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Actin gene expression visualized in chicken muscle tissue culture by using *in situ* hybridization with a biotinated nucleotide analog

(actin mRNA/cell differentiation/nucleic acid localization)

ROBERT H. SINGER* AND DAVID C. WARD†

*Department of Anatomy, University of Massachusetts Medical School, Worcester, Massachusetts 01605; and †Department of Human Genetics, Yale University School of Medicine, New Haven, Connecticut 06510

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ABSTRACT **The chicken muscle tissue culture system has been used for visualizing actin gene expression after *in situ* hybridization. Cell differentiation is morphologically distinguishable in this system as the myoblasts fuse into myotubes. This differentiation involves the production of large amounts of actin required for myofibrils. The presence of actin mRNA has been observed in cells preserved with ethanol and paraformaldehyde by hybridizing a recombinant plasmid into which a biotinated analog of dUTP was incorporated by nick-translation. The biotin was then detected by using an anti-biotin antibody and a rhodamine-conjugated second antibody. Alternatively, avidin conjugated to rhodamine or avidin complexed to biotinated peroxidase has been used for mRNA detection. The procedure described preserved morphological detail yet is compatible with hybridization conditions and reveals the disposition of actin mRNA during gene expression.**

The method of *in situ* hybridization has been a powerful tool for the localization of specific sequences linearly arranged on a chromosome (1). This approach has been modified for the detection of RNA transcripts within individual cells. In some studies of this type total polyadenylylated RNA has been detected by using poly(U) probes (2–4). Other approaches have used complementary RNA or DNA reverse transcripts as probes either for viral sequences (5–7) or for specific messages within individual cells (8–10). More recently, a purified recombinant DNA clone has been used to detect specific cellular sequences (11). All of these approaches have used radiolabeled probes and the *in situ* hybridization has been detected by autoradiography. The inherent lack of resolution in tritium-labeled probes due to the track of the decay particle and the thickness of the emulsion makes the approach undesirable for precise morphological work. Recently, a number of workers have attempted the use of nucleic acid probes conjugated with fluorescent molecules for the detection of *in situ* hybridizations (12–15). Langer *et al.* (16) have developed a biotinated analog of dUTP that can be incorporated into DNA by nick-translation and contains an allylamine linker arm between the biotin molecule and the pyrimidine ring. Polynucleotides containing this analog have been used for *in situ* hybridization on *Drosophila* chromosomes with superior resolution and signal-to-noise ratios (17).

The investigation of gene expression by using recombinant DNA methods focuses on the molecular aspects of cell differentiation without consideration of the diversity of morphology inherent in the cell population containing these molecules. Because differentiation is often defined on the basis of cell morphology, we have attempted to develop a method relating cellular structure to the molecular aspects of gene expression. This method requires high-resolution *in situ* hybridization and preservation of morphological detail. This work reports the initial results we have obtained by using the muscle tissue culture system for the detection of actin gene expression *in situ* and a recombinant actin plasmid labeled with biotin. This culture system is well suited to the investigation because both the morphology of cell differentiation (fusion of myoblasts into a syncytial myotube) and gene expression during this fusion process (e.g., the actin gene) have been well defined (18–22).

MATERIALS AND METHODS

Preparation of Cells for *in Situ* Hybridization. Coverslips (22-mm diameter) were washed in acid/alcohol, coated with a layer of carbon, and sterilized by 70% (vol/vol) ethanol and UV light. Myoblasts isolated from the pectoralis muscles of 12-day chicken embryos were seeded into dishes containing sterilized coverslips at a density of 3×10^6 cells per 100-mm culture dish. At the appropriate times after incubation (usually 48 hr), the coverslips were removed, washed in Earle's balanced salt solution, fixed for 1 min in 2% (wt/vol) paraformaldehyde at pH 7.5, and placed in 70% ethanol and stored at 4°C until use. Under these conditions, cultures were stable for several months as determined by recovery of RNA into which tritiated uridine was incorporated (data not shown). At the time of use, the coverslips were rehydrated into phosphate-buffered saline (P_i/NaCl) containing 10 mM magnesium chloride (P_i/NaCl/Mg). All procedures were performed at room temperature unless otherwise noted. The coverslips were treated with 0.1 M HCl in P_i/NaCl for 10 min and washed in P_i/NaCl/Mg before being placed in proteinase K (Boehringer) at 50 μg/ml for 10 min. They were then treated with 4% paraformaldehyde, pH 7.5, in P_i/NaCl/Mg for 10 min at room temperature and washed in 1% acetylated bovine serum albumin in P_i/NaCl. The samples were acetylated according to the method of Hayashi *et al.* (23) and placed in 50% (vol/vol) deionized formamide and 2× standard saline/citrate buffer (NaCl/Cit; 1× NaCl/Cit is 0.15 M NaCl/0.015 M sodium citrate) for 10 min. Before hybridization, the coverslips were heated directly on a dry block in this solution for 1 min at 70°C.

