raphy, radioactivity in gel pieces was determined by scintillation counting. Approximately 1.5 × 10^8 of the 293 cells were centrifuged (JE-10X rotor, Beckman J-6M) at 1200 rpm (pump speed, 80 ml/min). The elutriation buffer (EB) was PBS containing 0.3 mM EDTA, fetal calf serum (1%), and glucose (0.1%). Cell fractions were collected in 500 ml by increasing the pump speed by 20 ml/min in a stepwise manner. The fractionated cells were centrifuged, washed, and counted with trypan blue dye exclusion. Approximately 75% of the cells were recovered, of which more than 90% were viable. Fractionated cells (1 × 10^6) were fixed 1 hour or overnight in 80% ethanol and then washed twice in PBS containing fetal calf serum (1%). Fixed cells were stained in PBS containing propidium iodide (PI) (4 μg/ml) and ribonuclease (1 μg/ml) at 37°C for 30 min. Stages of the cell cycle were assessed by analysis of stained cells with a Coulter EPICS C flow cytometer. The remaining cells in each fraction were extracted, immunoprecipitated, and assayed for histone H1 kinase activity.

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29 March 1991; accepted 11 June 1991

Requirement of Microfilaments in Sorting of Actin Messenger RNA

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Specific messenger RNAs (mRNAs) can be sequestered within distinct cellular locations, but little is known about how this is accomplished. The participation of the three major cellular filaments in the localization of actin mRNA was studied in chicken embryo fibroblasts. Movement of actin mRNA to the cell periphery and maintenance of that regionalization required intact microfilaments (composed of actin) but not microtubules or intermediate filaments. The results presented here suggest that actin-binding proteins may participate in mRNA sorting.

A FUNDAMENTAL QUESTION IN CELL biology concerns how molecules are targeted to their sites of function. A number of works have described the sequence-specific sorting of mRNA to distinct cellular regions (*1*). Often these mRNAs are sequestered in cellular locations where their corresponding proteins function (*2*), implying that localized synthesis may be a means to regionalize some proteins. Our studies address the mechanism of actin mRNA sorting to the actin-rich ruffling edges of motile chicken embryo fibroblasts (CEFs).

Information for the localization of β-actin mRNA is encoded in the nucleic acid sequence of the mRNA and not the amino acid sequence of the nascent actin polypeptide (*3*). In *Drosophila*, a sequence in the 3′ untranslated region of *bicoid* mRNA is necessary for its localization in the embryo (*4*). Therefore, a mechanism exists in cells to transduce nucleic acid information into spatial information. It is likely that this mechanism involves cellular filament systems. Cytoplasmic motor proteins have been described that implicate both microtubules and actin filaments in intracellular transport (*5*). Furthermore, evidence exists that a direct cytoskeletal interaction with mRNA (*6*) appears to be necessary for translation (7). In *Dictyostelium discoideum*, the translation elongation factor EF-1a was shown to be an actin-binding protein (*8*).

We have focused on the functions of the three cellular filament systems in the movement of actin mRNA to the cell periphery of CEFs, as well as the subsequent maintenance of that localization. Movement and maintenance of localization were distinguished experimentally with the use of spreading cells. In cells in suspension, actin mRNA is uniformly distributed; however, when these cells are transferred to tissue culture plates and allowed to spread, actin mRNA relocalizes to the cell periphery in minutes (*3*). Maintenance of this localization was studied in cells that had completed spreading and had peripherally localized their actin mRNA.

In order to correlate the spatial relation between actin message and cellular filaments, we visualized both simultaneously. A method was developed for detection of mRNA and specific proteins that preserved the intracellular location of the mRNA as well as the antigenicity of the protein (*9*). The three major filament systems—microtubules (MTs), intermediate filaments (IFs),

