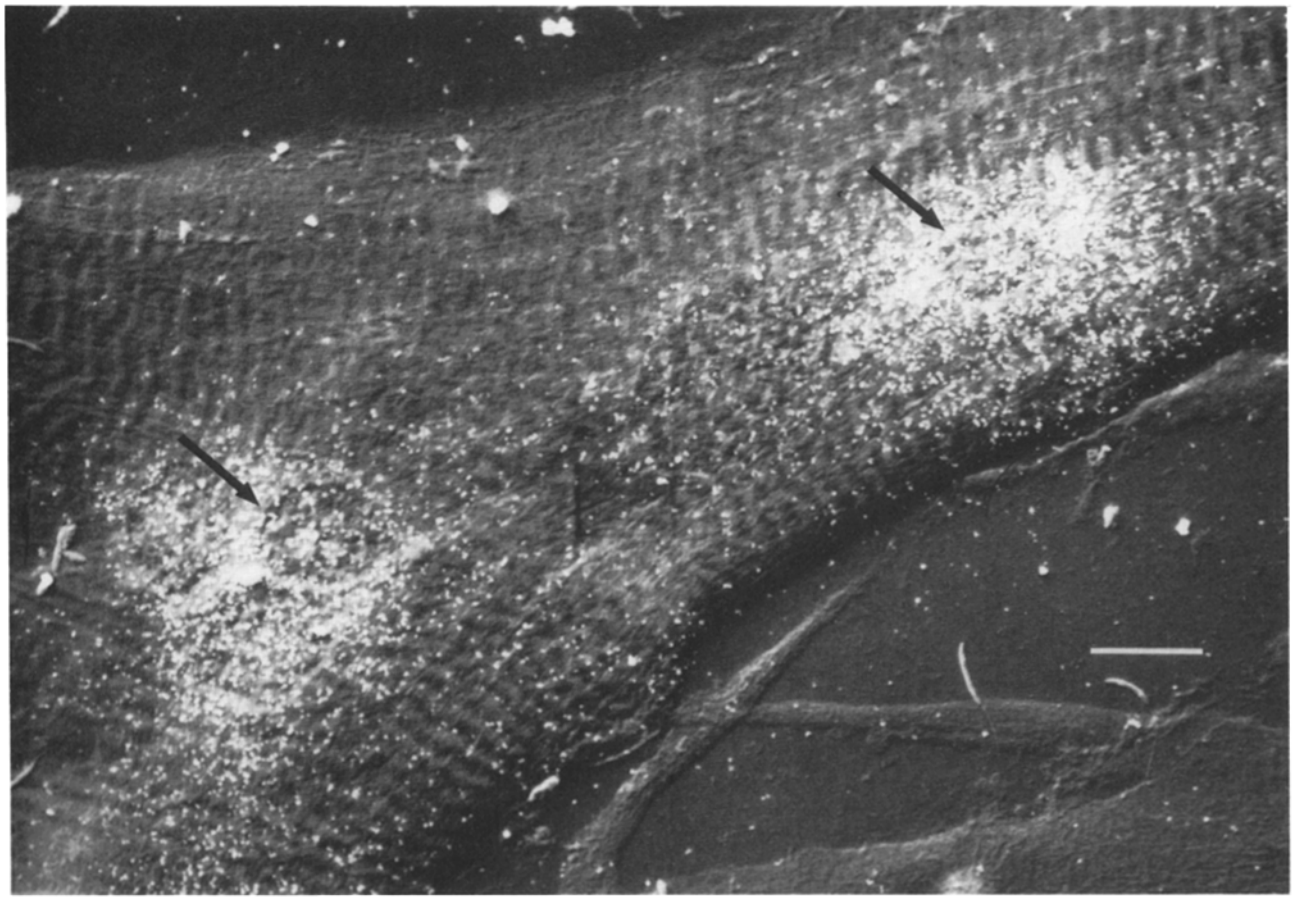

Figure 2. Low magnification autoradiogram of chick myotube hybridized with ^{35}S-labeled cDNA probe against the AChR α-subunit showing two clusters of AChR mRNA colocalized with clusters of nuclei (*arrows*) with very little spread into the cytoplasm. Autoradiograms were photographed using the scanning electron microscope. Bar, 10 μm.

It must be noted that even if the radioactivity were uniformly distributed in a tissue, the number of developed grains in the autoradiographic emulsion would fluctuate according to Poisson statistics. However, since in our experiments the mean grain density for both the AChR and AChE exon probes was >6 grains per nucleus (i.e., an average of about 12 and 20 grains per nucleus for the AChR α-subunit and the AChE, respectively), the Poisson fluctuations are not large and could not fully explain the width of either curve. For instance in Fig. 5 if the AChR had had a uniform label, on the average only 0.2% (instead of 35%) of the nuclei would have a density of <0.25 times the mean and only 5×10^{-4}% (instead of 10%) would have had a grain density >2.5 times the mean. For the AChE exon probe (since it had a higher mean grain density) a uniform label would have given only $\sim 3 \times 10^{-3}$% (instead of the observed ~3%) of the nuclei with <0.25 times the mean grain density and only 10^{-6}% (rather than 1%) of the nuclei with >2.5 times the mean. Thus for both the AChR and AChE probes the Poisson fluctuations are relatively unimportant and the experimentally observed grain distributions in Fig. 5 can be considered to approximate the true distribution of label. Thus if one can assume that the nuclear label represents mRNA transcribed by that nucleus, then, in the transcription of the AChR α-subunit, many nuclei (~30%) are relatively inactive, and ~10% are very active with the rest ranging in between. For the AChE on the other hand most of the nuclei are almost equally active. There is, however, a very small fraction (~1%) showing heavy activity.

