

# Intracellular and Surface Distribution of a Membrane Protein (CD8) Derived from a Single Nucleus in Multinucleated Myotubes

Evelyn Ralston and Zach W. Hall

Department of Physiology, School of Medicine, University of California, San Francisco, San Francisco, California 94143-0444

**Abstract.** We have investigated the contribution of an individual nucleus to intracellular and surface membranes in multinucleated muscle fibers. Using a retroviral vector, we introduced the gene encoding the human T-lymphocyte antigen CD8 into C2 mouse muscle cells to form a stable line expressing the human protein on its surface. The intracellular and surface distributions of the protein were then investigated by immunocytochemistry in hybrid myotubes contain-

ing a single nucleus expressing CD8. We show that the intracellular distribution of CD8 is limited to a local area surrounding the nucleus encoding it and several neighboring nuclei. On the cell surface, however, the protein is distributed over the entire myotube. Widespread distribution of a surface membrane protein in multinucleated myotubes can thus result from localized synthesis and processing.

**M**ANY membrane proteins are not distributed uniformly on the cell surface, but are restricted to structurally and functionally distinct domains. In adult muscle fibers, for example, >90% of the acetylcholine receptor (AChR)<sup>1</sup> is found in the postsynaptic membrane of the neuromuscular junction, which constitutes <0.1% of the total muscle cell surface (Salpeter, 1987). Recent experiments suggest that in muscle fibers, which contain hundreds of nuclei, differential gene expression among nuclei may contribute to the restricted distribution of the AChR. Thus both Northern blot analysis and *in situ* hybridization demonstrate that AChR subunit mRNA is concentrated near endplates of adult rat and chicken muscle fibers (Merlie and Sanes, 1985; Fontaine et al., 1988; Fontaine and Changeux, 1989).

One implication of these experiments is that surface membrane proteins are synthesized and processed near the nuclei that are the source of their mRNA, an idea that is supported by several recent observations (Pavlath et al., 1989; Ralston and Hall, 1989; Rotundo, 1989). A restricted surface distribution could then result: (a) if protein were inserted into the membrane locally; and (b) if movement of the protein were subsequently constrained, either by intrinsic barriers to diffusion in the membrane (Pavlath et al., 1989), or by attachment to the cytoskeleton or extracellular matrix.

To compare the intracellular and surface distributions of a well-defined membrane protein produced from a single nucleus in muscle cells, we introduced the gene encoding the human T-lymphocyte antigen, CD8, into the C2 muscle cell

line. CD8 is a 34-kD protein containing a large extracellular domain with immunoglobulin homology, a single hydrophobic transmembrane segment, and a short, highly charged cytoplasmic sequence (Littman et al., 1985; Littman, 1987). After infecting C2 muscle cells with a retroviral vector encoding CD8, we selected stable lines expressing the protein and characterized the synthesis, processing, and transport to the surface of CD8 in these lines. Hybrid myotubes were then formed with the infected and parental cells using cell ratios such that only one or a few nuclei per myotube expressed CD8. Labeling of these nuclei with [<sup>3</sup>H]thymidine allowed their identification by autoradiography so that the intracellular and surface distributions of the protein derived from a single identified nucleus could be determined by immunocytochemistry. Our results show that within hybrid C2 myotubes, CD8 is restricted to a region near the nucleus that encodes it, but that the protein is widely distributed on the surface. Localized synthesis and insertion of a membrane protein is thus not sufficient to produce a restricted surface distribution in myotubes.

## Materials and Methods

### Antibodies

Hybridoma cells producing the mouse monoclonal antibody OKT8 (Hoffman et al., 1980) were obtained from the American Type Culture Collection (Rockville, MD), and antibodies obtained from ascites fluid in CAF1 mice by standard procedures.

### Cell Culture

Cells of the C2C12 subclone of the C2 mouse muscle cell line (Yaffe and Saxel, 1977) were maintained as described in Ralston and Hall (1989).

1. *Abbreviations used in this paper:* AChR, acetylcholine receptor; GM, growth medium.

## Infection of C2 Cells with Retroviral Vector

Supernatant from cultures of PA12 cells (an amphotropic retrovirus packaging cell line) that had been transfected with the plasmid pMV7-CD8 was a generous gift of Dan Littman (Department of Microbiology and Immunology, University of California, San Francisco). pMV7-CD8 (Maddon et al., 1986) is derived from cloning the human CD8 gene (Littman et al., 1985) into the retroviral vector pMV7 (Kirschmeier et al., 1988). It also contains the gene for neomycin phosphotransferase, which makes mammalian cells resistant to the antibiotic G418. For infection, C2 myoblasts in growth medium (GM) were plated on 10-cm culture dishes at a density of  $3 \times 10^5$  cells/dish. 24 h later, the frozen viral supernatant was thawed, cleared by spinning for 10 min at 1,500 g, diluted in GM containing 4  $\mu$ g/ml polybrene (Aldrich Chemical Co., Milwaukee, WI) and added to the cells. After 6 h, the cells were rinsed twice with PBS and fed with GM. 2 d later the cells were split and replated at a density of  $3 \times 10^5$  cells/10 cm dish in GM supplemented with 600  $\mu$ g/ml active G418 (Gibco Laboratories, Grand Island, NY). Thereafter, fresh medium was added every second day. After 6 d, the concentration of G418 was reduced to 400  $\mu$ g/ml. Colonies became visible about a week after the infection and were assayed a few days later for surface expression of CD8 by a red blood cell rosetting assay (see below).

Since supernatants from the retroviral packaging cell line PA12 may contain helper virus (Miller and Buttimore, 1986), we tested selected cell lines and found helper virus activity in four out of five. The cell line that was free of helper activity was retained for our experiments.

## Red Blood Cell Rosetting

The dishes with G418-resistant colonies were washed with PBS, covered with 5 ml of a 1:1,000 dilution of OKT8 in PBS-5% FCS and left at room temperature for 1 h. They were washed with PBS. They were then incubated with a 0.4% suspension in PBS-5% FCS of red blood cells to which goat anti-mouse IgG had been coupled by the CrCl<sub>3</sub> method (Galfrè and Milstein, 1981). After 30 to 45 min, the plates were shaken by a mild lateral blow, rinsed with PBS, and examined under a tissue culture microscope.

## Surface Iodination of C2 Cells with Lactoperoxidase

Cells were iodinated according to the general procedures of Forsayeth et al. (1986). C2 cultures grown in 75 cm<sup>2</sup> flasks were washed three times with PBS and incubated in 10 ml PBS containing 0.02 M D-glucose, 0.1 mg/ml lactoperoxidase (Boehringer Mannheim Diagnostics, Inc., Houston, TX), .375 mg/ml glucose oxidase, and 2.5 mCi Na<sup>125</sup>I (Amersham Corp., Arlington Heights, IL). After 30 min at room temperature, the cells were washed 3 times with PBS and extracted with 750  $\mu$ l of 10 mM Tris (pH 7.4), 150 mM NaCl, 0.5% NP-40, 1 mM PMSF.

