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Distribution of Myosin Heavy Chain mRNA in Embryonic Muscle Tissue Visualized by Ultrastructural *in Situ* Hybridization

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We have localized myosin heavy chain (MHC) mRNAs in cells of intact embryonic chick muscle using high resolution *in situ* hybridization. Blocks of muscle were aldehyde-fixed prior to detergent treatment and hybridized with a biotinated cDNA probe, followed by colloidal gold-labeled antibodies, before embedment. Labeling was determined to represent MHC mRNA by extensive quantitative comparisons of electron micrographs from experimental and four different types of control samples. MHC mRNA was localized primarily to peripheral regions of 14-day chick pectoral muscle cells, where the majority of developing myofibrils were found. MHC mRNAs were consistently associated with the nonmyofibrillar cytoskeletal filaments which had diameters ranging from 4 to 10 nm. They were often oriented parallel to the longitudinal axis of the cell. The resolution of the ultrastructural approach allowed us to demonstrate that the mRNA molecules visualized were not directly associated with myofilaments, suggesting that nascent chains read from those messages do not assemble directly into myofilaments simultaneous with translation.

INTRODUCTION

An important step toward understanding cellular mechanisms involved in the sorting and assembly of a protein is identification of the precise site where its nascent chains are synthesized. This information would be directly provided by a coupling of *in situ* hybridization and electron microscopy to reveal the distribution of specific mRNA molecules with respect to other cellular components at the ultrastructural level.

In this paper we examine the distribution and cellular associations of myosin heavy chain (MHC) mRNA molecules in 14-day chick pectoralis muscle by electron microscopy. The mechanisms involved in the assembly of the complex structure of the sarcomere have long been a subject of interest. In addition to the biological importance of understanding myosin filament development in sarcomeric muscle, this system has specific features that make it particularly suitable for study. The myosin heavy chain is the major protein by weight of the developing myofibril. Furthermore, its mRNA is large (5.9 kb) (Molina *et al.*, 1987) and extremely abundant during myogenesis (>12,000 molecules/myonucleus) (Robbins and Heywood, 1978), which should increase the likelihood of reliable detection after thin sectioning. In addition, MHC protein is assembled into an identifiable myofilament which has a precise orientation within the developing myofibril. If there were relationships between mRNAs and specific cellular components of the developing muscle cell they might well be revealed, even in thin section, because of this clearly defined spatial organization.

A major obstacle to *in situ* hybridization studies at the electron microscopic level is the conflict between requirements for morphological preservation, particularly of embryonic tissue, and conditions suitable for penetration, hybridization, and detection of the nucleic acid probe. In modifying methodology we have therefore explored several changes that we anticipated might reduce these problems and more closely match the precision of the hybridization technique to the microscopic resolution. First, we chemically fixed the tissue prior to any treatment with detergents. By this change from methods previously used for cultured muscle cells (Singer *et al.*, 1989a; Silva *et al.*, 1989) we hoped to minimize artifacts that might be produced by extraction of soluble proteins from unfixed cells, while retaining the increased penetrability to reagents that detergents provide. Second, to avoid the barrier of the embedment we performed hybridization and detection on small slices of tissue before embedding and sectioning rather than afterward (Binder *et al.*, 1986; Webster *et al.*, 1987). Visualization of the mRNAs was accomplished with nonradioactive methodology, i.e., a biotinated probe detected with gold-labeled antibodies, which provides high resolution since the gold particles are located directly at the site of hybridization (see also Hutchinson *et al.*, 1982).

This paper has two interrelated purposes. First, to describe a hybridization protocol which provided quantitatively validated ultrastructural detection of MHC mRNA in developing chicken skeletal muscle cells. Second, to describe the location of those messages in rela-

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tion to developing myofibrils and the nonmyofibrillar cytoskeleton.

MATERIALS AND METHODS

Tissue

Pectoral muscle from 14-day chicken embryos was studied. Various techniques for handling tissue samples through 50 changes of solution without contamination while retaining orientation were investigated. These included vibratome sections, capillary tube tissue processing units, well units with membranes, attachment of tissues to coverslips, and individual handling of tissue blocks. Specimen sizes between approximately 1×1 mm and 2×5 mm were tested.

Fixation

Samples were immersion-fixed in 1% paraformaldehyde and 0.1% glutaraldehyde in 0.1 *M* $NaPO_4$ buffer, pH 7.4, for 15 or 30 min, or in buffered 2% paraformaldehyde, 0.2% glutaraldehyde for 15 or 30 min. Following fixation, the tissue was washed in three changes of the same buffer.

Probe

The probe consisted of a recombinant cDNA clone containing 5.9 kb of sequences complementary to all skeletal forms of myosin heavy chain mRNA (PCM-5, an extension of PCM-2) (Freyer and Robbins, 1983). The probe was biotin-labeled by nick translation (Langer *et al.*, 1981), and DNase concentrations utilized routinely yielded probe fragments from 100 to 400 nucleotides. Four different probe preparations were used in the course of these experiments; the selected protocol was repeated with two of these probes.

