

# Localization of all seven messenger RNAs for the actin-polymerization nucleator Arp2/3 complex in the protrusions of fibroblasts

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Accepted 9 March 2005

Journal of Cell Science 118, 2425-2433 Published by The Company of Biologists 2005

doi:10.1242/jcs.02371

## Summary

The actin-related protein 2/3 (Arp2/3) complex is a crucial actin polymerization nucleator and is localized to the leading protrusions of migrating cells. However, how the multiprotein complex is targeted to the protrusions remains unknown. Here, we demonstrate that mRNAs for the seven subunits of the Arp2/3 complex are localized to the protrusions in fibroblasts, supporting a hypothesis that the Arp2/3 complex is targeted to its site of function by mRNA localization. Depletion of serum from culture medium inhibits Arp2/3-complex mRNA localization to the protrusion, whereas serum stimulation leads to significant mRNA localization within 30 minutes. The effect of serum suggests that Arp2/3-complex mRNA localization is a

cellular response to extracellular stimuli. The localization of the Arp2/3 complex mRNAs is dependent on both actin filaments and microtubules, because disruption of either cytoskeletal system (with cytochalasin D and colchicine, respectively) inhibited the localization of all seven subunit mRNAs. In addition, myosin inhibitors significantly inhibit Arp2 mRNA localization in chicken embryo fibroblasts, suggesting a myosin motor dependent mechanism for Arp2/3-complex mRNA localization.

Key words: Actin cytoskeleton, Microtubules, mRNA targeting, Cell motility, Myosin

## Introduction

The actin-related protein 2/3 (Arp2/3) complex is a stable protein complex that nucleates actin assembly and creates a highly branched actin-cytoskeleton structure at leading protrusions in migrating cells (Machesky and Insall, 1999; Machesky et al., 1997; Pollard et al., 2000; Svitkina and Borisy, 1999; Welch et al., 1997). It consists of seven protein subunits of equimolar stoichiometry (Mullins et al., 1997). The function of the Arp2/3 complex is essential for cell migration – injection of a blocking antibody abolishes the nucleation of actin polymerization and protrusion formation (Bailly et al., 2001). The complex caps the pointed ends of actin filaments, exhibits nanomolar affinity and also binds to the sides of actin filaments (Mullins et al., 1998). This binding of the Arp2/3 complex to actin filaments is thought to be the molecular basis for the formation of highly branched actin-filament network at the leading edge, which is an effective structure to drive cell protrusion (Bailly et al., 1999; Mullins et al., 1998; Pollard et al., 2000). The Arp2/3 complex is localized to the leading edge, which is consistent with an important function in actin dynamics. However, it remains unknown how the Arp2/3 complex is assembled and targeted to the leading edge in the cell.

Emerging evidence demonstrates that asymmetric distribution of mRNA is an important mechanism to target proteins to their sites of function (Hesketh, 1996; Kislauskis et al., 1994; Simmonds et al., 2001; St Johnston, 1995; Wilhelm

and Vale, 1993; Wilkie and Davis, 2001). Since the first detection of mRNA localization in oocytes (Jeffery et al., 1983) and fibroblasts (Lawrence and Singer, 1986), mRNA localization has been detected in *Drosophila* and *Xenopus* oocytes, muscle cells, endothelial cells, yeast, neurons and oligodendrocytes (Bassell et al., 1999; Wilhelm and Vale, 1993). To date, the list of localizing mRNAs has become so extensive that it covers a diverse population and a broad array of systems. The physiological significance of mRNA localization is to ensure that proteins coded by the mRNAs are synthesized close to the site of function. Translation of mRNA only at the 'right place' can prevent nascent peptides from interacting with the 'wrong' partners to ensure optimal activity. The importance of making protein at the right place has been demonstrated by studies in which mislocalization of mRNA resulted in defects in, for example, embryo development (Berleth et al., 1988; Pokrywka and Stephenson, 1991; St Johnston et al., 1989). In fibroblasts,  $\beta$ -actin mRNA is localized to the leading edge and disruption of such localization results in slower cell motility, loss of directionality and corresponding delocalization of actin polymerization (Kislauskis et al., 1997; Shestakova et al., 2001).

Many cellular functions are performed by protein complexes that are segregated to different compartments of the cell. In addition to protein complexes that are assembled and disassembled rapidly in response to cellular signals, there are other protein complexes that are stable and function as an

entity. Targeting the latter type of protein complex in the cells via mRNA localization might have an additional advantage over targeting mRNAs for individually functional proteins. This is because localized protein synthesis of each subunit of a protein complex would provide a high concentration of the subunits to facilitate the assembly of the protein complex. Furthermore, the localized protein synthesis and complex assembly would overcome diffusion constraints of large complexes that are synthesized and assembled randomly in the cytoplasm and then targeted by protein transport.

In this work, we provide evidence to support this hypothesis. Using the stable Arp2/3 protein complex as a model system, we show that mRNAs for the seven members of the Arp2/3 complex are localized to protrusions in fibroblasts. This is the first evidence that all the mRNAs coding for a multisubunit protein complex are localized near its site of assembly and function. This localization depends on both actin filaments and microtubules, which is different from  $\beta$ -actin mRNA, whose localization is independent of microtubules, suggesting a more complex mechanism for the Arp2/3-complex mRNA localization.

