

# Intracellular Localization of Messenger RNAs for Cytoskeletal Proteins

Jeanne Bentley Lawrence and Robert H. Singer

Department of Anatomy  
University of Massachusetts Medical School  
55 Lake Avenue North  
Worcester, Massachusetts 01605

## Summary

**We have analyzed intracellular distributions of mRNAs for the cytoskeletal proteins actin, vimentin, and tubulin by in situ hybridization. Although polyadenylated RNA was homogeneously distributed throughout the cell, actin mRNA demonstrated a nonhomogeneous distribution in 95% of randomly selected chicken embryonic myoblasts and fibroblasts, as detected by isotopic and nonisotopic techniques. Actin mRNA concentrations were highest at cell extremities, generally in lamellipodia, where grain densities were up to 16-fold higher than in areas near the nucleus. Vimentin mRNA, unlike actin mRNA, was distributed near the nucleus. Tubulin mRNA appeared most concentrated in the peripheral cytoplasm. These results demonstrate that cytoplasmic mRNAs are localized in specific, nonrandom cellular patterns and that localized concentrations of specific proteins may result from corresponding localization of their respective mRNAs. Hence, actin mRNA distribution may result in increased concentration of actin filaments in lamellipodia of motile cells.**

## Introduction

The spatial distribution of specific mRNAs within intact cells has not been previously studied. In the work presented here we have used in situ hybridization to mRNAs for cytoskeletal proteins to address the possibility that mRNAs for different proteins exhibit characteristic patterns of localization. Knowledge of the intracellular distribution of specific mRNAs is important for understanding cellular organization and function. In particular, localization of a given mRNA provides insight into the site of synthesis of the corresponding protein. A central question in cell biology concerns how newly synthesized proteins come to reside in their appropriate locations within the cell. While our understanding of how proteins are targeted to membranes or the nucleus has progressed rapidly in recent years (Goldman and Blobel, 1978; Maccacchini et al., 1979; Moreland et al., 1985; Redman and Sabatini, 1966; Blobel, 1980), essentially nothing is known about how cytoplasmic proteins, constituting the bulk of the proteins in the cell, become localized at their site of function. Three mechanisms could serve to accomplish this localization: the protein may be transported after its synthesis is complete; the nascent chain may be transported during its synthesis; or, possibly, the protein may be synthesized close to the site of function due to localization of the corre-

sponding mRNA. Fundamental to discriminating among these mechanisms is the question of whether mRNAs exhibit differential localization within cells and, if so, how this localization relates to the distribution of the corresponding protein.

To determine whether mRNAs exhibit random or non-random distribution within cells, we have used an improved in situ hybridization methodology (Lawrence and Singer, 1985) optimized for the preservation of cellular morphology, RNA retention, and, consequently, preservation of the native configuration of RNAs. So that we might readily relate mRNA distribution with protein distribution, we have focused on localization of mRNAs for the filamentous proteins actin, tubulin, and vimentin because these proteins have been extensively studied and exhibit well-characterized cellular distribution. These major constituents of the cellular framework have preferred areas of localization; for instance, actin filaments tend to grow at the periphery of cells, in areas such as ruffles, lamellipodia, or filopodia (Ishikawa et al., 1969; Buckley, 1981; Small, 1981; and Stossel, 1984). In contrast, vimentin tends to encase the nucleus more heavily (Small and Celis, 1978; Lazarides, 1980; Osborn et al., 1982; Holtzer et al., 1982; Steinert et al., 1984), and microtubules are found to assemble from the organizing centers around the nucleus toward the cell boundaries (Brinkley et al., 1975; Osborn and Weber, 1976; Brenner and Brinkley, 1984). This cellular framework serves to illustrate the problem confronted by the cell in sorting newly synthesized proteins to the appropriate location. Thus, these proteins provide a good experimental system in which to study whether this asymmetric distribution of filaments may be effected by a localization of their corresponding messenger RNAs.

## Results

To determine whether the intracellular distribution of mRNA is homogeneous, or whether mRNAs for different proteins exhibit distinct patterns of localization within the cell, chicken embryonic muscle or fibroblast cultures were hybridized in situ with cloned DNA probes for specific cytoskeletal mRNAs, as well as for total poly(A) RNA. Except where otherwise indicated, experiments employed <sup>3</sup>H-labeled probes and autoradiography for detection and quantitation.

### Localization of Actin mRNA

Actin mRNA was localized with a full-length, 2 kb chicken  $\beta$ -actin probe, which detects mRNAs for all actin isoforms because of homologies in the coding region (Cleveland et al., 1980). Autoradiographs of cells hybridized with this probe revealed that the distribution of actin mRNA was highly nonhomogeneous, with many cells exhibiting discrete areas of high grain density. Labeling patterns for cells with varying morphologies are illustrated in Figures 1A-1C and Figure 2. In Figure 1A, two cells have been juxtaposed to emphasize that cells with similar morphologies