## Metabolic Labeling with [<sup>35</sup>S] Amino Acids

C2 cultures grown on 6-cm dishes were incubated for 15 min at 37°C in methionine- and cysteine-free DME H-16 (CMF) and subsequently labeled with 0.2 mCi/ml [<sup>35</sup>S]methionine (Tran-<sup>35</sup>S-label, ICN) in 1.2 ml of cysteine methionine-free. The cells were then washed once with complete medium supplemented with 2 mg/ml each of methionine and cysteine and incubated further in the same medium. For experiments without chase, cells were washed three times with cold PBS with 2 mg/ml cysteine and methionine. All cells were extracted with 500  $\mu$ l of 10 mM Tris (pH 7.4), 66 mM EDTA, 1% NP-40, 0.4% DOC, and 1 mM PMSF (NDET buffer).

For neuraminidase treatment, the cells were labeled for 15 min, washed as described above, and incubated for 1 h at 37°C. The pH of the medium was lowered to 6.0 by addition of morpholino-ethanesulfonic acid buffer (20 mM final concentration). One unit (2 mg) of type V neuraminidase (Sigma Chemical Co., St. Louis, MO) was added and the cells returned to 37°C for 1 h, then washed and extracted in NDET.

## Immunoprecipitations

Cell extracts were centrifuged for 5 min in a centrifuge (Eppendorf made by Brinkmann Instruments, Inc., Westbury, NY) at maximum speed. The supernatants were then incubated with OKT8 and SDS (to a final concentration of 0.2%) overnight at 4°C on a rotary shaker. After addition of 40  $\mu$ l of a 10% suspension of *Staphylococcus aureus* (Pansorbin; Calbiochem-Behring Corp., San Diego, CA) and incubation on a shaker at room temperature for 40 min, the Pansorbin suspension was layered on 900  $\mu$ l of 35% sucrose in half-strength NDET buffer with SDS (NDETS) in a tube (Eppendorf made by Brinkmann Instruments, Inc.). After centrifugation, the pellet

was washed with NDETS followed by distilled water. The pellet was then resuspended in 60  $\mu$ l sample buffer, heated for 3 min in a boiling water bath, and centrifuged. The supernatant was analyzed by electrophoresis on a 9% SDS polyacrylamide gel according to Laemmli (1970) using molecular mass standards from Bio-Rad Laboratories (Cambridge, MA). After electrophoresis, the gel was stained with Coomassie blue, enhanced for 15 min at room temperature with 1 M sodium salicylate, dried, and exposed to preflashed film (XRomat; Eastman Kodak Co., Rochester, NY).

## Formation of Hybrid Myotubes

Parental C2 cells and C2-CD8 cells were plated on separate 10-cm dishes at a density of  $7.5 \times 10^4$  cells/dish. On the next day, fresh growth medium was added and the C2-CD8 cells were supplemented with [<sup>3</sup>H]thymidine (6.7 Ci/mmol; ICN Radiochemicals, Irvine, CA) at a final concentration of 0.05  $\mu$ Ci/ml. 48 h later, the cells were trypsinized and plated together at a density of 60,000 cells/cm<sup>2</sup> in the wells of duplicate 4-well slides (Lab-Tek, Nunc Inc., Naperville, IL), pretreated with a 1:5 dilution of Vitrogen (Collagen Corp., Palo Alto, CA) in water. After 24 h, fusion medium was added, followed by fusion medium supplemented with cytosine arabinoside 24 h later to kill residual unfused myoblasts. 1-2 d later the cells were processed for immunocytochemistry.

## Immunocytochemistry

**Surface Staining of Live Cells.** Cells were grown on small glass coverslips in 24-well tissue culture dishes or on 4-well slides (Lab-Tek). OKT8 was added to a final dilution of 1:800. After 1 h at 37°C, the cells were washed with tissue culture medium, and incubated with fresh medium containing fluorescein (FITC)-conjugated goat anti-mouse (Cappel Laboratories, Malvern, PA) at 37°C for 1 h. The cells were then washed with PBS, fixed for 15 min with 2% paraformaldehyde, rinsed in PBS, stained with 2.5  $\mu$ g/ml bis-benzimide (Hoechst 33258, Sigma Chemical Co.) in distilled water for a few minutes and mounted in glycerol supplemented with paraphenylenediamine (Platt and Michael, 1983).

**Staining of Fixed, Permeabilized Cells.** Cells were stained as described in Ralston and Hall (1989).

**Autoradiography.** After the staining was completed, the slides were dried on a heating plate at low setting, dipped in autoradiographic emulsion (K5; Ilford, Moberley, UK), and exposed for 12-14 d at 4°C. They were then developed (D-19; Eastman Kodak Co.), fixed, and mounted in glycerol with paraphenylenediamine.

## Results

### Isolation of C2 Cells Expressing CD8

C2 myoblasts were infected with the retroviral vector pMV7-CD8 (Maddon et al., 1986) which contains the gene for the human T-cell lymphocyte antigen CD8 as well as the neomycin resistance gene. >95% of the G418-resistant colonies obtained after infection expressed CD8 on their surface as determined by a red blood cell rosetting assay (see Materials and Methods). 25 of the positive colonies were expanded and analyzed by immunofluorescence using monoclonal antibody OKT8 directed against CD8 (Hoffman et al., 1980). The five lines with highest expression for surface CD8 were tested further. Fluorescence microscopy and analysis by the fluorescence-activated cell sorter showed that virtually all cells in each of these lines express CD8 on their surface. Each of the five selected lines was compared with the parental line with respect to growth rate, time course of myoblast fusion, and expression and spontaneous clustering of the AChR. No differences were observed beyond those normally seen between subclones of the parental C2 line. One of the lines, termed C2-CD8, was chosen for further experiments (see Materials and Methods).

## Assembly and Intracellular Transport of CD8 in C2-CD8 Cells

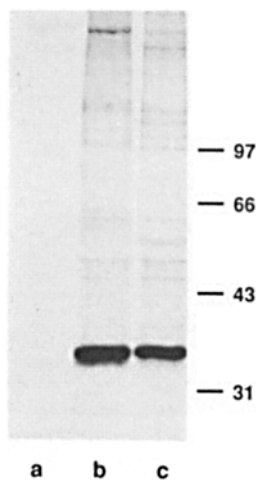
We then characterized the CD8 protein synthesized in C2 cells and compared its properties to those reported for the protein made in lymphocytes. We also characterized the intracellular processing of CD8 and its transport to the surface as such studies have not been previously described.

Myoblast and myotube cultures of C2-CD8 were incubated with  $^{35}\text{S}$ -labeled amino acids, and extracts of the cells were immunoprecipitated with OKT8. In both cases, a single band of apparent molecular mass 33 kD was observed (Fig. 1), close to the value of 34 kD obtained with CD8 immunoprecipitated from human peripheral blood lymphocytes (Snow and Terhorst, 1983). CD8 extracted from C2-CD8 cells was cleaved by neuraminidase but not by endoglycosidase F (data not shown), suggesting that in C2 cells, as in lymphocytes, CD8 has O-linked, but not N-linked, carbohydrates (Snow et al., 1984, 1985; Littman, 1987).

