ASSEMBLY OF THE SARCOPLASMIC RETICULUM

Localization by Immunofluorescence of Sarcoplasmic Reticulum Proteins in Differentiating Rat Skeletal Muscle Cell Cultures

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ABSTRACT

Immunofluorescent staining techniques were used to study the distribution of the Ca^{2+} + Mg^{2+}-dependent ATPase and calsequestrin in primary cultures of differentiating rat skeletal muscle cells, grown for different periods of time under various culture conditions.

In mononucleated myoblasts calsequestrin was detected after 45 h in culture whereas the ATPase was not detected until 60 h. After cell fusion began, both proteins could be identified in all multinucleated cells. Myoblasts grown for longer than 60 h in low Ca^{2+} medium contained calsequestrin and the ATPase, even though they were unable to fuse. These studies at the cellular level confirm biochemical findings on the biosynthesis of calsequestrin and the ATPase.

Immunofluorescent staining of myoblasts showed that calsequestrin first appears in a well-defined region of the cell near one end of the nucleus. At later times, the staining occupied progressively larger regions adjacent to the nucleus and took on a fibrous appearance. This suggests that calsequestrin first accumulates in the Golgi region and then gradually spreads throughout the cell. In contrast, the ATPase appeared to be concentrated in many small patches or foci throughout the cytoplasm and was never confined to one particular region, although some parts of the cell often stained more intensely than others. In multinucleated cells, alternating dark and fluorescent strands parallel to the longitudinal axis of the cells were evident.

Fluorescent staining with these antisera was not observed in fibroblasts which were also present in the cultures.

Sarcoplasmic reticulum is the intracellular membrane system in muscle cells which regulates contraction by modulating the intracellular concentration of calcium ions (3). The relationship between the individual components of this membrane system and their function at the molecular level has been studied extensively (13). Very little is known, however, about the assembly of this membrane system during the maturation of muscle cells. Examination of the microsomal fractions isolated from embryonic and neonatal muscle, for their ability to transport Ca^{++} in an ATP-depen-

dent reaction, has shown a progressive increase in Ca^{++} transport activity during development (2, 5, 10, 17).

Recently, we have undertaken an investigation of the synthesis of the two major sarcoplasmic reticulum proteins, the Ca^{++} + Mg^{++}-dependent ATPase (7) and calsequestrin (22). A study of the temporal pattern of biosynthesis of these proteins in differentiating rat skeletal muscle cells in culture showed that the initiation of synthesis of calsequestrin (an extrinsic component) preceded the initiation of synthesis of the ATPase (the major intrinsic protein) by about 20 h. Moreover, calsequestrin synthesis preceded myoblast fusion which began 50–60 h after plating (22). The maximal rate of synthesis of calsequestrin was reached at about 72 h after plating. The rate of synthesis of calsequestrin then decreased while the rate of ATPase synthesis remained high. If cells were transferred to a low Ca^{++} medium which inhibited fusion, the rate of calsequestrin synthesis decreased whereas the rate of ATPase synthesis increased sharply after a slight delay, even though no fusion occurred.

In order to understand the assembly of the sarcoplasmic reticulum, we have begun a morphological study of the biosynthesis of calsequestrin and the Ca^{++} + Mg^{++}-ATPase and their incorporation into this membrane system. In this report, we describe the appearance and distribution of calsequestrin and of the ATPase in developing rat skeletal muscle cells as determined by the immunofluorescent staining technique.

The results confirm, at the cellular level, our previous biochemical findings on the temporal sequence of initiation of synthesis of these two sarcoplasmic reticulum proteins. In addition, we have found that some myoblasts, cultured in standard medium, are capable of synthesizing the ATPase before fusion. The results obtained with the fluorescent antibody technique fully support the view that cell fusion is not essential for the initiation of synthesis of either the ATPase or calsequestrin. The localization of these two proteins in myogenic cells during maturation and cell fusion is described and the possible relationship between the observed staining patterns and organized subcellular structures is discussed.

MATERIALS AND METHODS

Cell Culture

Isolation of muscle cells from neonatal rats and their growth on standard medium, low Ca^{++} medium and normal Ca^{++} medium has been described previously (7, 22).

Purification of Rat ATPase and Rabbit Calsequestrin

Rat ATPase was prepared by procedures similar to those used for the purification of rabbit ATPase (11), except that the fractionation in ammonium acetate was carried out at pH 8.35. Rabbit calsequestrin was purified as previously described (14).

Preparation of Antibodies

The rabbit anti-rat ATPase serum previously characterized (7) was used. The specificity of this antiserum was demonstrated by Ouchterlony double-diffusion tests against purified rat ATPase or solubilized rat sarcoplasmic reticulum. Only a single precipitin line was obtained in both cases. No precipitin line was observed when normal sera were used. This antiserum did not cross-react with either purified rat calsequestrin or high affinity Ca^{++}-binding protein (7).

ATPase antibodies were isolated from the serum by absorption with insoluble purified ATPase. Since the anti-ATPase serum was prepared against an ATPase preparation which also contained phospholipid and proteolipid (15), the latter substances were removed from the absorbant by acetone extraction (6). The insoluble, acetone-extracted ATPase was then washed twice with phosphate-buffered saline (PBS), which contained 147 mM NaCl, 2.67 mM KCl, 0.49 mM $MgCl_2$, 0.9 mM $CaCl_2$, 8.1 mM Na_2HPO_4, and 1.47 mM KH_2PO_4, pH 7.2, extracted twice with 0.1 M glycine, pH 2.8, and washed again with PBS. For absorption, about 50 mg of insoluble ATPase and 4 ml of serum were incubated for 30 min at room temperature, and then the mixture was centrifuged for 10 min at 25,000 *g*. Ouchterlony double-diffusion tests were used to determine whether the antibody was removed from the serum. The insoluble ATPase-antibody complex was washed twice in PBS, and the specific antibody was recovered from the insoluble complex by two washes at 0°C in 0.1 M glycine, pH 2.8. The glycine extract was adjusted immediately to pH 7.5 with phosphate buffer and concentrated to 1 ml by pressure dialysis. About 3 mg of specific antibody were obtained from 4 ml of serum. This purified antibody gave only a single precipitin line in Ouchterlony tests.