Since nuclei are not exactly of equal size we also plotted the distributions for the grain density over only one defined area of ~39 μm^2 (i.e., the area of the ocular counting grid square) over the most heavily labeled regions of each nucleus, and found that the shape of the distribution of grain densities per unit area of nucleus was almost identical to that per whole nucleus for both the AChR α-subunit and AChE probes (data not shown).

Cytoplasmic Label

The distribution of developed grains into the cytoplasm was also radically different for the AChR α-subunit and AChE probes. Fig. 6 *A* gives a schematic drawing of how we assigned specified areas over and away from a nucleus for tabulating grain distributions. Fig. 6 *B* shows that the developed grains from the mRNA for the AChR α-subunit has a sharp gradient away from the peak label over nuclei, while that from the AChE has a much shallower gradient, confirming the qualitative observations of the autoradiograms in Figs. 2, 3, and 4. Some of this gradient is expected to be due to limitations in autoradiographic resolution. Studies on ^{14}C resolution (and thus for ^{35}S which has a similar energy decay) indicate that under our experimental conditions of using thin emulsion layers, the major factor limiting resolu-

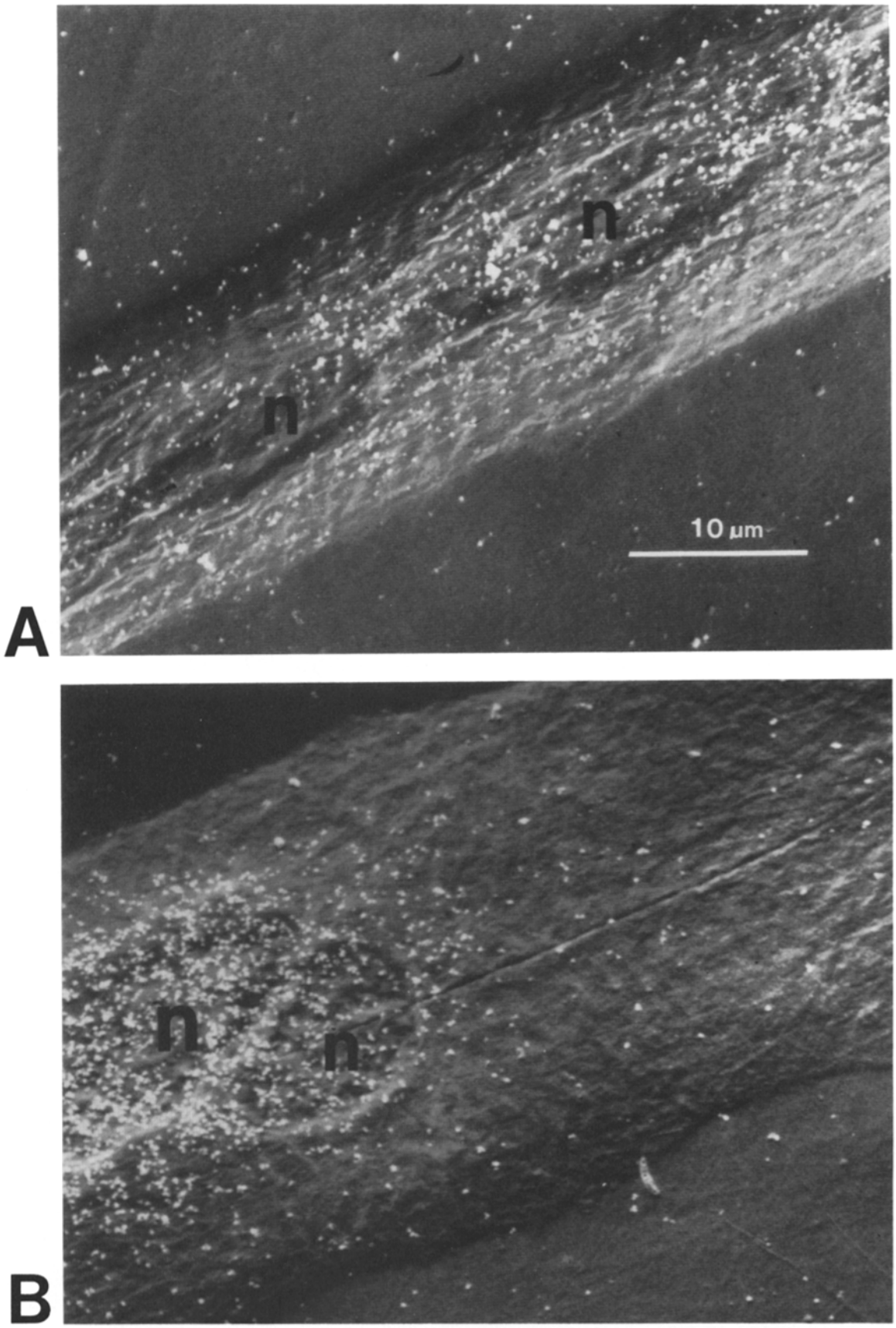

Figure 3. ScEM Autoradiograms of chick myotubes hybridized with ^{35}S-labeled cDNA probes against AChE (*A*) and against the AChR α-subunit (*B*), illustrate the different cytoplasmic distributions of the two mRNAs: that of the AChR α-subunit (*B*) is clustered over and around specific nuclei, and the two nuclei are not equally labeled, while that of the AChE (*A*) is more uniformly distributed in the cell. *n*, Nuclei. Bar, 10 μm.