Detergent Treatment and Hybridization

Prior to hybridization the tissue was treated with a solution of 0.5% Triton X-100, 0.5% Saponin in 0.1 *M* $NaPO_4$ buffer, pH 7.4, for 15 or 30 min, then rinsed thoroughly in three 5-min changes of the buffer (Singer *et al.*, 1989b). The tissue was pretreated first in 0.1 *M* glycine, 0.2 *M* Tris, pH 7.4, for 10 min, and then in 50% formamide, 2× SSC (1× SSC = 0.15 *M* NaCl, 0.015 *M* Na citrate, pH 5.5) at 60°C for 5–10 min. For the ribonuclease controls, tissue was suspended in 20-μl drops of a 100 μg/ml solution of equal parts RNase A, T_1, and T_2 in 2× SSC for 1 hr at 37°C before the two pretreatment steps.

A buffer containing 50% formamide, 10% dextran sulfate, 2× SSC, and 100 μg/μl each of tRNA and sheared salmon sperm DNA, 1% BSA, and 1 μg/ml of probe was used to hybridize the tissue (Lawrence and Singer, 1985). The nucleic acid components were heated to 90°C for 10 min in 100% formamide before adding the rest of the mixture. Two controls were run concurrently using the same buffer mixture but substituting biotinylated pBR322 (the probe vector) for the complete probe, or eliminating the probe. For hybridization, each piece of tissue was suspended in an individual 20-μl drop of hybridization mixture in a parafilm-covered well of a ceramic spot plate, covered with a parafilm-covered glass sheet, and wrapped in parafilm to prevent drying during overnight incubation at 37°C. For the remaining steps of the protocol each sample was isolated and precautions were taken to avoid contamination, including the use of a sterile instrument for each transfer of each sample.

Following hybridization the tissue was transferred back to vials and washed at 37°C for 30 min first in 2× SSC, 50% formamide, and then in 1× SSC, 50% formamide, and finally on a shaker for 30 min in 1× SSC at room temperature.

Detection

Individual samples were suspended in 20-μl drops of rabbit anti-biotin (Enzo Biochem., Inc., New York) in 1% BSA, 1% Triton X-100, 0.15 *M* NaCl, 0.05 *M* Tris, pH 7.4, at dilutions of 1:10, 1:4, 1:1, and undiluted, and incubated 4 to 5 hr at 37°C. Washing in three changes of 1× PBS (2.7 m*M* KCl, 1.5 m*M* KH_2PO_4, 137 m*M* NaCl, 4.3 m*M* $Na_2HPO_4 \cdot 7H_2O$, pH 7.4) for 30 min on a shaker followed incubation. After washing, tissue was incubated overnight in individual 20-μl drops of 10 nm gold-labeled goat anti-rabbit antibody (Janssen Life Science Products, Piscataway, NJ) in the same buffer, at the same dilutions, at room temperature. Incubation was followed by 1 hr of washing in three changes of 1× PBS on a shaker. As a fourth control, tissue was carried through all other steps, but was not treated with primary antibody.

Electron Microscopy

The tissue, processed as above, was then fixed for 30 min in 2% glutaraldehyde in 0.1 *M* $NaPO_4$, pH 7.4, buffer followed by 1% OsO_4 in the same buffer for 30 min. Following dehydration in a graded ethanol series, infiltration, and embedding in Polybed 812 (Polysciences, Inc., Warrington, PA), it was thin-sectioned using block faces up to 1×2 mm depending on sample size, stained with lead and uranyl acetate, and examined with a JEOL 100S electron microscope.

Evaluation of Hybridization Protocols

In 12 experiments to determine the most successful protocol for this combination of probe and tissue, the

following parameters were varied as detailed above: (1) tissue size and handling, (2) time and concentrations of initial fixatives, (3) time of treatment in detergents, and (4) concentrations of antibiotin and gold-labeled secondary antibody. The results were assessed by comparison of ultrastructural morphology in hybridized and conventionally prepared tissues, a semiquantitative estimate of signal or background labeling levels based on at least two different controls for each experiment, and an unavoidable measure of empirical judgment.

Analysis of Results from Selected Protocol

After the most successful conditions were selected, three independent experiments following the same protocol but utilizing two different probe preparations were evaluated in detail. From each experimental and control tissue block 60- to 90-nm sections were collected, photographed, and studied. Two thousand gold particles were initially counted in blocks of MHC probe tissue from each of the three separate experiments and in the equivalent tissue area in two different controls for each. The signal-to-noise ratio was computed by comparing the total number of gold particles over myotubes and early myofibers on experimental samples (signal) with the number over myotubes and early myofibers on the control samples (noise). Extensive analysis was based on the counting of ≥12,000 gold particles in the MHC probe tissue samples from one experiment, using photographic prints magnified ×45,000. After determining the tissue area examined, all gold particles were counted in the same area in the tissues run under the four different control conditions. Both the total number of gold particles and the number of clusters of gold particles of each size (1 to >30) were recorded. The distribution of gold particles could be best studied in these and other photographic prints since cellular components had low contrast. Penetration of the probe and detection antibodies into the tissue was estimated by determining the number and thickness of sections between the tissue face and the point where all labeling was absent in a series of thin sections. The same blocks were then turned approximately 90° to expose a new cut face and again sectioned in the same way.