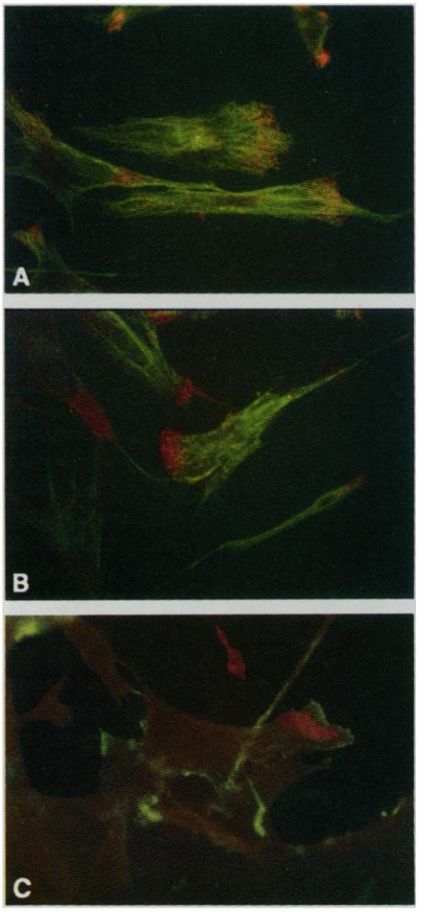

Fig. 1. Distribution of actin mRNA in relation to each of the three major cellular filament systems in CEFs. The simultaneous visualization of mRNA and protein was achieved by in situ hybridization with a digoxigenin-labeled actin cDNA probe in conjunction with mouse antibodies to tubulin, vimentin, and actin; detection was achieved with a rhodamine-conjugated antibody to digoxigenin (red) and fluorescein-conjugated secondary goat antibodies to mouse protein (green). (**A**) Actin mRNA, red; MTs, green; (**B**) actin mRNA, red; IFs, green; and (**C**) actin mRNA, red; MFs, green. All images were single exposures photographed with a Zeiss ×63 infinity-corrected objective with standard epifluorescence optics. A dual-wavelength filter set was used that allowed simultaneous visualization of rhodamine and fluorescein without optical shift.

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and microfilaments (MFs)—are composed largely of tubulin, vimentin, and actin, respectively. Fluorescent detection of actin mRNA revealed a high concentration in the distal regions of cellular lamellae in the form of punctate fluorescence (Fig. 1). Actin mRNA did not coincide with any of the filament systems, including MFs (Fig. 1), but was closely juxtaposed to the ruffling edges, regions devoid of ribosomes (*10*) that contain high concentrations of filamentous actin (MFs) (Fig. 1C). Because actin, unlike tubulin and vimentin, is also present in the form of 6-nm filaments, which are not resolvable by light microscopy, it is possible that actin mRNA coincides with a subset of these actin filaments. Figure 1 shows that the sites of synthesis of actin do not coincide with the entire steady-state actin filament network but are found closely juxtaposed to regions of the cell undergoing actin polymerization. This approach revealed the gross distribution of actin mRNA and cellular filaments but did not resolve their functional relation in terms of mRNA localization. In order to probe this interaction, we used the specific cytoskeletal-disrupting drugs Colcemid and cytochalasin D.

When fully spread cells (plated for 2 days) were exposed to Colcemid for 60 min, MTs depolymerized (Fig. 2, A and B) and IFs collapsed around the nuclei (Fig. 2, C and D). However, actin mRNA was still localized in the peripheral regions of cells. MFs were unaffected by Colcemid, as judged by the presence of stress fibers and high concentrations of filamentous actin in the ruffling edges. Some cells acquired a stellate morphology as a result of Colcemid treatment with actin mRNA concentrated to the periphery of cellular projections (Fig. 2H). The percentage of Colcemid-treated cells with peripherally localized actin mRNA was not significantly different than control cells (mean ± SD = 54 ± 12% for n = 900 cells treated with Colcemid for 60 min versus 51 ± 11% for n = 1800 control cells) (Figs. 2G and 3A) (*11*). Even after 3 hours in Colcemid, a significant number of cells had actin mRNA peripherally localized (40%, Fig. 3A). In contrast, cells treated with low doses of cytochalasin D for 10 min, which did not affect MTs or IFs, underwent a dose-dependent disruption of MFs with a corresponding loss of peripheral actin mRNA localization. The percent of cells with actin mRNA peripherally localized after treatment with cytochalasin D (0.25 μg/ml) decreased by half (Fig. 3B). Higher doses (10 μg/ml) resulted in more severe disruption, with the formation of short bundles of MFs (similar to those shown in Fig. 2F), rather than normal stress fibers (Fig. 2E) and only 10 ± 6%, (n = 600) of the cells had actin mRNA peripherally localized. The remaining cells had a uniform distribution of actin mRNA (Fig. 2I). These data demonstrate that actin mRNA can rapidly disperse throughout the cytoplasm after disruption of MFs and that maintenance of peripheral actin mRNA localization does not require intact MTs or IFs.