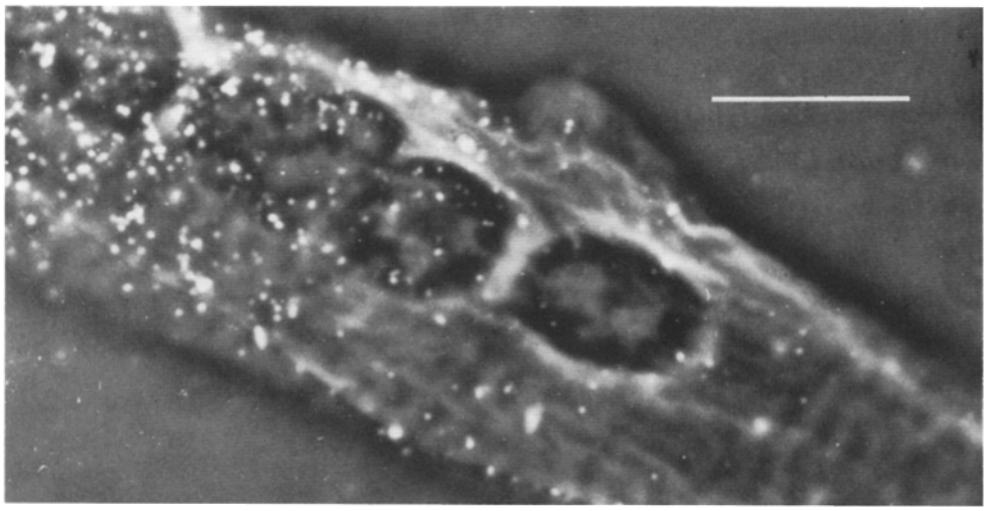

Figure 4. Light microscope autoradiograms of ^{35}S-labeled cDNA probe against the AChR α-subunit mRNA photographed using oil immersion anoptral (negative) contrast optics (Reichert Diapan microscope; Reichert Jung, Vienna) comparing nuclear label in a row of adjacent nuclei. Bar, 10 μm.

tion is the thickness of the tissue (Salpeter et al., 1969, 1974, 1987; Salpeter and Salpeter, 1971). From geometric considerations (Bachmann and Salpeter, 1965) and the above mentioned autoradiographic resolution studies we estimate a resolution half distance (HD) value of ~6 μm for the myotubes in our study. From Salpeter et al. (1969) we calculate that if the radioactivity were restricted to the area over the nuclei, the grain density would have dropped to about one-third its peak value by 6 μm from the edge of those nuclei. Thus even the steep grain density gradient for the AChR α-subunit mRNA cannot be purely radiation spread from the nucleus, but must represent true label outside the nucleus. We estimate that the observed grain density distribution for the AChR α-subunit mRNA is compatible with a uniformly labeled source which extends beyond the edge of the nucleus for ~3–4 μm. We define a nuclear domain as a disk containing the nucleus itself (5–6-μm radius) plus the radioactive annulus. The total label within the nuclear domain is larger than that of the nuclear label by the ratio of the two disk areas, i.e., for the AChR α-subunit it is $(5.5 \pm 3.5)^2/5.5^2 =$ 2.7-fold.

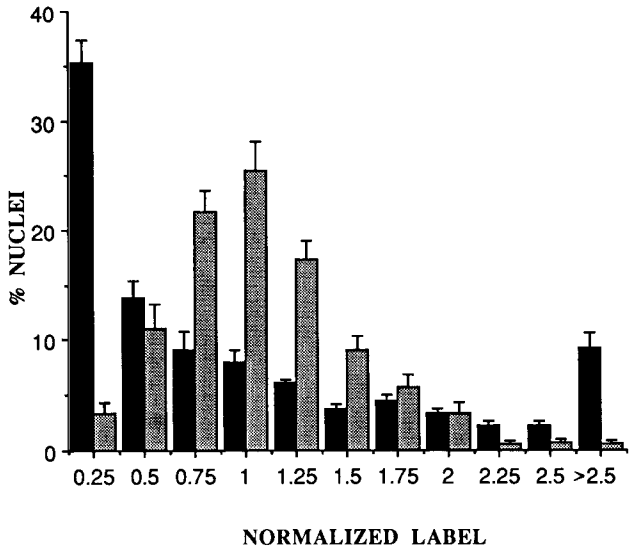

Figure 5. Frequency distribution of label over nuclei, hybridized with the cDNA exon probes for the AChR (■) α-subunit and for AChE (▨) The x-axis shows increasing label (as multiples of the mean value) and the y-axis gives the percentage of nuclei having that degree of label. The label is normalized so that the mean number of grains/nucleus for each dish is located in the fourth column and designated as 1 (see Materials and Methods for details of the normalization) and each histogram column represents a range of grains/ nucleus including approximately one quarter of the mean value. All columns above 10 were pooled and labeled >2.5 times the mean giving a total of 11 histogram columns. Note radical difference in distribution of nuclear label for AChE and AChR α-subunit. The distribution of developed grains over nuclei hybridized with cDNA for the AChR α-subunit is highly skewed with a peak at the origin. ~35% of nuclei having less than one quarter of the mean number of grains and >10% having >2.5 times the mean. The label for the AChE mRNA has a more nearly Gaussian distribution yet shows some (~1%) heavily labeled nuclei (>2.5 times the mean).

