Strategies for Ultrastructural Visualization of Biotinated Probes Hybridized to Messenger RNA In Situ

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1 Introduction

Our laboratory has emphasized in situ hybridization as a means of investigating the expression and distribution of specific mRNA molecules within single cells, particularly in developing chicken muscle cells in culture. Using an in situ hybridization protocol optimized for maintaining the structural integrity of the cell and the

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native configuration of RNA (LAWRENCE and SINGER 1985; SINGER et al. 1986b), we investigated the intracellular distribution of specific mRNAs within intact myoblasts and fibroblasts and provided evidence indicating that mRNAs for certain cytoskeletal proteins are localized within specific regions of the cell (LAWRENCE and SINGER 1986). In particular, actin mRNA was found to be associated with the mobile structure of the cell, the lamellipodia, shown to be site of active actin polymerization (WANG 1985).

An understanding of the molecular mechanism(s) governing this mRNA localization will require elucidation of the physical association of mRNA with the cytoskeleton, since this may clarify how mRNAs are held in place within the cell or are transported to their final destinations. The investigation of mRNA–cytoskeleton association would be greatly facilitated by development of an electron microscopic in situ hybridization methodology. An ultrastructural study would provide a higher resolution view of the intracellular distribution of mRNA molecules particularly as it relates to the disposition of the well-studied cytoskeletal proteins.

We have extended our work on nonisotopic detection to the resolution of the electron microscope using biotin-labelled probes (SINGER et al. 1986a, 1987b, 1989; SILVA et al. 1989). High resolution is possible since the detection of biotin occurs directly at the site of hybridization. Much previous work using biotin-labelled probes detected by indirect immunocytochemistry with colloidal gold-labelled antibodies has been done by B. HAMKALO and her associates (e.g., HUTCHINSON et al. 1982; RADIC et al. 1987) for high resolution studies on reiterated chromosomal sequences. BINDER et al. (1986) have employed in situ hybridization using biotinated probes to lowicryl-embedded thin sections to detect mitochondrial rDNA and small nuclear RNAs using biotin antibodies and protein A–colloidal gold. WEBSTER et al. (1987) have reported the detection of P_0 mRNA in Schwann cells and WOLBER et al. (1988) have reported the detection of cytomegalovirus sequences in infected fibroblasts.

Recent work (SINGER et al. 1989) has developed a method of analysis of electron microscopic data which gives extremely high signal-to-noise ratios. This approach, described in more detail later, utilizes colloidal gold antibodies to detect biotinated probe molecules hybridized to the target mRNAs. Since the mRNA acts as a template, the smaller probe molecules hybridize in an iterative way to the message strand. Analysis in this way allowed us to identify single mRNA molecules.

We undertook to continue our investigations of muscle proteins and messenger RNAs in developing myotubes, since we have analyzed the expression of similar genes in previous publications (PUDNEY and SINGER 1979, 1980; SINGER and PUDNEY 1984; LAWRENCE and SINGER 1985, 1986; SINGER et al. 1986a, b, 1987a, b), particularly in regard to the use of nonisotopic detection methods (i.e., biotinated probes). The use of biotinated oligonucleotides to investigate the expression of different isoforms of actin has revealed the asynchronous expression of particular genes representing the cardiac form of actin, the skeletal form, and the suppression of β-actin (LAWRENCE et al. 1989). Having elucidated the expression of actin, we wished to explore as well the expression of the myosin heavy-chain gene, particularly since this may shed light on the development of the sarcomere in early myogenesis. While whole critical-point dried cells viewed by electron micro-

scopy have proven valuable for detection of cytoskeletal mRNAs in single cells in culture, the more dense structure of the myofiber necessitates a thin sectioning approach. We then extended our observations using biotin-labelled probes in conjunction with a "preembedding" ultrastructural thin sectioning approach to detect mRNA. This provided further information on message distribution and its association with certain cellular components. In particular, the investigation of the distribution of myosin heavy-chain mRNA in sectioned myotubes can elucidate the relationship of protein synthesis to the developing sarcomere. Results in an earlier publication (SILVA et al. 1989) illustrated an approach to these questions and emphasized difficulties in analyzing technical parameters based on high magnification studies.