RESULTS

Selection of Hybridization Protocol

By the criteria described previously, the most successful of the experimental conditions we tested were: handling of tissue samples as individual 1 × 3 mm strips, fixation in 2% paraformaldehyde, 0.2% glutaraldehyde for 15 min followed by treatment in 0.5% Triton X-100, 0.5% Saponin for 15 min, and use of both detection antibodies undiluted. This protocol represents a delicate compromise, because when initial fixation was increased to 30 min, muscle morphology was improved but the tissue showed less labeling. Detergent treatment was necessary for good labeling, but 30 min significantly reduced tissue integrity. Fortunately, labeling levels increased as concentrations of detection antibodies were increased, while background remained acceptably low.

In brief, the three experiments following this protocol all gave qualitatively similar results in tissue ultrastructure and the distribution and pattern of gold particles. In each of the three experiments, controls (vector alone, no probe, no antibiotin, RNase) clearly had lower levels of labeling than did the experimentals, and overall signal-to-noise ratios were ≥6:1 (see below).

Morphology of Hybridized Tissue

The ultrastructure of myotubes and early myofibers prepared by standard methods has been described by a number of investigators (e.g., Allen and Pepe, 1965; Fischman, 1967). The particular features relevant to these hybridization studies (Fig. 1) were the wide lateral spacing of developing myofibrils, the close association of individual myofibrils with the cell's complex cytoskeletal network (Cooke, 1985), and the striking linear arrays of ribosomes (occasionally more than 70, but usually fewer) which were usually oriented parallel to the longitudinal axis of the cell and associated with the cytoskeletal filament system close to the developing myofibrils (Allen and Pepe, 1965).

In tissue hybridized by our most successful protocol (Fig. 2a), myotubes and early myofibers could be identified by characteristics including syncytial nature, nuclear characteristics, and presence of developing myofibrils. M, A, I, and Z bands were well delineated; myofilaments were held together more closely at the Z band than elsewhere in the sarcomere. The distribution and associations of nonmyofibrillar cytoskeletal components, both the longitudinally oriented 8- to 10-nm filaments and the more randomly arrayed 4- to 6-nm filaments, seemed unperturbed by the procedure. Effects of the detergent treatment that followed fixation were evident in the lack of prominence and, in some cases, rupture of plasma and inner and outer mitochondrial membranes; the nuclear envelope remained intact. Cytoplasmic aggregations of glycogen particles were removed. As has been found in other ultrastructural hybridization studies (Singer *et al.*, 1989a), free polysomes were either absent or altered so that they did not stain with the usual heavy metals (e.g., Fig. 2b), presumably due to extraction by the hybridization solution. Some mem-