Hybridization Conditions. DNA probes were nick-translated by the method of Rigby *et al.* (24) but using a biotinyl-dUTP derivative that contained an 11-atom spacer arm between the 5 position of the pyrimidine ring and the carboxyl group of the biotin moiety. The conditions used give an average probe length of 200–300 nucleotides, with some considerably smaller material, after separation on Sephadex G-50. Increasing the length of the linker arm over that initially reported (16) improves the ability of both the antibody and avidin to interact with the bio-

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Abbreviations: P_i/NaCl, phosphate-buffered saline; NaCl/Cit, 0.15 M NaCl/0.015 M sodium citrate.

tinated hybridization probes (unpublished data). The nick-translated probe (40 ng), 5 μg of sonicated herring sperm DNA, and 20 μg of tRNA were added to a silane-treated Eppendorf tube, lyophilized, resuspended in 5 μl of formamide, and heated for 10 min at 70°C. The hybridization solution was made up in these tubes to a final volume of 10 μl. The hybridization buffer was 50% deionized formamide, 4× NaCl/Cit with 1% bovine serum albumin, 10 mM vanadyl sulfate (25), and 10% dextran sulfate. The coverslips were placed upside down onto 10 μl of this hybridization mixture on Parafilm, covered with Parafilm, and incubated 48–72 hr at 34°C in a tissue culture incubator with 100% relative humidity. After hybridization, the coverslips were washed thoroughly in 50% formamide/2× NaCl/Cit at 37°C and then in P_i/NaCl for 1 hr. A 1:10,000 dilution of a goat anti-biotin antibody (45 mg/ml; Enzo Biochemicals, New York) was placed in 1% bovine serum albumin/P_i/NaCl with vanadyl in 10 μl and incubated for 2 hr with the coverslips. The coverslips were washed with P_i/NaCl for 1 hr, and then rabbit anti-goat antibody conjugated to rhodamine (Miles, repurified) was used for 1 hr at 37°C in the same manner. Alternatively, an avidin-biotin–peroxidase complex (ABC kit from Vector Laboratories, Burlingame, CA) or avidin directly conjugated to rhodamine (1:500 dilution of 4.6 mg/ml in 1% bovine serum albumin/P_i/NaCl) was used (Vector Laboratories) for mRNA detection. After an hour of washing in P_i/NaCl, the coverslips were mounted in glycerol/Tris·HCl, pH 8.0, 9:1 (vol/vol), on glass slides and sealed with nail polish. The cells were viewed through a Zeiss photomicroscope II with a ×63 Planapochromat phase objective and, where appropriate, with epifluorescent rhodamine optics. Photography was at ASA 400, using Kodak Tri-X panfilm, 45- or 120-sec exposure.

RESULTS

The pBR322 plasmid containing the actin sequences (26) and the same plasmid without the actin sequences were nick-translated in the presence of the biotinated analog dUTP and used in parallel as probes to investigate actin gene expression in developing chicken muscle cells in tissue culture. The morphological changes during this differentiation have been well described (18), and the fusion of single-celled myoblasts into a syncytium (myotube) is easily seen in phase-contrast microscopy. Investigation of gene expression during this phase of fusion indicates that muscle-specific gene products increase considerably (19). Actin is one of these major products, its mRNA increasing from about 3% to 15–20% of the average total cellular message population during this time (20–22). We have used a chicken β-actin clone 2 kilobases in size (26) in order to view this transition from single-celled myoblasts into myotubes. Figs. 1 and 2 show the results of the hybridization of biotinated probes to cultures of myotubes followed by use of the antibody detection system.