## Materials and Methods

### Materials

Diethyl-pyrocabonate (DEPC), *Escherichia coli* tRNA, 2,3-butanedione monoxime (BDM) and heparin were from Sigma (St Louis, MO). Bovine serum albumin (BSA, protease and nuclease free), digoxigenin-11-dUTP (DIG-11-dUTP), sheep anti-DIG antibody (peroxidase conjugated), sheep anti-fluorescein antibody (peroxidase conjugated) and RNase inhibitor were from Roche (Indianapolis, IN). Mouse anti-DIG antibody was from Jackson ImmunoResearch Laboratories (Westgrove, PA). Eagle's minimum essential medium (MEM), fetal bovine serum (FBS) and trypsin/EDTA were from Mediatech (Herndon, VA). Pathogen-free fertilized chicken eggs were purchased from Charles River SPAFAS (Franklin, CT). Tyramide signal amplification (TSA) reagents were purchased from Perkin Elmer (Boston, MA). H7 and ML-7 were from Calbiochem (La Jolla, CA). Blebbistatin was from BioMol (Plymouth Meeting, PA). Other general chemicals were from Sigma and Fisher (Pittsburgh, PA).

### Cell culture

Fertilized SPAFAS SPF chicken eggs (Charles River Laboratories, USA) were incubated for embryos according to the manufacturer's protocol. Primary chicken embryo fibroblasts (CEFs) were prepared from the breast muscle of 12-day chicken embryos and cultured as described previously (Kislauskis et al., 1993). Human foreskin fibroblasts (HFFs) were obtained from ATCC (Manassas, VA) and maintained in MEM (Mediatech) supplemented with 10% FBS, 1 mM sodium pyruvate and 0.5% penicillin/streptomycin. HFFs were used between passages 9 and 18. For immunofluorescence staining or fluorescent in situ hybridization (FISH), the cells were plated on glass coverslips coated with 0.5% gelatine for ~50% confluence 24 hours later. For drug treatment, fibroblasts were treated with 5  $\mu$ M colchicine, 0.4  $\mu$ M cytochalasin D or medium alone for 90 minutes or 2 hours before fixation. Myosin inhibitors 2,3-butanedione monoxime (BDM, 20 mM), H7 (100  $\mu$ M), blebbistatin (100  $\mu$ M) or myosin-light-chain-kinase inhibitor ML-7 (40  $\mu$ M) were used to treat the cells for 2 hours before fixation.

### Indirect immunofluorescence staining

Cells plated on coverslips were fixed and stained by standard methods

as described (Liu et al., 2002). For Arp2, we used rabbit anti-Arp2 antibodies (H-84) from Santa Cruz Biotechnology. This antibody can be used for formaldehyde-fixed cells, which result in better preserved cell morphology than methanol-fixed cells, which are required by other anti-Arp2 antibodies. Mouse anti- $\alpha$ -tubulin antibody was from Sigma and fluorescein-phalloidin was from Molecular Probes (Eugene, OR).

### Cloning of chicken Arp3 cDNA

A DIG-labelled DNA probe for cloning of chicken Arp3 cDNA was prepared using a 138 bp fragment of human Arp3 coding region. A 1:2 ratio of DIG-11-dUTP to dTTP was used in a PCR reaction. A phage cDNA library from adult chicken brain (Clontech, Palo Alto, CA) was used for screening using a standard cloning process recommended by the company. Six positive clones were obtained and sequenced. A 1.7 kb fragment of clone 5.2 contains the whole coding region of chicken Arp3, flanked by ~80 bases of untranslated region (UTR) 5' of the open reading frame and ~350 bases of UTR 3' of the open reading frame. All of the other five clones contain different sizes of fragment of chicken Arp3 that are redundant to the 1.7 kb of clone 5.2. The open reading frame of the cloned chicken cDNA codes for a polypeptide that is 98% identical to human Arp3. This chicken Arp3 cDNA sequence has been deposited in GenBank (accession number AF288600). The complete 3' UTR was obtained later by using a 3'-RACE system (Invitrogen) and deposited (accession number AF498322).

### RT-PCR cloning of cDNA fragments encoding chicken Arp2 and human Arp2/3 complex

Total RNA was isolated from HFFs and CEFs by Trizol extraction (Invitrogen, Carlsbad, CA). cDNA was produced with an oligo-dT primer using ThermoScript reverse transcription (RT) system (Invitrogen). PCR reactions were performed using the following primers.

Chicken Arp2: 5'-GATACAACATTGAGCAGGAG-3' and 5'-CGTC-TAGAACCCAATTGCCTACTGGGAAGG-3'.

Chicken Arp3: 5'-GCAGATCTATGGCAGGGCGGCTTCCGGCC-3' and 5'-TAGGATCCAAAGGTACAACCTGAAGCATT-3'.

