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Actin mRNA Localizes in the Absence of Protein Synthesis

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Abstract. Actin mRNA is localized in chicken embryo fibroblasts to the distal regions of leading lamellae, but not within the ruffling edges. In this investigation we have addressed the role of actin translation in this process. The translocation of actin mRNA to the cell periphery was studied by monitoring the distribution of actin mRNA in cells during spreading. Within 90 min, actin mRNA moved from a perinuclear to a peripheral distribution. Formation of lamellipodia preceded actin mRNA localization, indicating that localization is not a prerequisite for this event. Neither puromycin (which dissociates ribosomes from mRNA) nor cycloheximide (which stabilizes ribosomes on mRNA) had any effect on this movement of actin mRNA. Anchoring of actin mRNA was studied using cells with peripherally localized actin mRNA. No change in actin mRNA localization was observed for 30 min in the same inhibitors. These data indicate that the presence of the nascent polypeptide is not necessary for translocation of actin mRNA to the cell periphery, or anchoring at that site. This suggests that the mRNA contains information concerning its spatial distribution within the cytoplasm.

Actin mRNA in chicken embryo fibroblasts is localized to the distal regions of leading lamellae and filopodia (Lawrence and Singer, 1986). This localization is sequence specific since other messages display different distributions within these cells. Nonhomogeneous distributions of specific mRNAs have also been described in oocytes (actin mRNA, Jeffrey et al., 1983; Vgl, Anl, An2, and An3 mRNAs, Rebagliati et al., 1985), embryos (bicoid mRNA, Frigerio et al., 1986; Berleth et al., 1988), skeletal muscle (nicotinic acetylcholine receptor mRNA, Fontaine et al., 1988), and neurons (MAP2 mRNA, Garner et al., 1988; Papandrikopoulos et al., 1989).

Which components associated with the mRNA are involved in its sequence-specific localization? Two possibilities are the nascent polypeptide and/or sequence-specific ribonucleoproteins. A localization mechanism involving the nascent polypeptide is supported by several observations. The nascent polypeptide in conjunction with the signal recognition particle can direct mRNAs for secreted and membrane proteins to the endoplasmic reticulum (Walter and Blobel, 1981). Nascent polypeptides can also associate with mRNAs for some cytoskeletal proteins with filaments ("cotranslational assembly," Isaacs and Fulton, 1987). This could account for actin mRNA localization near lamellipodia which are regions undergoing rapid actin polymerization (Wang, 1985). Consistent with this, actin mRNA is associated with actin filaments (Ornelles et al., 1986; Singer et al., 1989).

We have investigated the role of the nascent polypeptide in two aspects of message localization: (a) translocation of the mRNA to its site of localization and (b) anchoring at that site. The movement of actin mRNA to the cell periphery was investigated by observing the redistribution of actin mRNA which occurred as freshly plated cells spread on a substrate. The anchoring of actin mRNA within leading lamellae was examined using cells that had established peripheral actin mRNA localization. To address the role of the nascent polypeptide in these events, two types of protein synthesis inhibitors were used: those that dissociate nascent polypeptides from the mRNA-ribosome complex (puromycin and NaF), and those that retain nascent polypeptides on mRNA (cycloheximide and emetine).

Here we present evidence that the association of the nascent polypeptide with mRNA is not required for the dynamic process of actin mRNA localization or its maintenance. This suggests that the nucleic acid sequence of the mRNA contains cellular spatial information.