It is apparent that the fluorescent signal obtained with the actin probe (Fig. 1*B*) is considerably stronger than the background fluorescence seen with pBR322 control DNA (Fig. 1*D*). The fluorescence generated by the actin probe is nonuniform in distribution and often appears as discrete foci distributed

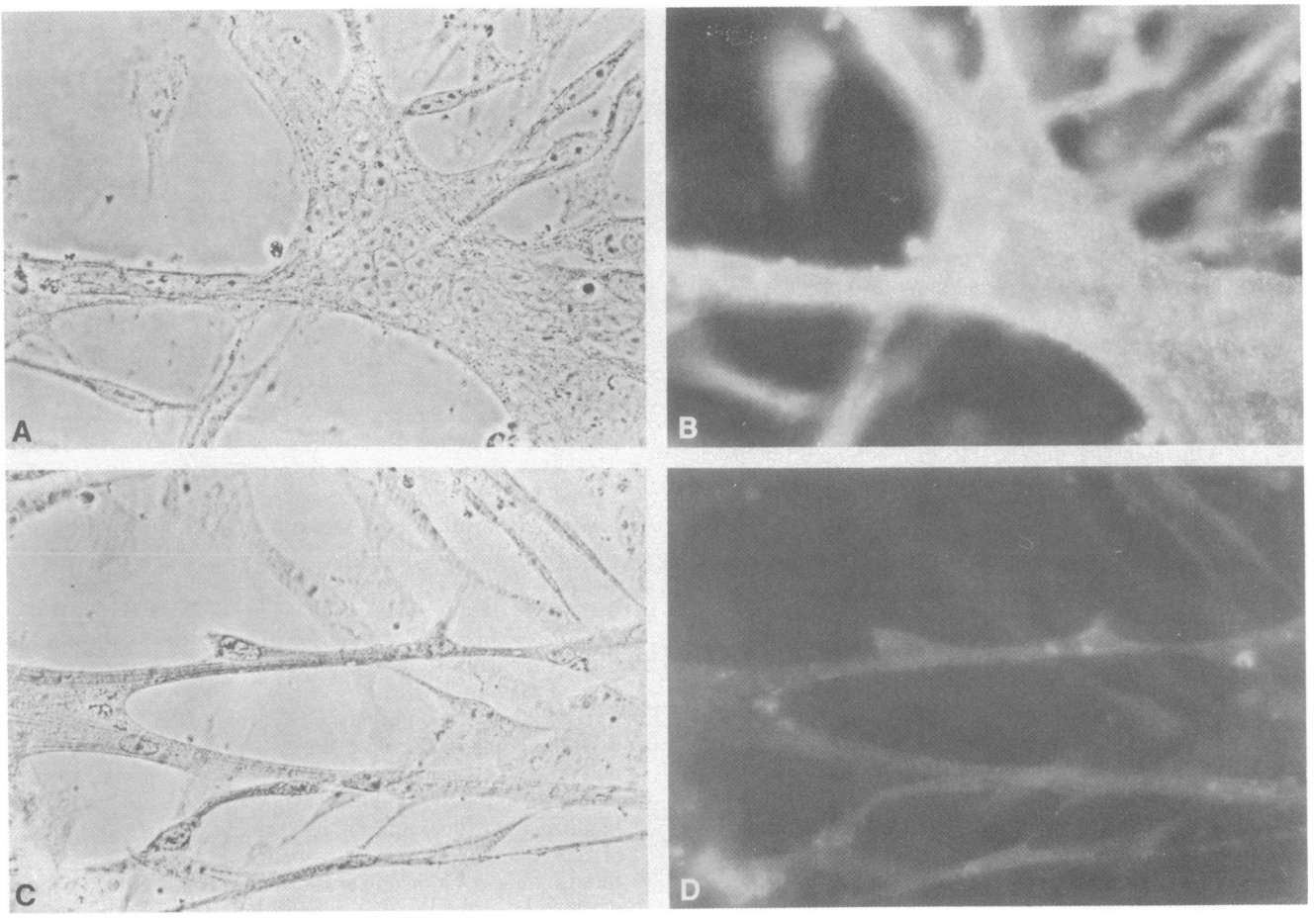

FIG. 1. Hybridization *in situ* of the plasmid pBR322 with and without an actin insert. Hybridization was detected with goat anti-biotin antibodies followed by rhodamine-conjugated rabbit antibodies to goat immunoglobulin. *A* and *B* are phase-contrast and fluorescence micrographs, respectively, of a culture hybridized to the biotinated actin recombinant plasmid. *C* and *D* illustrate a parallel culture treated identically but hybridized to the biotinated plasmid pBR322. (×600.)