For the AChE mRNA, Fig. 6 *B* gives the grain distribution for the small fraction of nuclei (~15%) which are >40 μm from another nucleus. An analysis of radiation spread similar to that for the AChR α-subunit described above, assigns a labeled annular cytoplasmic region of ~9–10-μm-wide around nuclei, i.e., an AChE nuclear domain larger than the nucleus by a factor of $(5.5 + 9.5)^2/5.5^2 =$ 7.4-fold. However, our measurements show that nuclei are on average ~20 μm apart and thus the average nuclear domains touch or can slightly overlap (even apart from the radiation induced spread of developed grains which must have a considerable overlap). Therefore the actual factor by which the label in the nuclear domain exceeds the nuclear label must be less than the factor of ~7 given above. For another estimate of what that factor might be, we determined that the surface area of the cell is six times that of the nuclei, and that the average grain density over the cell as a whole is about two thirds that over nuclei. The label due to AChE mRNA per nuclear domain by this estimate is $2/3 \times 6 = 4$ times larger than the nuclear label. The exact size of the AChE nuclear domain still needs to be determined.

It is not known why the AChE mRNA is distributed more widely over the cytoplasm than is the AChR α-subunit mRNA. Some speculations are given in the Discussion. Nevertheless whatever the reason, if one can assume that the cytoplasmic location of mRNA mainly represents where the protein is being translated on the ribosomes of the RER, our

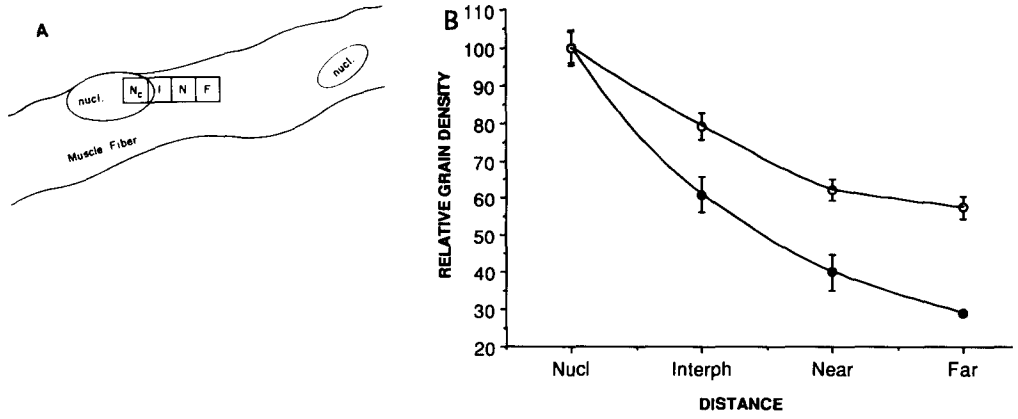

Figure 6. Cellular distribution of the mRNA for AChE and AChR α-subunit. (*A*) Diagram of how grain densities were measured from a nucleus, using 39-μm^2-unit-area consecutive grid squares. Areas tabulated were: over grid square with highest grain density in nucleus (N_c) (normalized to 100); at interphase spanning the nuclear envelope (*I*); and into cell cytoplasm, near (*N*) and far (*F*) from the nucleus. The far area goes up to ~18 μm from the edge of a nucleus. To minimize overlapping distributions only nuclei further than 40 μm from an adjacent nucleus in the direction of the grain density, were used for this tabulation. This meant that only singly located nuclei, or nuclei at the edge of a cluster were chosen (and constitute only 15% of the nuclear population). (*B*) Distribution of label from nucleus into cytoplasm due to AChR α-subunit mRNA (●) or for AChE mRNA (○), obtained as diagrammed in Fig. A. Note shallower gradient for the distribution of AChE than AChR α-subunit mRNA. To be compared with Figs. 3 and 4.

results indicate that a broader population of RER segments may be involved in processing AChE than is involved in processing the AChR α-subunit.

Distribution of Nuclear Unprocessed mRNA for AChE

To eliminate the possibility that rapid diffusion and slow turnover of AChE mRNA from only a few active nuclei might overlap adjacent nuclei and give an erroneous appearance that most of the nuclei are equally active, we labeled the unprocessed mRNA by in situ hybridization using an intron fragment of the AChE gene. Fig. 7 shows an autoradiogram obtained after hybridization with the AChE intronic probe. There was significant label only over nuclei (label over cytoplasm was not above background). The grains overlying these nuclei seemed randomly distributed over the nucleoplasm and showed no preferential localization, in contrast to that suggested for the AChR α-subunit by Berman et al. (1990). When the grains per nucleus over individual nuclei were tabulated for the intronic probe, the frequency distribution of nuclear label was found to be essentially identical to that for the exonic probe (Fig. 8). This shows that