Materials and Methods

Cell Culture

Skeletal myoblasts and fibroblasts obtained from pectoralis muscles of 12-d-old chicken embryos were cultured using standard techniques. Cells were plated onto gelatin-coated coverslips at a density of 2 × 10⁶ cells/100-mm dish in minimal essential medium supplemented with 10% heat-inactivated FBS and 2% embryo extract (derived from 12-d-old chicken embryos). In experiments requiring previously spread cells, 2-d muscle cultures were used that consisted of myoblasts, fibroblasts, and early myofibers. To fix...
cells, the coverslips were washed in HBSS and then immersed for 15 min in 4% paraformaldehyde in PBS. After fixation, cells were stored in 70% ethanol at 4°C. For the cell-spreading experiments, 1 ml of 0.25% trypsin in 0.2% EDTA solution (Bethesda Research Laboratories, Gaithersburg, MD) was added to cells in a 150-mm tissue culture flask. As soon as cells started to round up, a 20-fold excess of medium was added to the flask to inhibit the trypsin activity. Cells were then transferred (density of ~4 x 10^6 cells/dish) into 100-mm tissue culture dishes containing gelatin-coated coverslips and fixed at various times after plating.

**DNA Probe Preparation**

The actin cDNA probe used in these experiments was a 1.382-kb segment (nt 95-1477) of a full-length β-actin cDNA probe (Cleveland et al., 1980) that was subcloned into Bluescript KSII+ (Stratagene Cloning Systems, La Jolla, CA) in order to remove GC-rich regions. The removal of GC-rich regions from the cDNA probe was important since they resulted in hybridization to GC-rich RNA in nuclei. This β-actin cDNA probe was nick transcribed with a dUTP analog conjugated to digoxigenin via an 11-atom linker (Bio-Rad Laboratories, Richmond, CA). Digoxigenin-labeled probes immobilized to coverslips were then treated with DNase I (400 ng/ml; Worthington Biochemical Corp., Freehold, NJ) to remove any nicked or linearized probe DNA. The digoxigenin-labeled probe was not detected when hybridized to an excess of radioactive actin cDNA. The use of nick-translated probe was also essential since it resulted in labeled fragments of 50-500 nucleotides long. Unincorporated nucleotides were removed by centrifugation through 1 cc Sephadex G50 columns. The probes were then sized and assessed for digoxigenin incorporation by electrophoresis on 1% agarose denaturing gels, and subsequently blotted onto Zeta-probe filters (Bio-Rad Laboratories, Richmond, CA). Digoxigenin-labeled probes immobilized to coverslips were then treated with an antidigoxigenin antibody conjugated to alkaline phosphatase (Boehringer Mannheim Biochemicals), washed for 15 min in buffer 1 (150 mM NaCl and 100 mM Tris-Cl, pH 7.5). Alkaline phosphatase activity was detected colorimetrically by incubation of the filters at 37°C in 15 ml of buffer 3 (100 mM Tris-Cl, pH 9.5, 100 mM NaCl, 50 mM MgCl2) containing 3 mg of nitroblue tetrazolium (Boehringer Mannheim Biochemicals) and 3.25 mg of bromochloroindolyl phosphate (Boehringer Mannheim Biochemicals).

**Hybridization and Detection**

Probe DNA (20 ng/coverslip) was dried down with Escherichia coli tRNA (20 µg/coverslip) and sonicated salmon sperm DNA (20 µg/coverslip), and then resuspended in 100% formamide (10 µl/coverslip). Probe DNA, E. coli tRNA, and salmon sperm DNA were then heated to 90°C for 10 min and combined with 10 µl of hybridization mix for a final probe concentration of 1 µg/ml. The final hybridization solution consisted of 50% formamide, 2× SSC, 0.2% BSA, 10% dextran sulfate, and 1 mg/ml each of E. coli tRNA and salmon sperm DNA. Fixed cells on coverslips were rehydrated in PBS + 0.5% Triton X-100 for 10 min and then placed in 50% formamide/2× SSC (0.3 M sodium chloride, 0.03 M citric acid, pH 7.4) at room temperature for 30 min. The coverslips were then placed cellside down on a parafilm containing 20 µl of the hybridization solution and incubated for 3 h at 37°C in a humidified chamber. Cells were then washed in 10 ml of coplin jars in 50% formamide/2× SSC at 37°C for 30 min, followed by three washes in 1× SSC at room temperature, with agitation, for 10 min each. Cells were then subsequently equilibrated in buffer 1 for 2 min and then incubated in 20 µl of antidigoxigenin/alkaline phosphatase conjugate (Boehringer Mannheim Biochemicals) diluted 1:250 in antibody buffer (buffer 1 + 0.3% Triton X-100 + 1% BSA) at 37°C for 30 min. Cells were then washed twice, for 15 min each, at room temperature in buffer 1, followed by equilibration for 1 min in buffer 3. Cells on coverslips were then transferred to six-well tissue culture cluster dishes with each well containing 2 ml of nitroblue tetrazolium/bromochloroindolyl phosphate solution (3 mg of nitroblue tetrazolium and 2 mg of bromochloroindolyl phosphate in 10 ml of buffer 3) at room temperature. The color development was monitored by bright-field microscopy, and stopped by addition of PBS when sufficient reaction product was observed in the cell periphery (30-90 min).