In this chapter, we detail the strategies we have employed to approach the superimposition of precise molecular information onto high resolution ultrastructural morphology. In addition, we summarize the progression of our ultrastructural investigations from Triton-extracted single cells to Triton-extracted thin-sectioned myotubes, both of these in tissue culture; and finally we report the development of a method for in situ hybridization to fixed tissue. This method has been used to investigate the relationship of myosin heavy-chain mRNA to the developing sarcomere in chicken muscle tissue.

2 Materials and Methods

2.1 Biotination of Probes

During nick translation with biotinated nucleotide, the probe is reduced to fragments that preferably should average about 200 nucleotides per molecule. The probe size is controlled by the DNase concentration in the reaction. Probe size was monitored by alkaline agarose gel electrophoresis, using alkaline phosphatase detection on probes transferred to nitrocellulose (BRL, Gaithersburg, MD, DNA detection kit). Incorporated into this probe was a biotinated analog of thymidine [biotin-11-deoxyuridine triphosphate (dUTP) Enzo Biochemicals New York, N.Y] with several biotins per 100 nucleotides. Biotin-specific activity was measured by incorporating phosphorus-labelled deoxycytidine triphosphate (dCTP) into the probe simultaneously with biotinated nucleotide [60 μm, diluted 1:10 with unlabelled thymidine triphosphate (TTP)]. Assuming the biotinated nucleotide was incorporated at 80% of the rate of the equivalent unlabelled nucleotide (LANGER et al. 1981) and that the total incorporation of dCTP was equimolar to TTP, we calculate that the nick-translated probe was substituted approximately 2% with biotinated nucleotides.

Probes used were the full-length β-actin clone (1.9 kb) and the full-length sarcomeric myosin heavy-chain clone (a gift of J. Robbins, 5.9 kb).

2.2 Treatment Through Hybridization

2.2.1 Cells for Whole-Mount Electron Microscopy

2.2.1.1 Cell Culture

Skeletal myoblasts and fibroblasts were isolated from the pectoral muscles of 12-day-old chicken embryos and cultured by standard techniques. Cells were plated

at a density of 2×10^5/well in Costar six-well tissue culture plates containing grid assemblies: carbon–formvar-coated electron microscope grids on 22-mm circular coverslips. Grids were London 200 mesh or honeycomb gold finder types (Fullam, Laham, N.Y.). Carbon rods and formvar were purchased from Tousimis (Rockville, MD). Grid assemblies were made by floating a formvar film onto glass coverslips supporting between four and nine gold grids. After drying, the formvar ist stabilized with a thin layer of carbon. Grid assemblies in multiwell dishes were sterilized by γ irradiation from a cesium 137 source in a Gammcell (Atomic Energy of Canada, Ltd.). All pretreatments and washes were performed in the six-well tissue culture plates.

Three-day old cultures were extracted and fixed by a modification of the method of LENK et al. (1977). Cells were briefly washed with isotonic buffer (0.3 *M* sucrose, 0.1 *M* NaCl, 10 m*M* 1,4 Piperazinediethanol sulfonic acid (PIPES), 3 m*M* $MgCl_2$, 10 μM Leupeptin (Sigma, St. Louis, MO). 1:40 dilution of vanadyl complex (4 m*M* adenosine, 0.2 *M* $VOSO_4$, pH 7), then extracted for 90 sec in the same buffer plus 0.5% Triton X-100 (Boehringer Mannheim, W. Germany) followed by another brief wash in the first buffer, fixed with 4% glutaraldehyde in phosphate-buffered saline, 5 m*M* magnesium, and passed through an ethanol series (30%, 50%, 70% for 5 min each). Each solution was removed by aspiration. Cells were stored in 70% ethanol at 4 °C until used. In order to preserve microtubes, all solutions were used at room temperature and ethylene glycol tetra-acetic acid was included to eliminate calcium.