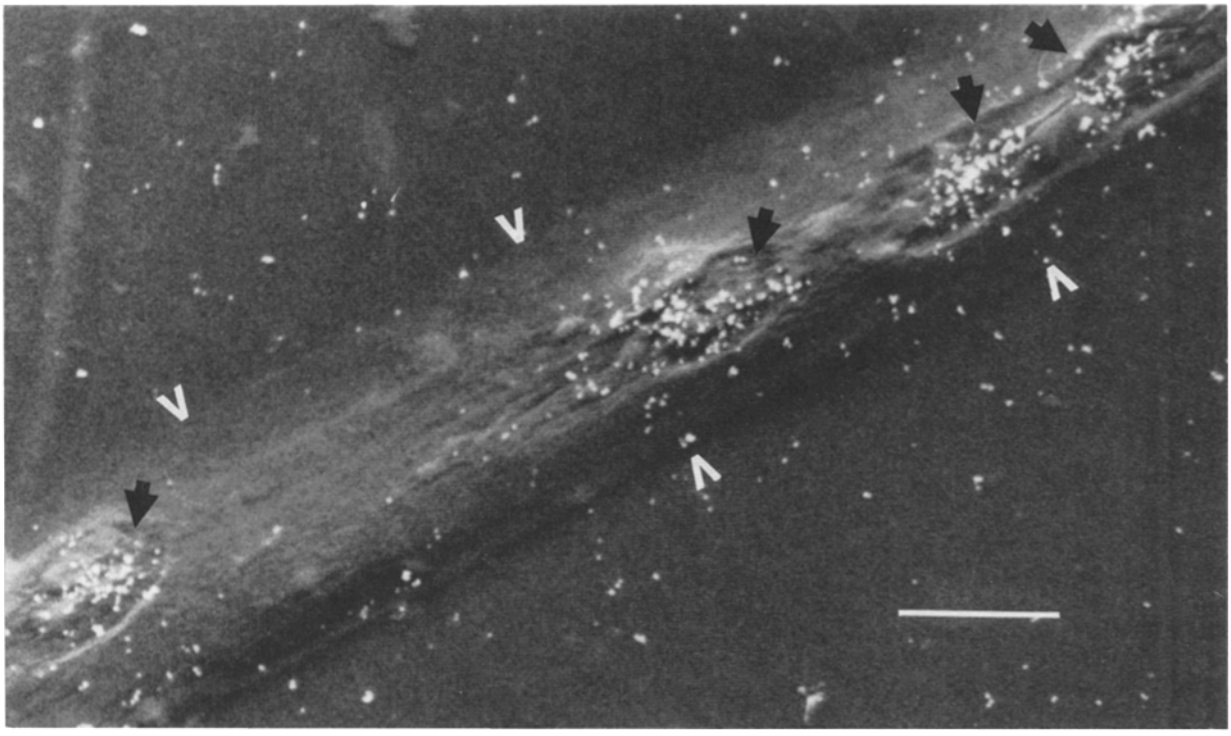

Figure 7. ScEM autoradiogram of myotubule labeled with ^{35}S-labeled intronic AChE probe. Note uniform distribution of label within the nuclei (*black arrows*). To illustrate the extent of label, autoradiogram was heavily overexposed, which explains the high background. Label over cytoplasm is not however significantly above off tissue background. Edge of cell is marked by white arrowheads. Bar, 10 μm.

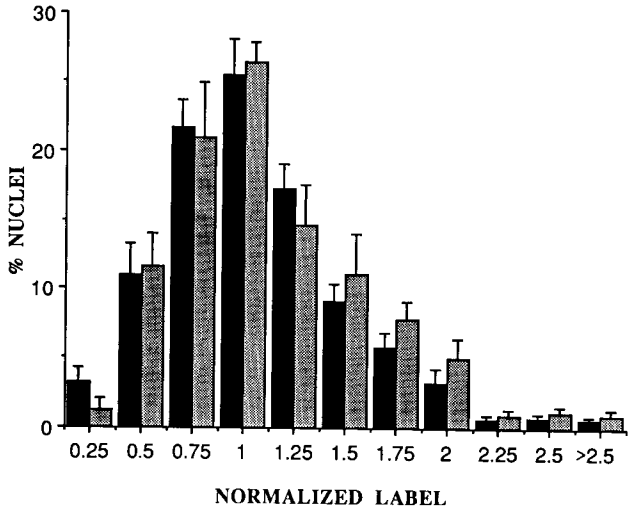

Figure 8. Comparison of label in nuclei hybridized with exonic (■) and intronic (▨) AChE probes. Histogram prepared and normalized as in Fig. 5. The distributions are not significantly different.

the spread of mature mRNA to overlap adjacent nuclei is not a factor in causing the near Gaussian distribution of nuclear label that we see after hybridizing with the AChE exonic probe.

RNase Controls

Autoradiograms of controls, treated with RNase, before hybridization gave little label above background indicating the specificity of the labeling to RNA with all three probes.

Quantitation of mRNA Transcripts for AChE and AChR α-Subunit

By In Situ Hybridization Autoradiography. Autoradiograms prepared as described here can in principle be quantified to yield information on the amount of probe (and thus mRNA transcripts) that have been hybridized in different parts of the cell. To obtain absolute quantitation, several criteria must be met. The specific activity of the probe and the sensitivity of the emulsion must be known, and the probe must saturate the available cellular mRNA. The emulsions must not be overexposed so that linear grain yields can be obtained, and the tissue must be thin enough so that no self-absorption of the ^{35}S β-decays occurs before they hit the emulsion (or the extent of self-absorption must be known).

The amount of probe hybridized in the tissues can then be obtained using the following equation:

$$P = \frac{G \times d}{(SA/k)(e^{-kt_s})(1{,}440)(1-e^{-kt_e})} \qquad \text{(Eq. 1)}$$

Where P = μg of probe bound in the tissue; k = e fold rate constant (for ^{35}S it is 0.00793/d); SA = specific activity of probe on day of hybridization (dpm/μg DNA) × 1,440 min/d (and SA/k give the total number of potential decays); G = grain density; d = number of decays to give one developed grain (determined by autoradiography sensitivity studies, e.g., Harris and Salpeter, 1980); t_s = time between hybridizing the cells and coating with emulsion; and t_e is the exposure time.