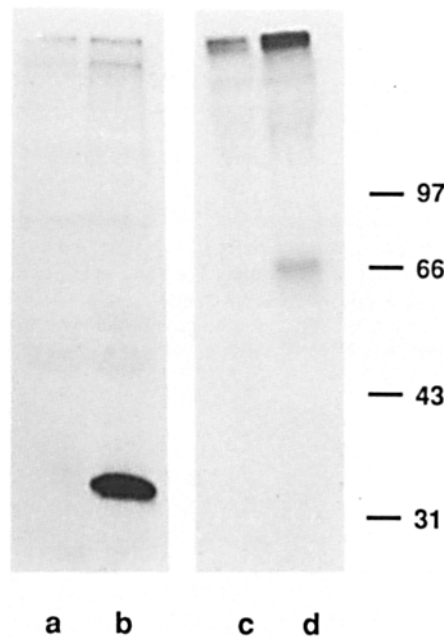
To investigate the structure of CD8 in the plasma membrane of C2-CD8 cells, we radioiodinated the surface proteins of C2-CD8 myoblast and myotube cultures, immunoprecipitated membrane protein extracts and analyzed the precipitates on reducing and nonreducing gels (Fig. 2). Although a single band of 33 kD was observed in reducing gels, this band was not present in nonreducing gels. Rather, a band of 66 kD was seen, along with a large amount of high molecular mass material that did not penetrate the gel. CD8 expressed in C2 cells thus assembles into dimers and higher molecular mass complexes, as it does in human lymphocytes (Snow and Terhorst, 1983).

Processing of CD8 in C2-CD8 cells was examined in pulse-chase labeling experiments with  $^{35}\text{S}$  amino acids. After a 5-min pulse, newly synthesized CD8 migrated as a 24-kD band (Fig. 3), in good agreement with the molecular weight of 23,550 predicted by cDNA sequencing (Littman et al., 1985). At 10 min, a 27-kD band appeared that was subsequently rapidly chased into the mature 33-kD protein. By 15 min, all of the newly synthesized protein had been processed to the mature form.

The time course of transport to the surface was investigated using the susceptibility of CD8 on the cell surface to cleavage by trypsin (Snow et al., 1985). Cells in myoblast and myotube cultures were trypsinized at various times after



**Figure 1.** Immunoprecipitation of CD8 from C2-CD8 cultures labeled with  $^{35}\text{S}$  amino acids. Cultures of control C2 myoblasts (lane a) and of C2-CD8 myoblasts (lane b) and C2-CD8 myotubes (lane c) were labeled for 4 h with 0.5 mCi/ml Tran- $^{35}\text{S}$ -label. Cell extracts prepared as described in Materials and Methods were immunoprecipitated with monoclonal antibody OKT8 and analyzed by SDS-PAGE.

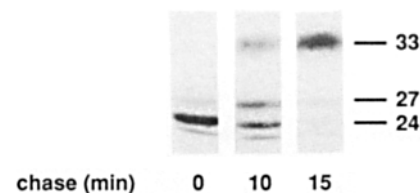


**Figure 2.** Analysis of CD8 expressed on the surface of C2-CD8 cells. Cell surface proteins of control C2 myoblasts (lanes a and c) and of C2-CD8 myoblasts (lanes b and d) were radioiodinated (see Materials and Methods), extracted, and immunoprecipitated with OKT8. The precipitates were divided into two aliquots. One was resuspended in sample buffer containing 5% DTT as reducing agent (lanes a and b), and the other in sample buffer without reducing agent (lanes c and d). Samples were run on a 9% SDS-polyacrylamide gel.

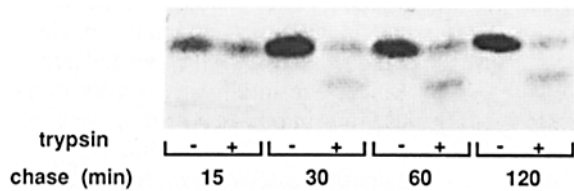
a 5-min pulse-label with  $^{35}\text{S}$  amino acids and the immunoprecipitated CD8 compared with immunoprecipitates from nontrypsinized cells taken at the same time (Fig. 4). Newly synthesized CD8 first became susceptible at 30 min after the pulse, when trypsin caused a decrease in the intensity of the 33-kD band with the concomitant appearance of a lower molecular mass band. Not all of the CD8 was cleaved, even after 2 h of chase, indicating that some of the protein does not reach the surface. From 2 to 6 h, the level of surface CD8 remained constant (data not shown).

## Localization of CD8 in C2-CD8 Cells

The surface and intracellular distribution of CD8 in C2-CD8 was examined by indirect immunofluorescence (Fig. 5). When intact, unfixed cultures (Fig. 5, a and b) were stained to visualize the surface distribution, bright patches of CD8 were seen. These were uniformly distributed over the entire



**Figure 3.** Posttranslational processing of CD8 in C2-CD8 cells. Cultures of C2-CD8 myoblasts were labeled with a 5-min pulse of Tran- $^{35}\text{S}$ -label, chased for the time indicated, extracted, immunoprecipitated with OKT8, and analyzed on a 9% SDS-polyacrylamide gel.



**Figure 4.** Time course of CD8 appearance on the cell surface. Cultures of C2-CD8 myoblasts were labeled with a 5-min pulse of Tran-<sup>35</sup>S-label and chased for various times. For each time point, two dishes were extracted, one of them after treatment with 100  $\mu$ g/ml trypsin for 30 min at 4°C. The extracts were immunoprecipitated with OKT8 and analyzed by SDS-PAGE on a 9% gel.

surface of both myoblasts (Fig. 5 *a*) and myotubes (Fig. 5 *b*). Capping (formation of a single aggregate) of the antigen was occasionally observed shortly after plating myoblasts at low density, but was otherwise not observed.