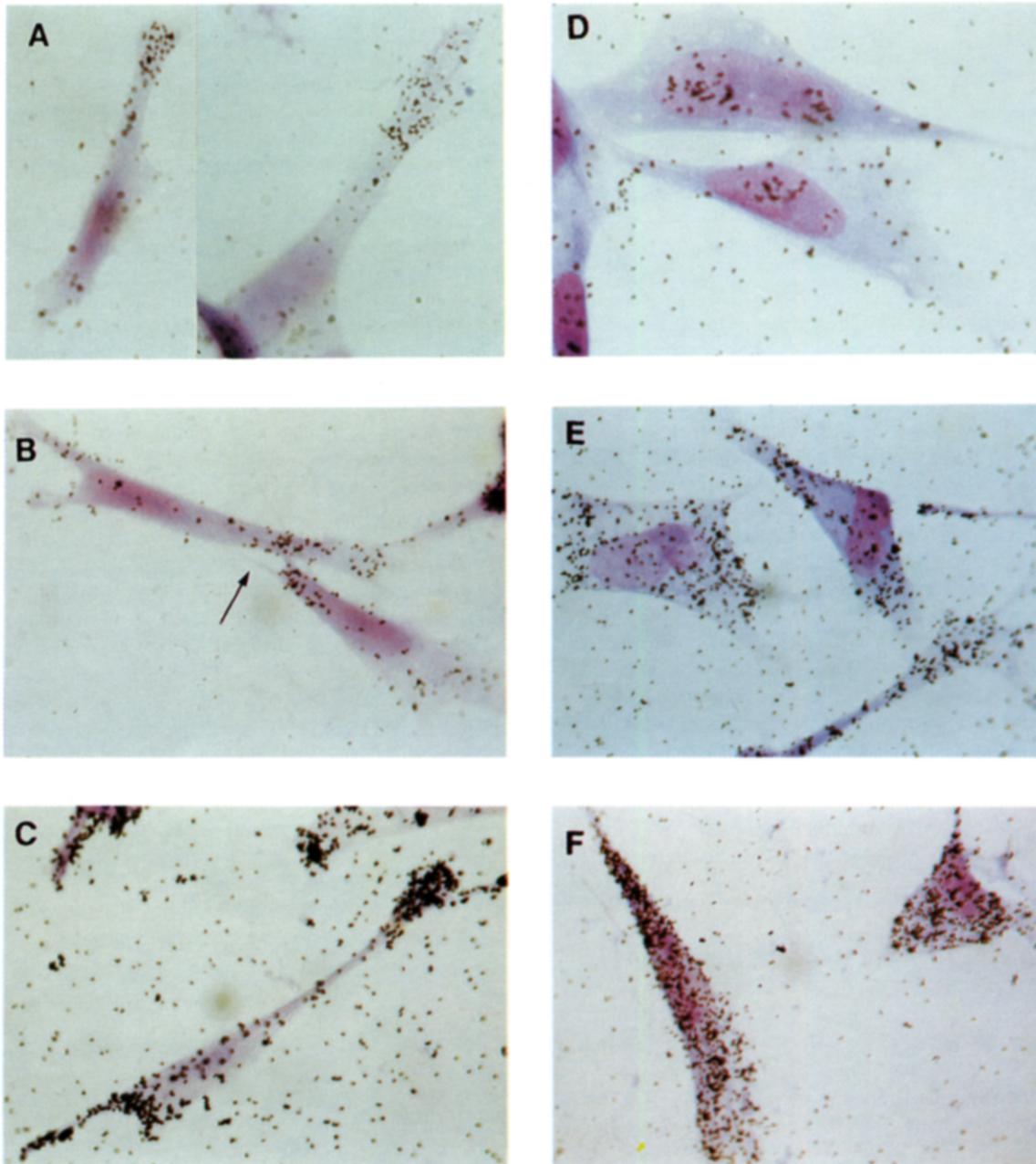


Figure 1. Autoradiographs of Cells Hybridized In Situ with  $^3\text{H}$ -Labeled Probes to mRNAs for Cytoskeletal Proteins and to Poly(A) mRNA. Shown are Giemsa-stained cells at 2000 $\times$  magnification. Specific activities of nick translated probes ranged from  $1\text{--}3 \times 10^7$  cpm/ $\mu\text{g}$  and exposure times were from 8–13 weeks. See Experimental Procedures for description. (A–C) actin probe; (D) vimentin probe; (E) tubulin probe; (F)  $^3\text{H}$ -labeled poly(U) probe (New England Nuclear, 5.1 Ci/mmol).

often showed similar labeling patterns. The highly labeled regions were consistently at the cell periphery, in lamellipodia, indicating that actin mRNA was concentrated in these parts of the cell. (We define a lamellipodium as a flattened protrusion extending from the cell body and often containing a ruffling membrane at its leading edge, as described by Bard and Hay, 1975.) In contrast to the labeling of the lamellipodia, the nuclear region generally exhibited little label. Typical distribution of actin mRNA is further illustrated in Figure 2A. The cell in the upper part

of this figure shows the classic morphology of a motile cell, and the lamellipodium containing the apparent forward-leading edge (arrow) is highly labeled relative to the rest of the cell. At the opposite end of the cell there is a cell process, presumably undergoing retraction, that has elevated actin mRNA levels, but to a lesser extent than the larger lamellipodium. This unequal, bipolar distribution of actin mRNA was the most frequently observed pattern in cells with this characteristic morphology (see also cells in Figures 1C and 2B). A highly pronounced and much more

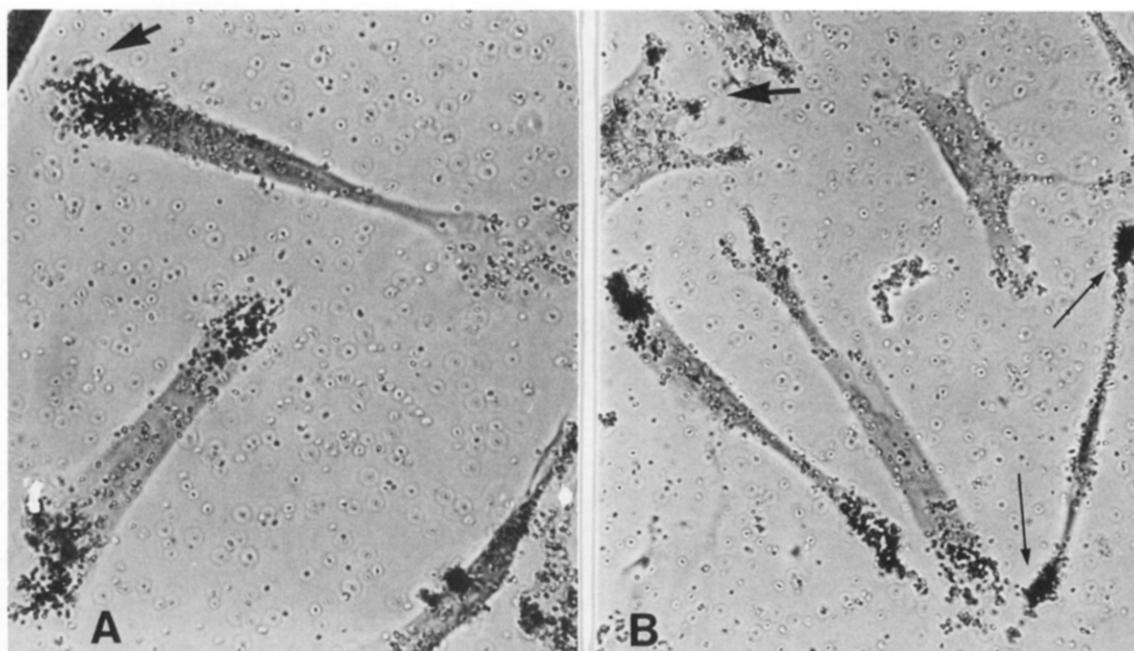


Figure 2. Autoradiographs of Cells Hybridized In Situ with  $^3\text{H}$ -Labeled Probe to Actin mRNA, Phase-Contrast Optics

(A) Bipolar distribution of actin mRNA (2000 $\times$ ). The cell in the upper part of this photograph exhibits classical morphology of a cell in motion. The arrow indicates the forward leading edge.