The sheep anti-rabbit calsequestrin serum previously characterized (22) was used. The specificity of this antiserum was demonstrated by Ouchterlony double-diffusion tests against purified rat calsequestrin or solubilized rat sarcoplasmic reticulum. Only a single precipitin line was obtained in both cases. No precipitation line was obtained when normal sera were used. This antiserum did not cross-react with either purified rat ATPase or high affinity Ca^{++}-binding protein (22).

Specific calsequestrin antibodies were prepared from the serum by absorption with an insoluble calsequestrin-albumin complex. To prepare insoluble calsequestrin, 40

mg of bovine serum albumin and 10 mg of calsequestrin were dissolved in 1 ml of 0.2 M sodium acetate, pH 5.0. 0.2 ml of 2.5% glutaraldehyde was added dropwise, and the mixture was stirred for 3 h at room temperature. The material was then diluted with 20 ml of 1 M glycine, 0.1 M sodium phosphate, pH 7.5, and washed twice by centrifugation in glycine-PO_4 buffer, twice by centrifugation in PBS, twice by centrifugation in 0.1 M glycine, pH 2.8, and, finally, twice in PBS (1, 8). The insoluble calsequestrin-albumin complex was then added to 2 ml of sheep anti-calsequestrin serum. All of the antibody was removed from the serum in two incubations of 30 min at room temperature. The specific antibody-calsequestrin complex which was obtained upon centrifugation was dissociated by glycine buffer, and the specific antibody was recovered by the same procedures used for recovery of the specific ATPase antibodies. This purified antibody gave only a single precipitin line in Ouchterlony tests.

Absorption

Solutions of specific antibodies were absorbed by incubation with the appropriate antigen. Calsequestrin antibody (100 μg) was incubated with 6 μg of rat calsequestrin for 72 h at 4°C in 0.3 ml of PBS. The supernatant solution obtained after centrifugation was used in place of the specific antibody in immunofluorescence tests. Similarly, 25 μg of specific ATPase antibody was incubated for 72 h at 4°C with 5 μg of purified, lipid-free rat ATPase dissolved in 0.5% Triton X-100. The supernatant solution obtained after centrifugation was used in immunofluorescence tests.

Preparation of FITC-Conjugated Antibodies

Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit immunoglobulin was obtained from Hyland (F/P ratio 2; 13.5 mg/ml). FITC-conjugated rabbit anti-sheep immunoglobulin was prepared by labeling the immunoglobulin fraction from rabbit anti-sheep IgG serum (Josef De Rose Assoc., Downsview, Ontario, Canada) with FITC as described by The and Feltkamp (19) (F/P ratio 2.7; 10 mg/ml).

Indirect Fluorescent Antibody Staining

Primary rat skeletal muscle cells were grown either in Labtek chambers (Miles Laboratories Inc., Miles Research Products, Elkhart, Ind.) or on glass cover slips placed in 55-mm diameter plastic Petri dishes. Each 100 mm^2 Labtek chamber was filled with 0.5 ml of medium and 1.2×10^5 cells. Each Petri dish was filled with 3 ml of medium and 3×10^6 cells. Media were changed daily. After various time intervals up to 140 h after plating, the medium was removed and the cells on the glass slide or cover slip were rinsed with PBS, pH 7.2, and fixed for 30 min at room temperature in 1% formaldehyde in PBS, pH 7.2, air dried, and stored in a desiccator at −20°C for up to a week.

The fixed cells were incubated with specific antibody for 30 min at room temperature (anti-ATPase, 15 μg/ml in PBS, pH 7.2; anti-calsequestrin, 100 μg/ml in PBS, pH 7.2) and then rinsed four times with PBS, pH 7.2. Cells previously treated with specific rabbit ATPase antibodies were incubated with the FITC-conjugated immunoglobulin fraction of goat anti-rabbit IgG (3.5 mg/ml) for 30 min at room temperature. Cells previously treated with specific sheep calsequestrin antibodies were incubated with the FITC-conjugated immunoglobulin fraction of rabbit anti-sheep IgG (0.5 mg/ml) for 30 min at room temperature. Finally, the cells were washed four times in PBS, pH 7.2, and mounted in 50% glycerol in PBS. The cells were examined in a Zeiss fluorescence microscope provided with an Epi-fluorescence attachment and interference filters. The photographs were taken on Ilford FP-4 film.

For a specific time point, the labeling of cells with ATPase and calsequestrin antibodies was carried out by dividing a cover slip in half and using one half of the cover slip for treatment with ATPase antibodies and the other half for treatment with calsequestrin antibodies. When cells were grown in Labtek chambers, one chamber at each end of the same slide was used for the labeling of cells with each of the two antibodies.

Analysis of Sugars in Calsequestrin

The sugar content of calsequestrin was determined by the method of Zanetta et al. (21). To confirm the absence of fucose and galactose, a sample, after methanolysis, was passed through a Dowex 50 (^+H) column to remove amino acids. Glucosamine was also measured by a colorimetric technique (9) after acid hydrolysis in 4 N HCl at 100°C for 4 h.