When cells were fixed and permeabilized to reveal the intracellular distribution of CD8 (Fig. 5, *c* and *d*) the dominant pattern of staining was similar to that seen in muscle cells with Golgi markers (Tassin et al., 1985; Miller et al., 1988; Gu et al., 1989). Thus, in myoblasts (Fig. 5 *c*), a coarsely granular staining was seen at one pole of the nucleus, and in myotubes (Fig. 5 *d*) a discontinuous, but nonpolar, perinuclear staining was observed, coupled with a coarsely gran-

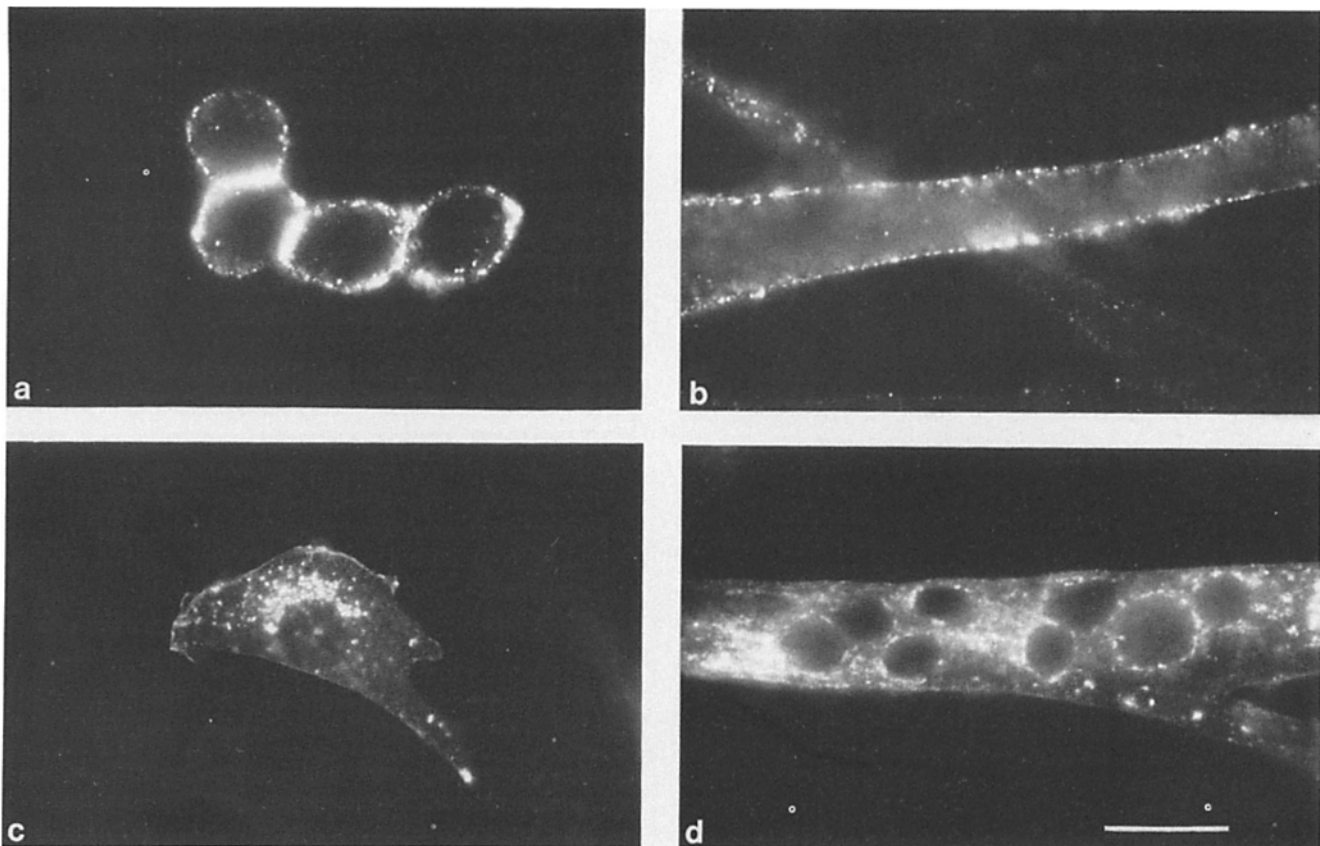
ular pattern between nuclei. In addition to the Golgi pattern, a fine-grained staining was observed that appeared in linear arrays aligned with the myotube axis (data not shown).

To determine if CD8 seen by intracellular staining represents precursor to cell surface CD8, we treated cultures of C2-CD8 myotubes for 2 h with cycloheximide (0.4 mM) before fixation and subsequent staining. Such treatment essentially abolished the Golgi-like staining, but did not affect the linear arrays of fine-grained staining (not shown). Thus, the Golgi staining seen in untreated cells presumably corresponds to CD8 destined for the surface. Similar results were obtained with myoblast cultures.

#### *Localization of CD8 in Hybrid Myotubes*

Hybrid myotubes were formed using mixtures of myoblasts from the parental C2 line and from C2-CD8. The C2-CD8 cells were incubated with [<sup>3</sup>H]thymidine before plating with C2 myoblasts to label their nuclei. We have shown previously that >98% of nuclei are labeled under the conditions used (Ralston and Hall, 1989). Autoradiography of hybrid myotubes formed using various ratios of C2 to C2-CD8 myoblasts showed that myotubes containing a single C2-CD8 nucleus could be obtained when ratios of 20:1 or 50:1 were used.

When such hybrid myotube cultures were stained, autoradiographed and examined for immunofluorescence, CD8 was seen to be distributed over the entire surface of the my-



**Figure 5.** Surface and intracellular localization of CD8 in C2-CD8 cells. OKT8, followed by FITC-conjugated goat anti-mouse (GaM) was used to stain cultures of C2-CD8 myoblasts (*a* and *c*) or myotubes (*b* and *d*). Either intact cells (*a* and *b*) or cells that had been fixed and permeabilized (*c* and *d*) were stained. Bar, 20  $\mu$ m.