(B) Low magnification view (800 $\times$ ) showing several cells in a field exhibiting localization of actin mRNA. Small arrows indicate a bipolar myoblast with symmetrical labeling. Large arrow indicates a fibroblast with three lamellipodia, each of which exhibits increased label.

symmetrical localization of actin mRNA was observed in a minor fraction of cells consisting of extremely elongated bipolar myoblasts, as shown by the cell at the extreme right of Figure 2B (small arrows). Cells with a more spread morphology frequently had several lamellipodia, each of which was densely labeled toward the outermost region of the projection (indicated by large arrow, upper left of Figure 2B). As shown in Figure 1B, high concentrations of actin mRNA were commonly observed in areas of cell contact. Grains were often concentrated at the base of thin processes extending toward another cell, while the process itself was essentially devoid of label (see arrow). Similar patterns of actin mRNA localization were observed regardless of whether cells were grown on plastic or glass. The only cells that characteristically exhibited relatively uniform grain distributions were myotubes and small mononucleated cells believed to be proliferative myoblasts. RNAase-treated controls or cells hybridized with a pBR322 plasmid lacking the actin insert showed no hybridization.

We used two general approaches to verify that the non-homogeneous patterns of grain distribution observed after in situ hybridizations to actin mRNA were not artifacts, either of hybridization or of autoradiographic detection of  $^3\text{H}$ -labeled probes. First, we confirmed the peripheral distribution of actin mRNA using alternative detection techniques, and second, we showed that the same detection technique (tritium) used with different probes produced results specific for the probes.

Similar patterns of actin mRNA distribution were ob-

served using three alternative methods for detection of hybridized probe. Two nonisotopic methods were employed whereby the probe was labeled by incorporation of biotinylated nucleotides and the hybridized probe was detected either histochemically, using a reaction in which biotinylated alkaline phosphatase is conjugated to streptavidin (Leary et al., 1983; Singer et al., in press), or by fluorescence, using fluorescein-labeled avidin (fluorescein-avidin) (Singer and Ward, 1982). Detection of actin mRNA by alkaline phosphatase staining is shown in Figure 3A: the staining is darkest at what appears to be the forward-leading edge of the cell. Essentially no staining was observed in control samples that were treated identically except that the probe was pBR322 lacking the actin insert (Figure 3B). Localization of actin mRNA using fluorescein-avidin for detection yielded similar results, with prominent staining frequently observed in cell projections and extremities (see Figure 3C). This fluorescence pattern contrasts with the much more generally dispersed fluorescence produced by acridine orange, which stains total RNA (Fulton et al., 1980) (Figure 3D). In some cells acridine orange fluorescence was less intense in the lamellipodia, presumably due to decreased cell thickness in these regions. In addition to the hybridizations by nonisotopic methods, hybridizations were performed with an actin probe labeled with  $^{35}\text{S}$ , which has a longer path length of radioactive emission than does  $^3\text{H}$ ; in principle, actin mRNA from the entire cell would be detected. The presence of heavily labeled lamellipodia in many cells was still evident with  $^{35}\text{S}$ -labeled probes, although the

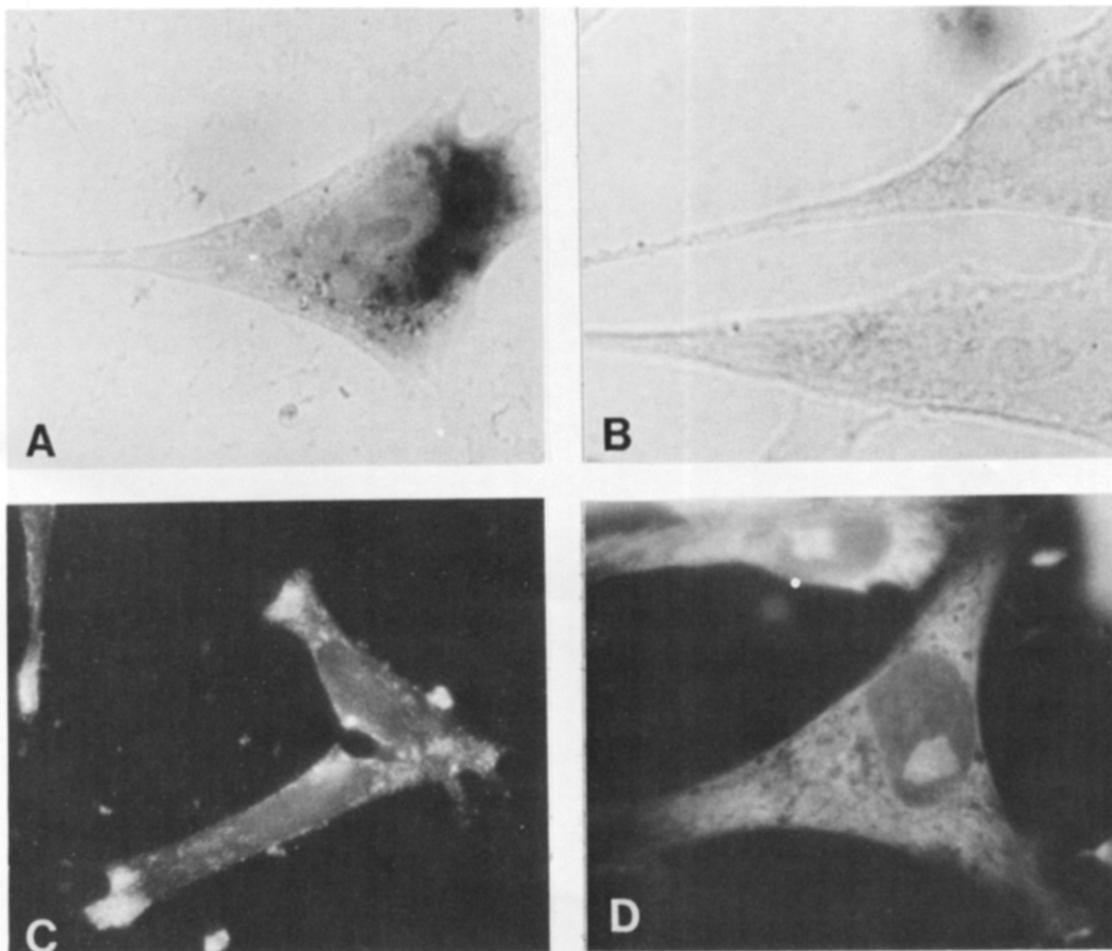


Figure 3. Nonisotopic Detection of Actin mRNA In Situ, 2000x Magnification

- (A) The actin probe was biotinylated and hybridization was detected using the alkaline phosphatase method (see Experimental Procedures).  
(B) Same as (A) except a biotinylated control probe (pBR322 without actin insert) was used.  
(C) Hybridization of biotinylated actin probe detected using fluorescein-avidin. Epifluorescence optics.  
(D) Total RNA stained with the fluorescent acidophilic dye, acridine orange. Epifluorescence optics.

resolution was lower than with  $^3\text{H}$ -labeled probes (data not shown). These observations with alternative detection methods, coupled with the results presented below, support the conclusion that the localized grain distributions resulting from in situ hybridizations with actin cDNA probes represent actual intracellular distributions of actin mRNA. A more defined resolution of message distribution within the three-dimensional architecture of the entire cell would require analysis of sectioned cells.