RESULTS

To determine the time of appearance and the distribution of the ATPase and calsequestrin in cells at various times during differentiation, primary cultures of rat skeletal muscle were examined by the indirect fluorescein antibody technique. In cultures prepared for microscopy, only mononucleated bipolar myoblasts and flat irregularly shaped mononucleated fibroblasts were present when cells were grown in standard medium up to 70 h. Fusion started after about 70 h, and subsequently the number of multinucleated muscle cells progressively increased. This is in contrast to the growth pattern observed with higher density plating in Petri dishes where fusion began after about 50 h in culture (7, 22).

Localization of Calsequestrin

In cultures grown in standard or normal Ca^{2+} medium, immunofluorescent staining of cells with antibody to calsequestrin was not observed before

45 h (Fig. 1*a*). Immunofluorescent staining in some myoblasts was first detected at about 45 h, localized in one region of the cytoplasm near the nucleus (Fig. 1*b* and 1*c*), which we believe is the Golgi region. The nucleus often appeared indented near the stained region (Fig. 1*b*). The staining appeared homogeneously distributed within this region, and no structural features could be resolved at this stage. The cytoplasm outside this region was negative. Later, myoblasts were observed in which the fluorescent staining occupied increasingly larger regions adjacent to the nucleus (Figs. 1*d*, 2*a* and 2*b*) and took on a distinctly fibrous appearance. With time, an increasing number of myoblasts were specifically stained with antibodies to calsequestrin, but all of the above stages could still be recognized. After 65 h in culture, some myoblasts were specifically stained throughout the cytoplasm.

Following fusion, all of the bi- and multinucleated myotubes were specifically labeled with calsequestrin antibody (Figs. 2*c* and 4*a*). In the vast majority of the myotubes, fluorescent strands running parallel to the longitudinal axis of the cell could be distinguished (Fig. 4*a*). Occasionally, bi- or trinucleated cells were encountered in which the staining was absent from the cytoplasm surrounding one nucleus while that surrounding the other nucleus was stained (Fig. 2*d*).

Localization of ATPase

In cultures grown in standard or normal Ca^{++} medium, immunofluorescent staining with the ATPase antibody was not observed before 65 h (Fig. 3*a*).

Specific staining was first detected in some mononucleated myoblasts after 65 h, just before fusion began (Fig. 3*b*, 3*c*, and 3*d*). This contrasts with the much earlier appearance of calsequestrin in cells grown in the same cultures and is in agreement with results from earlier biochemical studies (22).

The fluorescent staining in myoblasts with antibodies to the ATPase was present throughout the cytoplasm (Fig. 3*b* and 3*c*) and was granular, indicating that the ATPase was concentrated at certain foci rather than homogeneously distributed. Occasionally, however, one region did stain more intensely than the rest of the cytoplasm, but there was no sharp line of demarcation between these two regions (Fig. 3*d* and 3*c*) as was seen with antibodies to calsequestrin (Fig. 1*b* and 1*c*). At later times, after cell fusion began, immunofluorescent staining with the ATPase antibodies was observed in all multinucleated cells (Figs. 3*e*, 3*f*, and 4*b*).

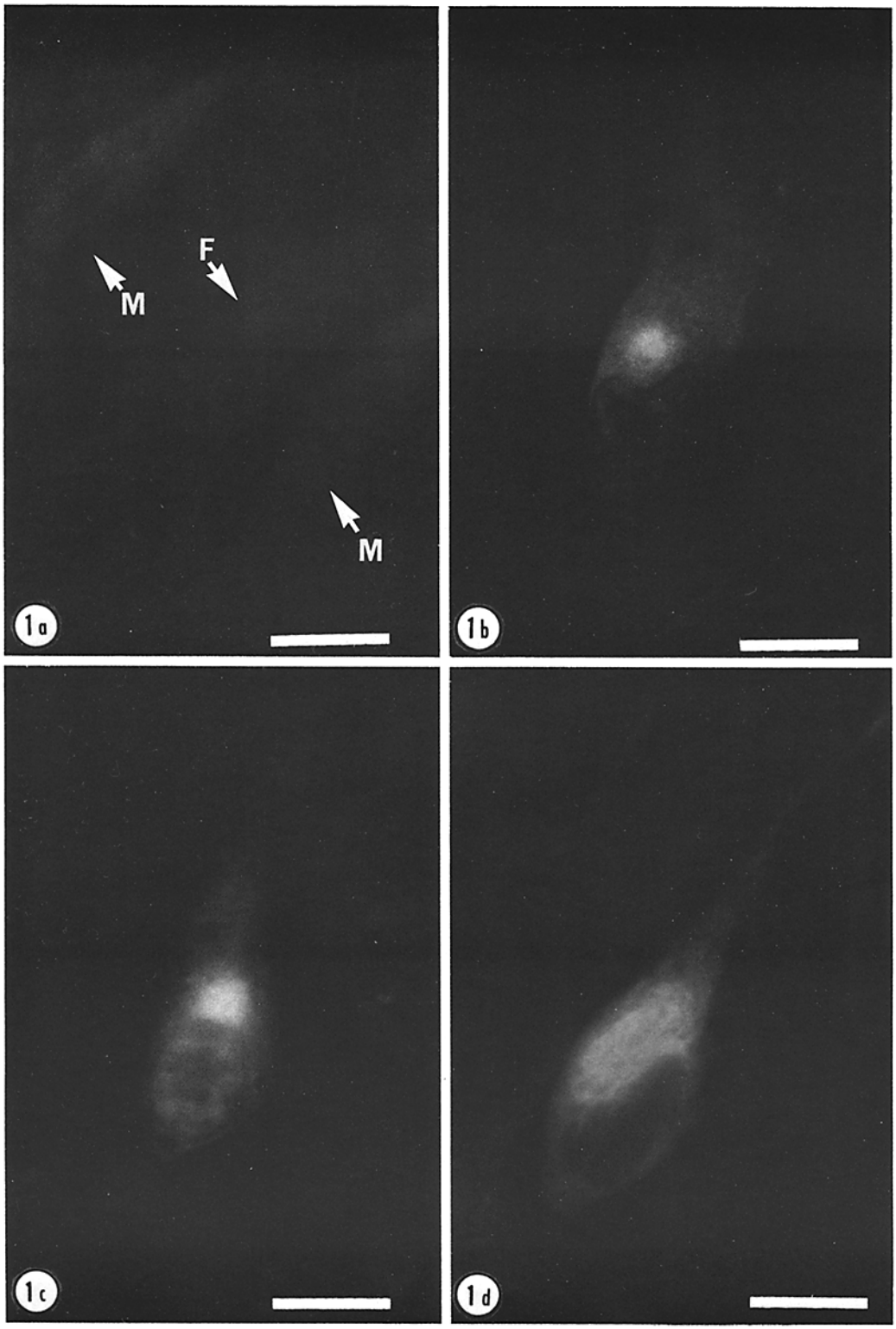
The staining pattern observed in myotubes with ATPase antibodies (Fig. 4*b*) was very similar to that observed after staining with the calsequestrin antibody (Fig. 4*a*), in that positively stained strands running parallel to the longitudinal axis of the cell could be detected. The fluorescent strands observed with anti-ATPase, however, appeared more granular than those detected with anti-calsequestrin. When cells grown in low Ca^{++} medium were treated with the ATPase antibody, the staining pattern observed was again indistinguishable from that of the myoblasts grown in standard medium.