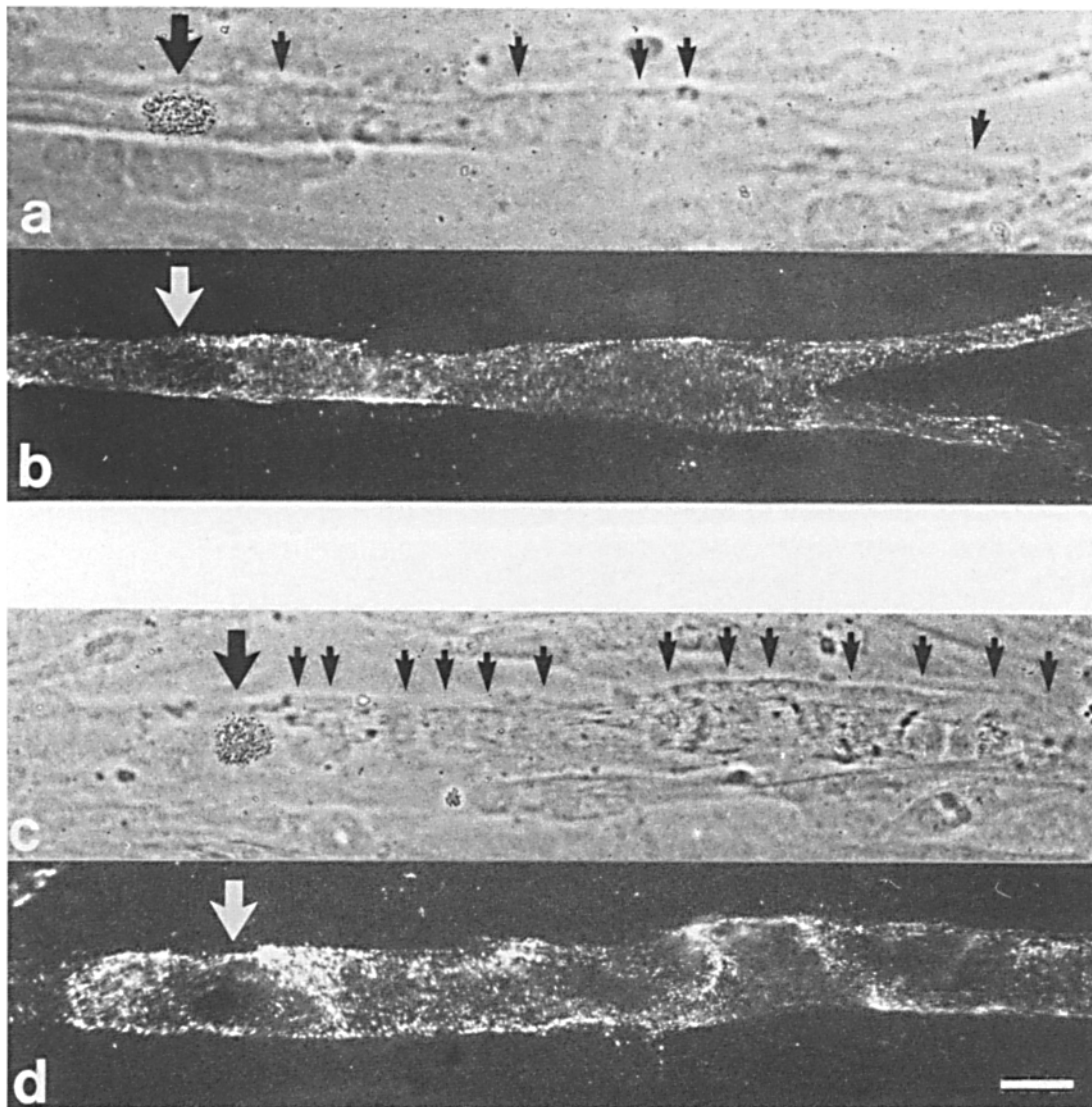
otubes, without respect to the position of the nucleus encoding CD8 (the source nucleus) (Fig. 6). The diffuse localization of CD8 was independent of myotube size and of C2:C2-CD8 ratio over the range 1:1-50:1.

Occasionally, local aggregates of CD8 were seen. In cultures that were also stained with rhodamine-conjugated  $\alpha$ -bungarotoxin, the aggregates of CD8 were invariably found to correspond to clusters of AChR (Fig. 7). When such aggregates were observed on the surface of hybrid myotubes, they were not preferentially localized in close proximity to the source nucleus.

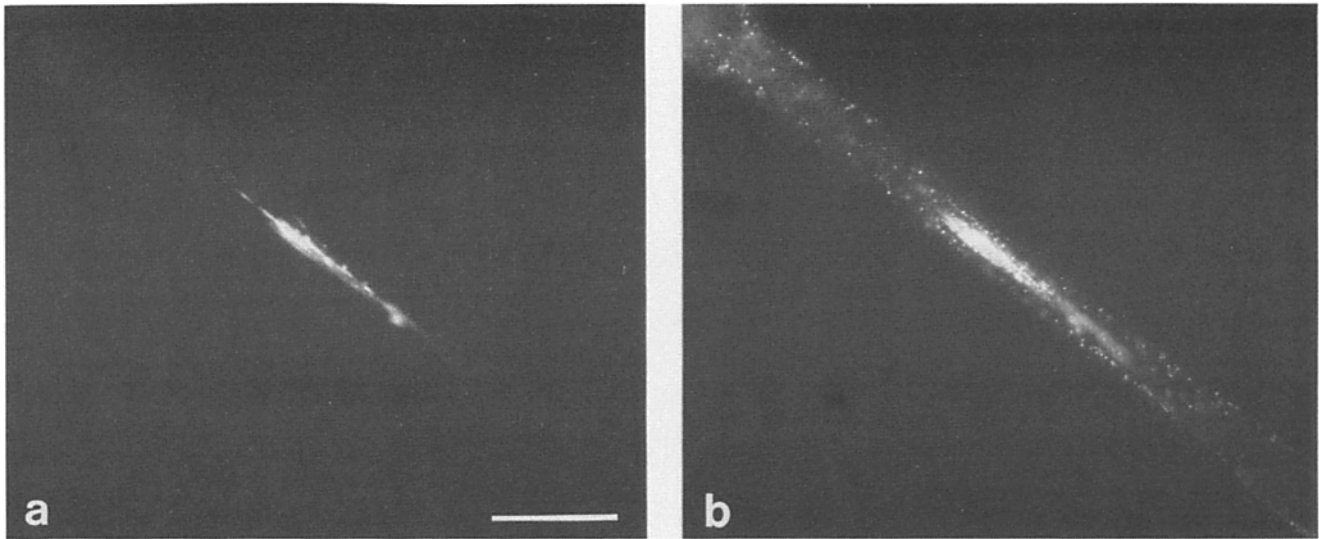
To determine the intracellular distribution of CD8, cultures were permeabilized before staining and subsequent autoradiography. In this case a Golgi-like pattern of immunofluorescence was seen near the source nucleus (Fig. 8). In

myotubes containing a single source nucleus, immunofluorescence was not confined to the region around this nucleus, but generally extended over a region containing several nuclei. In some cases these nuclei were in close proximity to the source nucleus (Fig. 8 *b*), but in other cases they were one or several nuclear diameters away (Fig. 8, *a* and *b*).

To express these results quantitatively, we measured the range of CD8 staining, both within and at the surface of hybrid myotubes containing a single [ $^3$ H]-positive nucleus. For surface staining, we measured 16 myotubes, whose length averaged  $420 \pm 119$  (SD)  $\mu$ m. The average extent of CD8 staining in these myotubes was  $416 \pm 118$   $\mu$ m. Thus,  $99 \pm 3\%$  of the myotube length ( $\sim 42$  nuclear diameters) was covered with CD8. By contrast, the extent of intracellular staining, measured on 11 myotubes, ranging from 217 to 605  $\mu$ m



**Figure 6.** Surface localization of CD8 in hybrid myotubes. Hybrid myotubes were formed by plating together C2 myoblasts and [ $^3$ H]thymidine-labeled C2-CD8 myoblasts in a 20:1 ratio. Intact myotubes were incubated with OKT8 followed by FITC-GaM, fixed, stained with Hoechst 33258, and processed for autoradiography. Two examples of hybrid myotubes are shown. Each one contains a single [ $^3$ H]-positive nucleus (*large arrow*), which is clearly identified by the autoradiographic grains in phase optics (*a* and *c*). The other nuclei in the same myotube were identified by observing the field with the filter appropriate for Hoechst (not shown). We have indicated the position of these nuclei on the figure by small arrows. The same field, examined in FITC optics (*b* and *d*) shows the surface distribution of CD8. Bar, 20  $\mu$ m.