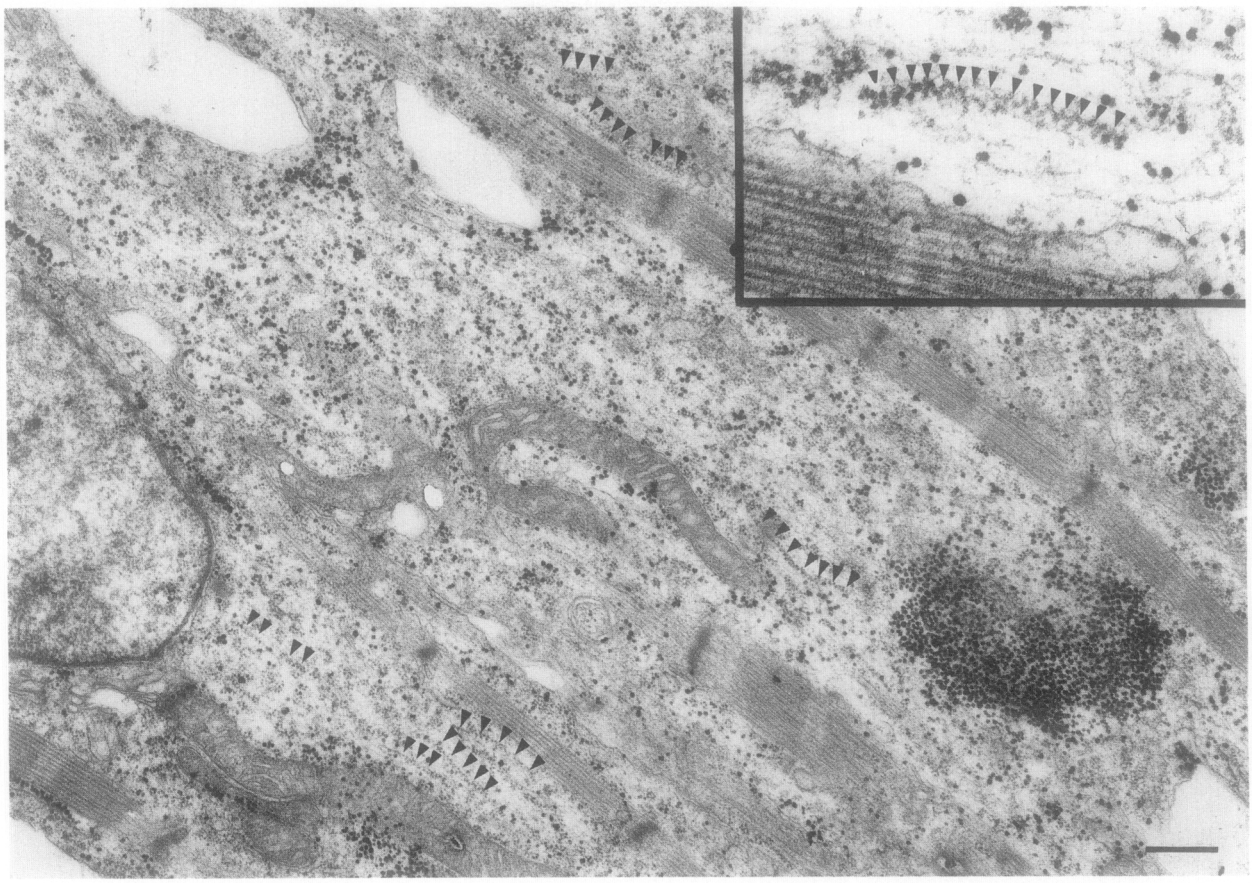

FIG. 1. Unhybridized chick muscle at the same developmental stage at which we have examined MHC mRNA localization. Myofibrils are widely spaced; the cytoplasm between them contains mitochondria, glycogen, and long chains of polysomes (arrowheads) which are oriented in the longitudinal axis of the cell and are closely associated with a complex cytoskeletal filament system (inset). Bar, 0.5 μm for main figure, 0.2 μm for inset.

brane-bound polysomes could be identified, mostly in nonmuscle cells.

Quantitative Considerations

The signal-to-noise ratio for each of the three separate experiments, based on counting 2000 particles for signal, was ≥6:1. Based on the amount of tissue area surveyed to achieve the 2000-particle count, we selected one experiment for complete quantitation. In that experiment we counted 13,345 gold particles in a tissue area of 2300 μm^2 divided approximately equally between the two MHC probe blocks, and all particles in the 2300 μm^2 of area from the four controls, again divided between two different specimens in each case. In reviewing the sections and micrographs, we believe that we examined portions of at least 1000 cells under each condition. Our estimates suggested that the probe and/or the detection system antibodies penetrated the tissue approximately 10 μm from the nearest cut surface, but that deeper penetration was not reliably achieved. Therefore, all sections used for quantitation came from within 5 μm of a cut tissue surface.

The overall signal-to-noise ratio for this experiment computed using an average of the four controls as "noise" was >7:1. This means that >85% of the gold particles over the developing muscle cells represent MHC mRNAs. The ratios computed using each individual control as noise ranged between 6:1 (no probe) and >10:1 (vector only). This result is essentially equivalent to the preliminary signal-to-noise ratios which we had calculated (based on counting 2000 gold particles for signal).

No single type of control adequately represents all possible sources of experimental error; furthermore several controls overlap each other in the nonspecific signals they reveal. In these experiments nonspecific sticking of probe was evaluated by using a biotinylated pBR probe lacking the MHC insert. Omitting the probe altogether permitted evaluation of nonspecific sticking of the antibiotin and/or the gold-conjugated antibody, while omitting the antibiotin revealed nonspecific stick-