**The Localization Assay**

The percentage of cells with localized actin mRNA was determined by two individuals each counting at least 600 cells in randomly selected fields from at least two different experiments using a 40X objective and bright-field optics. Cells were scored as having localized actin mRNA if actin mRNA was more concentrated over leading lamellae and filopodia than elsewhere in the cytoplasm.

**Protein Synthesis Inhibitors**

For experiments requiring protein synthesis inhibitors, cells were treated with puromycin (200 µg/ml), cycloheximide (5 µg/ml), emetine (50 µM), or NaF (10 mM) for at least 30 min. These doses and exposures were sufficient to inhibit protein synthesis 90-96% as determined by [35S]methionine incorporation into TCA-precipitable protein. The kinetics of puromycin inhibition of protein synthesis were determined by comparing [35S]methionine incorporation into TCA-precipitable protein in control cells vs. treated cells. 10 min after [35S]methionine addition, puromycin (200 µg/ml) was added to half the cells and incorporation using puromycin-treated cells determined after 1, 5, 10, 15, and 20 min. Puromycin was found to inhibit protein synthesis within 1 min of application to cells.

**Results**

**Intracellular Localization of Actin mRNA**

Using the nonisotopic detection method described above, actin mRNA in chicken embryo fibroblasts was localized to the distal regions of leading lamellae but excluded from lamellipodia or ruffling edges (Fig. 1, a and b). Actin mRNA was concentrated within a band of cytoplasm set back from the leading edges by ~3 µm (Fig. 1, a and b). Often the actin mRNA signal revealed by the alkaline phosphatase reaction appeared punctate and occasionally formed linear arrays as if associated with filamentous cellular structures. In cells without a polarized morphology (e.g., stellate cells), actin mRNA was still found at the distal ends of filopodia (Fig. 1, a and b). In these structures, as in leading lamellae, mRNA was absent from the most distal few micrometers. The absence of actin mRNA within the ruffling edge is consistent with electron microscopic studies that indicate the exclusion of ribosomes and cellular organelles from this region (Abercrombie et al., 1971).

Approximately 60% of single cells had actin mRNA localized to leading lamellae or filopodia (see Fig. 4); the remainder had either very low actin mRNA signal, or a more uniform distribution. Parameters that may influence assessment of intracellular actin mRNA distribution are the presence of differentiated single cells (i.e., myocytes) in the cell population that contain low levels of β-actin (Lawrence et al., 1989), cells in different stages of the cell cycle, or cells in different stages of commitment to movement in a given direction.