2.2.1.2 Hybridization

The details and derivation of the hybridization protocol have been published elsewhere (LAWRENCE and SINGER 1985; SINGER et al. 1986b, 1987a). The salient features of this method are that cell treatments that disrupt morphology or cause loss of mRNA have been minimized. However, Triton extraction of the cells was used initially in order to facilitate satisfactory probe penetration (see Sect. 3). Briefly, cells on grid assemblies were rehydrated in phosphate saline with 5 m*M* $MgCl_2$ for at least 10 min, followed by 0.1 *M* glycine, 0.2 *M* TRIS, pH 7.4 for 10 min. Afterwards cells were kept in 50% formamide, 2X SSC at 37 °C for 15 min prior to hybridization. For ribonuclease controls, 100 µl of a 100 µg/ml solution of RNase A in 2X SSC was applied to samples for 1 h at 37 °C prior to the 50% formamide 2X SSC prehybridization treatment. Probes used were biotinated by nick translation (LANGER et al. 1981) and were approximately 100–200 nucleotides in length. Cells were hybridized in buffer containing 50% formamide, 2X SSC with 100 µg/µl each of tRNA and sheared salmon sperm DNA, 1% bovine serum albumin (BSA) and 1 µg/ml of probe. The nucleic acid components were heated to 90 °C for 5 min in 100% formamide before adding the other components.

Each grid assembly was placed cell-side down onto a 10 µl drop of the hybridization mixture on parafilm, covered with an additional sheet of parafilm to prevent drying, and incubated for 3 h in a humidified chamber of 37 °C. After hybridization, coverslips were gently removed from the parafilm and placed in individual wells in a clean multiwell dish containing 2.5 ml 50% formamide, 1X SSC, and finally 1X SSC, each for 30 min. The cells were left in 1X SSC overnight at 4 °C to be stained the next day with antibodies.

2.2.2 Cultured Myotubes for Thin-Section Electron Microscopy

2.2.2.1 Cell Culture

Cells grown on glass or plastic coverslips were used, Triton-extracted as described above, and postfixed in 2% glutaraldehyde; other fixatives, when used appropriately, can also give good results (SILVA et al. 1989). The cells were either processed immediately after fixation or stored in 70% ethanol or phosphate-buffered saline for brief periods of time (days) if antibodies to protein were to be used.

2.2.2.2 Hybridization

Cells were rehydrated in phosphate-buffered saline plus 5 m*M* $MgCl_2$ for 10 min in Coplin jars, followed by 0.1 *M* glycine, 0.2 *M* TRIS-HCl, pH 7.4, for 10 min. The cells were then placed in 50% formamide (Fluka, Switzerland) 2X SSC (0.3 *M* NaCl in sodium citrate buffer) for 10 min prior to hybridization. The probe, *E. coli* tRNA, and salmon sperm DNA were lyophilized and then resuspended in formamide and melted at 90 °C for 10 min. Just prior to placing on the cells, the probe, tRNA, and DNA were combined with the hybridization mix, so that the final probe concentration was 1 μg/ml and the final hybridization solution consisted of 50% formamide 2X SSC, 0.2% BSA, 10 m*M* vanadyl sulfate ribonucleoside complex (BERGER and BIRKENMEIER 1979; this can be eliminated if all solutions are autoclaved and RNase-free), 10% dextran sulfate (Sigma), and 1 mg/ml each of *E. coli* tRNA and salmon sperm DNA. Cells on the coverslips were incubated in 20 μl hybridization solution for 3 h at 37 °C by putting the coverslips cell-side down on parafilm. After hybridization, coverslips were placed in 10-ml Coplin jars (VWR) and rinsed three times with shaking for 30 min each in 2X SSC, 50% formamide at 37 °C; SSC, 50% formamide at 37 °C; and SSC at room temperature.

2.2.3 Aldehyde-Fixed Developing Muscle for Thin-Section Electron Microscopy

2.2.3.1 Tissue Preparation

Hand-cut thin strips of pectoral muscle from 14-day-old chicken embryos were immersed in 2% paraformaldehyde 0.2% glutaraldehyde for 15 min. They were then rinsed and extracted for 15 min in 0.5% Triton and 0.5% saponin, followed by 0.1 *M* glycine, 0.2 *M* TRIS-HCl (pH 7.4) for 10 min. Strips were placed in 50% formamide (Fluka), in 0.3 *M* NaCl in sodium citrate buffer for 10 min.

2.2.3.2 Hybridization

Strips were incubated overnight in individual 20 μl drops of a hybridzation solution which differed from that used for cultured cells only by the absence of $VOSO_4$ ribonucleoside complex.