Movement of actin mRNA to the cell periphery was studied in spreading cells. Cells were able to spread when plated in media that contained Colcemid (5 μg/ml) but were unable to generate a characteristic polarized morphology. MTs were not seen in these cells and a diffuse distribution of tubulin (similar to that shown in Fig. 2B), as well as IFs that were collapsed around the nuclei, were observed (similar to those shown in Fig. 2D). The polymerization of MFs was unaffected. Despite the disruption of two of the three major filament systems, actin mRNA was still localized to the cell periphery (Fig. 2K) as in control cells (Fig. 2J) [48 ± 19% (n = 600) cells plated in Colcemid for 180 min had actin mRNA peripherally localized, as compared to 48 ± 17% (n = 900) control cells] (Fig. 3C). Furthermore, the kinetics of actin mRNA relocalization was similar to that observed for untreated cells (Fig. 3C).

In contrast, cells plated in the presence of low doses of cytochalasin D (0.5 μg/ml) spread abnormally; often these cells extended thin sheets of cytoplasm that resembled lamellae but lacked ruffling edges. MFs appeared as small clusters throughout the cytoplasm of these cells (Fig. 2F), whereas MTs and IFs were unaffected. Significantly fewer spreading cells had actin mRNA localized to the periphery after 180 min in cytochalasin D compared to control or Colcemid-treated cells [7 ± 3% (n = 900) cells compared to 48 ± 17% (n = 900) control cells] (Fig. 3C). The majority of the cells had actin mRNA concentrated in the perinuclear region with lamellae-like structures completely devoid of actin mRNA (Fig. 2L). These data indicate that, in addition to their function in the maintenance of actin mRNA localization, intact MFs are also required for the movement of actin mRNA to the cell periphery.

The simplest mechanism that explains our results is that transport and subsequent anchoring of actin mRNA in the cell periphery is MF-dependent. Free diffusion of message is improbable because we have found the same percentage of actin mRNA associated with the detergent-extracted cytoskeleton at both early and late stages of spreading (*12*).