**Actin mRNA Localization during Cell Spreading**

We examined the distribution of actin mRNA within cells that were trypsinized, and then replated onto gelatin-coated coverslips and allowed to spread for various periods of time. Immediately after trypsinization, cells were centrifuged directly onto microscope slides, fixed, and actin mRNA distribution assayed. These cells had a round morphology and actin mRNA was found to be uniformly distributed by in situ hybridization (i.e., 0% of cells had actin mRNA localized; not shown). Cells that spread for 30 min exhibited a variety of cell morphologies (Fig. 1, c and d), and actin mRNA was peripherally localized in 14% of cells that had begun to extend lamellae (see Fig. 3). Cells were not scored as having localized actin mRNA if they had either very low actin mRNA signal, a uniform distribution of actin mRNA, or actin mRNA that was localized within the perinuclear region (Fig. 1, c and d). In the 14% of cells with localized actin mRNA...
Figure 1. Actin mRNA distribution within chicken embryo fibroblasts. (a) Cells were hybridized with a digoxigenin-labeled probe to actin mRNA, and detected with an antidigoxigenin antibody conjugated to alkaline phosphatase. Actin mRNA revealed by the alkaline phosphatase reaction appears as opaque, punctate signal concentrated to the distal region of leading lamellae and filopodia (black arrowhead) in this phase-contrast micrograph. Actin mRNA was not detected within the lamellipodia or ruffling edges (white arrowheads). Bar, 10 μm. (b) Bright-field micrograph of the same cell illustrates the low background associated with digoxigenin-labeled probes and the localization of actin mRNA to the leading lamellae and filopodia. Same magnification as a. (c and d) Phase-contrast and bright-field micrographs of actin mRNA distribution in cells that were trypsinized, replated, and allowed to spread on a substrate for 30 min. At this time, some of the cells had actin mRNA localized peripherally (middle cell), while other cells had very low actin mRNA signal (cell on left), or a perinuclear distribution of actin mRNA (cell on right). Bar, 10 μm. (e and f) Phase-contrast and bright-field micrographs of actin mRNA distribution within cells allowed to spread for 90 min. Bar, 10 μm. Actin mRNA distribution within these cells was indistinguishable from cells cultured for 2 d.

mRNA, the degree of regionalization was not as great as in cells cultured for 2 d, i.e., substantial amounts of actin mRNA were found throughout the cytoplasm (Fig. 1, c and d). These data suggest that reextension of lamellae can precede actin mRNA localization. By 90 min after plating, actin mRNA distribution was characteristic of cells that had been cultured for 2 d (Fig. 1, e and f), and was quantitatively identical (58% had actin mRNA localized; compare Fig. 3 with Fig. 4). This regionalization also occurred in the presence of the transcription inhibitor actinomycin (5 μg/ml) (data not...
Figure 2. The effect of protein synthesis inhibitors on actin mRNA localization. (a) Actin mRNA distribution in cells plated in the presence of puromycin (200 µg/ml) and allowed to spread for 30 min. (b) Low-power bright-field and phase-contrast micrographs of actin mRNA distribution in cells that spread in the presence of puromycin for 90 min. (c) Actin mRNA distribution in cells after spreading for 90 min in the presence of cycloheximide (5 µg/ml). (d) Actin mRNA distribution in cells that were already spread before treatment for 30 min with puromycin. (e) Actin mRNA distribution in a cell already spread before treatment with cycloheximide for 30 min. (f) Actin mRNA distribution in previously spread cells treated with puromycin for 3 h. In this group of cells, actin mRNA delocalized, exhibiting a perinuclear distribution with filopodia (arrowheads) devoid of actin mRNA. Bars, 10 µm.

shown) indicating that messages existing before trypsinization were relocalized.