2.3 Detection of Hybridization

2.3.1 Cells on Grids or Coverslips

Grids or coverslips were pretreated with 8% RNase-free BSA (Boehringer, FRG), TRIS-buffered saline (TBS), pH 7.4, for 10 min. Excess fluid was removed with

bibulous paper and each grid was transferred to 100 µl drops of the primary antibody staining solution: rabbit antibiotin (Enzo Biochemicals) diluted 1:100 in 1% BSA, 0.5% Triton X-100, TRIS-buffered saline, pH 7.4 (BTTBS) and incubated for 2.5 h at room temperature. They were washed for 30 min on a stirring platform with three changes of 3 ml/grid or coverslip of the same buffer. Incubation with the secondary antibody, colloidal gold-conjugated goat antirabbit (GAR 10, Janssen Pharmaceutica, Belgium) diluted 1:2 to 1:10 in the same BTTBS buffer, was overnight at room temperature. The secondary antibody was centrifuged on a tabletop centrifuge for 9 min (tap 2) to remove gold clusters before using. When protein was labelled as well as the mRNA, mouse monoclonal antibodies were used in conjugation with a 5-nm colloidal gold-conjugated goat-antimouse antibody (Janssen). For double labelling procedures, both primary (antiprotein and antibiotin) and both secondary antibodies (antimouse and antirabbit) were treated simultaneously. The grid assemblies or coverslips were placed in multiwell dishes on parafilm and sealed tightly to prevent evaporation. After incubation, the coverslips were washed in PBS three times at room temperature. They were then placed in 2% glutaraldehyde in PBS for 2 h at room temperature and then rinsed in PBS for 10 min (three times) at room temperature on a stirring platform in the multiwell dishes.

2.3.2 Muscle Tissue

Detection methods were identical to those described for cells, except that primary and secondary antibodies were used undiluted and the time in primary antibody was increased to 4 h.

2.4 Electron Microscopy

2.4.1 Whole-Mount Cells

Cells on the grid assemblies were critical-point dried after dehydration in graded ethanol and observed with a JEOL 100S or 100CX electron microscope at an accelerating voltage of 100 kV without further contrast enhancement.

2.4.2 Cells for Thin Sectioning

Coverslips containing hybridized myotubes were postfixed in freshly dissolved 2% OsO4 in Sørensen's phosphate buffer for 30 min. After rinsing in distilled H_2O (four times, 10 min each, room temperature), they were dehydrated in a series of graded ethanol (50%, 70%, 95%, twice each for 5 min). They were then placed in fresh 100% alcohol three times for 10 min each. After treatment with 100% alcohol/fresh Epon (1:1) for 30–60 min at room temperature, they were placed in 100% Epon for 1 h at room temperature. Beem capsules were filled with fresh 100% Epon and quickly inverted onto the surface of the coverslips and oven baked for 48 h at 60 °C. The petri dish was immersed in liquid nitrogen for a few seconds, then gently but firmly tapped from behind to remove the Beem capsule and attached coverslip (this was much easier with glass than with plastic coverslips). The Beem capsules were then routinely processed for ultrathin sectioning. Transmission electron microscopy was then performed on a JOEL 100S electron microscope at an accelerating voltage of 60 kV.

2.4.3 Muscle Tissue

Strips were further fixed after hybridization in 2% glutaraldehyde for 1 h, rinsed briefly in buffer, dehydrated in a graded series of ethanols, infiltrated, and embedded in Epon. The tissue was initially cut in strips rather than minced to aid in orientation when embedding and sectioning.

2.5 Quantitative Electron Microscopic Studies on Cultured Cells

A standardized approach was instituted for the quantitation of the 10-nm gold particles counted from the electron microscopic prints. Multiple cells on grids or multiple sections placed on grids were studied from each experiment. Single cells in whole-mount or well-developed myotubes (those with obvious myofiber development) were studied and micrographs at a standard magnification were taken from regions showing the greatest gold concentrations. The gold particles were manually counted from each print and the total number of clusters of 2, 3, 4 particles etc., was also determined. Gold particles at the edge of the cell (i.e., at the cell membrane) were not counted. Quantitation of gold particles was expressed in mean $\pm$ standard deviation and statistical analysis was performed utilizing the Student t test determination. In addition, values are expressed using the total number of gold particles counted as clusters. A large number of prints were studied for quantitation in each experiment.