This equation gives the potential sensitivity of the in situ technique. e.g., If one uses a double (deep red) layer of Ilford L4 emulsion (d = 5);^{35}S-labeled probes with a specific activity of 1×10^9 dpm/μg DNA at time of emulsion coating; and a 1-wk exposure, then one developed grain would reflect the presence of 5×10^{-13} μg of probe hybridized in the tissue.

Our experiments were not initially designed to give absolute quantitation of mRNA transcripts, and we used the hexamer primer method for labeling DNA probes which does not lend itself easily to an accurate determination of the specific activity of the DNA that actually binds to the tissue. The nature of the random primer method for labeling the probes produces both a nonradioactive template and small strands of labeled DNA in the hybridization mixture with different probabilities of being involved in the hybridization. We estimate that the specific activity of our probes may not be accurate to much better than a factor of ~2–4. However most of the other conditions were met. The sensitivity 'd' value is about eight and five decays, respectively, for a monolayer (purple interference color) and double layer (red interference color) of Ilford L4 emulsion developed with D19 (see Harris and Salpeter, 1980; Salpeter, 1981). Furthermore preliminary unpublished results with a ^{35}S-labeled test specimen suggest that not much self-absorption is expected in a myotube ~10–15 μm thick, as in our dishes. We have also established that the mRNA available for hybridization is saturated by our probe since the same grain density was obtained after using 2 or 4 ng of DNA per hybridization. One important uncertainty is whether any of the mRNA in the cell is hidden from the probe and not available for hybridization. If such mRNA exists and is not uniformly distributed in the tissue, it would of course present a problem for all in situ hybridization studies and not just for absolute quantitation. In any case, any values obtained from autoradiography do not include any such potentially hidden mRNA.

Within these limitations we calculated the average nuclear label separately for each dish and then averaged them. For the exonic probe the average nuclear label from all experiments was ~1.87×10^{-11} μg of probe DNA hybridized to AChE mRNA and ~1.05×10^{-11} μg probe DNA hybridized to AChR α-subunit mRNA. The statistical SEM values combining dishes from all experiments were ~ ±25% and within each experiment were ~ ±10%, indicating that the autoradiographic reproducibility is very good and in principle could give highly reliable results on the amount of probe hybridized in different cellular domains.

Once a value for P is obtained as μg of DNA, then dividing by the weight of the probe (e.g., 1.8 kb for the AChR and 0.79 kb for the AChE exonic probes times 5.42×10^{-16} μg per base) gives the number of probes that hybridized in the tissue, assuming of course, that all the small segments of the probes hybridize fully to the transcript. Since one probe hybridizes to one mRNA transcript, this calculation gives the number of transcripts hybridized in the tissue. We obtained values of 44 AChE and 11 AChR α-subunit transcripts per nuclear label and thus a four-fold higher AChE than AChR α-subunit nuclear label. These average values can be used with Fig. 5 to determine the frequency distribution of transcripts for different nuclei. For the cell as a whole, label in the total nuclear domains is larger than the nuclear label by the additional cytoplasmic labeled rims described above. For the AChR α-subunit, this increases the labeled area and

thus the number of transcripts by 2.7-fold, giving an average of ~30 AChR α-subunit transcripts per nuclear domain. We argued above that for the AChE the number of transcripts per nuclear domain is about four times that over the nucleus itself. Thus the number of AChE transcripts per nuclear domain would be about 176, and thus about six times that for the AChR α-subunit. A comparison of total tissue AChE and AChR α-subunit mRNA was also determined using Northern blot analysis to be described below.

For the AChE intron probe we obtained a value of $2.59 \pm 0.3 \times 10^{-12}$ μg DNA hybridized per nucleus. Since we used a 1.3-kb probe, this means an average of $\sim 3.68 \pm 0.4$ transcripts per nucleus. As indicated above there may be systematic errors that would limit the accuracy of the absolute transcript number obtained. Future studies will have to determine the extent of this limitation. However, assuming that the systematic errors are the same for all the probes, the reproducibility of the autoradiographic grain counts in the different experiments argues that the relative values must be accurate to $\pm 25\%$. We therefore conclude that per nucleus and per nuclear domain the AChE transcripts exceed the AChR α-subunit transcripts by a factor of about four and six, respectively, and that the processed AChE mRNA transcripts exceed the unprocessed transcripts by ~50-fold.

By Northern Blot Analysis. Northern blot analysis in two experiments on mRNA isolated from chick myotubes (Fig. 9), confirmed that our probes hybridized to the AChE transcripts of 4.8 and 6 kb (Gough, N. R., M. Rimer, and W. R. Randall, manuscript in preparation) and the 3.2-kb AChR α-subunit transcript (Moss et al., 1987). When the relative intensity of the autoradiograms of the hybridized blots (see Materials and Methods) in the two experiments were corrected for the differences in size and specific activity of the two probes, the AChE transcripts were found to exceed the AChR transcripts by about three- to sixfold. Thus the Northern blot values are similar to those obtained from our in situ hybridization autoradiographic analysis.