#### Localization of Poly(A) mRNA, Vimentin mRNA, and Tubulin mRNA

Using  $^3\text{H}$ -labeled probes and autoradiographic detection, we compared the intracellular distribution of actin mRNA with that of other messages in identical cell cultures. The distribution of total poly(A) RNA was evaluated using a tritiated poly(U) probe (New England Nuclear). As can be seen in Figure 1F, the distribution of grains over chicken myoblasts and fibroblasts was generally uniform after hy-

bridization with the poly(U) probe, in marked contrast to the often highly localized distribution observed for actin mRNA. Hybridization to poly(A) RNA resulted in essentially equivalent grain densities over the central and peripheral regions of the cell, while lamellipodia only rarely exhibited significantly increased grain densities. This relatively uniform distribution of poly(A) RNA is consistent with the generalized distribution of total RNA observed with acridine orange staining, as described above. Control hybridizations with a tritiated poly(A) probe produced little label above background. The consistent difference in results between hybridizations with the poly(U) probe and the actin probe demonstrates that the observed localization of actin mRNA is specific for this particular message and is distinct from the general distribution of poly(A) and total RNA within the cell.

The labeling patterns observed in cells with diverse morphologies are best described in qualitative terms. However, we wished to derive a means of quantitatively

Table 1. Quantitative Summary of mRNA Distribution

Message	Average LI* (Range)	% of Cells with LI > 2.1	% of Cells with Highest Grain Density over Nucleus	% of Cells with Highest Grain Density over Lamellipodia
Poly(A)	1.4 (1.0-2.8)	6	22 <sup>†</sup>	28 <sup>†</sup>
Actin	5.9 (1.1-16.3)	95	0	100
Vimentin	3.7 (1.1-8.4)	72	89	8
Tubulin	2.4 (1.0-4.5)	56	2	67

\* LI = Localization Index =  $\frac{\text{Number of grains in most densely labeled region}}{\text{Number of grains in least densely labeled region}}$ , where grain counts were performed at 1000 $\times$  magnification in areas representing 6 ( $\mu\text{m}$ )<sup>2</sup>. Averages are based upon data from a minimum of 50 cells taken from two or more independent experiments.

<sup>†</sup> As indicated by the low LI for poly(A), observed increases in grain densities were not significant (see text).

representing and comparing the degree to which different messages are localized within cells. Grain counts were performed using 1,000 $\times$  magnification on an ocular grid where one grid square was equivalent to six square micrometers. The most densely and least densely labeled regions of the cell were determined in areas corresponding to one grid square. The ratio of the highest grain density to lowest grain density in a given cell is referred to as the Localization Index (LI) in Table 1. By definition, this index is always greater than or equal to 1.0. An index of 1.0 indicates perfectly uniform grain distribution throughout a cell, whereas increasingly higher indices indicate increasing degrees of localization. Of the probes tested, hybridization of a poly(U) probe resulted in the most homogeneous distribution of grains, yielding an average LI of 1.4 for 50 randomly selected cells. This contrasts with the striking localizations observed for actin mRNA, for which this ratio averaged 5.9 for randomly selected single cells, with a range of 1.1 to 16.0. We also analyzed the percentage of cells in the population that showed nonuniform distribution of an mRNA. Using the range and average LI for cells hybridized for poly(A) RNA, we adopted the criterion that an LI of 2.1 or above indicated nonrandom grain distribution. Hence, only 6% of cells hybridized for poly(A) RNA exhibited nonhomogeneous distribution, as indicated in Table 1. The distribution of highest grain densities for poly(A) RNA, 22% over the nucleus and 28% over the lamellipodium, is consistent with a random grain distribution since these numbers approximate the fractions of the cell area represented by these structures. For actin mRNA, 95% of randomly selected cells showed localization by the criterion stated above. In cells hybridized for actin mRNA, the highest density of label was never over the nucleus but was in 100% of the cells over the lamellipodium, generally in the outermost region of the structure.

We next evaluated the distribution of messages for proteins comprising two other major components of the cytoskeleton, intermediate filaments and microtubules. Cells were hybridized with <sup>3</sup>H-labeled probes for either chicken vimentin or chicken tubulin. A representative example of the distribution of vimentin mRNA is presented in Figure 1D and quantitative results are presented in Table 1. In striking contrast to actin mRNA, vimentin mRNA was centrally located, with the highest density of grains over the nucleus. Label was frequently evident as clusters

over a region of the nucleus near, but not over, the nucleolus. As indicated in Table 1, the average LI for vimentin in randomly selected cells was 3.7, and 72% of the cells were judged, by the same criterion used above, to have a non-uniform distribution of message. The important difference between vimentin mRNA and actin mRNA distribution was that in 89% of cells hybridized with the vimentin probe, the nuclear region exhibited the densest label, whereas this was not true for virtually any of the cells analyzed for actin mRNA hybridization.

Tubulin mRNA exhibited more regionalization than poly(A) RNA, with an average LI for randomly selected cells of 2.4 and with 56% of the cells exhibiting non-homogeneous distribution. The pattern was similar to that of actin mRNA in that grain densities were higher over the cytoplasm. Label in the nuclear region was occasionally observed for tubulin mRNA. In general, localizations of tubulin mRNA were less pronounced. This is represented quantitatively by the difference in LI between these two messages (Table 1). As illustrated in Figure 1E, after hybridization with a chicken  $\alpha$ -tubulin probe, grains were diffuse but densest over lamellipodia in 67% of cells. Often there were two areas of increased grain density in opposite parts of the cell, although the striking localizations observed for actin mRNA were not paralleled in tubulin samples.

We conclude that the three mRNAs investigated, each corresponding to a different component of the cytoskeleton, exhibit patterns of intracellular distribution that are distinct from one another as well as from that of total poly(A) mRNA. Actin mRNA is localized toward the cell periphery, whereas vimentin mRNA is most concentrated near the nucleus. Tubulin mRNA is less sharply localized, but it appears to be more concentrated in areas peripheral to the nucleus than is poly(A) RNA.