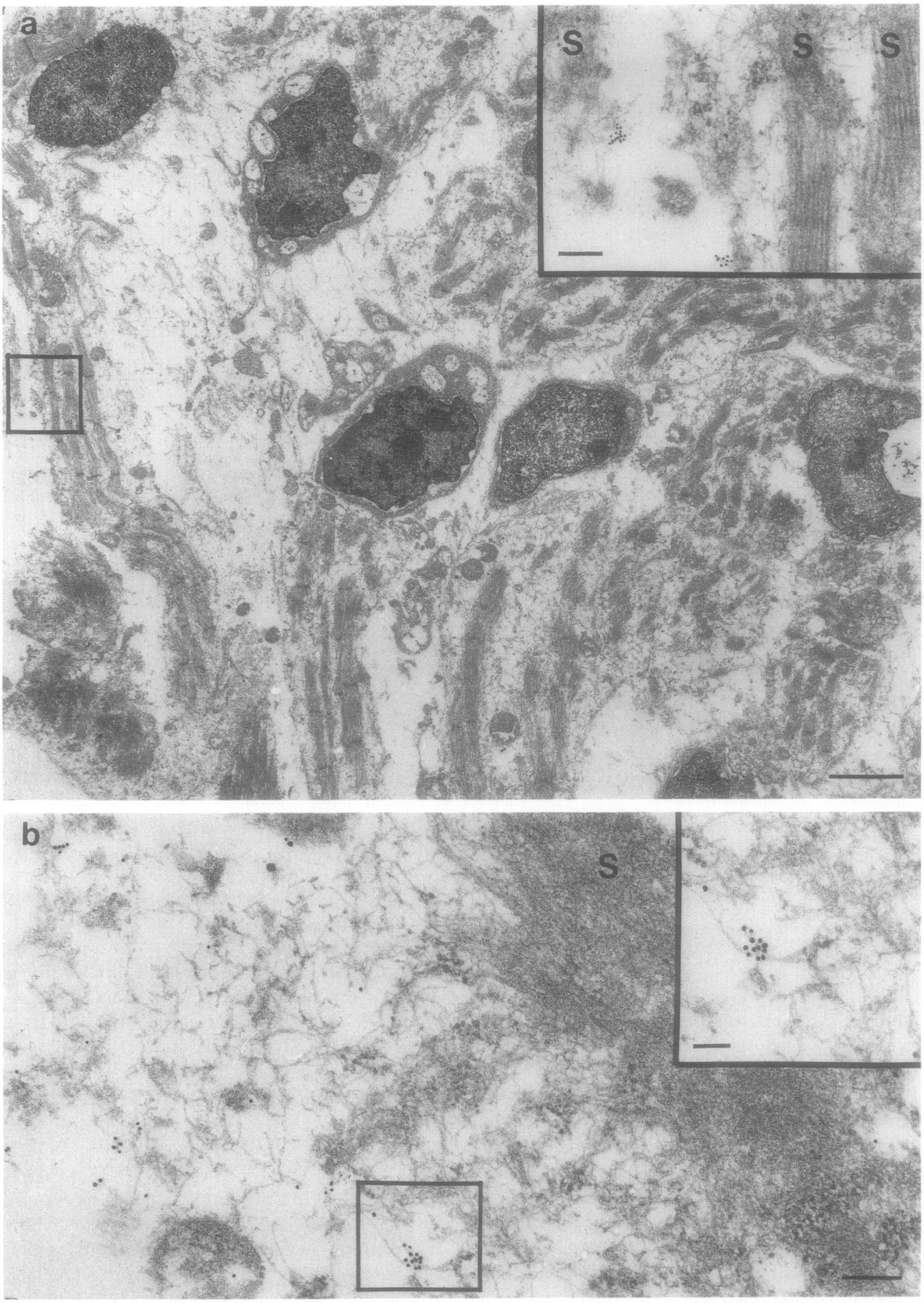

FIG. 2. Localization of MHC mRNA in 14-day chick pectoralis detected by colloidal gold-labeled antibodies in thin sections. (a) Overall structure of a typical block of embryonic tissue which was fixed prior to detergent treatment and hybridized before embedment is shown. The boxed area enlarged in the inset shows two clusters of 9 and 18 gold particles which are near but not apposed to myofilaments in developing sarcomeres (S). Bar, 2.0 μm; inset bar, 0.2 μm. (b) Usual pattern of gold labeling following this protocol. The largest cluster of 12 particles (enlarged in the inset) sits atop the intersection of several cytoskeletal filaments. Bar, 0.2 μm; inset bar, 0.1 μm.

TABLE 1
SIZES OF GOLD PARTICLE CLUSTERS IN EXPERIMENTAL AND CONTROL TISSUES

	Number of clusters in 2300 μm^2 of tissue				
		Controls			
Cluster size	MHC probe	Vector only	No probe	No antibiotin	RNase
1-2	4772	390	1135	935	928
3-4	736	28	107	173	88
5-6	251	8	34	49	24
7-8	123	1	17	18	19
9-10	63	2	4	10	3
11-12	35	2	3	4	0
13-14	30	0	1	5	0
15-16	19	0	0	1	0
17-18	10	0	0	1	0
19-20	6	0	1	0	0
21-22	6	0	1	0	0
23-24	3	0	0	0	0
25-26	6	0	0	0	0
>30	6	0	0	0	0

ing of the gold-conjugated antibody. Digestion in a mixture of three RNases provides yet another control, but this control is often less reliable due to incomplete digestion or to increased background across the entire sample. One of the most troublesome errors involves random sticking of the detection system and this is reflected in one way or another in each control. Therefore we have used an average of the four controls for simplicity. A fifth extremely valuable control is provided by the presence of nonmuscle cells (primarily fibroblasts) within the hybridized specimens. No difference was observed between "signal" and "noise" over nonmuscle cells (based on analysis of approximately 30 cells).

More specific than the signal/noise ratio based on comparison of total gold particle numbers is a consideration of clusters of gold particles—the number in each size category, and the arrangement of individual gold particles within a cluster, as Singer *et al.*, (1989a) have previously discussed in detail. Because many probe molecules hybridize to one mRNA template, bona fide hybridization should produce larger groupings of gold particles; furthermore, if the gold particles are close enough together on the mRNA template, they should reflect its 3-D configuration.