3 Results

3.1 In Situ Hybridization Visualized in Triton-Extracted Whole Mounts of Single Cells

Isotopic experiments done previously (LAWRENCE and SINGER 1985) indicated a signal-to-noise ratio greater than 50:1 for hybridization to actin mRNA even in Triton-extracted cells (SINGER et al. 1989). This demonstrated that the extraction, fixation and hybridization parameters used provided a strong hybridization signal, appropriate for pursuing further experiments using nonisotopic detection of biotin-labelled probes. The use of nonisotopic detection would be expected to reduce the signal-to-noise ratio, since each additional step has the potential to introduce background noise and to diminish the efficiency of detection. Since the gold particles give a pictorial representation of in situ hybridization (such as silver grains do in autoradiography), and are quantifiable, they allow a more rigorous analysis of signal to noise. The method of data analysis was established in initial experiments, all of which utilized Triton-extracted whole mounts of chicken fibroblasts and myoblasts hybridized with an actin probe. The most direct method is to count gold particles within cells on experimental (biotinated actin probe) samples compared to those within cells on negative control samples (biotinated pBR322 probe without actin insert, hybridized under identical conditions). Quantitation of overall gold densities for at least 12 cells in four experiments demonstrated an average signal-to-noise ratio higher than 6:1. Therefore, when the actin probe is used, more than 80% of the total gold particles represent bona fide hybridization. Further inspection of

gold particle distribution and theoretical considerations of labelling and detection made it evident that individual sites of hybridization could be identified with very high confidence. Since our intent is to visualize hybridization with nanometer resolution at the high magnification of the electron microscope, this is a key element in the methodology and subsequent data analysis. The strategy derives from the fact that many of these small probe molecules hybridize along the larger mRNA template, resulting in a tightly clustered array of gold particles qualitatively distinct from adventitious noise.

Since the target site for in situ hybridization was the β-actin mRNA of 1816 nucleotides (KOST et al. 1983), or the myosin heavy chain, 6 kb (MOLINA et al. 1987; see Sects. 3.2, 3.3), many probe molecules will hybridize at random positions, *dependent* on the target mRNA. Subsequent detection of biotin by indirect immunocytochemistry would result in this iterated array of biotins being detected by a parallel array of antibodies, thereby producing consecutive electron-dense 10-nm images representing the templating of the target molecule. Noise, however, would have different characteristics, because any nonspecific sticking of the small probe molecules or of the antibodies would be very unlikely to form an array of gold particles, since they would be individual events *independent* of the templating effect of the target molecules. Hence, the signal would be expected to be both quantitatively and qualitatively different from noise. Statistical evidence presented elsewhere (SINGER et al. 1989) supports this interpretation.

Antibody background noise was measured by following the protocol but without the probe. The number of colloidal gold particles per unit cellular area in control samples reacted with biotinated pBR322 probe was significantly less than in samples reacted with pBR322 containing either myosin or actin insert. More importantly, the presence of hybridizing probe increased significantly the number and size of the colloidal gold *clusters*. The largest number of gold particles were single, and for single particles signal-to-noise ratios were seen to be about 3:1. However, for increased number of particles in a cluster, progressively fewer clusters were observed using the control probe relative to the experimental probe. It was apparent that hybridization with the probe sequence yields a significant number of larger-sized (greater than 6) clusters, such that sizable clusters of gold-labelled antibodies are detected. This results in the expected proportional increase in signal-to-noise ratio increasing logarithmically to 30:1 for clusters of eight colloidal gold particles. Therefore, when as many as eight particles are seen in a cluster, the probability is 97% that this signal results from the dependent interaction of the probe and the mRNA.