Quantitative Studies

To quantitate the changes in the staining patterns observed, after staining with calsequestrin antibody, the myogenic cells were classified according to four types of staining: Golgi-associated staining (Fig. 1*b* and 1*c*); staining in Golgi region and in surrounding region (Figs. 1*d*, 2*a* and 2*b*); whole cytoplasmic staining; and staining in multinucleated cells (Figs. 2*c*, and 4*a*). Similarly, after treatment with ATPase antibody, stained cells were classified as either myoblasts (Fig. 3*b*, 3*c*, 3*d* and 3*e*) or myotubes (Fig. 3*e*, 3*f* and 4*b*). The results obtained are presented in Figs. 5*a*, *b* and *c*.

Before fusion (Fig. 5*a*), most of the calsequestrin antibody-stained myoblasts showed Golgi-as-

FIGURE 1 Rat skeletal muscle myoblasts in culture sampled at various times after plating and treated with antibodies against calsequestrin; 1*a*, *b*, and *c* are from cultures grown in standard medium; *d* from a culture grown in normal Ca^{++} medium. Scale bar, 10 μm. (*a*) 40 h. Specific staining is absent in both myoblasts (*M*) and fibroblasts (*F*). (*b*) and (*c*) 45 h. Specific, homogeneous staining is present in a localized area of the cytoplasm near the nucleus. Note that the nucleus is indented near the stained region in *b*. (*d*) 55 h. Positively stained myoblast in which the stained perinuclear region occupies a larger portion of the cytoplasm than in (*b*) and (*c*) and has a fibrous appearance.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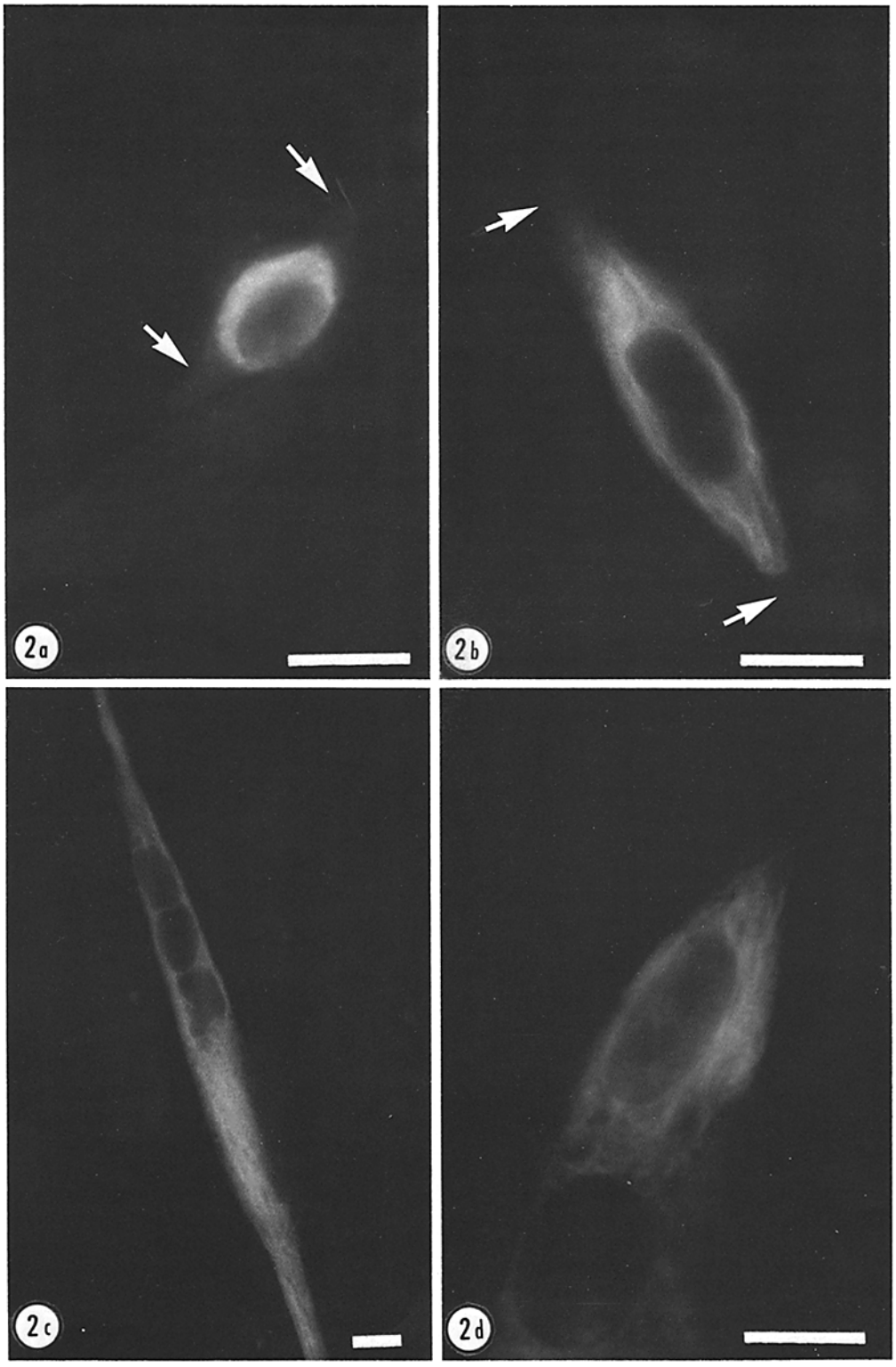
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sociated staining. The proportion of cells with this type of staining decreased rapidly as differentiation proceeded. The proportion of myogenic cells showing additional staining in cytoplasm surrounding the Golgi region reached a maximum at a later time and then decreased. The proportion of myoblasts showing whole cytoplasmic staining increased steadily throughout the period examined. Since the relative number of multinucleated cells also increased steadily, the proportion of stained myoblasts reached a maximum and then decreased.