**Figure 7.** Occasional clusters of CD8 are colocalized with sites of AChR clustering. Intact C2-CD8 myotubes were double-stained with rhodamine-conjugated  $\alpha$ -bungarotoxin to detect AChR and with OKT8 followed by FITC-GaM. (a) rhodamine optics; (b) FITC optics. Bar, 20  $\mu$ m.

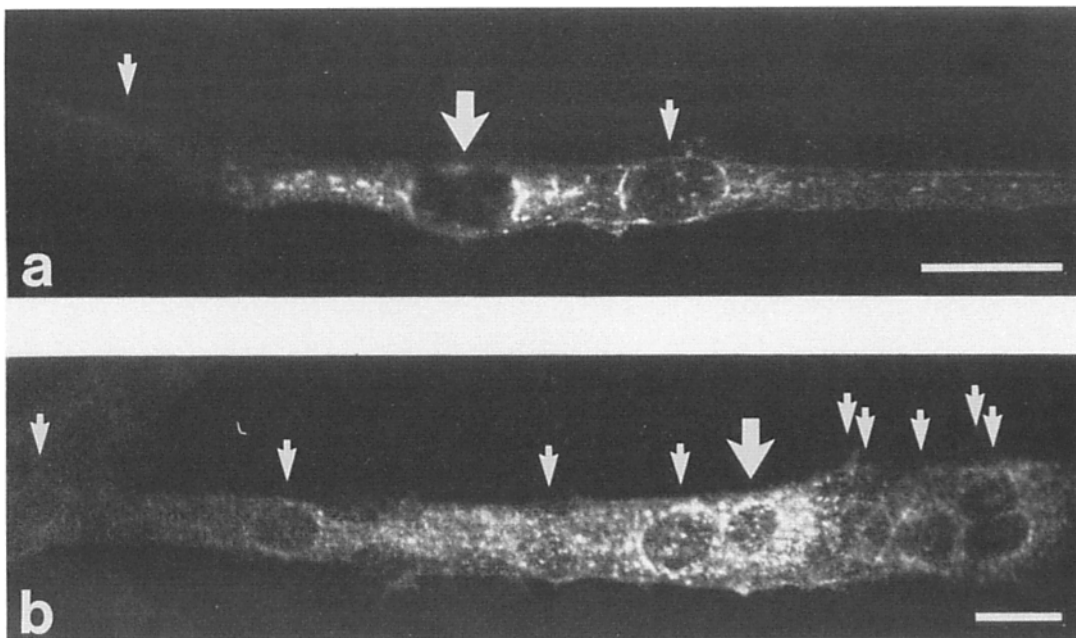
in length (average  $365 \pm 131 \mu\text{m}$ ), was  $68 \pm 31 \mu\text{m}$ , representing  $20 \pm 13\%$  of the total length. In hybrid myotubes, CD8 encoded by a single nucleus thus occupies a restricted intracellular region, but after insertion into the membrane covers virtually the entire cell surface.

### Discussion

The major aim of this work was to examine CD8 distribution in hybrid myotubes containing a single nucleus expressing

the foreign gene. Our experiments yielded two results: (a) that CD8 has a restricted intracellular distribution that encompasses the area around the source nucleus as well as several neighboring nuclei; and (b) that CD8 has an unrestricted distribution on the cell surface. From this we conclude that membrane proteins that are synthesized and processed locally can occupy the entire muscle cell surface after they are inserted into the surface membrane.

The conclusion that CD8 is synthesized and processed locally is based on the pattern of immunocytochemical staining



**Figure 8.** Intracellular localization of CD8 in hybrid myotubes. Hybrid myotubes, formed as described in the legend to Fig. 6, were fixed, permeabilized, stained with OKT8, FITC-GaM, and Hoechst 33258, and processed for autoradiography. Two examples (a and b) are shown under FITC optics to reveal the intracellular distribution of CD8. In each case, the single nucleus labeled with [ $^3\text{H}$ ]thymidine (identified under phase optics as in Fig. 6) is indicated by a large arrow, whereas the other nuclei in the field (identified by Hoechst staining) are indicated by small arrows.

around the source nucleus in hybrid myotubes. The major component of this staining appears to be the Golgi apparatus, although there is also a fine-grained component that may represent the endoplasmic reticulum or lysosomes. Metabolic labeling experiments demonstrated that CD8 undergoes extensive intracellular processing after synthesis (Fig. 3), part of which presumably occurs in the Golgi apparatus. The protein synthesized by muscle cells appears to be O-glycosylated, as in lymphocytes, and O-glycosylation has been thought to take place in the Golgi (Hanover et al., 1982; Abeijon and Hirschberg, 1987; see, however, Pathak et al., 1988). After treatment with cycloheximide, staining in the Golgi apparatus disappears, indicating that the protein there is part of a transient precursor population.

The pulse-chase metabolic labeling experiments deserve special comment because such experiments have not been reported previously for CD8. The protein is synthesized in C2-CD8 cells as a 24-kD precursor, and is subsequently processed via a 27-kD intermediate, to a final product of 33 kD (Fig. 3). As the final product has properties that are similar to those reported for CD8 produced by lymphocytes, it is likely that the pathway in lymphocytes is similar to that seen in C2-CD8 cells. Previous experiments based on enzymatic and chemical cleavage of the final product in lymphocytes have suggested that carbohydrates contribute  $\sim 2$  kD to the molecular mass of the mature protein (Snow et al., 1985). Our results are in agreement with the values of 9–10 kD suggested by *in vitro* translation experiments (Littman et al., 1985).

The pulse-chase experiments indicate that the final steps of intracellular processing in C2-CD8 cells occur within 15 min, presumably in the Golgi apparatus, and that CD8 first appears on the surface within 30 min of synthesis. Surface CD8, which is assembled into dimers and larger aggregates, is not removed rapidly, but is relatively stable. A fraction of the newly synthesized CD8 apparently is not transported to the surface, but remains in an intracellular compartment, inaccessible to trypsin. This compartment could correspond to the "hidden pool" that has been seen in studies of the AChR (Devreotes et al., 1977).

The finding that intracellular CD8 is near its source nucleus is consistent with previous results from our own and other laboratories showing that proteins targeted to intracellular structures are distributed in a local region near the nucleus from which the mRNA originates (Miller et al., 1988; Pavlath et al., 1989; Ralston and Hall, 1989; Rotundo, 1989). This region is not confined to the area of a single nucleus, but encompasses that of several nuclei, so that compartments associated with neighboring nuclei may be partially shared. Intracellular CD8, for example, extended on average  $\sim 70$   $\mu\text{m}$  from its source nucleus, and was almost always associated with several nuclei. Although these nuclei were often clustered, physical contiguity or close juxtaposition to the source nucleus was not required (see Fig. 8). In this respect our results may differ from those of Pavlath et al. (1989).