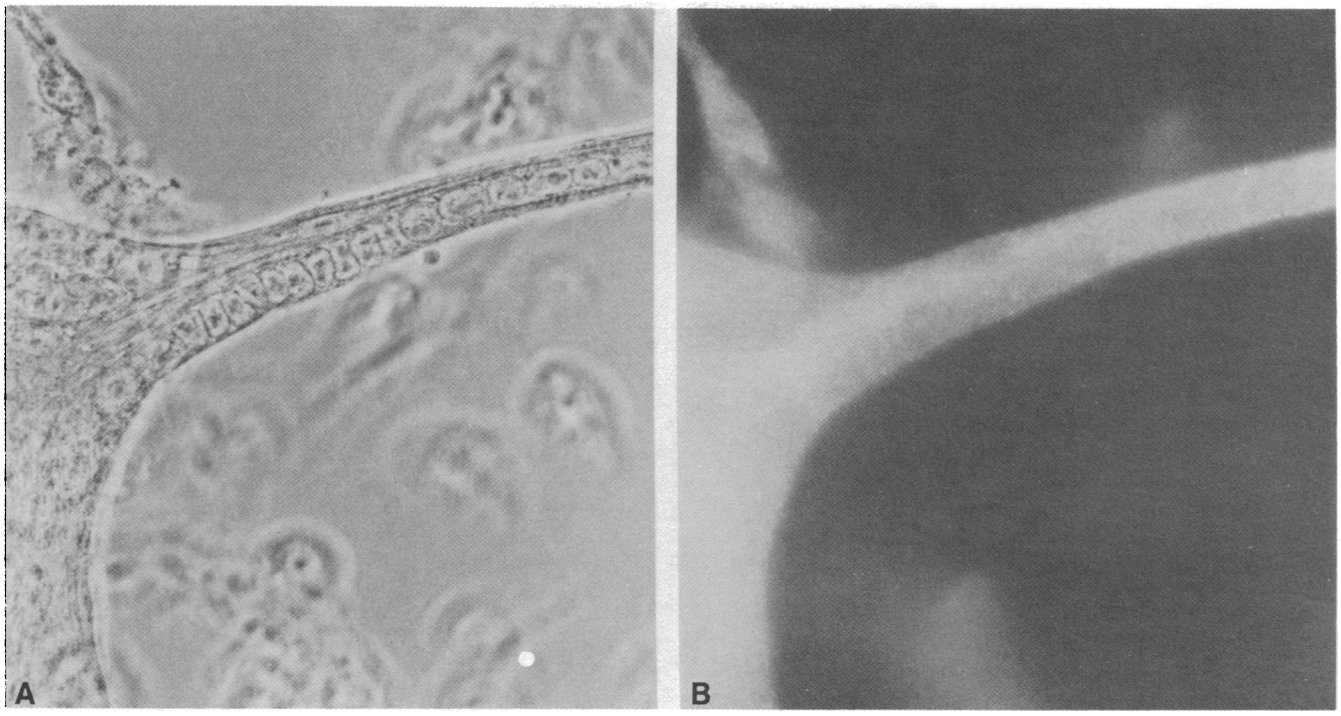

FIG. 2. Hybridization *in situ* to a myotube of a biotinated recombinant actin plasmid detected as in Fig. 1. (*A*) Phase-contrast micrograph; (*B*) fluorescence micrograph. (×1,200.)

throughout the cytoplasm. Fig. 2*A* illustrates a myotube at higher magnification, a linear array of nuclei is juxtaposed with a myofilament bundle. The quality of morphological preservation is evident in the resolution of nuclear membranes, nucleoli, and fibrillar matrix, despite the exposure to the hybridization protocol. Fig. 2*B* shows the fluorescent signal resulting from the hybridization, which indicates a preferential localization of actin mRNA over the fibrillar bundles. This figure also demonstrates that the fluorescence intensity of the myotube is significantly stronger than that generated by the peripheral myoblasts seen in the phase-contrast picture (Fig. 2*A*). Because of the narrow depth of field of the lens, these myoblasts are somewhat out of focus when the thicker myotube is examined, and this may account for some of the decrease in fluorescence. However, an observed difference in fluorescence intensity between myotube and myoblast is consistent with an increased production of actin mRNA subsequent to cell fusion (19–22).