The data in Table 1 show that the number of clusters of gold particles in each size category was significantly higher using the MHC probe compared with any of the controls. Additionally, clusters containing more than 10 gold particles occurred very rarely in any of the controls. This resulted in signal/noise that increased from 6:1 for 1-2 particles to 15:1 for clusters of 13-14. The noise level diminished essentially to zero at cluster sizes > 14 so that signal/noise approached infinity (Fig. 3).

Locations and Associations of MHC Messages in Individual Myotubes

Most of the gold particles indicating presence of MHC mRNA were located in peripheral regions of the developing muscle cells near both myofibrils and myonuclei. A lower total amount of gold was present in the central cores of the cells. No gold labeling was found between the myofilaments of an individual myofibril (Figs. 2a, 4a), associated with intact mitochondria, or within myonuclei (Fig. 4c). Controls showed some gold particles within the central regions of the cell, but almost none in the latter three structures. While clusters of gold particles were located in the neighborhood of myofibrils, they were usually about 0.1–0.5 μm from the nearest myofilament that was visualized in that section (Figs. 2, 4). They were associated with the complex cytoskeletal network which pervaded the cytoplasm. Most commonly they sat atop filaments which measured 4–10 nm in diameter (Ishikawa *et al.*, 1968). Some gold particles occupied regions of intersection between several filaments, others covered part of a single filament (Figs. 2b, 4).

The largest clusters of gold (>10 particles) almost all touched one or more cytoskeletal filaments, but their

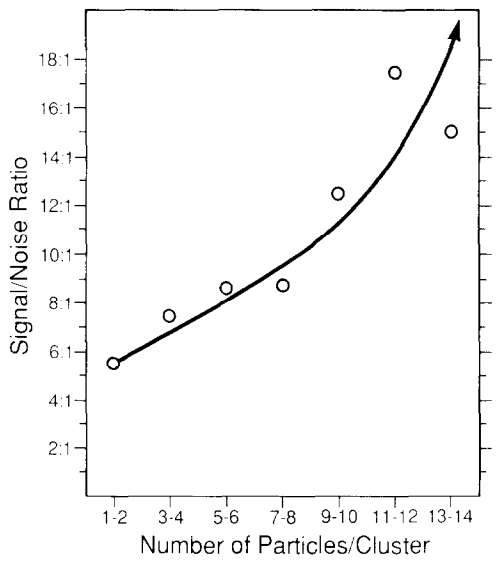

FIG. 3. Signal-to-noise ratios for gold cluster sizes ranging between 1 and 14 particles, based on analysis of 2300 μm^2 of tissue. For cluster sizes > 14 particles the ratio approaches infinity, as the arrowhead indicates. In each case the noise level is based on an average of the four controls (vector only, no probe, no antibiotin, RNase) as discussed in text.