The Effect of Protein Synthesis Inhibitors on Actin mRNA Localization in Spreading Cells

Actin mRNA was redistributed to the cell periphery even after dissociation of nascent polypeptides using puromycin, which inhibited protein synthesis within 1 min of application (see Materials and Methods). At 30 min after plating, actin mRNA was localized in ~15% of puromycin-treated cells compared with 14% of control cells. After 90 min this increased to ~49% of puromycin-treated cells compared with 58% of control cells (see Fig. 3). The puromycin-treated
cells with localized message were visually indistinguishable from control cells at 30 and 90 min after plating (Fig. 2, a and b). Similar results were also obtained for cells spreading in the presence of cycloheximide (Fig. 2 c), despite the different mechanism of action. Cycloheximide results in ribosomes and nascent polypeptides accumulating on mRNA (Godchaux et al., 1967; Lodish, 1971), unlike puromycin, which dissociates ribosomes and nascent chains from the mRNA (Yarmolinsky and de la Haba, 1959; Joklik and Becker, 1965). The percent of cells with localized message decreased minimally after exposure to these inhibitors for 3 h. These data indicate that translation of actin mRNA is not required for its anterograde movement to leading lamellae.

The Effect of Protein Synthesis Inhibitors on Maintenance of Actin mRNA Localization

After actin mRNA is translocated to its site, localization must be maintained. To determine whether the nascent polypeptide anchors actin mRNA within leading lamellae, the effects of puromycin, NaF, cycloheximide, and emetine were evaluated on cells with localized actin mRNA. NaF disaggregates polysomes by inhibiting translation initiation (Marks et al., 1965). Emetine inhibits elongation, and like cycloheximide, results in accumulation of ribosomes and nascent polypeptides on mRNA (Jimenez et al., 1977; Lodish, 1971). If the nascent polypeptides were involved in anchoring actin mRNA, puromycin and NaF would be expected to result in message delocalization, whereas cycloheximide and emetine would be expected to inhibit delocalization. No significant visual delocalization of actin mRNA, or decrease in the percentage of cells with localized actin mRNA was observed after exposures to puromycin (Fig. 2 d), NaF, cycloheximide (Fig. 2 e), or emetine for 30 min (Fig. 4). Even after 3 h in puromycin or cycloheximide, actin mRNA remained localized in some cells although a significant number displayed a perinuclear actin mRNA distribution (Fig. 2 f). The fact that actin message remained localized in cells despite these various inhibitors suggests that the nascent polypeptide does not tether actin mRNA at its localization site.

Discussion

We present evidence that the nascent actin polypeptide is not necessary for either the translocation of actin mRNA to the leading lamellae of chicken embryo fibroblasts or in the anchoring of actin mRNA within this region. For this work a model system was developed for studying each of these two aspects of mRNA localization separately. Translocation of actin mRNA was studied in spreading cells wherein actin mRNA became redistributed from the perinuclear region to the cell periphery. Anchoring of actin mRNA was studied in cells after peripheral actin mRNA localization had been established.

We found that relocalization of actin mRNA to the cell periphery during spreading occurred even when nascent polypeptides were dissociated from mRNA by puromycin. The fact that the number of puromycin-treated cells with regionalized actin mRNA increased from 30 to 90 min qualitatively and quantitatively like control cells indicates that localization clearly occurs in the presence of this drug. However, the percentage of cells with localized actin mRNA that had spread in puromycin for 90 min was consistently slightly lower than control cells (49% or puromycin-treated cells compared with 58% for control cells). A slightly lower percentage of cells with localized actin mRNA was also found after cycloheximide treatment. The slight decrease in the percent of cells with actin mRNA localized after prolonged exposure (3 h) to both puromycin and cycloheximide, despite their disparate mechanisms of action, suggest that this results from secondary effects of these drugs. The rapid relocalization of actin mRNA in spreading cells in either the absence or presence of inhibitors is further evidence that ongoing protein synthesis is not required for actin mRNA localization, especially when considering that suspension of cells can reduce translation, albeit over longer times than in this experiment (Farmer et al., 1983).