When the individual clusters were examined, it became evident that there were additional qualitative differences between the mRNA probe and the control probe. Significant numbers of clusters were found where the gold particles were not simply in an aggregate, but rather in a characteristic array with relatively constant spacing and a linear dimension proportional to the number of gold particles involved. This is consistent with the theory of the probe hybridizing to a template. Examples of the visualization of individual clusters denoting hybrids are given in Fig. 1. This micrograph shows the arrangement of gold particles in clusters for which the number of particles is such that it represents bona fide hybridization to an mRNA molecule. The structure of the cluster appears to be circular. In many cases, circular arrangements prove to be actually spiral when examined by stereo microscopy

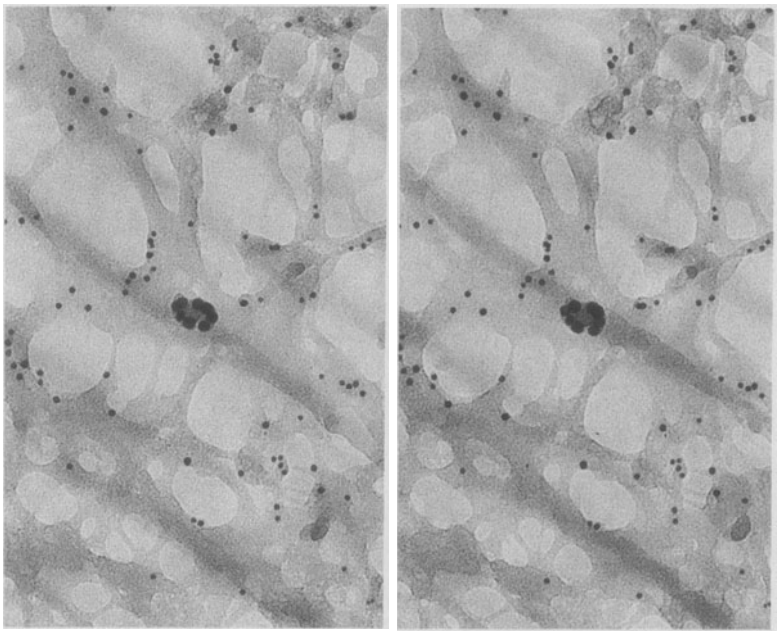

Fig. 1. Stereo pair of β-actin mRNA detected by in situ hybridization. Actin protein was detected in the same samples using 5-nm gold conjugated antibodies indirectly. Triton-extracted myoblasts fixed in glutaraldehyde were used for the hybridization, after having been grown on grids. Biotinylated actin DNA probe was used and detected by 10-nm colloidal gold conjugated antibodies using indirect immunocytochemistry. × 165000

(a stereo pair is provided for illustration). The sample has also been exposed to monoclonal antibodies to actin which have been detected by indirect immunocytochemistry using 5-nm colloidal gold. This allows the high-resolution visualization of both the message and its cognate protein in the same area. It is evident that actin protein is distributed throughout the area occupied by the mRNA. The association of the messages with the cytoskeleton can be seen clearly in these preparations. In almost all cases examined, the mRNA detected sits astride several filaments, very often at the base of, or along, a long, single filament with the diameter of actin filaments (6 nm).

3.2 In Situ Hybrids Visualized in Triton-Extracted, Cultured Myotubes: A Preembedment Procedure

We found that the Triton extraction followed by glutaraldehyde fixation afforded a satisfactory combination of probe penetration and preservation of the structure of our particular interest: the developing sarcomere. It should be emphasized that membranes or membrane-associated structures are solubilized in this procedure. The particular method emphasizes a preembedment technique. It follows from previous work on whole-mount cells that, in order to investigate the developing myotube — a structure too dense to penetrate in its entirety with the electron beam —

it would be necessary to section this structure after hybridization. In contrast to previous work, we used a probe for myosin heavy chain instead of actin, because the 6 kb message offered a bigger target and thus would be more likely to withstand the signal reduction involved in partitioning the myotube into thin sections. In addition, whether myosin mRNA would be associated with developing sarcomeres is, in itself, an interesting question.