The range of intracellular CD8 staining places an upper limit on the migration of the CD8 mRNA from its source within the muscle fiber. This limit is in general agreement with a previous, similar estimate that we have made for the mRNA encoding a protein targeted to the nucleus (Ralston and Hall, 1989). CD8 mRNA could have a more restricted

distribution than that of the intracellular protein if there were exchange between the compartments associated with each nucleus at the level of the endoplasmic reticulum and/or the Golgi apparatus. Exchange of membrane proteins at the level of the Golgi has been shown to occur both *in vivo* and *in vitro* (Rothman et al., 1984).

In contrast to its restricted intracellular location, CD8 occupies the entire surface of hybrid myotubes. Although inhomogeneities in its distribution were observed, these bore little relation to the position of the source nucleus. Interestingly, CD8 sometimes formed clusters; these were always associated with clusters of AChR, and possibly represent nonspecific trapping of CD8. The widespread distribution of CD8 in the membrane presumably results from diffusion of the protein from its local site of membrane insertion (Frye and Edidin, 1970). Nonclustered acetylcholine receptors are able to diffuse in the muscle membrane (Axelrod et al., 1976; Poo, 1982; Stya and Axelrod, 1983), and it is likely that CD8 does as well. In lymphocytes, CD8 is uniformly distributed on the surface and freely redistributes in the presence of antibodies (A. Kupfer, personal communication). Our results are thus consistent with the idea that there are no intrinsic barriers to diffusion in the myotube membrane.

The most prominent membrane protein that is nonuniformly distributed in myotubes is the AChR, which is concentrated in patches on the myotube surface, both in aneural cultures and at sites of nerve–muscle contact in co-cultures (Schuetze and Role, 1987). Recent experiments suggest that sites of nerve–muscle contact may also be sites of preferential synthesis and insertion of the AChR (Role et al., 1985; Merlie and Sanes, 1985; Fontaine et al., 1988; Fontaine and Changeux, 1989). Our results suggest that retention of the AChR near these sites is not simply the result of local insertion, but must involve specific mechanisms, such as attachment to the extracellular matrix or cytoskeleton, to prevent its dispersion.

In contrast to the results reported here, Pavlath et al. (1989) have reported that the antigen recognized by 5.IH11, a monoclonal antibody that reacts with a surface protein of human cells (Walsh and Ritter, 1981; Hurko and Walsh, 1983; Walsh et al., 1982), is in some cases retained near the source nucleus in interspecific hybrids. The antigen recognized by 5.IH11 has recently been reported to be human N-CAM (Walsh et al., 1989). Because the antibody recognizes secreted and glycosyl-phosphatidylinositol-linked forms of N-CAM, as well as the transmembrane form, the significance of the different results found in the two studies is unclear. The restricted distribution of human N-CAM in interspecific hybrids could arise because of its interaction with extracellular matrix or cytoskeleton.

Our experiments and those of others suggest the following model for the localization of proteins in multinucleated muscle cells. Both soluble and membrane proteins are synthesized and processed in an area closely surrounding their source nucleus. Soluble proteins are then free to diffuse through the cytoplasm (Mintz and Baker, 1967; Ralston and Hall, 1989), unless they are targeted to a subcellular organelle such as the nucleus (Ralston and Hall, 1989), mitochondria, lysosomes, or to a local macromolecular assembly such as the myofibrils (Pavlath et al., 1989) or the cytoskeleton. Their range, in these cases, will reflect the competition between the rates of diffusion and of local uptake. The situa-

tion is similar for membrane proteins: after insertion into the plasma membrane near their source nucleus, they are free to diffuse, unless they become associated with cytoskeletal or extracellular matrix elements. The range of a membrane protein will thus, like that of a soluble protein, reflect a competition between the kinetics of diffusion and of local entrapment.

Understanding the contribution of an individual nucleus to the intracellular and surface organization of muscle cells may be important in several contexts. Recent experiments on the distribution of AChR subunit mRNA show surprising heterogeneity among nuclei even in uninervated or denervated cells in which AChR is evenly distributed on the surface (Harris et al., 1989; Fontaine and Changeux, 1989). These results raise the possibility that other uniformly distributed proteins in muscle cells are derived from only a subset of nuclei scattered throughout the muscle fiber that express mRNA for their synthesis. Finally, knowing the range over which the products of a single nucleus extend will have obvious importance for attempts to obtain phenotypic rescue of diseased muscle fibers by fusing normal cells into them (Partridge et al., 1989).

We thank Dan Littman for generously providing the vector and for advice and encouragement throughout this work; Herman Gordon (University of California, San Francisco) for continuous help and suggestions; Helen Blau (Stanford University) for stimulating discussions; Annie Moriarty and Anne Norment (University of California, San Francisco), for reagents and technical advice; Richard Rotundo (University of Miami School of Medicine) for sharing his unpublished results; and Richard Myers, Ron Vale (both at the University of California, San Francisco), and members of our laboratory for constructive comments on the manuscript.

Supported by grants from the National Institutes of Health and the Muscular Dystrophy Association.

Received for publication 13 June 1989 and in revised form 31 July 1989.