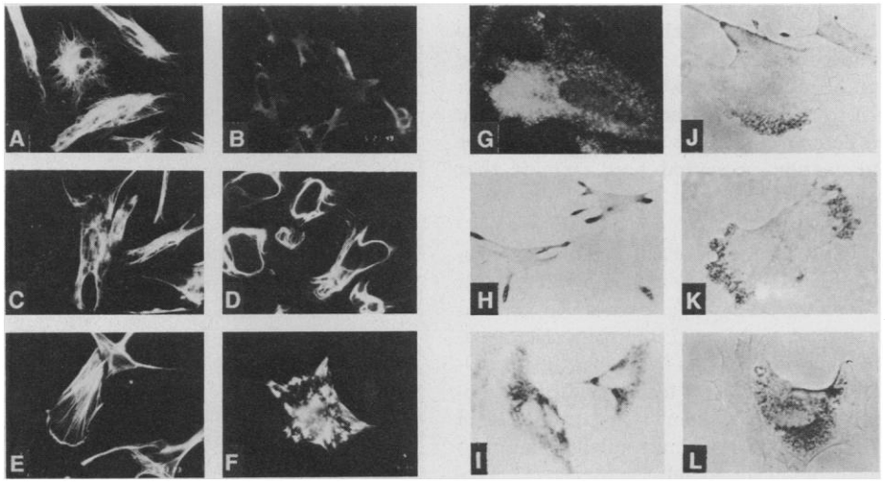

Fig. 2. The effect of disruption of cellular filament systems by Colcemid (5 μg/ml) and cytochalasin D (0.1 μg/ml) on the maintenance and establishment of actin mRNA localization in CEFs. The CEFs were obtained from pectoral muscles of 12-day-old chicken embryos and cultured for 2 days unless otherwise noted. (**A** through **F**) Fluorescent micrographs obtained with standard epifluorescence optics and camera mounted on a Zeiss IM 35 microscope. (**G**) This fluorescent image was obtained with a liquid nitrogen–cooled charged-couple device camera mounted on the same microscope. The image was corrected for camera dark current and background fluorescence. Actin appears as white punctates in this black and white micrograph. (**H** and **I**) Micrographs obtained with bright-field optics. (**J** through **L**) Micrographs obtained with phase-contrast optics. (A) Normal cells stained for the detection of MTs. (B) As in (A), but after a 60-min exposure to Colcemid. (C) Normal cells stained for the detection of IFs. (D) As in (C), but after a 60-min exposure to Colcemid. (E) Normal cells stained for the detection of MFs. (F) As in (E), but after a 60-min exposure to cytochalasin D. (G) Actin mRNA distribution in normal cells revealed by in situ hybridization coupled with immunofluorescent detection. (H) Actin mRNA distribution in CEFs treated with Colcemid for 90 min before fixation was revealed by in situ hybridization coupled with immunoenzymatic detection. (I) Actin mRNA distribution in cells treated with cytochalasin D for 10 min before fixation. (J) Actin mRNA distribution in normal cells that spread for 3 hours, (K) in cells that spread for 90 min in the presence of Colcemid, and (L) in cells that spread for 3 hours in the presence of cytochalasin D. In (I) through (L) actin mRNA was detected by the same method as in (H).

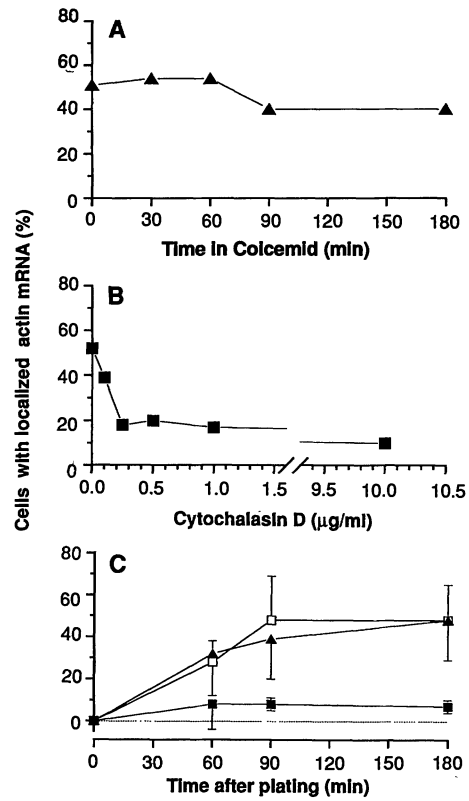

Fig. 3. Effect of Colcemid and cytochalasin D on actin mRNA localization in cells under steady-state conditions (fully spread) (**A** and **B**) and in cells in the process of spreading (**C**). (A) The effect of time in Colcemid on the percent of cells that display peripheral actin mRNA localization. Cells were treated with Colcemid (5 μg/ml) for various times, fixed, hybridized, and then detected with the alkaline phosphatase reaction. The percent of cells with localized actin mRNA was then determined. For 0 min, there were six experiments, 1800 total cells counted and an SD of 8%. For 30 min, there were three experiments, 900 total cells counted and an SD of 12%. For 60 min, there were three experiments, 900 total cells counted and an SD of 18%. For 90 min, there were two experiments, 600 total cells and a range of 4%. For 180 min, one experiment was performed with 300 total cells. (B) The effect of a 10-min exposure to various cytochalasin D doses on the percent of cells with peripherally localized actin mRNA. Cells were treated with varying doses of cytochalasin D for 10 min after which actin mRNA distribution was analyzed. Each point is the average of two experiments (600 cells counted); the range was less than 7% at all concentrations except for 0.1 μg/ml where the range was 19%. (C) The effect of Colcemid (triangles) or cytochalasin D (solid squares) on the relocalization of actin mRNA that occurs during spreading (control, open squares). Cells were plated in the presence of Colcemid (5 μg/ml) or cytochalasin D (0.5 μg/ml) and allowed to spread for various periods of time. For the control, each time point is the average of three experiments ± SD (900 cells counted). For Colcemid, each point is the average of two experiments ± range (600 cells counted). For cytochalasin D, each point is the average of three experiments ± SD (900 cells).

This finding indicates that actin mRNA is not free to diffuse in the cytoplasm when most cells are in the process of relocalizing actin mRNA or when localization is completed. Furthermore, the relocalization of actin mRNA is not passively associated with the redistribution of MFs because it lags behind the re-extension of cellular lamellae (*3*). The association of actin mRNA with MFs is not unique to this message because vimentin and tubulin mRNAs are also associated with MFs (*13*) and the majority of polyadenylated mRNA is associated with MFs as well (*14*). Furthermore, because actin mRNA continues to be sorted after inhibition of protein synthesis, this association with MFs is not related to an affinity of nascent actin polypeptides for MFs as a result of cotranslational assembly (*3, 15*).