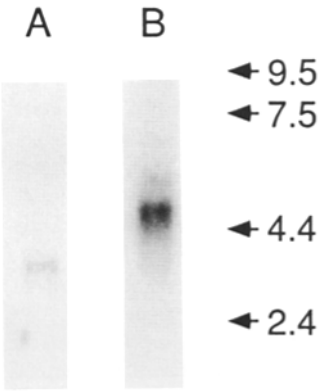

Figure 9. Northern blot analysis of AChE and AChR α-subunit transcripts in chick myotubes. Five micrograms of poly(A)$^+$ RNA prepared from myotube cultures were hybridized with ^{32}P-labeled probe of AChR α-subunit (*A*) and AChE catalytic subunit (*B*) (see Materials and Methods). At least two transcripts (4.8 and 6 kb) are visible with the probe for AChE and one transcript (3.2 kb) labels with the AChR probe. Markers are RNA ladder. The same amount of mRNA was used for each hybridizing probe as determined by absorbance (260) and obtained from the same mRNA preparation. Northern results were confirmed by two independent experiments.

Discussion

This study draws four major conclusions: (*a*) the percentage of nuclei per myotube that expresses AChE is higher than that expressing AChR; (*b*) the frequency distribution of nuclear label is radically different for the two transcripts, although a distinct overlap of the two populations must exist with many of the nuclei transcribing AChR α-subunit constituting a subset of the nuclei transcribing AChE; (*c*) there are about six times more AChE than AChR α-subunit mRNA transcripts in the cell per nuclear domain, and the total AChE mature transcripts exceed the unprocessed nuclear transcripts by ~50-fold; and (*d*) the cytoplasmic distributions of the mature transcripts differ for the two genes, with AChE transcripts having a broader distribution around active nuclei (i.e., a larger nuclear domain) than AChR α-subunit transcripts.

We should first consider the nature of the mRNA hybridized by our AChE probe. Although AChE is found in muscle in several molecular forms, all are products of a single gene (Schumacher et al., 1988; Sikorav et al., 1988; Rotundo et al., 1988; Maulet et al., 1990). The enzyme is assembled into multimeric catalytic subunits consisting of dimers and tetramers. The asymmetric form may be anchored to basal lamina components through a disulfide-bonded collagen-like peptide (Taylor et al., 1987; Krejci et al., 1991). The AChE subunits of the globular forms can also associate with the plasma membrane through either a disulfide-linked small membrane inserted peptide (Inestrosa et al., 1987; Roberts et al., 1991) or through a covalently attached glycophospholipid anchor (Futerman et al., 1985; Maulet et al., 1990). Recent studies on *Torpedo* electric organ AChE have shown that the catalytic subunits forming the glycophospholipid-anchored form and those of the peptide-anchored forms arise from alternative splicing of the exons encoding the carboxyl terminus of the catalytic subunits (Sikarov et al., 1988; Maulet et al., 1990). There is at present no indication that these glycophospholipid-anchored forms exist in the chicken. However, current information regarding chick muscle AChE indicates that both globular and asymmetric forms of AChE are present in muscle cultures taken from 11-day chick embryos (Massoulié et al., 1984), as used here, and that they colocalize with AChR at high density clusters in myotubes (Wallace, 1986) and at neuromuscular junctions (Massoulié and Bon, 1982). Since the exon probe for AChE used in the present study encodes a region common to the catalytic subunits of all known forms of the enzyme, it should have detected all AChE transcripts expressed in the myotubes and not distinguished between them.

When comparing the relative number of the AChR α-subunit with that of the AChE mRNAs we should keep in mind that the AChR molecule is a pentameric structure with subunits in stoichiometry $\alpha_2\beta\gamma\delta$ and that the α subunit represents only two fifths of the molecular structure of the AChR molecule. The relative abundance of the mRNA for the other AChR subunits is not known, although it has been suggested that in the chick the α subunit mRNA may be the least abundant (Harris et al., 1988), Thus, the amount of mRNA encoding the total AChR molecule could be closer to that for the AChE than is apparent from only the α subunit data. A similar study for the other subunits would resolve this issue.

Values for the number of AChE and AChR α-subunits hybridized in our tissue is not intended to claim full quantitation of cellular transcripts. As stated in Results, we recognize that it may represent an underestimation of the actual mRNA in the cell and be subject to other systematic errors which

could be as large as two- to fourfold. We are gratified however by the surprisingly high reproducibility in the quantitative results (i.e., to ~ ±25% between experiments and ~ ±10% between the dishes of the same experiment) making the relative values considerably more accurate than the absolute values. It is also noteworthy that using the very different technique of nuclease protection, Harris et al. (1988) estimate ~40 AChR α-subunit mRNA transcripts in chick myotube per nuclear domain, which is very close to the value of 30 mRNA transcripts obtained by us. There is at present no nuclease protection data for the AChE transcripts. The similarity in our AChR α-subunit values with those from the nuclease protection assay leads us to be optimistic that the in situ hybridization technique may not be missing many AChR α-subunit mRNA transcripts in the cell.

We confirm and extend the finding of Harris et al. (1989) that there is a skewed distribution of nuclei active in transcribing AChR α-subunit mRNA in cultured chick myotubes. More than one third of the nuclei have little or no label and ~10% have >2.5 times the mean value. The result for the AChE is dramatically different. With both intronic and exonic probes, nuclear label due to AChE mRNA was distributed in near Gaussian fashion although with a slight skewness in the direction of the heavily labeled range. Thus, in the multinucleated myotubes, even though the number of labeled nuclei suggests that there must be considerable overlap in the nuclei transcribing both AChE and AChR, the distribution of label indicates that nuclei are not likely to express both AChR α-subunit and AChE transcripts to the same extent. Our conclusion that in cultured chick myotubes the two species of mRNA are expressed by an overlapping but not fully coincident set of nuclei is consistent with that derived from the study of Porter-Jordan et al. (1986). These latter authors used a density shift technique, and demonstrated that 70–90% of the coated vesicles contain at least one or more molecules of AChE, but only ~1 in 60 contained AChR.