In cultures grown in low Ca^{++} medium, cells with the staining patterns described above appeared in the same sequence. The proportion of cells having these patterns, however, changed with time in a different manner (Fig. 5 b). The relative number of myoblasts showing Golgi-associated staining decreased with time but less rapidly. Whereas in normal Ca^{++} the proportion of myoblasts showing additional staining in the region surrounding the Golgi apparatus reached a maximum and then rapidly declined, in low Ca^{++} medium this number kept increasing during the period examined. Myogenic cells with whole cytoplasmic staining appeared 20 h later than in normal Ca^{++}, and their proportion increased only slightly with time.

Previous results obtained from biochemical studies indicated that the rate of calsequestrin synthesis declined rapidly with time when muscle cells were transferred from standard medium to low Ca^{++} medium (22). This decrease was also reflected in the results obtained by immunofluorescence. The fraction of stained myogenic cells was lower in low Ca^{++} medium and, in addition, most of the stained myogenic cells showed Golgi-associated staining or, in addition, staining in the cytoplasm surrounding the Golgi region. Those cells grown in normal Ca^{++} showed whole cytoplasmic staining in mono- and multinucleated cells. Thus, the amount of calsequestrin per myogenic cell appears to be lower in low Ca^{++} medium than in normal Ca^{++} medium.

The results presented in Fig. 5 *c* show that ATPase-positive myoblasts increased to a maximum and then declined. Meanwhile, the number of myotubes increased steadily. Biochemical studies showed that although the initiation of ATPase synthesis could be delayed by transfer to low Ca^{++}, the rate of ATPase synthesis still increased to relatively high values (7, 22). Our present results show that the appearance of ATPase-positive myoblasts in low Ca^{++} medium was delayed but that once initiated, the increase in number of stained myoblasts in low Ca^{++} was as rapid as the increase in number of stained myotubes grown in normal Ca^{++} (Fig. 5 *c*).

Control Studies

To test the specificity of the staining pattern observed with the two antibodies, the supernate from the ATPase antibody, absorbed with ATPase, and the supernate from the calsequestrin antibody, absorbed with calsequestrin, were used in immunofluorescent staining tests. In both cases, the specific immunofluorescent staining patterns were almost completely abolished.

Specific immunofluorescent staining with antibody to calsequestrin or antibody to the ATPase was not detected in the spindle region or elsewhere in mitotic cells that were frequently observed in these cultures. Likewise, no staining was observed in the flat, irregularly shaped fibroblasts which could be readily distinguished from the bipolar myoblasts.

Sugar Analysis of Calsequestrin

Although it was evident from earlier studies (12, 14, 18) that calsequestrin is a glycoprotein, accurate analysis of its sugar content has not yet been reported. The data presented in Table I show that calsequestrin contains only *N*-acetylglucosamine and mannose in a molar ratio of 1:2:3. This indicates that only a "core" carbohydrate is pres-

FIGURE 2 Rat skeletal muscle myoblasts and myotubes in culture sampled at various times after plating and treated with antibodies against calsequestrin; 2*a*, *c*, and *d* are from cultures grown in standard medium; (*b*) from culture grown in normal Ca^{++} medium. Scale bar, 10 μm. (*a*) and (*b*) 55 h. Myoblasts showing staining limited to the perinuclear area. The cytoplasm at both ends of the myoblasts remains unstained (arrows). The fibrous appearance of the positively stained regions is best seen in (*b*). (*c*) 90 h. Positively stained multinucleated myotube. (*d*) 90 h. A binucleated cell in which the staining is limited to the cytoplasm around only one of the two nuclei.