Actin gene transcripts in cultures of myoblasts, an average

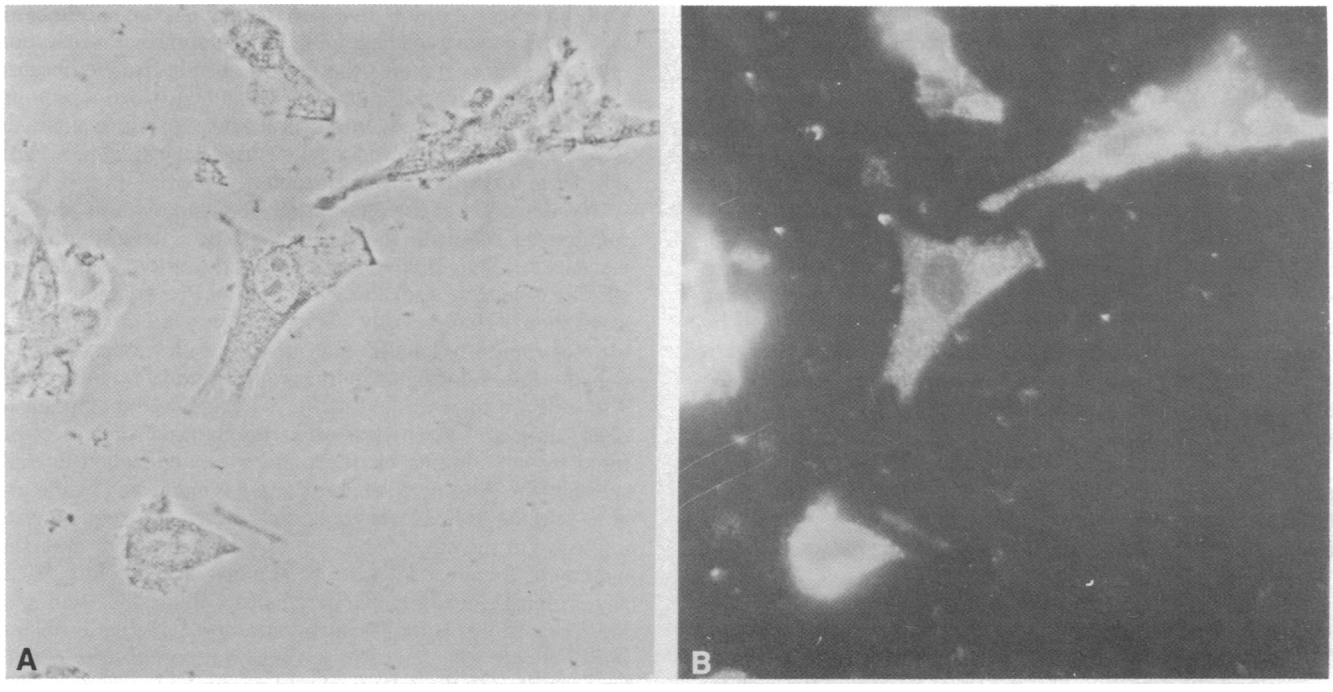

FIG. 3. Hybridization *in situ* to a myoblast culture of biotinated recombinant actin plasmid detected as in Fig. 1. (*A*) Phase-contrast micrograph; (*B*) fluorescence micrograph. (×1,500.).