Our results on the extent of the cytoplasmic label due to exonic AChR α-subunit mRNA differ from those of Bursztajn et al. (1989) who reported that these transcripts are randomly distributed in the cytoplasm of chick myotubes. Instead we find a sharp grain density gradient around active nuclei, again as previously reported for chick and rodent muscle (Harris et al., 1989; Horovitz et al., 1989). Our estimates from resolution studies indicate that the AChR α-subunit nuclear domain extends into the cytoplasm for ~3–4 μm around the nucleus. The distribution of the AChE mRNA was less sharply localized around active nuclei. We calculate from data as in Fig. 6 *B* that the AChE nuclear domain, with its ~9–10-μm-thick cytoplasmic rim around the nucleus, which, however, in many cases could overlap that of adjacent nuclei. Rotundo (1990), analyzed the assembly of AChE in quail myotube cultures and concluded that AChE oligomers in muscle fibers are preferentially translated and assembled in the vicinity of the nucleus of origin, with a limited diffusion of the mRNA encoding the AChE proteins. Thus, a specific domain of AChE mRNA exists in cultured myotubes, presumably associated with RER membranes. Our estimate that this domain can be ~9–10 μm around a nucleus argues that the extent of the AChE domain overlaps but is larger than that of the AChR α-subunit. This is noteworthy since both the AChR α-subunit and AChE transcripts are translated on membrane bound ribosomes and are destined for delivery to the surface of the cell, where they are both diffusely localized as well as localize in high density clusters. They could thus have been expected to distribute within the perinuclear regions in a similar manner. Since in cultured muscle cells RER membranes may not be restricted to regions near a nucleus but are diffusely distributed throughout the cell (Horovitz et al., 1989) there is no a priori reason why AChR and AChE need to be translated in the same location on the RER.

The difference in cellular distribution of the two transcripts may reflect a different diffusion, or turnover rate of the two mRNA transcripts. Alternatively (or in addition), it may reflect different locations of the RER ribosomes with which the two mRNA transcripts are associated. Whatever the reason for the broader distribution of the AChE transcripts, the simplest assumption is that their location represents their site of translation. We cannot exclude the possibility that not all transcripts are equally translated and only those transcripts near the nuclei or those overlapping the AChR domains are active. Our study is not able to address that possibility but it would raise interesting questions about the regulation of the process of translation.

An unanswered question is whether there is any selective insertion of AChR and AChE molecules into myotube surface clusters, and whether, if it exists, such insertion is in the vicinity of the nuclei expressing these proteins. More information is available on how AChRs form clusters than on how AChE molecules do. It has been well established that AChR clusters form by a redistribution of AChRs that are preexisting all over the muscle membrane (reviewed in Salpeter, 1987) and does not need new protein synthesis (Wallace, 1988). Yet, both preferential insertion (Bursztajn and Fischbach, 1984; Role et al., 1985) and preferential association of muscle nuclei with AChR clusters have been reported (Englander and Rubin, 1987). No similar information is available for AChE. We also have unpublished results which show no change in the number and frequency distribution of nuclear label for either AChE or AChR α-subunit mRNA after cells were treated with agrin, a molecule that causes a drastic redistribution and increase in AChR/AChE clusters (Wallace, 1986). We suggest that in cultured myotubes a colocalization of AChR and AChE at AChR/AChE clusters is a posttranslational event. This is most likely regulated by cluster-inducing molecules and probably involves a different regulation for AChE than for AChR molecules. However some copackaging of AChR and AChE proteins in Golgi vesicles for local insertion at preformed clusters cannot be ruled out.

Selective insertion has been more convincingly suggested at the neuromuscular junction in innervated vertebrate muscle where there exists a high concentration of AChR, AChE, and other synaptic specialized molecules. It is believed that AChR transcription is largely limited to the sole-plate nuclei of the adult neuromuscular junction, and that the high AChR concentration is maintained by preferential insertion of newly synthesized receptors under or near the postsynaptic membrane. These suggestions are strengthened by the finding that there is a higher level of AChR α-subunit mRNA in subsynaptic cytoplasm (Merlie and Sanes, 1985; Fontaine et al., 1988; Goldman and Staple, 1989; Klarsfield et al., 1991; Witzemann et al., 1991) than in extrajunctional cytoplasm. Thus, it is reasonable to believe that local inser-

tion may be the mechanism for maintaining the AChR at the neuromuscular junction. The evidence for AChE is not yet as firm. However, preliminary in situ hybridization results from our laboratory indicate that there is an accumulation of AChE mRNA as well as AChR α-subunit mRNA at the chick neuromuscular junction. This is consistent with the conclusion from polymerase chain reaction analysis (Lee, R. K., B. J. Jasmin, and R. L. Rotundo. 1991. *Soc. Neurosci.* 946(Abstr.)) that there is a preferential distribution of AChE mRNA at the junction. It therefore appears that, during development, cues associated with the formation of the neuromuscular junction may change the pattern of expression of the nuclei involved in both AChR and AChE transcription. What these factors are, remains to be determined.

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