#### Actin mRNA Localization: Relationship to Cell Motility or Myogenesis?

The pattern of localization for actin message is particularly noteworthy, because it so closely and consistently mimics the well-documented peripheral distribution of growing actin filaments in the lamellipodia of motile cells (Pollard and Korn, 1971; Goldman et al., 1976; Lazarides, 1976; Wolosewick, 1984; Wang, 1984). Quantitation of the amounts of actin mRNA in different parts of the cell revealed that, in some cells, as much as 80% of the mRNA

detected was in the outer lamellipodia, which represent less than 25%–30% of the cell's area. For example, in the cell at the left of Figure 1A, 62% of the grains reside in a distal region of the lamellipodium that represents only 14% of the cell area. Because lamellipodia are flattened processes, and the thinnest region of the cell is at the periphery, this area represents significantly less than 14% of the cell's volume.

This heavy enrichment of actin message in intracellular regions known to be involved in cell locomotion, for which actin polymerization provides the major mechanical force, suggests that localization of actin mRNA may be related to cell motility. Since all of the above observations were on myogenic cultures containing myoblasts and fibroblasts, we analyzed pure fibroblast cultures to confirm that actin mRNA localization is not strictly a function of myogenic differentiation. Essentially the same peripheral distribution of actin mRNA was observed in cultures of motile embryonic fibroblasts, which yielded an average LI of 4.3 with 86% of the cells exhibiting a nonhomogeneous distribution of this message. In addition, we analyzed cultures of rat L8 myoblasts, less motile cells which fuse into myotubes only at confluence. These cells exhibited a much more generalized distribution of actin mRNA, with a tendency for the thin periphery of cells to exhibit fewer grains than the rest of the cell. These results provide further support for the suggestion that localization of actin mRNA at the cell periphery is closely related to cell motility and is not strictly a property of cells that are undergoing myogenic differentiation.

## Discussion

The work presented here establishes for the first time that mRNAs exhibit specific and distinct patterns of intracellular localization in intact somatic cells. In particular, our results define differences in the spatial distribution of the mRNAs for actin, vimentin and tubulin in intact cultured cells and therefore indicate that these intracellular proteins may be synthesized in specific cellular regions. These observations have implications for the relationship of protein synthesis, and hence gene expression, to cell structure and motility. Some of these considerations are discussed below.

We feel that the observed distributions of mRNA are morphologically and physiologically significant in that they are related to cell polarity and, most likely, to cell motility. Actin mRNA is greatly enriched in the lamellipodia and projections of 95% of cultured cells. Since up to 80% of actin mRNA is concentrated in the structures, it is reasonable to assume that these cellular projections are sites of increased actin synthesis, although no evidence is presented here on the translatability of the localized mRNA. Much evidence indicates that these are the structures where the polymerization of actin filaments, one of the major mechanical forces responsible for cell movement, is occurring (Pollard and Korn, 1971; Goldman et al., 1976; Lazarides, 1976; Huang et al., 1978; Stossel, 1984; Wang, 1984; Woloszewick, 1984). Current models of actin filament assembly are generally based on the polymeriza-

tion of preexisting monomer pools (Allen and Taylor, 1975; Bray and Thomas, 1976; Tilney, 1976; Pollard and Craig, 1982; Taylor et al., 1982; Stossel, 1984). Therefore, the polymerization of actin at the cell periphery may be significantly facilitated by the localized synthesis of new actin monomers. Our observation of an asymmetric, bipolar distribution of actin mRNA in most cells is consistent with suggested models of actin assembly in cell movement in which actin polymerization and synthesis are most active at the forward protruding edge of the cell, but are also increased in the rear of the cell where actin filaments facilitate cell retraction (Taylor et al., 1982; Hay, 1985; Wang, 1984). The most distinct and dramatic localizations we observed are for actin mRNA in chicken embryonic myoblasts and fibroblasts, both highly motile cell types. We have observed a less peripheral distribution of actin mRNA in less motile L8 rat myoblasts. All of these observations suggest a possible relationship between the localization of actin message and cell motility.

Similarly, the nuclear localization of vimentin mRNA suggests a relationship between the site of synthesis of this protein and its functional disposition within the cell, since intermediate filaments are most concentrated around the nucleus (Small and Celis, 1978; Lazarides, 1980; Osborn et al., 1982; Holtzer et al., 1982; Steinert et al., 1984). The observed clustering of grains in a region of the nucleus resulting from hybridization to vimentin mRNA is consistent with the existence of a proposed intermediate filament organizing center in this area (Eckert et al., 1982). Another possibility is that these clusters may represent hybridization to a messenger RNA that is associated with the nucleus and is highly homologous to vimentin; such amino acid homology has been recently reported for nuclear lamins (McKeon et al., 1986). We are currently investigating distribution of vimentin mRNA in more detail.

Our results imply that some molecular mechanism exists whereby the mRNAs for different proteins are differentially sorted within a cell. One possibility is that the mRNA is sorted by some signal on the nascent polypeptide chains, in a manner analogous to membrane proteins (Blobel and Sabatini, 1971; Blobel and Dobberstein, 1975; Blobel, 1980). For instance, the nascent actin polypeptide could have an affinity for the tips of growing actin filaments. An alternative model would suggest that the localization of the mRNA is directly dependent on its nucleotide sequence and is not mediated by protein synthesis. For instance, distribution of mRNA might conceivably be achieved via some sequence-recognition ribonucleoprotein complex that confers localization on mRNA. Since the mRNA sequences of the well-conserved actin family differ primarily in their untranslated regions (Cleveland et al., 1980; Ordahl et al., 1980), these sequences could possibly contain information for intracellular localization. This may have implications for the role of actin isoform gene expression during development.

A structural system exists within the cell that may serve to sequester or transport messages. Much evidence indicates that cytoplasmic mRNAs are translated while associated with the cytoskeletal framework of the cell (Lenk

et al., 1977; Fulton et al., 1980; Cervera et al., 1981; Bonneau et al., 1985). A nonrandom distribution of actin mRNA has been reported in the ascidian, *Styela* (Jeffrey et al., 1983; Jeffrey, 1984); actin mRNA is more heavily concentrated in the ectoplasm and myoplasm of ascidian eggs and remains with the triton-insoluble cellular framework. Cell fractionation studies have shown that myelin basic protein mRNA is translated on non-membrane-bound polyribosomes, which are enriched in the myelinating processes of oligodendrocytes and which may be held in place by association with cytoskeletal elements (Colman et al., 1982). Quantitative studies in our laboratory (unpublished data) have shown that 60%–75% of actin mRNA and 85%–100% of tubulin and vimentin mRNA remain with the triton-insoluble cytoskeletal framework. Therefore, it is likely that the mechanism whereby these mRNAs are held in a particular intracellular location involves attachment to the cytoskeleton, although the mechanism whereby specific mRNAs are recognized and differentially localized remains to be determined.