Yisraeli and Melton (1988) demonstrated that synthetic Vgl mRNA lacking a translation-start codon when microinjected into Xenopus oocytes still became localized to the vegetal pole. MacDonald and Struhl (1988) demonstrated a sequence in the 3' untranslated region of bicoid mRNA
necessary and sufficient for localization to the anterior region of Drosophila embryos. The results presented in this study on somatic cells support the conclusions from oocytes and embryos that translation of message is not required for localization. We have also shown that localization occurs much more rapidly in somatic cells than in these other systems (within minutes after cells begin to spread on a substrate). Differences between molecular aspects of message localization exist as well between actin mRNA in somatic cells and these other systems: actin is synthesized in the cytoplasm whereas Vgl is associated with membranes during its synthesis and bicoid message is asymmetrically introduced into the embryo. Hence message localization may occur by a variety of mechanisms.

We have also shown that the nascent actin polypeptide is not required for anchoring actin mRNA within the cell periphery. Exposure to puromycin, as well as NaF, emetine, and cycloheximide had no effect on the percent of cells with actin mRNA localized after 30 min and in a significant percent of cells after 3 h. It is likely that 30 min would be sufficient for actin mRNA to diffuse throughout the cell if it were no longer tethered to cellular structures by the nascent polypeptide. A fusion protein of equivalent molecular weight to actin mRNA expressed in myofibers has been calculated to diffuse 50 μm in 20 min within the cytoplasm (Ralston and Hall, 1989). The maintenance of actin mRNA localization in the absence of nascent polypeptides suggests a direct interaction of the actin mRNA and its associated proteins with cellular structures, presumably the cytoskeleton. The cytoskeleton may be an important component in the translocation of mRNA to its localization site and in anchoring at that site (Lenk et al., 1977; Cervera et al., 1981; Bonneau et al., 1985; Ornelles et al., 1986). Recent evidence suggests that anchoring of mRNA involves actin filaments (Singer et al., 1989; Yisraeli et al., 1990). These data rule out a cotranslational mechanism for anchoring actin mRNA to cytoskeletal structures (Fig. 6 B in Singer et al., 1989) as has been observed for other structural proteins (Isaacs and Fulton, 1987).

What may be the possible physiological significance of message localization? In the case of actin mRNA, its proximity to the lamellipodium suggests a role in cell motility. Localized actin protein synthesis might facilitate regionalized actin nucleation and polymerization. Evidence from this work indicates that cells spread and form lamellipodia in the absence of protein synthesis, an indication that localized actin protein synthesis is not a prerequisite for lamellipodial formation. However, the relationship between motility and actin mRNA localization requires further investigation to determine whether localized protein synthesis can facilitate cell migration. Messenger RNA localization may be a general mechanism for targeting some cytoplasmic proteins to specific regions within the cytoplasm. It may also facilitate the assembly of macromolecular complexes at their functional sites thereby preventing protein associations from occurring in a spatially random manner. This regionalization would also allow a more efficient use of message and protein since they would be concentrated at their sites of function and not diffuse throughout the cytoplasm.

Since the completion of this work, Yisraeli et al. (1990) have suggested that Vgl mRNA localization in Xenopus oocytes occurs by a two-step process composed of microtubule-dependent translocation and microfilament-dependent anchoring at that site. It remains to be seen whether a similar mechanism operates in the localization of actin mRNA in fibroblasts. An important direction for future experiments will be the elucidation of specific nucleic acid sequences and/or the proteins involved in mRNA interaction with the cytoskeleton and localization within the cytoplasm.

We wish to acknowledge Dr. Jeanne Bentley Lawrence for her many contributions to this work, and in particular her idea of using spreading cells. We would also like to thank Yol-Li Wang, Ken Carter, Merrill Wolf, Gary Bassell, Krishan Taneja, and Edward Kislauskis for critical review of the manuscript; Dr. Laura F. Steel (Fels Institute, Temple University, Philadelphia, PA) for many helpful discussions; Kevin Byron for excellent technical assistance; and Marie Picard Craig for photography and preparation of figures.

This work was supported by a National Institutes of Health (NIH) National Research Service Award fellowship to C. L. Sundell, and NIH Grant HD18066 to R. H. Singer.

Received for publication 20 April 1990 and in revised form 24 July 1990.

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