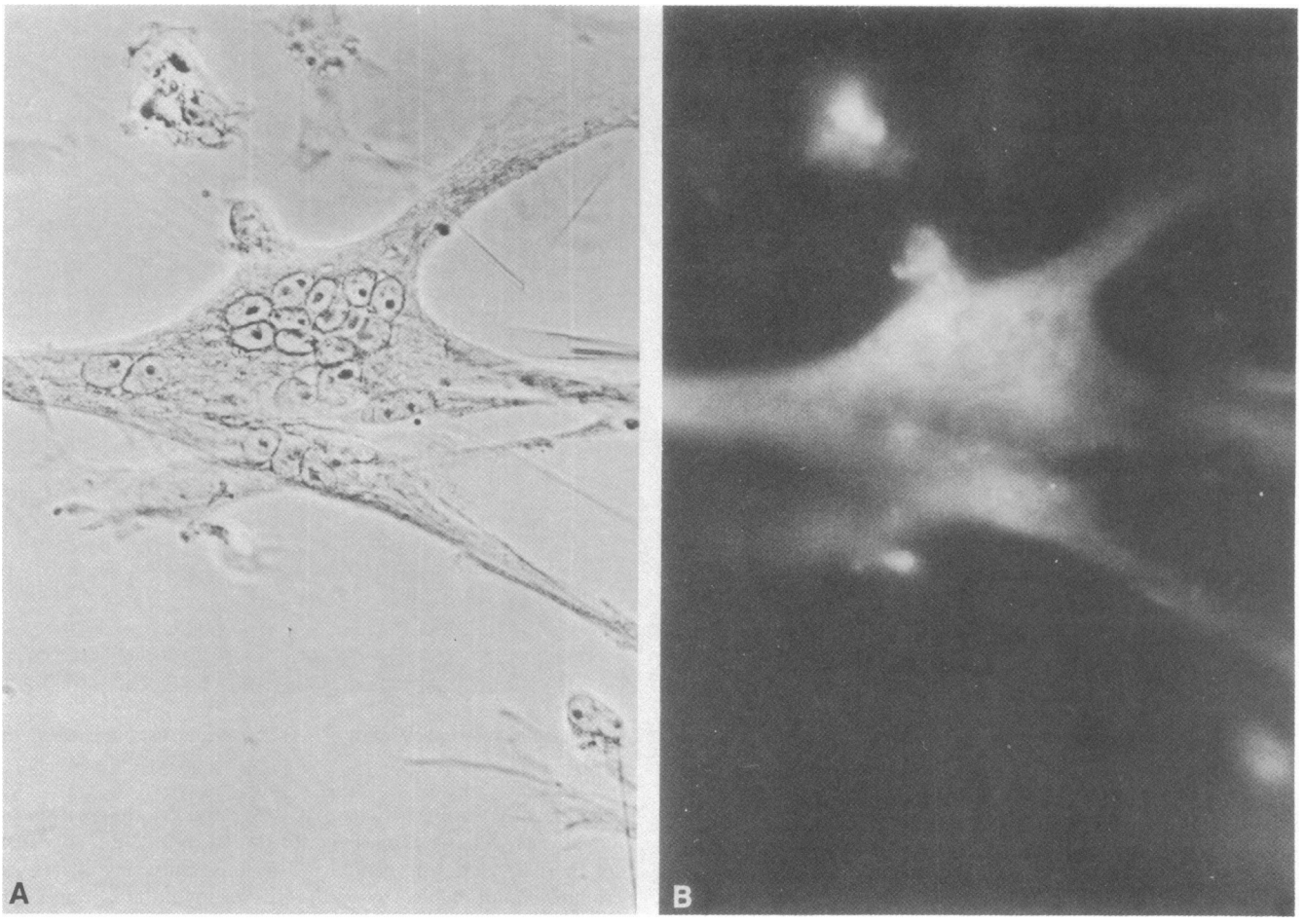

FIG. 4. Hybridization *in situ* of a biotinated recombinant actin plasmid to a myotube detected by avidin conjugated to rhodamine. (*A*) Phase-contrast micrograph; (*B*) fluorescence micrograph. (×1,000.)

of 1,000 copies per cell, can be observed by this general technique, although longer exposure times are required. The punctate nature of cytoplasmic fluorescence, detected by the indirect antibody method, is more readily observed in myoblast cultures (Fig. 3), possibly because the cells are considerably thinner than the myotubes. This observation might suggest that actin mRNA is dispersed throughout the cytoplasm in discrete "packets" or "clumps," although at this time we cannot eliminate the possibility that these foci are artifacts of the technique.