Our ability to detect intracellular distribution of mRNAs by *in situ* hybridization may be a function of the particular hybridization method employed. The technique used was previously optimized such that steps that would tend to disrupt cellular morphology or cause degradation and diffusion of mRNAs (e.g., proteinase and acid treatment, long hybridization times) have been eliminated or minimized (Lawrence and Singer, 1985). This improved methodology is likely to be essential for preserving the native distribution of mRNAs and may produce results qualitatively different from those obtained previously. In earlier work describing the fluorescence detection of biotinylated probes, which preceded the development of this improved hybridization methodology, specific localization of actin mRNA was not observed (Singer and Ward, 1982). In the current work, most of our analysis employed tritiated probes and autoradiography for detection, since this method reproducibly provided the best combination of sensitivity, quantitation, and resolution. The 1–2  $\mu\text{m}$  track length of tritium decay (Rogers, 1979) is advantageous not only because of increased resolution compared with other radionuclides, but also because it minimizes differences in labeling due to differences in cell thickness. Hence we observed homogeneous grain distributions after hybridization to poly(A) mRNA despite differences in cell thickness, and we detected higher concentrations of actin mRNA at the cell periphery even though this is generally the thinner region of the cell. However, interpretations of autoradiographic labeling patterns from tritiated probes must take into consideration the short path length of this radioisotope, since molecules in the more ventral regions of the cell may escape detection. Although similar localization of actin mRNA was confirmed using alternative detection techniques that detect probe throughout the entire cell, resolution of mRNA distribution in the dorsoventral axis would require sectioned material. Current work in our laboratory using biotinylated probes for electron microscopic detection will provide further information on message distribution and its association with cellular components.

## Experimental Procedures

### Cell Culture

Skeletal myoblasts were isolated from the pectoral muscle of 12-day chicken embryos and cultured by standard techniques. Cells were plated at a density of  $2 \times 10^6/100$  mm plate in minimum essential medium, 10% heat inactivated fetal calf serum, and 2% chicken serum. Cells were plated into dishes containing glass or plastic coverslips previously autoclaved in 0.5% gelatin. Cells were fixed after 2–3 days of incubation, when cultures consisted of a mixture of fibroblasts, undifferentiated myoblasts, and early myofibers. Coverslips containing cells were rinsed twice in Hank's balanced salt solution and fixed for 15 min in 4% paraformaldehyde (Fisher) in phosphate-buffered saline with 5 mM  $\text{MgCl}_2$ . To make up the fixative, paraformaldehyde was dissolved in PBS with low heat for 2–4 hr,  $\text{MgCl}_2$  was then added, and the solution was filtered. After fixation, cells were placed in 70% ethanol at 4°C until later use. A large number of coverslips with cells of uniform density were prepared from each cell culturing so that parallel samples could be examined with different probes.

### Probes and Nick Translation

The actin probe consisted of a full-length (2 kb) transcript-coding region for chicken  $\beta$ -actin inserted into pBR322 (Cleveland et al., 1980). This probe hybridizes with the mRNAs of different actin isoforms under the hybridization conditions employed. The vimentin probe contained a 750 base doublet of chicken vimentin cDNA cloned into the same plasmid (Zehner and Paterson, 1983). The tubulin probe contained 1400 bp of cDNA for chicken  $\alpha$ -tubulin in pBR322 (Cleveland et al., 1980; Valenzuela et al., 1981). The control probe used was pBR322 without any insert.

Plasmid DNA was routinely nick translated using three  $^3\text{H}$ -labeled nucleotide triphosphates (New England Nuclear, 54–100 Ci/mmol). Specific activity of  $^3\text{H}$ -labeled probes ranged from  $1 \times 10^7$  to  $3 \times 10^7$  cpm/ $\mu\text{g}$ . For probes nick translated with  $^{35}\text{S}$ -dCTP the specific activity ranged from  $1.7 \times 10^8$  to  $2.9 \times 10^8$  cpm/ $\mu\text{g}$ . For nonisotopic detection, probes were labeled by nick translation with biotinylated dUTP (Enzo Biochemical). The probe fragment length after nick translation was approximately 300–600 nucleotides for radioactive probes and 150–300 nucleotides for biotinylated probes. Probe fragment size was controlled by varying the amount of DNAase (Worthington) in the nick translation reaction, and the size was monitored using 1.5% alkaline agarose gel electrophoresis. For probes labeled with radioisotopes, probe size was determined by autoradiography of gels treated for fluorography (Enhance, New England Nuclear). Biotinylated probes were sized using alkaline phosphatase detection of probes transferred to nitrocellulose (see below).

### Hybridization

The details and derivation of the hybridization protocol have been published elsewhere (Lawrence and Singer, 1985). The salient features of this method are that cell treatments that remove cellular constituents, such as proteinase, acid, or acetic anhydride, have been omitted and that incubation in the hybridization solution is short (3 hr) so that any possible diffusion of mRNA is minimized. Cells fixed in paraformaldehyde and stored in 70% EtOH, as indicated above, were rehydrated in phosphate-buffered saline plus 5 mM  $\text{MgCl}_2$  for ten min, followed by 0.1 M glycine, 0.2 M Tris-HCl, pH 7.4, for 10 min. Cells were then placed in 50% formamide (Fluka),  $2 \times$  SSC (0.3M sodium chloride in sodium citrate buffer) for 10 min at 60°C 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 ten minutes. 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  $\mu\text{g}/\text{ml}$  and the final hybridization solution consisted of 50% formamide  $2 \times$  SSC, 0.2% BSA, 10 mM vanadyl sulfate ribonucleoside complex (Berger and Birkenmeier, 1979), 10% dextran sulfate (Sigma), and 1 mg/ml each of the *E. coli* tRNA and salmon sperm DNA. For hybridizations with  $^{35}\text{S}$ , 10 mM DTT was added to the hybridization solution. Cells on coverslips were incubated in 20  $\mu\text{l}$  of hybridization solution for 3 hr at 37°C by putting 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  $2 \times$  SSC, 50% formamide at 37°C;  $1 \times$  SSC, 50% formamide at 37°C; and  $1 \times$  SSC at

room temperature. Control samples were incubated in 100 µg/ml RNAase in 2× SSC for 1 hr at 37°C prior to hybridization.