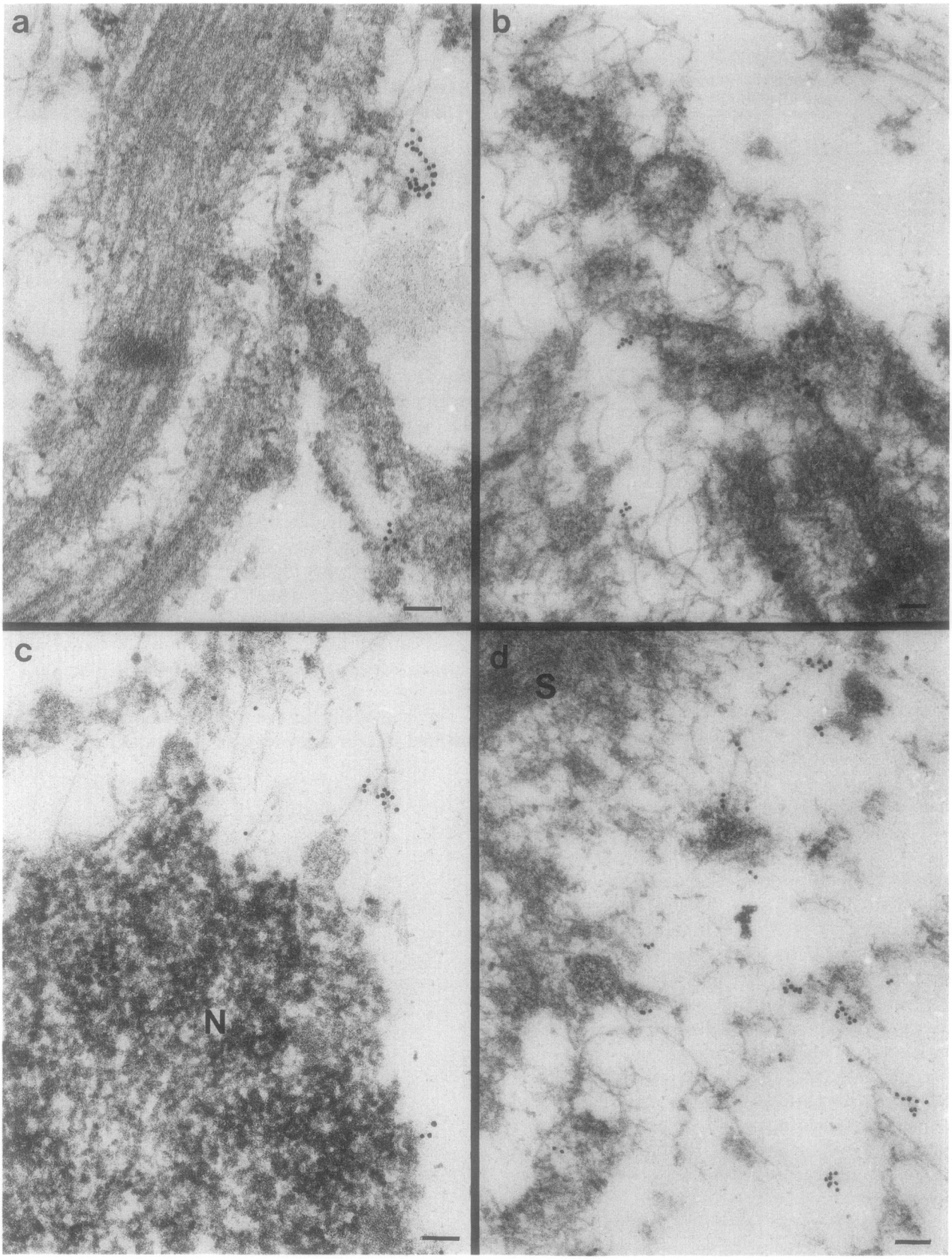

FIG. 4. Gallery of micrographs showing the intracellular localization of MHC mRNA in myotubes and early myofibers of intact muscle which is demonstrated by the reported hybridization protocol. Most colloidal gold particles are associated with cytoskeletal filaments. (a) Curving array of 24 gold particles is more than 0.3 μm from the nearest myofilament visualized. (b, c, and d) The arrangements of gold particles are less easily described, perhaps because of interruptions produced by sectioning. None is directly associated with formed myofilaments, although they are often in the neighborhood of developing sarcomeres (a, b, d). Label is occasionally associated with cytoskeletal filaments near myonuclei (N) as shown in c. In each panel bar, 0.1 μm.

shapes did not necessarily closely conform to that of the filaments (Fig. 4a). Based on size, these filaments could be either actin or intermediate filaments or both (Bennett *et al.*, 1979; Anderton, 1981; Steinert *et al.*, 1984). Clusters of gold were not associated with 14-nm filaments or with the small population of microtubules visualized in the hybridized cells. It should be emphasized that we visualized associations of gold particles with filaments of the complex cytoskeleton near myofibrils, but not with identifiable myofilaments, either individual (which were rare in our material) or associated with myofibrils.

Gold particles within clusters sometimes were regularly spaced apart, but their arrangement could not usually be described as having the well-defined circular or spiral patterns visualized for gold particles labeling actin mRNA in whole mounts of cultured chick myoblasts by Singer *et al.* (1989a). The size of the clusters revealed by the MHC probe seemed to be related to the plane in which the myotubes were cut. The largest size clusters were seen in approximately longitudinal sections (as judged with reference to the configuration of myofibrils) (Fig. 4a), as were the longest polysomal arrays (Fig. 1). They were almost never seen in sections that were nearly transverse.

DISCUSSION

We have demonstrated a high resolution *in situ* hybridization protocol which has made it possible to visualize MHC mRNA in developing cells of intact muscle. The conclusion that colloidal gold particles represent MHC message is based on both an overall signal/noise ratio of over 7:1 determined by rigorous quantitation and the fact that the signal/noise ratio increased steadily with increasing cluster size. Virtually no clusters of 10 or more gold particles were seen in any of the five different control preparations, including the internal control provided by nonmuscle cells in experimental sections. We have visualized cellular elements with which these MHC messages are associated, and have related the location of these MHC mRNAs to the cells as a whole and to mature myofilaments. This study extends previous electron microscopic hybridization methodology to intact tissues which have been aldehyde-fixed prior to detergent treatment, and are hybridized before embedment.

The majority of gold particles detecting MHC mRNA were associated with nonmyofibrillar cytoskeletal filaments which ranged in diameter between 4 and 10 nm and corresponded in size and distribution to actin and/or intermediate filaments. This specific association of MHC mRNA with the cytoskeleton, and not with other cytoplasmic components, supports previous biochemical and cell fractionation studies, indicating a physical association of cellular mRNA with the cytoskeleton (Lenk *et al.*, 1977; Fulton *et al.*, 1980; Pramanik *et al.*, 1986; Pudney and Singer, 1979, 1980). Since proteins and other cellular components in our tissue were fixed in aldehydes prior to treatment with the Triton-Saponin mixture, this feature of our method answers the criticism that disruption of the cytosol by detergents might alter critical intracellular relationships in unfixed cells. Our results therefore support a number of previously reported studies which have demonstrated the retention of mRNAs for nonsecreted proteins with the Triton-insoluble cytoskeletal framework.