Chicken  $\beta$ -actin: 5'-CCGGCCTGTATACCAACACCC-3' and 5'-GAACCTGGTCTTAAGTCAGAG-3'.

Human  $\beta$ -actin: 5'-ATGACTTAGTTGCGTTACAC-3' and 5'-GTG-CACCTTTTATTCAACTGG-3'.

Human Arp2: 5'-GTAATGTTTGAAACTTACCAG-3' and 5'-CGAT-ACCAAGGAATACCGAC-3'.

Human Arp3: 5'-AGAGATTTGAAAAGAAGTGTAG-3' and 5'-ACTGGTCCAACACTCTTGTC-3'.

Human p16 subunit of Arp2/3 complex: 5'-CGCTGGTCGGG-ATTGGGATG-3' and 5'-ATAGAATTTCTGCACCAGTTTG-3'.

Human p20 subunit of Arp2/3 complex: 5'-CAGCCAGCGCCCGC-GATGAC-3' and 5'-ACAAGCACAGAGTTTAAGTTT-3'.

Human p21 subunit of Arp2/3 complex: 5'-TTGAAACCCGGCGC-CGCCA-3' and 5'-CACTGTCCAGGTCCTGAAAGA-3'.

Human p34 subunit of Arp2/3 complex: 5'-GCACGAGCTCTCC-CTCCGTC-3' and 5'-ATTCTTGGGAAGTATGAAGTC-3'.

Human p41 subunit of Arp2/3 complex was obtained by PCR sequentially using three pairs of primers: 5'-GCGCGGGAGGCCAAGC-3' and 5'-GCACGTATTCCTTGAATAGGT-3'; 5'-GCCATCAGCT-GCCACGCC-3' and 5'-AAACATTTCAGCAAAGCAACCA-3'; and 5'-TGGAACAAGGACCGCACCCAGATT-3' and 5'-GCCACTGGT-GCAGAACTGCGAGC-3'. The PCR products were then ligated into the pGEM-T or pGEM-TE vector (Promega, Madison, WI) and confirmed by DNA sequencing.

### Probe preparation

Plasmids containing the interested insert were linearized and the RNA

probes were prepared (with DIG or fluorescein-labelled dUTP) using a Maxiscript in-vitro transcription kit (Ambion, Austin, TX). The RNA probes were quantified by comparison with standard RNA for ethidium-bromide fluorescence intensity. Results of Southern dot blotting indicated that each probe interacted specifically with its target with no cross-reaction to other targets. Poly-dT oligonucleotide probe was synthesized and labelled with DIG as described previously (Liu et al., 2002).

#### FISH and tyramide-signal amplification

Cells grown on glass coverslips were fixed with 4% paraformaldehyde in PBS for 20 minutes and then washed with 1× PBS. Cells were permeabilized with 0.5% Triton X-100 in 1× PBS for 5 minutes and then washed with 1× PBS containing 5 mM MgCl<sub>2</sub>. Poly-A RNAs were detected using chemically synthesized DNA oligo poly-dT probe as previously described (Chicurel et al., 1998). For specific mRNAs that were detected with RNA probes, the cells were prehybridized with hybridization buffer (HYB) (50% formamide, 5× sodium chloride-sodium citrate (SSC), 50 μg ml<sup>-1</sup> heparin, 100 μg ml<sup>-1</sup> *E. coli* tRNA, 0.1% Tween 20, pH adjusted to 6.5-6.6 with 1 N HCl) for at least an hour at 70°C in a moisture chamber. Cells were hybridized with RNA probes (concentration of 0.2 ng μl<sup>-1</sup> to 1.5 ng μl<sup>-1</sup> depending on the probe) for 3 hours or overnight at 70°C in a moisture chamber. The coverslips were washed extensively with HYB and PBT (1× PBS, 0.1% Tween 20) at 70°C. Coverslips were then blocked with blocking buffer [0.5% blocking reagent supplied with the TSA kit from Perkin-Elmer, 1% bovine serum albumin (BSA), 0.3% Triton X-100, 1-4% FBS in 1× PBS]. Cover slips were incubated with 0.25 units ml<sup>-1</sup> peroxidase-conjugated sheep anti-DIG antibody or peroxidase-conjugated sheep anti-fluorescein antibody for 1 hour at room temperature. After washes, cover slips were incubated with tetramethylrhodamine-tyramide or fluorescein-tyramide at a 1:100 dilution for tyramide-signal amplification (TSA) in a TSA-PLUS system (Perkin-Elmer) for 3-30 minutes at room temperature (depending on the mRNA).