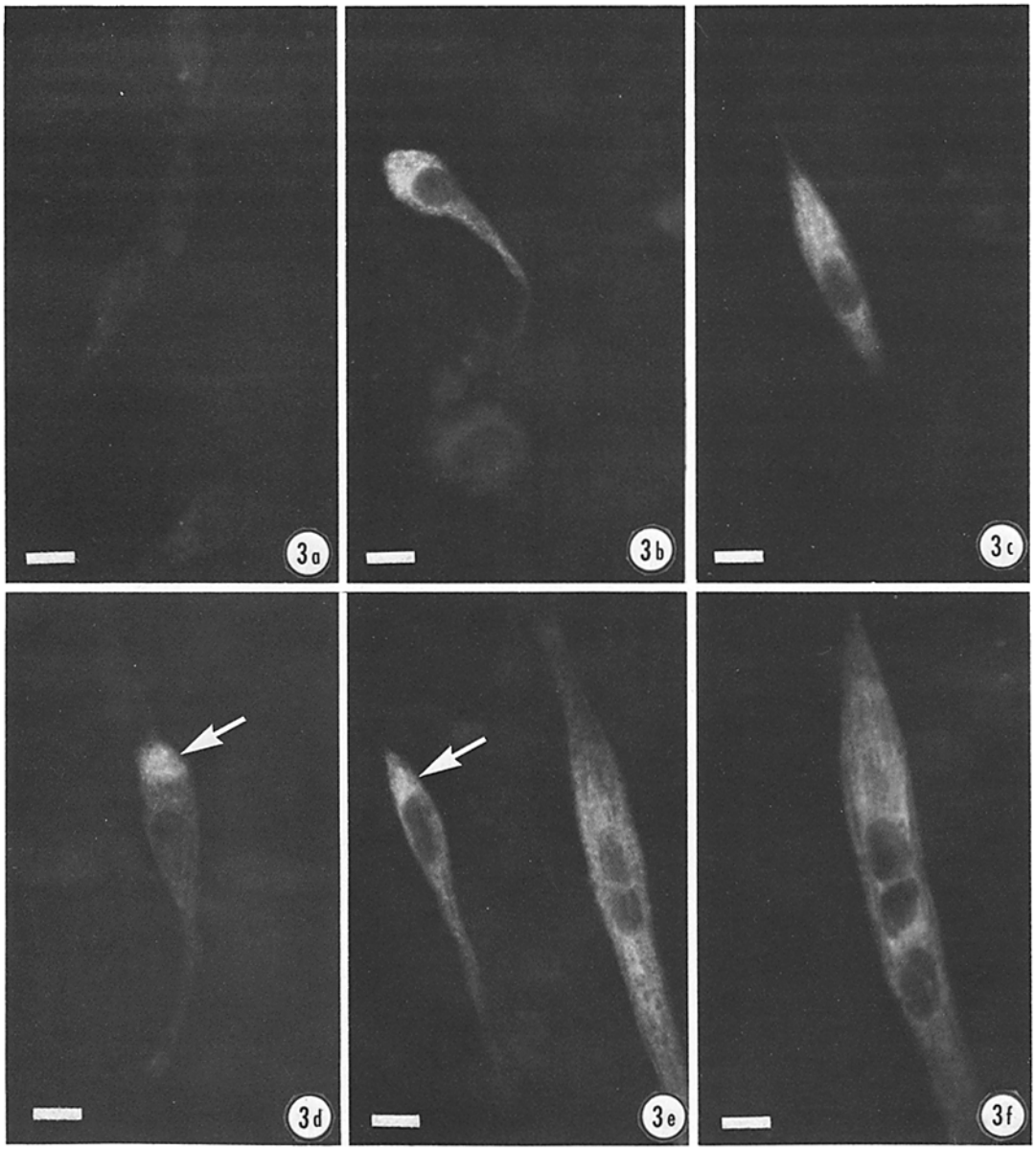

FIGURE 3 Rat skeletal muscle myoblasts and myotubes grown in standard media, sampled at various times after plating, and treated with ATPase antibody. Scale bar, 10 μm. (*a*) 55 h. Specific staining is absent from myoblasts. (*b*) and (*c*) 65 h. Staining in myoblasts when first detected is present throughout the cytoplasm. The granular appearance of this staining is best seen in (*b*). (*d*) 65 h. A myoblast showing more intense staining near one end of the cell (arrow). (*e*) 75 h. A myoblast showing more intense staining near one end of the nucleus (arrow), and a stained binucleated cell. (*f*) 90 h. Stained myotube, in which strands running parallel to the longitudinal axis of the cell can be distinguished.

ent in this glycoprotein (16). Since this core structure is so widely found and since its linkage is so invariant, it is probable that the linkage of sugars to the protein through asparagine (Asn) is identical to that occurring in other glycoproteins and is as follows:

$$(\text{Man})_2 \xrightarrow{\alpha} \text{Man} \xrightarrow{\beta} \text{GlcNAc} \xrightarrow{\beta} \text{GlcNAc} \longrightarrow \text{Asn}.$$

DISCUSSION

The use of specific antibodies to the ATPase and calsequestrin in the fluorescein-labeled antibody technique has permitted us to follow the appearance of these proteins and to determine their distribution during the differentiation of muscle cells in culture.

Calsequestrin was first detected in mononucleated myoblasts after 45 h in culture while the ATPase was not observed in these cells until 20 h later. These results complement our previous biochemical studies which established that the initiation of calsequestrin synthesis in skeletal muscle cultures precedes cell fusion and initiation of ATPase synthesis. Because initiation of ATPase synthesis and cell fusion occurred almost simultaneously in cells grown in standard medium, it could not be determined by biochemical analysis alone whether mononucleated myoblasts or only myotubes synthesized this enzyme. The present results clearly show that some myoblasts, grown for 65 h in standard medium, develop the capacity to synthesize the ATPase before cell fusion.