#### Detection of Hybridization

Samples hybridized with <sup>3</sup>H- or <sup>35</sup>S-labeled probes were dehydrated through 70%, 95%, and 100% ethanol and air dried. Coverslips were mounted cell side up on slides using Permount. To facilitate comparisons, samples hybridized with different probes were mounted together on one slide and dipped into Kodak NTB-2 emulsion in complete darkness. Air dried slides were placed in lighttight boxes with Drierite and were stored at 4°C. Exposure times for <sup>3</sup>H-labeled probes were from 8–12 weeks and for <sup>35</sup>S-labeled probes were from 3–12 days. Slides were processed through D-19 developer for 5 min, 1% acetic acid for 30 sec, Kodak Fixer for 5 min, and a water rinse for 30 min. Slides were immersed for 20–30 minutes in a freshly prepared solution of 5% Giemsa stain (BDH Chemicals) in 0.05 M Tris-HCl, pH 6.8. Alternatively, slides were stained with the DNA fluorochrome DAPI (4'-6'diamidino-2-phenylindole) for 10 min at 1 µg/ml in PBS. For staining of total RNA, slides were incubated in 0.1% acridine orange for 15 min in 1× PBS, 3 mM EDTA, pH 8.0, and then rinsed in running water for 1 hr. Slides were viewed through a Zeiss ICM photomicroscope equipped with epifluorescence optics. Grain counts were performed using 1,000× magnification. Photographs were taken with either Tri-X ASA 400 or Ektachrome 50 film.

For detection of probes labeled with biotin, two alternative techniques were employed. For fluorescence detection, samples hybridized with biotinylated probe were reacted with 2 µg/ml of fluorescein-avidin in 4× SSC for 30 min (Singer and Ward, 1982). Samples were then rinsed for 1 hr in PBS, mounted in antibleach mounting medium, and viewed with epifluorescence optics. Alternatively, biotinylated probes were detected by a colorimetric reaction using alkaline phosphatase (Leary et al., 1983; Singer et al., in press). Samples hybridized with biotinylated probes were reacted with streptavidin and then with a biotinylated alkaline phosphatase complex (Bethesda Research Laboratories). The streptavidin provides a bridge between the biotinylated DNA and the biotinylated enzyme. Alkaline phosphatase is then detected by incubation of cells in a mixture of 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium, which produces a dark purple precipitate at sites of hybridization.

#### Acknowledgments

We wish to acknowledge Carol Villnave for her excellent technical contribution. We would also like to thank Marie Giorgio and Susan Billings-Gagliardi for patient help with photography, Roya Rashtchian for technical support, Elayn Byron for secretarial assistance, and Kevin Byron for sacrificing Saturday mornings to tissue culture. The actin and tubulin probes were from original clones by Don Cleveland and the vimentin probe was obtained through the generosity of Bruce Paterson. This work has been supported by grant HD18066 from the National Institutes of Health and an MDA fellowship to J. B. L.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received November 29, 1985; revised March 3, 1986.

#### References

- Allen, R. D., and Taylor, D. L. (1975). The molecular basis of amoeboid movement. In *Molecules and Movement*, S. Inoue and R. Stephens, eds. (New York: Raven Press), pp. 239–257.
- Bard, J. B. L., and Hay, E. D. (1975). The behavior of fibroblasts from the developing avian cornea. *J. Cell Biol.* 67, 400–418.
- Berger, S. L., and Birkenmeier, C. S. (1979). Inhibition of intractable nucleases with ribonucleoside-vanadyl complexes: isolation of messenger ribonucleic acid from resting lymphocytes. *Biochemistry* 18, 5143–5139.
- Blobel, G. (1980). Intracellular protein topogenesis. *Proc. Natl. Acad. Sci. USA* 77, 496–1500.
- Blobel, G., and Dobberstein, B. (1975). Transfer of proteins across

membranes. I. Presence of proteolytically processed and unprocessed nascent immunoglobulin light chains on membrane-bound ribosomes of murine myeloma. *J. Cell Biol.* 67, 835–851.

Blobel, G., and Sabatini, D. D. (1971). Ribosome membrane interaction in eukaryotic cells. In *Biomembranes*, Volume 2, L. A. Manson, ed. (New York: Plenum Press), pp. 193–195.

Bonneau, A. M., Darveau, A., and Sonenberg, N. (1985). Effect of viral infection on host protein synthesis and mRNA association with the cytoplasmic cytoskeletal structure. *J. Cell Biol.* 100, 1209–1218.

Bray, D., and Thomas, C. (1976). Unpolymerized actin in tissue cells. In *Cell Motility*, Book B, Cold Spring Harbor Conferences on Cell Proliferation, Vol. 3, R. Goldman, T. Pollard, and J. Rosenbaum, eds. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory), pp. 461–473.

Brenner, S. L., and Brinkley, B. R. (1984). Tubulin assembly sites and the organization of microtubule arrays in mammalian cells. *Cold Spring Harbor Symp. on Quant. Biol.* 46, 241–253.

Brinkley, B. R., Fuller, G. M., and Highfield, D. P. (1975). Cytoplasmic microtubules in normal and transformed cells: analysis of tubulin antibody immunofluorescence. *Proc. Natl. Acad. Sci. USA* 72, 4981–4985.

Buckley, I. K. (1981). Fine structural and related aspects of non muscle-cell motility. In *Cell and Muscle Motility*, R. M. Dowben and J. W. Shay, eds. (New York: Plenum Press), pp. 135–203.

Cervera, M., Dreyfuss, G., and Penman, S. (1981). Messenger RNA is translated when associated with the cytoskeletal framework in normal and VSV-infected HeLa Cells. *Cell* 23, 113–120.

Cleveland, D. W., Lopata, M. A., MacDonald, R. J., Cowan, N. J., Rutter, W. J., and Kirschner, M. W. (1980). Number and evolutionary conservation of  $\alpha$ - and  $\beta$ -tubulin and cytoplasmic  $\beta$ - and  $\gamma$ -actin genes using specific cloned cDNA probes. *Cell* 20, 95–105.

Colman, D. R., Kreibich, G., Frey, A. B., and Sabatini, D. D. (1982). Synthesis and incorporation of myelin polypeptides into CNS myelin. *J. Cell Biol.* 95, 598–608.

Eckert, B. S., Daley, R. A., and Parysek, L. M. (1982). Assembly of keratin into PtK1 cytoskeletons: evidence for an intermediate filament organizing center. *J. Cell Biol.* 92, 575–578.

Fulton, A. B., Wan, K. M., and Penman, S. (1980). The spatial distribution of polyribosomes in 3T3 cells and the associated assembly of proteins into the skeletal framework. *Cell* 20, 849–857.

Goldman, B. M., and Blobel, G. (1978). Biogenesis of peroxisomes: intracellular site of synthesis of catalase and uricase. *Proc. Natl. Acad. Sci. USA* 75, 5066–5070.

Goldman, R. D., Schloss, J. A., and Starger, J. M. (1976). Organizational changes of actin like microfilaments during animal cell movement. In *Cell Motility*, Book A, Cold Spring Harbor Conferences on Cell Proliferation, Vol. 3, R. Goldman, T. Pollard, and J. Rosenbaum, eds. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory), pp. 217–246.

Hay, E. D. (1985). Interaction of migrating embryonic cells with extracellular matrix. *Exp. Biol. Med.* 10, 174–193.