#### Quantitative RT-PCR for chicken Arp2 expression in CEFs during serum depletion and stimulation

CEFs that were cultured in 150 mm tissue-culture dishes were serum starved for 0, 4, 8 or 16 hours. After the 16 hours of serum starvation, the cells were stimulated with 10% FBS for 0, 0.5, 1 or 2 hours. At each time point, CEFs were trypsinized and cell number was counted before total RNA extraction using Trizol (Invitrogen) according to the manufacturer's instructions. Following isolation, RNA was suspended in DEPC water (Millipore) such that 1 μl solution contains RNA extracted from 100,000 cells. We found that the expression of the genes encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin is greatly affected by serum starvation and stimulation, and so they are not useful as loading controls. Instead, we used cell number as the equal-input control. 1 μl RNA was reverse transcribed using the Superscript III First-Strand Synthesis system from Invitrogen with a chicken Arp2-specific antisense primer GCCTCGAGTCCCTGCTCAATGTTGTATCC. 2 μl corresponding cDNA was then used in a PCR reaction with chicken Arp2-specific sense primer GTGAGCTCGGTTTCTCTCTCCCTCATCT and the aforementioned antisense primer for 23 cycles. The amplified products were then run on a 1% agarose gel and analysed with Bio-Rad gel documentation system with Quantity One software. Each time point was normalized to time zero.

#### Double detection of mRNAs in the same cells using sequential FISH-TSA

In order to detect multiple low-abundance mRNAs in the same cells, we used sequential FISH-TSA. Two different RNA probes (one

labelled with DIG and the other with fluorescein) were hybridized to the same population of cells and washed as described in the previous section. Sheep anti-DIG antibody (peroxidase-conjugated) was used and the fluorescence signal was amplified with TSA (using tetramethylrhodamine-tyramide). The peroxidase activity was quenched with 0.01 N HCl at room temperature for 10-20 minutes, then washed three times for 10 minutes each with 1× PBS. Sheep anti-fluorescein antibody (peroxidase-conjugated) was then used as the second probe and the fluorescence signal was amplified with TSA (using fluorescein-tyramide).

#### Fluorescence microscopy

All the cell samples were viewed and images acquired with an Olympus microscope equipped with a cooled CCD camera (SensiCam from Cooke) and Slidebook software (Intelligent Imaging Innovation, Denver, CO). Image processing was performed with Adobe Photoshop (version 7.0, Adobe Systems, Mountain View, CA).

#### Quantification of mRNA localization

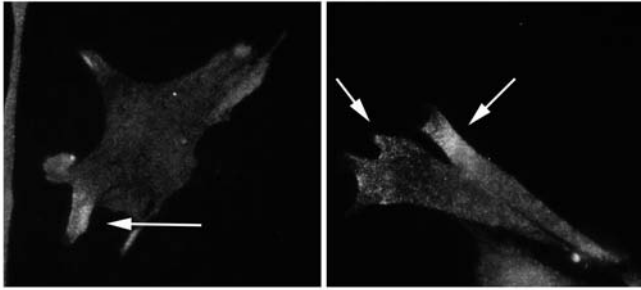
Localization of mRNA was quantified visually according to the following criteria. A cell was deemed to have localized mRNA if most (>60%) of the mRNA was found at the leading lamella. In some cases, in addition to being localized to the leading protrusions, a small proportion of the mRNA is concentrated at the trailing edge. Such a cell will be scored as localized if the mRNA at the leading and trailing edge combined is >70% of the total mRNA in the cell. A cell that failed to meet the above criteria was scored as not localized. To validate the visual scoring of mRNA localization, fluorescence pixel intensity in the cell protrusions was quantified and compared with that of the whole cell. A cell with fluorescent-pixel intensity in the protrusions at least 1.5 times that of the whole cells would be scored as localized. By analysing a total of 56 randomly selected cells, both methods showed similar results in scoring chicken Arp2 mRNA localization to the protrusion (41.1% versus 46.4% of the cells with Arp2 mRNA localization in the cell protrusion as quantified by visual scoring and fluorescent-pixel intensity, respectively). Because visual scoring is faster and is more suitable for scoring large numbers of cells, it was chosen as the method of routine scoring of mRNA localization in the cell. All the cells were scored in randomly selected fields. At least 300-500 cells from three independent experiments were scored for each mRNA. Statistical analysis was performed using the Student's *t*-test.

## Results

### mRNAs for all the seven subunits of the Arp2/3 complex are localized to the protrusions

The actin-polymerization nucleator Arp2/3 complex exists in the cells as a stable complex with equimolar stoichiometry (Mullins et al., 1997). It is localized to the leading protrusions of a variety of migrating cells (Machesky and Insall, 1999; Pollard et al., 2000; Svitkina and Borisy, 1999). By using a polyclonal antibody against the Arp2 subunit, we have confirmed the localization of the complex in the protrusions of cells used in this study (Fig. 1).

Because the Arp2/3 complex comprises seven protein subunits, its localization to the leading protrusions of migrating cells raises the question of how the complex is assembled and targeted to the site of function. We hypothesized that localized protein synthesis via mRNA localization could be an effective mechanism to facilitate complex assembly and to overcome the diffusion constraints imposed by its size and interaction with other proteins. We started by investigating the intracellular

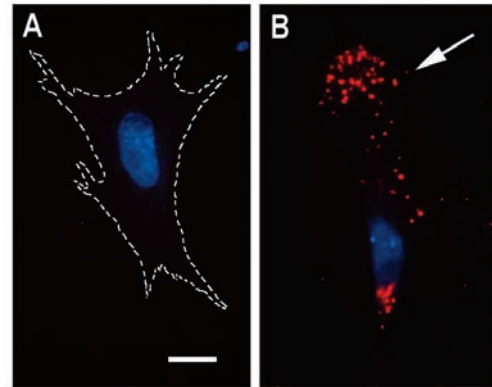


**Fig. 1.** The Arp2/3 complex is localized to protrusions of chicken embryo fibroblasts. The localization of Arp2 was used to infer the location of the Arp2/3 complex and confirmed in chicken embryo fibroblasts to be in a location like that seen in other cell types. Arrows point to the protrusions.