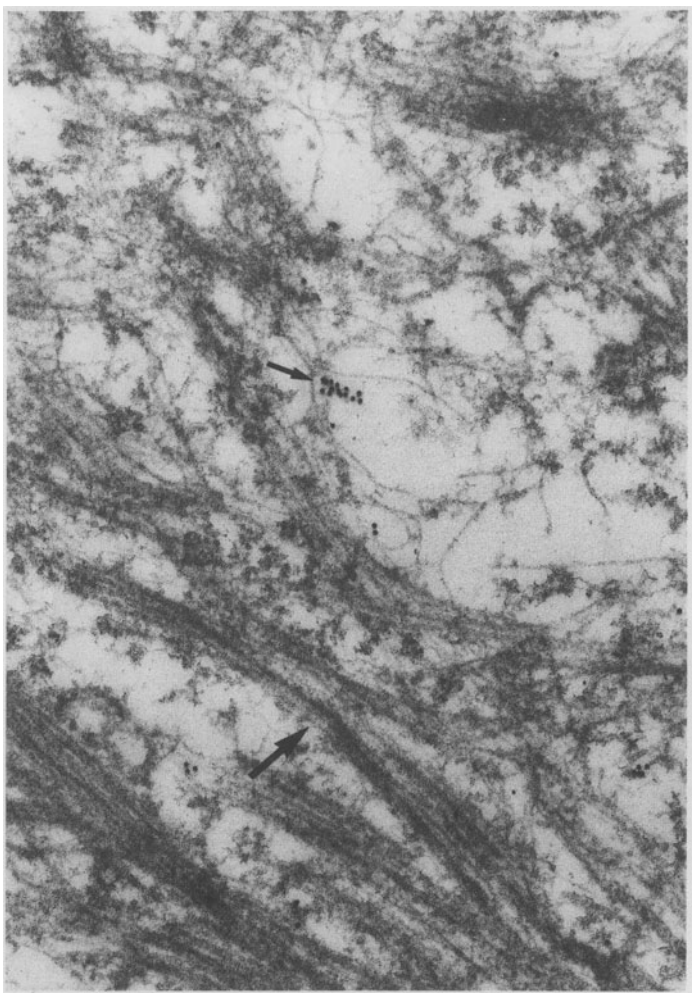

Fig. 2. Detection of myosin heavy-chain mRNAs in Triton-extracted myotubes, hybridized and thin sectioned. Cultured myotubes were Triton-extracted and fixed with 2% glutaraldehyde for 15 min at room temperature before hybridization and sectioning. A cluster of colloidal gold particles can be seen associated with an electron-dense filamentous protein of the diameter of myosin (*small arrow*). The number of colloidal gold particles indicates that it is a myosin heavy-chain message (see text), presumably in the act of translating a myosin protein. A developing myofiber is within 260 nm (*large arrows*). ×77000

The quantitative approach to the data analysis was identical to that described previously which involved the counting of colloidal gold particles used to detect the biotinated probe, both individually and as various cluster sizes. This allowed a critical evaluation of signal-to-noise ratio when comparing the MHC probe cloned into pBR322 versus the vector alone.

Besides the numbers of colloidal gold particles, morphology of the cells was assessed in each sample, as well as the localization of the signal, i.e., if myosin mRNA was located near assembling myofibers. Finally, the size of the colloidal gold clusters was evaluated for evidence of templating onto messages.

Cluster conformations appear to be consistent with the idea of hybridization to a message template. Increase in cluster size with the MHC probe was significantly greater than with the control probe in most experiments. Although this method requires extensive analysis of sections, it yields images which give a high degree of certainty that a message for myosin is visualized. In many of these images, the message is associated with an electron-dense filamentous structure which may have the characteristics of myosin (i.e., diameter of 14 nm). A good double-labelling protocol would be more conclusive in identifying this filament. Because of the sectioning of the tissue, it is difficult to assess the three-dimensional structure of the message, as was done on whole-mount cells.

Figure 2 illustrates an example of the results obtained from this approach. Sarcomeres in developing myofibrils can be easily seen in the region of the mRNA detected, but the area immediately surrounding consists of loose filamentous material where the signal for myosin mRNA is predominant. The mRNA can be seen associated with what appears to be a growing myosin polymer.

Recent work by ISAACS and FULTON (1987) and FISCHMAN (personal communication) have shown that a population of myosin heavy chains is either cotranslationally assembled into filaments, or is very rapidly assembled after translation. We have observed evidence of clusters of gold (i.e., mRNA for MHC) near or on filaments which may be in the process of assembly. Since these represent very few examples, most likely the result of fortuitous sections, it is not possible to ascertain whether or not this is a general occurrence. The approach, however, illustrates the potential for characterizing the relationship between the synthesis of cellular filament systems and their assembly, such as occurs during sarcomerogenesis.