In myoblasts, calsequestrin was first detected in a small region of the cytoplasm close to, and often within an indentation of, the nucleus. Since this site is characteristic of the position of the Golgi apparatus in many cell types, we believe that this calsequestrin-containing region corresponds to the Golgi apparatus. The possibility that calsequestrin accumulates in the Golgi region is strengthened by previous (12, 14, 16) and present studies showing that calsequestrin is a glycoprotein. Thus, the Golgi apparatus would be expected to play a role in the processing of calsequestrin before it is incorporated into sarcoplasmic reticulum membranes. The qualitative and quantitative changes in the staining pattern with time are consistent with a gradual spreading of calsequestrin from this region until it becomes distributed throughout the cytoplasm. The confinement of calsequestrin to a specific region of the cytoplasm at early times and the fibrous appearance of the staining at later times

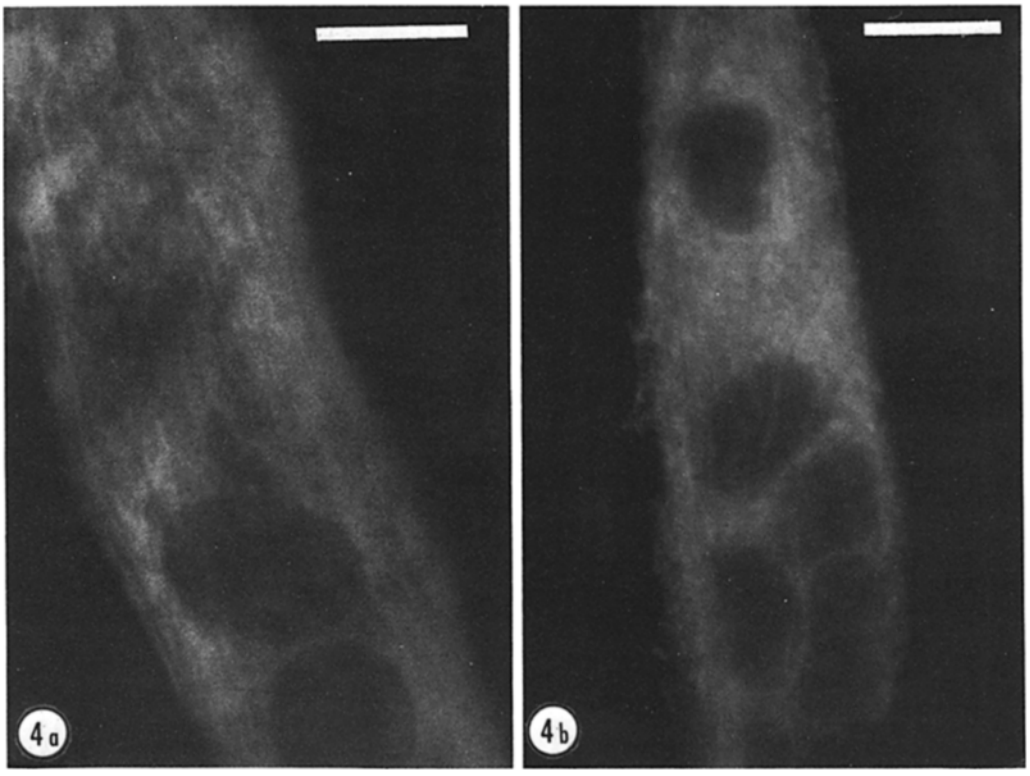

FIGURE 4 Parts of myotubes treated with antibody against calsequestrin (*a*) and ATPase (*b*) from cultures sampled 114 h after plating. Positively stained strands running parallel to the longitudinal axis of the cell can be seen in both myotubes. Staining with ATPase antibody gives a more granular appearance than staining with antibody to calsequestrin. Scale bar, 10 μm.