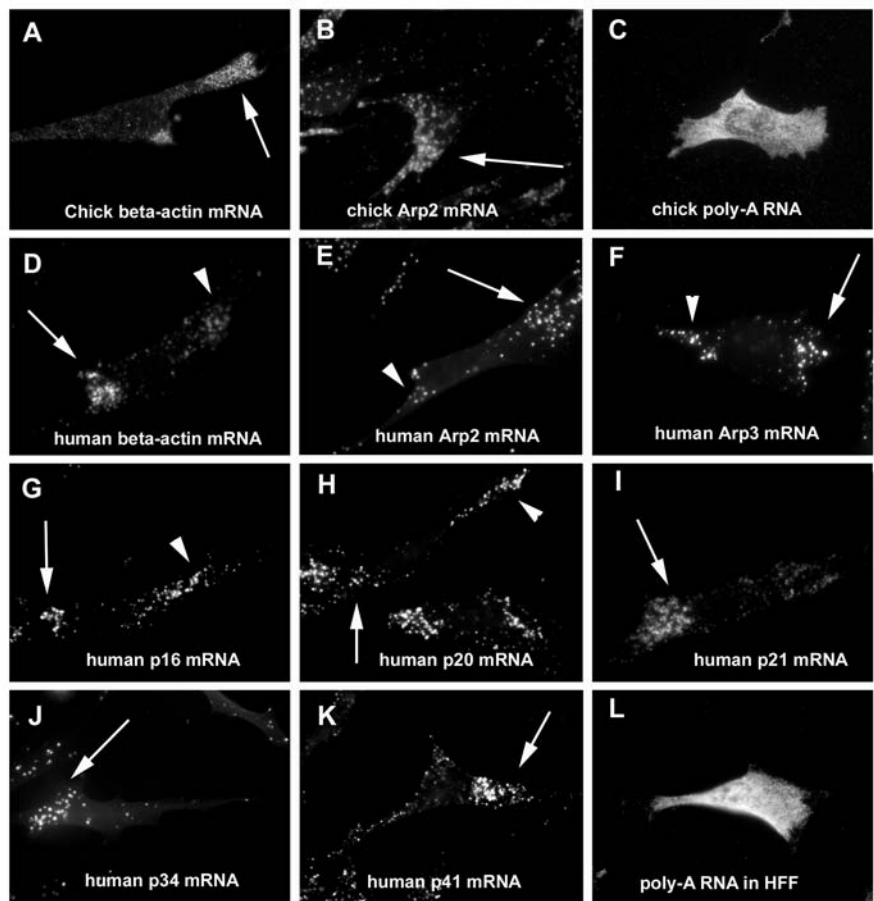
mRNA distribution for the Arp3 subunit in the CEFs using a conventional FISH protocol that has been successfully applied in the study of  $\beta$ -actin mRNA localization (Taneja and Singer, 1987; Taneja and Singer, 1990). However, because of the relatively low abundance of the Arp2/3-complex mRNAs in the cell, it was difficult to obtain sufficient signal for routine quantitative study for mRNA localization. We then used TSA, which can amplify signal up to 1000-fold with good spatial resolution (van de Corput et al., 1998). The combination of FISH and TSA (FISH-TSA) allowed us to have an excellent signal:noise ratio to score mRNA localization. A sense RNA probe for chicken Arp3 mRNA showed no signal, whereas an antisense RNA probe for chicken Arp3 mRNA showed a strong fluorescence signal (Fig. 2). In contrast to the fairly uniform distribution of poly-A RNA in the cells, a significant portion of the CEFs showed Arp2 and Arp3 mRNA localization to protrusions (Figs 3, 4). Only Arp2 and Arp3 were investigated in CEFs because they were the only two subunits of the complex that had been cloned at the time. We then prepared probes for all the subunits of the human Arp2/3 complex and studied their intracellular distribution in HFFs. All mRNAs for the seven subunits of the Arp2/3 complex localized to the protrusions in HFFs, compared with the uniform distribution of poly-A RNA (representing total cellular mRNA) (Figs 3, 4).