3.3 In Situ Hybridization on Unextracted Tissue: Preembedment Procedure

Work currently underway in our laboratories involves the extension of this approach to muscle tissue from developing embryos. Enhancement of signal-to-noise ratios has been a major concern, since quantitation of many samples is extremely time-consuming and cumbersome. Obviously, if the control probe gave no background at all, signal would be significant at any level and many fewer samples would require screening!

Preliminary quantitation suggests a signal-to-noise ratio of at least 6:1 using the methods described here for fixed tissue. Two advances are made by this procedure: the first is that fixation prior to detergent extraction produces morphological preservation more comparable with conventional methods, and circumvents the

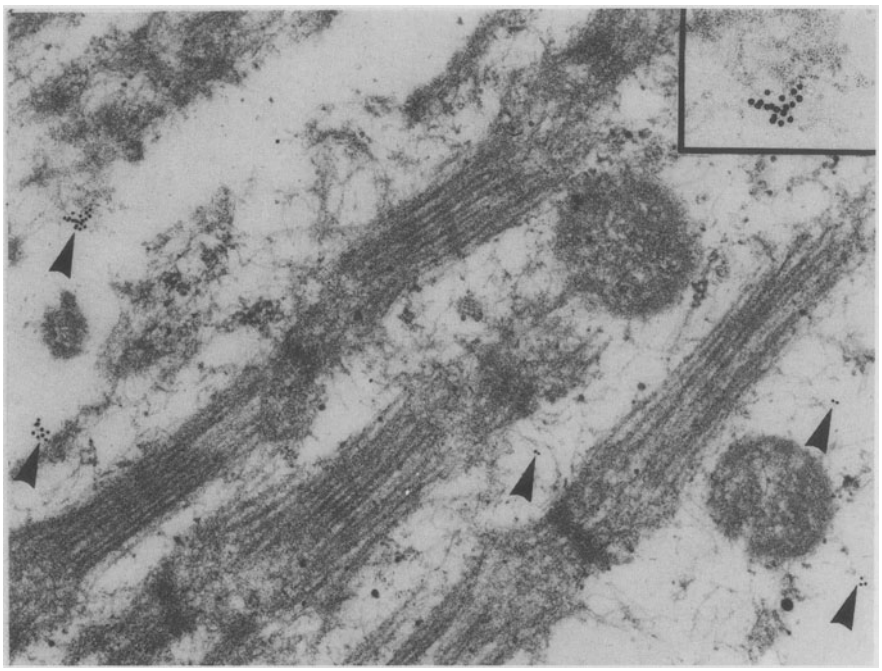

Fig. 3. Detection of myosin heavy-chain mRNA in fixed tissue. Pectoral muscle tissue from 14-day-old chicken embryos was fixed prior to detergent extraction and hybridization with myosin heavy-chain probe. Colloidal gold particles, which almost certainly indicate the position of myosin heavy-chain message, are associated with cytoplasmic filaments adjacent to developing myofibrils (*arrow heads*). At higher magnification, a group of 18 gold particles in this section has the configuration of a twisted string. Controls probe showed no clusters of a size greater than three colloidal gold particles. ×36000; inset ×72000

potential problem of structural rearrangement during detergent treatment of unfixed cells. The second is that molecular information is obtained from cells developing in vivo rather than in vitro. Therefore, possible artifacts of tissue culture are eliminated while maintaining the quality of the molecular results. Figure 3 illustrates the quality of the data obtainable for tissue material prepared and hybridized as in Sect. 2. Much the same results are obtained as with Triton-extracted myotubes, i.e., the mRNA appears to be intimately associated with cytoplasmic filaments immediately adjacent to developing myofibrils. However, in this case, the events described are occurring in developing chicken muscle in ovo and the morphological context of these events is much better preserved.

4 Summary

A progressive development of the application of in situ methodology to ultrastructural procedures has resulted in the ability to detect individual molecules of mRNA with high probability. Beginning with whole-mount cells and then developing myotubes,

both in culture and detergent extracted before fixation, we were able to progress to methods which allow detection of mRNA in tissue sections. Initial results confirm that the detection of mRNA in thin-sectioned tissue is very similar to observations on the extracted, cultured cells, and that the same methods of data analysis apply. Current work is devoted to the application of the methodology to other cellular structures, such as the nucleus, and to other tissue-probe systems, such as brain.

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