Holtzer, H., Bennett, G. S., Tapscott, S. J., Croop, J. M., and Toyama, Y. (1982). Intermediate-size filaments: changes in synthesis and distribution in cells of the myogenic and neurogenic lineages. *Cold Spring Harbor Symp. on Quant. Biol.* 46, 317–329.

Huang, H. L., Singer, R. H., and Lazarides, E. (1978). Actin-containing microprocesses in the fusion of cultured chick myoblasts. *Muscle and Nerve* 1, 219–229.

Ishikawa, H., Bischoff, R., and Holtzer, H. (1969). Formation of arrow-head complexes with heavy meromyosin in a variety of cell types. *J. Cell Biol.* 43, 312–328.

Jeffrey, W. R. (1984) Spatial distribution of messenger RNA in the cytoskeletal framework of Ascidian eggs. *Dev. Biol.* 103, 482–492.

Jeffrey, W. R., Tomlinson, C. R., and Brodeur, R. D. (1983). Localization of actin mRNA during early ascidian development. *Dev. Biol.* 99, 408–417.

Lawrence, J. B., and Singer, R. H. (1985). Quantitative analysis of in situ hybridization methods for the detection of actin gene expression. *Nucl. Acids Res.* 13, 1777–1799.

- Lazarides, E. (1976). Aspects of the structural organization of actin filaments. In *Cell Motility, Book A, Cold Spring Harbor Conferences on Cell Proliferation, Vol. 3*, R. Goldman, T. Pollard, and J. Rosenbaum, eds. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory), pp. 347–360.
- Lazarides, E. (1980). Intermediate filaments as mechanical integrators of cellular space. *Nature* 283, 249–256.
- Leary, J. J., Brigati, D. J., and Ward, D. C. (1983). Rapid and sensitive colorimetric method for visualizing biotin-labeled DNA probes hybridized to DNA or RNA immobilized on nitrocellulose: bio-blots. *Proc. Natl. Acad. Sci. USA* 80, 4045–4049.
- Lenk, R., Ransom, L., Kaufman, Y., and Penman, S. (1977). A cytoskeletal structure with associated polyribosomes obtained from HeLa cells. *Cell* 10, 67–78.
- Maccocchini, M. L., Rudin, Y., and Schatz, G. (1979). Transport of proteins across the mitochondrial membrane. *J. Biol. Chem.* 254, 7468–7471.
- McKeon, F. D., Kirschner, M., and Caput, D. (1986). Homologies in both primary and secondary structure between nuclear envelope and intermediate filament proteins. *Nature* 319, 463–468.
- Moreland, R. B., Nam, H. G., Hereford, L. M., and Fried, H. W. (1985). Identification of a nuclear localization signal of a yeast ribosomal protein. *Proc. Natl. Acad. Sci. USA* 82, 6561–6565.
- Ordahl, C. P., Tilghman, S. M., Ovitt, C., Formwald, J., and Largen, M. T. (1980). Structure and developmental expression of the chick  $\alpha$ -actin gene. *Nucl. Acids Res.* 8, 4989–5005.
- Osborn, M., and Weber, K. (1976). Cytoplasmic microtubules in tissue culture cells appear to grow from an organizing structure toward the plasma membrane. *Proc. Natl. Acad. Sci. USA* 73, 867–871.
- Osborn, M., Geisler, N., Shaw, G., Sharp, G., and Weber, K. (1982). Intermediate filaments. *Cold Spring Harbor Symp. Quant. Biol.* 46, 413–429.
- Pollard, T. D., and Craig, S. W. (1982). Mechanism of actin polymerization. *Trends Biochem. Sci.* 7, 55–58.
- Pollard, T. D., and Korn, E. D. (1971). Cytoplasmic filaments of amoeba proteins. I. The role of filaments in consistency changes and movement. *J. Cell Biol.* 46, 267–289.
- Redman, C. M., and Sabatini, D. D. (1966). Vectorial discharge of peptides released by puromycin from attached ribosomes. *Proc. Natl. Acad. Sci. USA* 56, 608–615.
- Rogers, A. W. (1979). *Techniques of Autoradiography*, 3rd ed., (New York: Elsevier/North Holland Biomedical Press).
- Singer, R. H., and Ward, D. C. (1982). Actin gene expression visualized in chicken muscle tissue culture by using in situ hybridization with a biotinylated nucleotide analog. *Proc. Natl. Acad. Sci. USA* 79, 7331–7335.
- Singer, R. H., Lawrence, J. B., and Rashtchian, R. (1986). Toward a rapid and sensitive in situ hybridization methodology using isotopic and nonisotopic probes. In *In Situ Hybridization: Application to the Central Nervous System*, K. Valentino, J. Eberwine, and J. Barchas, eds. (New York: Oxford University Press), in press.
- Small, J. V. (1981). Organization of actin in the leading edge of cultured cells: influence of osmium tetroxide and dehydration of the ultrastructure of actin networks. *J. Cell Biol.* 97, 695–705.
- Small, J. V., and Celis, J. E. (1978). Direct visualization of the 10-nm (100-Å)-filament network in whole and enucleated cultured cells. *J. Cell Sci.* 37, 393–409.
- Steinert, P. M., Jones, J. C. R., Goldman, R. (1984). Intermediate filaments. *J. Cell Biol.* 99, 22S–32S.
- Stosel, T. P. (1984). Contribution of actin to the structure of the cytoplasmic matrix. *J. Cell Biol.* 99, 15S–21S.
- Taylor, D. L., Heiple, J., Wang, Y-L., Luna, E. J., Tanasugara, L., Brier, J., Swanson, J., Feckheimer, M., Amato, P., Rockwell, M., and Daley, G. (1982). Cellular and molecular aspects of amoeboid movement. *Cold Spring Harbor Symp. on Quant. Biol.* 46, 101–111.
- Tilney, L. G. (1976). The polymerization of actin. III. Aggregate of non-filamentous actin and its associated proteins: a storage form of actin. *J. Cell Biol.* 69, 73–89.
- Valenzuela, P., Quiroga, M., Zaldivar, J., Rutter, W. J., Kirschner, M. W., and Cleveland, D. W. (1981). Nucleotide and corresponding amino acid sequences encoded by alpha and beta tubulin mRNAs. *Nature* 289, 650–655.
- Wang, Y-L. (1984). Reorganization of actin filament bundles in living fibroblasts. *J. Cell Biol.* 99, 1478–1485.
- Wolosewick, J. J. (1984). Distribution of actin in migrating leukocytes in vivo. *Cell Tissue Res.* 236, 517–525.
- Zehner, Z. E., and Paterson, B. M. (1983). Characterization of the chicken vimentin gene: single copy gene producing multiple mRNAs. *Proc. Natl. Acad. Sci. USA* 80, 911–915.