#### Localization of Arp2/3 complex and $\beta$ -actin mRNAs

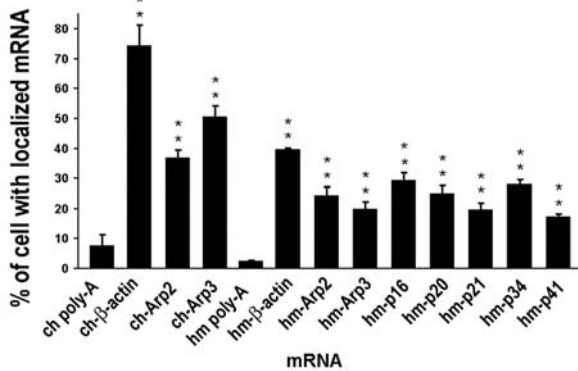
It has been shown that different mRNAs that are localized to the same cellular compartment can be transported together in a protein-RNA complex (Barbarese et al., 1995; Simmonds et al., 2001; Wilkie and Davis, 2001). Having observed that Arp2/3



**Fig. 2.** Effective detection of low-abundance mRNA using FISH-TSA. Images show representative chicken embryo fibroblasts processed using DIG-labelled sense (A) or antisense (B) RNA probe for chicken Arp3 mRNA. White dots in A mark the cell border. Blue indicates DAPI staining of nucleus, red indicates Arp3 mRNA in the cell and the arrow points to the mRNA localized to protrusions. Bar, 10  $\mu$ m.



**Fig. 3.** The mRNAs for the Arp2/3 complex are localized to protrusions in fibroblasts. Images show representative poly-A RNA and  $\beta$ -actin and Arp2/3-complex mRNA distributions in chicken embryo fibroblasts and human foreskin fibroblasts as detected with FISH-TSA. The fibroblasts have enriched Arp2/3-complex mRNA in their protrusions (arrows). In some cells, a proportion of the  $\beta$ -actin and Arp2/3-complex mRNA can also be found at the trailing protrusion of the cells (arrowheads). Poly-A RNA appears to be distributed uniformly.



**Fig. 4.** Quantification of Arp2/3-complex mRNA localization in fibroblasts. The proportion of the cells with localized mRNA was quantified by counting all the cells in randomly selected fields in the fluorescence microscope. 300-500 cells were scored for each mRNA from three independent experiments. Error bars indicate s.e.m. \*\*, statistically significant ( $P < 0.01$ ) differences from control basal level of poly-A RNA.

complex and  $\beta$ -actin mRNAs are localized to the protrusions of fibroblasts, we further asked whether the mRNAs of the Arp2/3 complex physically associate with each other or with  $\beta$ -actin mRNA. If the subunit mRNAs of the Arp2/3 complex were co-transported, they should be found to be associated with each other. To address these questions, double detection of two types of mRNAs simultaneously in the same cells was performed by sequential FISH-TSA. As shown in Fig. 5A-C, Arp3 mRNA appears not to be precisely colocalized with  $\beta$ -actin mRNA, although they both concentrate together in the protrusions. In addition, the Arp2 and Arp3 mRNAs also do not colocalize, although they are both concentrated together in the protrusions (Fig. 5D-F).

#### Expression and localization of Arp2 mRNA are serum dependent

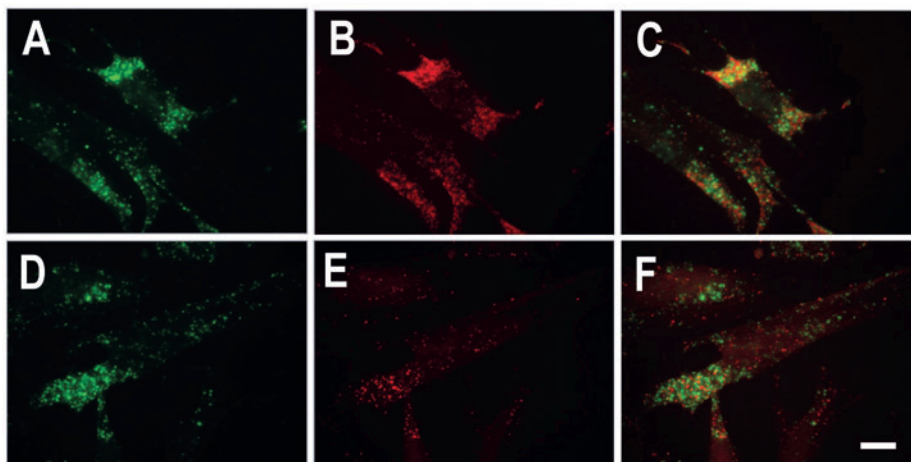
It was not clear whether Arp2/3-complex mRNA localization is an intrinsic property of fibroblasts by default or is a cellular response to extracellular signals. To address this question, we investigated the effects of serum depletion and stimulation on Arp2 mRNA expression and localization in CEFs. CEFs were

chosen as the cell model for this study because of their more prominent mRNA localization than the human cells. The excellent signal:noise ratio of FISH-TSA and the punctate nature of the mRNA signal allowed the number of mRNA spots in each cell to be counted in order to study the expression profile of the Arp2/3-complex mRNAs in cells. By using FISH-TSA and this method, we studied the effect of serum on Arp2 mRNA expression and localization. As shown in Fig. 6, the number of Arp2 mRNA fluorescence spots in the CEFs decreased after FBS depletion from the culture medium. 16 hours after serum starvation, the average number of Arp2 mRNA spots per cell is less than half of the number at time zero. Addition of 10% FBS to the culture medium stimulated the expression of Arp2. After 2 hours of FBS stimulation, the number of Arp2 mRNA spots per cell was about 70% of that at time zero (Fig. 6B). Although this approach is based on the observation that mRNA particles contain a single mRNA in the CEFs (Fusco et al., 2003), there is no direct evidence that one spot of Arp2 mRNA fluorescence is one mRNA molecule. Therefore, this expression profile was examined and confirmed by conventional semiquantitative RT-PCR (Fig. 6C). This is consistent with the observation that levels of related mRNAs, such as  $\beta$ -actin, are decreased after serum depletion (Latham et al., 1994).