Fig. 4 illustrates the hybridization results with the actin probe upon using a direct detection procedure; rhodamine-labeled avidin. Again, a nonuniform distribution of fluorescence is observed, with a preferential localization peripheral to the central nuclei cluster. Similar results have been obtained by using avidin complexed to biotinated peroxidase; in this variation of the method the sites of hybridization are localized through the deposition of insoluble enzyme products (data not shown).

The procedures of cell fixation described here give excellent preservation of cell structure even after the rigorous hybridization. Hence, high-resolution mapping of mRNA populations intracellularly should be possible with this technique.

DISCUSSION

The understanding of the relationship of cell morphology to gene expression requires a method that preserves the cellular structural integrity while using conditions promoting hybridization of the macromolecules. Investigations by Brahic and Haase (5) provided the basis for this method, although cell morphology was not their interest. A finer analysis of gene expression, however, requires that one distinguish the subtle cytological features of a cell beginning to differentiate. Preservation of these features requires the use of a strong fixative because the subsequent hybridization steps will tend to disrupt proteins that are not crosslinked. In our procedure, paraformaldehyde is adequate for this purpose when used in conjunction with ethanol to make the cells permeable.

The detection of the appropriate sequences within the cell requires an adequate signal-to-noise ratio. Background fluorescence resulting from nonspecific hybridization, adventitious sticking of nucleic acids to the cellular matrix, and nonspecific association of fluorescently labeled marker proteins all contribute to the noise. When the cells are fixed well with paraformaldehyde, the endogenous biotin groups tend to be destroyed. The cells are then washed with 1% bovine serum albumin to block unreacted aldehydes. Adventitious association is countered by the addition of tRNA and sonicated boiled herring sperm DNA. Nonspecific hybridization is monitored by the use of the vector pBR322 not containing the cloned sequences of interest. On the other hand, the signal is enhanced, after the method of Brahic and Haase, by the use of hydrochloric acid and proteinase K to remove the nucleoproteins. The timing of these procedures is important because it is possible to release the RNA from the cell. The procedure reported here results from monitoring the release of acid-precipitable tritiated RNA (uridine labeled) from cells during each step of the procedure. On the average, 50% of the RNA remains in the cells throughout

the entire procedure. Fixing too long with formaldehyde, however, makes it impossible to remove any nucleoproteins at all and hence the mRNA will not be available for hybridization. RNA degradation may occur at several points in the procedure, particularly in the incubations with antibodies. We have taken the precaution of sterilizing and deionizing all nonprotein solutions and adding vanadyl (25) to the antibody incubations. We have decreased the time of incubation to minimal periods. We use rhodamine fluorescence because it is more easily detected by film, its bleach period is longer, and there is minimal autofluorescence at its exciting wavelength.

Studies of actin gene expression in muscle (21) indicate that the copy number of actin mRNA increases 10-fold over a 36-hr period to about 10,000 per nucleus in the myotube from about 1,000 per single-celled myoblast. We have detected, therefore, in the myoblast, with the use of fluorescence or peroxidase, approximately 1,000 copies of a nucleic acid sequence per cell. A marked decrease in fluorescence signal is apparent when comparing myoblasts to myotubes. The limit of detectability is not approached in this work, but we estimate it to be about 200 copies per cell of a message. Improvement of the signal-to-noise ratio will make the limit of detectability even lower. For instance, current use of the peroxidase technique allows maximization of the signal-to-noise ratios by visually monitoring the reaction as it occurs. Additionally, a computerized video enhancement system and more sensitive detection systems can be developed. Quantitation of the fluorescence data presented here would yield valuable information on the correlation of gene expression with cell morphology.

Finally, observations made by this approach appear to indicate a morphological disposition of actin mRNA. The myotubes and myoblasts occasionally are seen to have a nonuniform distribution of these nucleic acids, which sometimes concentrate around nuclei or along filament bundles. Association of polysomes with cytoskeletal elements has been shown previously in HeLa cells (27) and myoblasts (28) by use of electron microscopy. A nonrandom distribution of a particular mRNA species would have important implications for the construction of cytological features such as filament systems.

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