## References

- Abeijon, C., and C. B. Hirschberg. 1987. Subcellular site of synthesis of the N-acetylgalactosamine ( $\alpha$  1-0) serine (or threonine) linkage in rat liver. *J. Biol. Chem.* 262:4153-4159.
- Axelrod, D., P. Ravdin, D. E. Koppel, J. Schlessinger, W. W. Webb, E. L. Elson, and T. R. Podleski. 1976. Lateral motion of fluorescent-labelled acetylcholine receptors in the membrane of developing muscle fibers. *Proc. Natl. Acad. Sci. USA.* 73:4594-4598.
- Devreotes, P. N., J. M. Gardner, and D. M. Fambrough. 1977. Kinetics of biosynthesis of acetylcholine receptor and subsequent incorporation into plasma membrane of cultured chick muscle. *Cell.* 10:365-373.
- Fontaine, B., and J.-P. Changeux. 1989. Localization of nicotinic acetylcholine receptor  $\alpha$ -subunit transcripts during myogenesis and motor endplate development in the chick. *J. Cell Biol.* 108:1025-1037.
- Fontaine, B., D. Sassoon, M. Buckingham, and J.-P. Changeux. 1988. Detection of the nicotinic acetylcholine receptor  $\alpha$ -subunit mRNA by in situ hybridization at neuromuscular junctions of 15-day-old chick striated muscles. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:603-609.
- Forsayeth, J., B. Maddux, and I. D. Goldfine. 1986. Biosynthesis and processing of the human insulin receptor. *Diabetes.* 35:837-846.
- Frye, L. D., and M. Edidin. 1970. The rapid intermixing of cell surface antigens after formation of mouse-human heterokaryons. *J. Cell Sci.* 7:319-335.
- Galfre, G., and C. Milstein. 1981. Preparation of monoclonal antibodies: strategies and procedures. *Methods Enzymol.* 73:3-46.
- Gu, Y., E. Ralston, C. Murphy-Erdosh, R. A. Black, and Z. W. Hall. 1989. Acetylcholine receptor in a C2 muscle cell variant is retained in the endoplasmic reticulum. *J. Cell Biol.* 109:729-738.
- Hanover, J. A., J. Eltins, G. R. Mintz, and W. J. Lennarz. 1982. Temporal aspects of the N- and O-glycosylation of human chorionic gonadotropin. *J. Biol. Chem.* 257:10172-10177.
- Harris, D. A., D. L. Falls, and G. D. Fischbach. 1989. Differential activation of myotube nuclei following exposure to an acetylcholine receptor-inducing factor. *Nature (Lond.)*. 337:173-176.
- Hoffman, R. A., P. C. Kung, W. P. Hansen, and G. Goldstein. 1980. Simple and rapid measurement of human T lymphocytes and their subclasses in peripheral blood. *Proc. Natl. Acad. Sci. USA.* 77:4914-4917.
- Hurko, O., and F. S. Walsh. 1983. Human fetal muscle-specific antigen is restricted to regenerating myofibers in diseased adult muscle. *Neurology.* 33:737-743.
- Kirschmeier, P. T., G. M. Housey, M. D. Johnson, A. S. Perkins, and I. B. Weinstein. 1988. Construction and characterization of a retroviral vector demonstrating efficient expression of cloned cDNA sequences. *DNA.* 7:219-225.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)*. 227:680-685.
- Littman, D. R. 1987. The structure of the CD4 and CD8 genes. *Annu. Rev. Immunol.* 5:561-584.
- Littman, D. R., Y. Thomas, P. J. Maddon, L. Chess, and R. Axel. 1985. The isolation and sequence of the gene encoding T8: a molecule defining functional classes of T lymphocytes. *Cell.* 40:237-246.
- Maddon, P. J., A. G. Dalgleish, J. S. McDougal, P. Clapham, R. A. Weiss, and R. Axel. 1986. The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain. *Cell.* 47:333-348.
- Merlie, J. P., and J. R. Sanes. 1985. Concentration of acetylcholine receptor mRNA in synaptic regions of adult muscle fibres. *Nature (Lond.)*. 317:66-68.
- Miller, A. D., and C. Buttimore. 1986. Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production. *Mol. Cell Biol.* 6:2895-2902.
- Miller, S. C., G. K. Pavlath, B. T. Blakely, and H. M. Blau. 1988. Muscle cell components dictate hepatocyte gene expression and the distribution of the Golgi apparatus in heterokaryons. *Genes & Dev.* 2:330-340.
- Mintz, B., and W. W. Baker. 1967. Normal mammalian muscle differentiation and gene control of isocitrate dehydrogenase synthesis. *Proc. Natl. Acad. Sci. USA.* 58:592-598.
- Partridge, T. A., J. E. Morgan, G. R. Coulton, E. P. Hoffman, and L. M. Kunkel. 1989. Conversion of mdx myofibers from dystrophin-negative to -positive by injection of normal myoblasts. *Nature (Lond.)*. 337:176-179.
- Pathak, R. K., R. K. Merkle, R. D. Cummings, J. C. Goldstein, M. S. Brown, and R. G. W. Anderson. 1988. Immunocytochemical localization of mutant low density lipoprotein receptors that fail to reach the Golgi complex. *J. Cell Biol.* 106:1831-1841.
- Pavlath, G. K., K. Rich, S. G. Webster, and H. M. Blau. 1989. Localization of muscle gene products in nuclear domains. *Nature (Lond.)*. 337:570-573.
- Platt, J. L., and A. F. Michael. 1983. Retardation of fading and enhancement of intensity of immunofluorescence by p-phenylenediamine. *J. Histochem. Cytochem.* 31:840-842.
- Poo, M. 1982. Rapid lateral diffusion of functional ACh receptors in embryonic muscle cell membrane. *Nature (Lond.)*. 295:332-334.
- Ralston, E., and Z. W. Hall. 1989. Transfer of a protein encoded by a single nucleus to nearby nuclei in multinucleated myotubes. *Science (Wash. DC)*. 244:1066-1069.
- Role, L. W., V. R. Matossian, R. J. O'Brien, and G. D. Fischbach. 1985. On the mechanism of acetylcholine receptor accumulation at newly formed synapses on chick myotubes. *J. Neurosci.* 5:2197-2204.
- Rothman, J. E., R. L. Miller, and R. L. Urbani. 1984. Intercompartmental transport in the Golgi complex is a dissociative process: facile transfer of membrane protein between two Golgi populations. *J. Cell Biol.* 99:260-271.
- Rotundo, R. L. 1989. Nucleus-specific translation and assembly of acetylcholinesterase in multinucleated muscle cells. *J. Cell Biol.* In press.
- Salpeter, M. 1987. Vertebrate neuromuscular junctions: general morphology, molecular organization, and functional consequences. In *The Vertebrate Neuromuscular Junction*. M. Salpeter, editor. Alan R. Liss Inc., New York. 1-54.
- Schuetz, S. M., and L. W. Role. 1987. Developmental regulation of nicotinic acetylcholine receptors. *Annu. Rev. Neurosci.* 10:403-457.
- Snow, P. M., and C. Terhorst. 1983. The T8 antigen is a multimeric complex of two distinct subunits on human thymocytes but consists of homomultimeric forms on peripheral blood T lymphocytes. *J. Biol. Chem.* 258:14675-14681.
- Snow, P. M., G. Keizer, J. E. Coligan, and C. Terhorst. 1984. Purification and N-terminal amino acid sequence of the human T cell surface antigen T8. *J. Immunol.* 133:2058-2066.
- Snow, P. M., J. E. Coligan, and C. Terhorst. 1985. Analysis of the structure of the human T cell surface antigen T8 by limited proteolysis and chemical cleavage. *J. Biol. Chem.* 260:2700-2708.
- Stya, M., and D. Axelrod. 1983. Mobility and detergent extractability of acetylcholine receptors on cultured rat myotubes: a correlation. *J. Cell Biol.* 97:48-51.
- Tassin, A. M., M. Paintrand, E. G. Berger, and M. Bornens. 1985. The Golgi apparatus remains associated with microtubule organizing centers during myogenesis. *J. Cell Biol.* 101:630-638.
- Walsh, F. S., and M. A. Ritter. 1981. Surface antigen differentiation during myogenesis in culture. *Nature (Lond.)*. 289:60-64.
- Walsh, F. S., C. A. Quinn, R. Yasin, E. J. Thompson, and O. Hurko. 1982. Human muscle antigens during development. In *Muscle Development: Molecular and Cellular Control*. M. L. Pearson and H. F. Epstein, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor NY. 535-542.
- Walsh, F. S., G. Dickson, S. E. Moore, and C. H. Barton. 1989. Unmasking N-CAM. *Nature (Lond.)*. 339:516.
- Yaffe, D., and O. Saxel. 1977. Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. *Nature (Lond.)*. 270:725-727.