In addition to Arp2 expression, we also investigated the effect of serum on Arp2 mRNA localization in the same cells. As shown in Fig. 6D, Arp2 mRNA localization in CEFs decreased dramatically even after 4 hours of serum starvation when the total Arp2 mRNA level was moderately reduced, suggesting a redistribution of the mRNA after serum depletion. Addition of 10% serum to the cell medium after 16 hours of serum starvation significantly stimulated Arp2 mRNA localization after 30 minutes. Arp2 mRNA localization peaked after 2 hours of serum stimulation. Arp3 mRNA showed similar results (not shown). These data suggest that Arp2/3-complex mRNA localization is a consequence of cellular response to the extracellular stimuli.

#### Localization of Arp2/3-complex mRNAs is dependent on both actin filaments and microtubules

Although most of the reports of localization of mRNAs show dependence on the cytoskeleton for transport and/or anchoring, it was not known whether Arp2/3-complex-mRNA localization



**Fig. 5.** Arp2/3-complex and  $\beta$ -actin mRNAs are localized to protrusions in the cell but do not colocalize. Double detection of two types of mRNAs in the same cell was performed by using both DIG-labelled and fluorescein-labelled probe for FISH. TSA was performed sequentially. (A) Chicken Arp3 mRNA. (B) Chicken  $\beta$ -actin mRNA. (C) Merge of A and B. (D) Chicken Arp3 mRNA. (E) Chicken Arp2 mRNA. (F) Merge of D and E. Bar, 10  $\mu$ m.

**Fig. 6.** Arp2 mRNA expression and localization are serum dependent. (A) Representative cells during serum starvation and stimulation. (a) Cells continuously exposed to serum. (b-d) Cells after 4, 8 or 16 hours of serum starvation (without stimulation), respectively. (e-g) Cells after 16 hours of serum starvation, stimulated with 10% of FBS for 0.5, 1 and 2 hours, respectively. Scale bar, 10  $\mu$ m. (B) Expression of Arp2 mRNA in CEFs after serum starvation and stimulation. The number of Arp2 mRNA fluorescence spots per cell was counted. At least 120 cells were counted for each time point from at least three independent experiments.

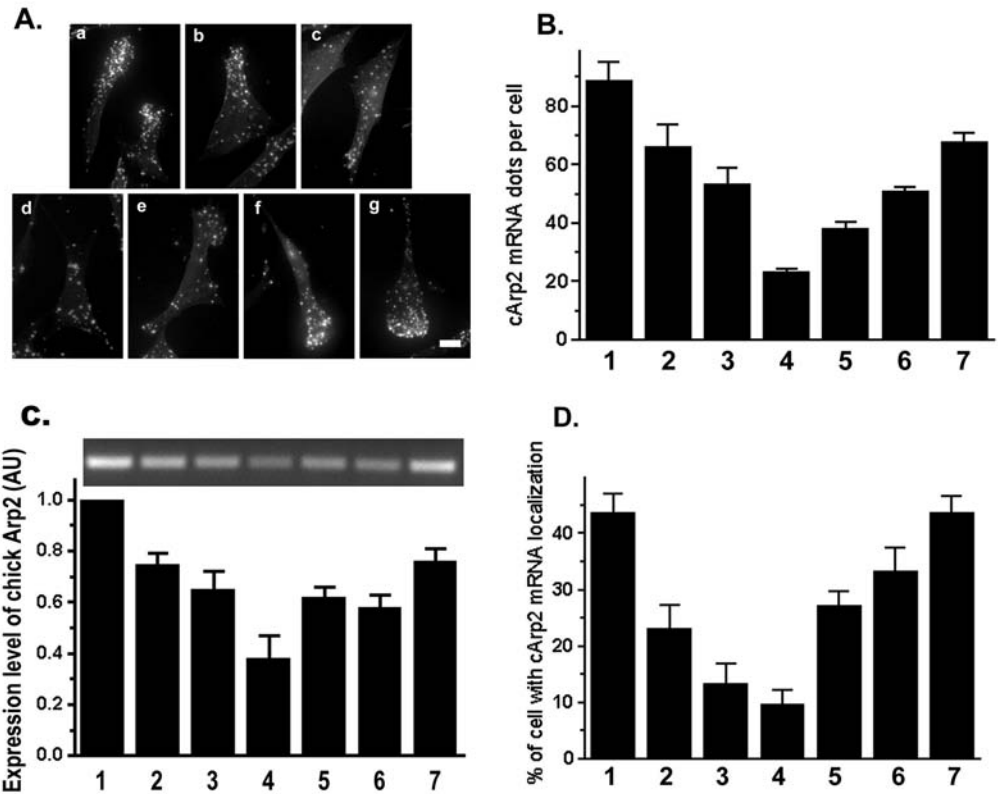
(C) Semiquantification of Arp2 expression by RT-PCR. CEFs were cultured in 150 mm dishes and then serum starved and stimulated as in A and B. Total RNA was isolated and reverse transcribed into cDNA. The cDNA from equal numbers of cells was amplified by PCR for chicken Arp2. Data represent three independent experiments and are all normalized to time zero (AU, arbitrary unit). Error bars indicate s.e.m. (Inset) Representative image of the RT-PCR product. (D) Localization of Arp2 mRNA to protrusions of CEFs after serum depletion and stimulation. Localization of mRNA was scored using the criteria described in Materials and Methods. For each time point, 300-600 cells were scored from three experiments. Error bars indicate s.e.m. (B-D) 1, always in medium with 10% FBS; 2, 4-hour serum starvation; 3, 8-hour serum starvation; 4, 16-hour serum starvation; 5, 0.5-hour FBS stimulation; 6, 1-hour FBS stimulation; 7, 2-hour FBS stimulation.