The size of even the largest clusters observed was consistent with the association of an individual cluster with a single MHC mRNA molecule. Since MHC mRNA is 5.9 kb, one could theoretically fit as many as 100 of the 10-nm gold particles used for detection along just one molecule (if antigen-binding sites were spaced 150 A apart). In fact, most clusters are much smaller than this theoretical limit. Presumably sectioning the hybridized tissue at 60–90 nm for electron microscopy would cut MHC mRNA molecules into shorter fragments, thus limiting the theoretical number of very large clusters. If the mRNA molecules were aligned in a specific plane relative to myofibrils and to the myotube or early myofiber as a whole, then one would expect cluster sizes to vary as a function of the angle between the long axis of these highly polarized cells and the plane of section. That is exactly what we observed. The largest clusters are seen specifically in sections that are longitudinal with respect to myofibrils. They are remarkably similar in size, location, and orientation to long polysomes in conventionally prepared developing chick muscle cells (Fig. 1) that have been thought by some to be reading MHC messages simply on the basis of their size and abundance (Allen and Pepe, 1965). The longitudinal orientation of these largest clusters conforms to that of intermediate filaments which contain both desmin and vimentin in early myotubes (Tokuyasu *et al.*, 1985; Gard and Lazarides, 1980). However, other filaments such as titin or nebulin (Wang, 1982; Wang and Wright, 1988) could also provide this orientation.

Interpretation of *in situ* hybridization studies must take into account the accessibility of specific cellular areas to the detection system. One useful indication is provided by the nonspecifically attached gold in the "no probe" and "no antibiotin" controls because the gold particles are the largest component of the system. Since central regions of the developing muscle cells are accessible to the gold particles, based on these two controls, then the lower labeling in central as compared to peripheral areas with MHC probe demonstrates a localization of MHC mRNAs. On the other hand, myonuclei, mitochondria, and regions within formed myofibrils are ap-

parently less accessible to the detection system; therefore, we cannot determine whether MHC mRNAs are present or absent in these regions. This does not alter the validity of the localization and cellular associations that we have reported; however, the possibility remains that MHC mRNAs may be found in additional locations.

Dix and Eisenberg (1988) localized MHC mRNA to myonuclei as well as intermyofibrillar regions in adult rabbit skeletal muscle by light microscopy using cRNA probes. We cannot comment on nuclear labeling and found no specific concentrations of cytoplasmic labeling close to the nuclear envelope. However, occasional messages can be seen in the vicinity of the nucleus (Fig. 4c) possibly in the process of transport from the nucleus to the sites of translation.

Studies based on filament isolation (Etlinger *et al.*, 1975) and EM autoradiography (Morkin, 1970) in postnatal or adult mammalian skeletal muscle suggest that newly synthesized myofilaments are preferentially added to the periphery of myofibrils. During elongative myofiber growth they are likely also added at their ends. Our observation of many MHC messages located near the periphery of myofibrils in developing chick pectoral muscle cells is consonant with those earlier findings. Actin mRNA is also found in the area of the cell actively undergoing actin protein polymerization (Lawrence and Singer, 1986); this suggests that the regional colocalization of protein synthesis and protein assembly may be a general phenomenon.

While MHC messages are located near developing myofibrils, the majority are 0.1–0.5 μm from the nearest myofilaments identifiable in the same sections. This spatial information, provided by high resolution hybridization, implies that nascent chains read from those messages do not assemble directly into growing myofilaments simultaneous with translation. Instead, it suggests an intervening process promoting or allowing the MHC proteins to assemble myofilaments, which at this stage are almost all part of developing myofibrils. It is possible that the MHC may diffuse to sites of assembly in a dynamic equilibrium model as suggested by Bouche *et al.* (1988); locating its mRNA near myofibrils could raise the critical concentration of MHC in this region, thereby promoting assembly. Microinjection of fluorescent myosin (Johnson *et al.*, 1988) indicates that correct assembly can occur post-translationally. Alternatively, as suggested by Isaacs and Fulton (1987) the nascent chains may be associated with and/or transported by the cytoskeletal filament system rather than diffusing freely. Pavlath *et al.* (1989) have shown that MHC and presumably its mRNA does not diffuse far from the nucleus activated for its synthesis. Sarcomere assembly could be facilitated by interaction of the growing sarcomere with filaments transporting the MHC proteins. Our observations are compatible with either of these models but, as suggested above, would appear to rule out models where proteins are incorporated directly into myofilaments of the developing sarcomere simultaneous with their synthesis. In future work it should be possible to obtain simultaneous *in situ* hybridization and immunogold detection of sarcomeric or filament proteins. This should provide more information about the specific filaments with which MHC mRNAs associate, and the events that may intervene between synthesis of MHC nascent chains and myosin filament assembly.

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