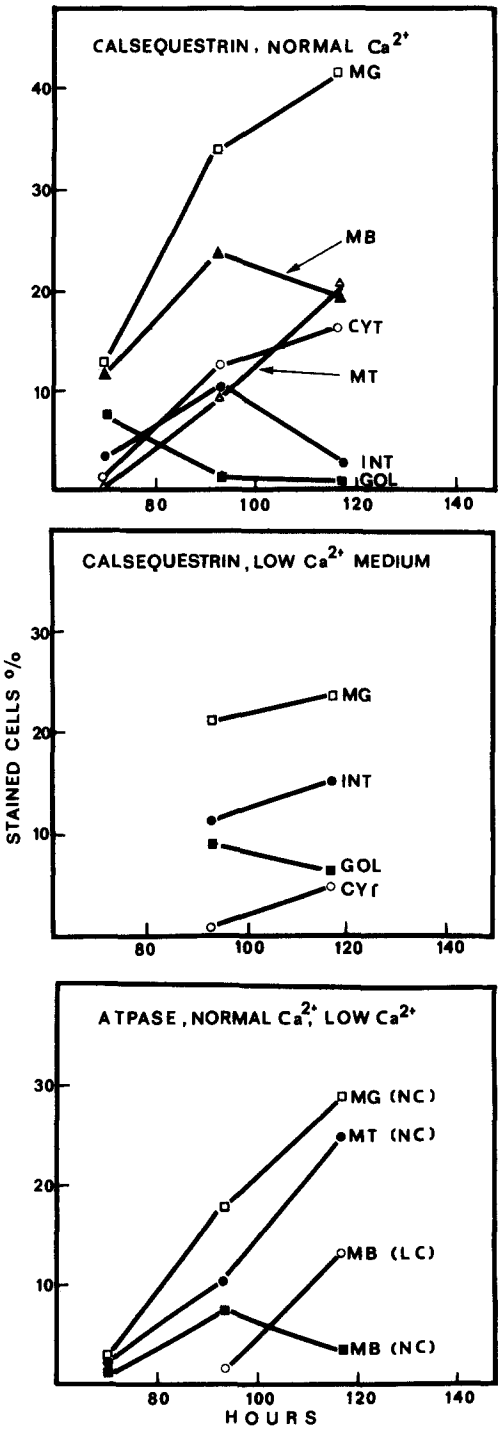

FIGURE 5 Changes in the distribution of cells with various staining patterns in rat skeletal muscle cultures after labeling with antibodies to either calsequestrin or ATPase. (For each time point, a total of 400-500 myogenic cells was counted in four different areas of the cover slip, classified as described in Results, and expressed in percent of the total number of myogenic cells counted. Percentage in the myotube category is based on the number of nuclei present in the myotubes counted. Total myogenic cells include total myoblasts as well as the total number of nuclei in myotubes.) Top and middle graphs: Cultures grown in normal Ca^{++} medium (top graph) or in low Ca^{++} medium (middle graph) labeled with calsequestrin antibodies. Percentage of myogenic cells showing staining in Golgi region *GOL* (■—■); staining of Golgi region and cytoplasm surrounding Golgi region *INT* (●—●); whole cytoplasmic staining (mononucleated *CYT* (○—○); whole cytoplasmic staining (multinucleated) *MT* (△—△). Total myoblasts stained *MB* (▲—▲), total myogenic cells stained *MG* (□—□). Bottom graph: Cultures grown in normal Ca^{++} medium (*NC*) or low Ca^{++} medium (*LC*) labeled with ATPase antibodies. Percentage of stained myoblasts in normal Ca^{++} *MB* (*NC*) (■—■) and in low Ca^{++} medium *MB* (*LC*) (○—○). Percentage of stained myotubes in normal Ca^{++} *MT* (*NC*) (●—●). Total myogenic cells in normal Ca^{++} medium *MG* (*NC*) (□—□).

suggest that calsequestrin is always associated with organized subcellular structures and that it is not free to diffuse throughout the cytoplasm. Attempts are now being made to identify the ultrastructural basis of these staining patterns.

In some multinucleated cells, staining was found in the cytoplasm around some but not all of the nuclei. This raises the possibility that a myoblast which has not yet begun to synthesize calsequestrin may be capable of fusing with myoblasts and myotubes which have already initiated calsequestrin synthesis.

The granular staining pattern observed in myoblasts and myotubes after treatment with ATPase antibodies suggests that the ATPase, when it first appears, accumulates in many regions of the cyto-

TABLE I

Sugar Content of Calsequestrin

Sugar	Content	
	nmol/mg	*mol/mol**
Mannose	75.5	3.33
Glucosamine	51.6	2.26
Fucose	<5	0
Galactose	<5	0
Sialic acid	<5	0

* Assuming a molecular weight of 44,000 for calsequestrin (10).

plasm. Ultrastructural analysis of the biogenesis of sarcoplasmic reticulum in differentiating chick skeletal muscle cultures has indicated that the sarcoplasmic reticulum is formed by the budding of membranous vesicles from the rough endoplasmic reticulum (4). Assuming that the development of the sarcoplasmic reticulum in differentiating rat skeletal muscle cells in culture is similar, it is possible that the granular appearance of the ATPase staining pattern observed in myoblast and myotubes marks the sites where the ATPase-containing membranes are being assembled.

Strands running parallel to the longitudinal axis of the cell were present in some myoblasts and became more prominent in myotubes after staining with either calsequestrin or the ATPase antibody. They are probably due to the separation of the calsequestrin- and the ATPase-positive regions by unstained, newly developing myofibrils. The similarity in staining patterns obtained with both antibodies suggests that both proteins become components of the same subcellular structure at later stages.

The processes whereby the ATPase and calsequestrin become constituents of the same membrane system are still unknown. We assume that the sarcoplasmic reticulum does not exist without the ATPase since this protein constitutes 95% of the intrinsic protein mass of the membrane (13, 22). On the basis of this assumption, we have previously proposed that the incorporation of calsequestrin into sarcoplasmic reticulum occurs only after the formation of the ATP-ase containing membrane structures. The time of appearance and the distribution of the ATPase and calsequestrin during the differentiation of skeletal muscle cells, as determined in the present studies, are consistent with this view. The polypeptide chain of calsequestrin may be synthesized on the rough endoplasmic reticulum and then transferred intraluminally to the Golgi apparatus where its final processing into a glycoprotein occurs. As a result of this process, calsequestrin accumulates in the Golgi region and becomes detectable by the immunofluorescence technique. During the period when calsequestrin is processed and accumulates in the Golgi region, the synthesis of the ATPase and the assembly of the sarcoplasmic reticulum are initiated at multiple foci throughout the cytoplasm. After the formation of the sarcoplasmic reticulum, calsequestrin is transferred to the lumen of this membrane system by a mechanism which is as yet unknown. A more precise ultrastructural identification of the subcellular structures labeled by the two antibodies will be required in order to understand the role of the endoplasmic reticulum and the Golgi apparatus in the synthesis and processing of the ATPase and calsequestrin and in order to determine how these two proteins assemble into a functional sarcoplasmic reticulum.

We thank Drs. J. R. Wilson and H. Schachter of the Department of Biochemistry, University of Toronto, for carrying out the sugar analysis of calsequestrin. The expert technical assistance of Elizabeth Zachwieja and Michael Wassmann is gratefully acknowledged.

A. O. Jorgensen is a fellow of the Canadian Heart Foundation and E. Zubrzycka is a fellow of the Muscular Dystrophy Association of Canada currently on leave of absence from the Nencki Institute of Experimental Biology, Warsaw, Poland.

This research was supported by grant MT-3399 from the Medical Research Council of Canada and by a grant from the Muscular Dystrophy Association of Canada to D. H. MacLennan, and by grant MT-3302 from the Medical Research Council of Canada and by grant from the Ontario Heart Foundation to V. I. Kalnins.

A preliminary report of this work was presented at the International Congress on Cell Biology, Boston, September 5-10, 1976 (Abstract No. 836). This is paper 3 of a series. Papers 1 and 2 are references 7 and 22, respectively.

Received for publication 9 November 1976, and in revised form 15 February 1977.

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