However, it remains to be seen whether other messages may use different filament systems for transport. In *Xenopus* oocytes, the transport of Vg1 mRNA to the vegetal pole has been shown to be MT-dependent because it is inhibited by Colcemid (*16*). It is possible that the failure of Vg1 mRNA to transport to the vegetal pole in that study resulted from prolonged exposure to the drug (5 days) rather than a direct effect on MTs. The rapidity of actin mRNA localization in our studies allows for short drug exposures. Hence, the sensitivity of actin mRNA localization to cytochalasin D and its resistance to Colcemid occur within physiologically relevant time periods. There is substantial evidence that MFs may function in transport of vesicles and organelles. Our studies suggest that they may participate in message transport as well. The finding that transport and anchoring of actin mRNA both occur on MFs suggests that different actin-binding proteins may participate in these two components of actin mRNA localization.

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9. CEFs obtained from pectoral muscles of 12-day-old embryos were plated onto gelatin-coated cover slips. Cultures were fixed after 2 days unless otherwise noted. For immunofluorescence or in situ hybridization, cells were fixed in paraformaldehyde (4%) in phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$ for 10 min. MTs and IFs were visualized after hybridization with monoclonal antibodies to α- and β-tubulin (Amersham) or vimentin (Boehringer Mannheim), followed by a fluorescein-conjugated goat antibody to mouse immunoglobulin G (IgG). MFs were visualized with either rhodamine-conjugated phalloidin (Molecular Probes) or a monoclonal antibody to rabbit skeletal actin (East-Acres Biologicals) in conjunction with a fluorescein-conjugated goat antibody to mouse IgG. In order to develop a protocol for the simultaneous staining of mRNA and protein, we optimized fixation and hybridization conditions for best labeling. We achieved simultaneous visualization of protein and mRNA by performing in situ hybridization as described (*2*), followed by antibody detection of protein and mRNA. Actin antigenicity was not preserved through the hybridization, so we used an antibody to actin before hybridization. In this case, cells were first labeled with the antibody to actin, followed by the fluorescein-conjugated goat antibody to mouse IgG. Cells were then fixed again in paraformaldehyde in PBS (4%, 5 min). After briefly washing the cells in PBS, we processed them for in situ hybridization as described, except that 50 ng of the actin cDNA probe was used (per 20 μl hybridization volume) and cells were hybridized for 1 hour. The insert and plasmid were labeled by nick translation with deoxyuridine triphosphate conjugated to digoxigenin. Hybridized probe was detected by an antibody to digoxigenin conjugated to alkaline phosphatase (Boehringer Mannheim) or by fluorescence with an antibody to digoxigenin conjugated to rhodamine (Boehringer Mannheim). The alkaline phosphatase reaction product appeared as opaque punctates when viewed with phase or bright-field microscopy.
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11. We determined the percentage of cells with localized actin mRNA by counting 300 cells in randomly selected fields from at least two different experiments in which we used a ×40 objective and bright-field optics. We scored cells as having localized actin mRNA if the mRNA was more concentrated over the distal regions of leading lamellae and filopodia than elsewhere in the cytoplasm.
12. We ascertained the percentage of actin mRNA associated with the cytoskeleton by extracting cells for 1 min in Triton X-100 at early and late stages of spreading. Actin mRNA associated with the cytoskeleton was determined by quantitative in situ hybridization with a ^{32}P-labeled actin cDNA probe. The actin mRNA signal in extracted cells was compared to that in unextracted cells after normalization for cell number. Cells were labeled with [^3H]thymidine (1 μC/ml) for 24 hours. After correction for total RNA lost during the hybridization procedure (20%), ~71% of actin mRNA was associated with the cytoskeleton 30 min after plating, as compared to 74% after 3 days of plating.
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17. We thank J. Lawrence for her contributions to this work; G. Bassell, K. Taneja, and E. Kislauskis for helpful discussions; F. Fay and D. Bowman of the University of Massachusetts Biomedical Imaging Group for their assistance with Fig. 2G; C. Dunshee for photography; and K. Byron for tissue culture. Supported by NIH grant HD18066 (R.H.S.) and by an NIH National Research Service Award (C.L.S.).

25 March 1991; accepted 12 June 1991