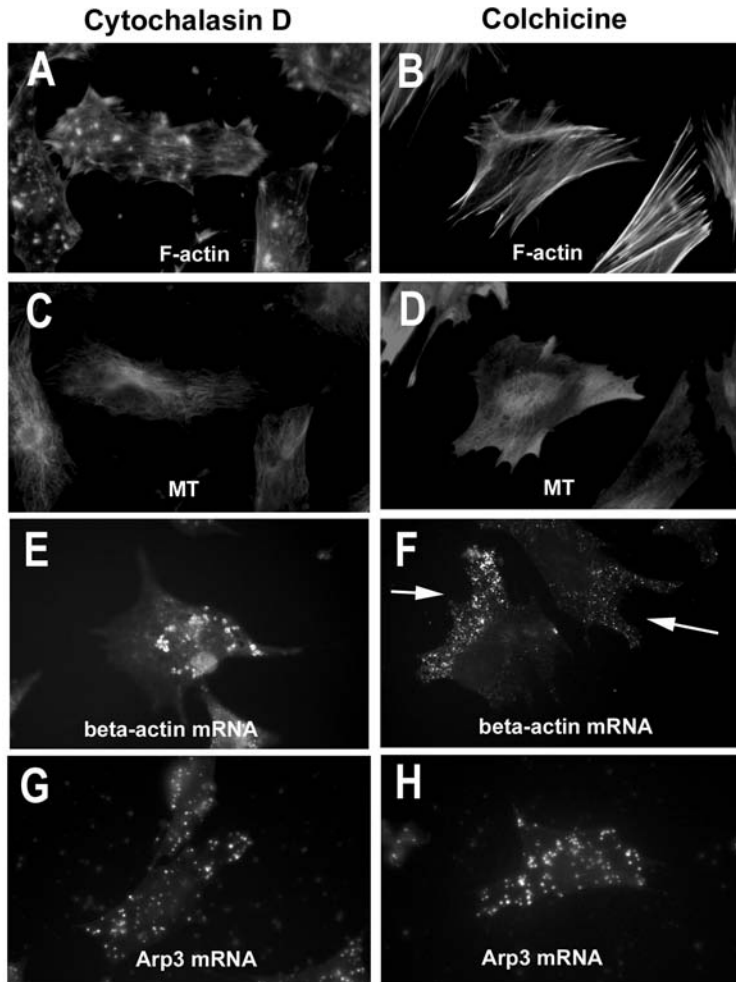
is dependent on the cytoskeleton. To investigate the mechanism of Arp2/3-complex mRNA localization in fibroblasts, we selectively disrupted either the actin or the tubulin-based cytoskeleton. As shown in Fig. 7, the actin cytoskeleton was effectively disrupted by 0.4  $\mu$ M cytochalasin D, whereas microtubules were not affected. Application of 5  $\mu$ M colchicine on the cells disrupted the microtubules but not the actin filaments. Disruption of either actin filaments or microtubules is sufficient to inhibit Arp2/3-complex mRNA localization significantly (Figs 7, 8). These results differ from those obtained with  $\beta$ -actin mRNA: the localization was affected by the disruption only of actin filaments, not microtubules (Sundell and Singer, 1991) (Figs 7, 8). This different dependency on the cytoskeleton for localization between  $\beta$ -actin and Arp2/3 complex mRNAs might reflect differences in their respective localization mechanisms.

**Myosin motors play an important role in Arp2/3-complex mRNA localization**

The dependency of mRNA localization on the cytoskeleton indicates a potential role for motor proteins in transport. To investigate further the mechanisms by which the Arp2/3-complex mRNAs are targeted to the protrusions, we used BDM (a widely used inhibitor for muscle myosin motors) to treat the muscle-derived CEFs in normal growth medium for

2 hours. The cells were then fixed for FISH-TSA to determine the effects of BDM treatment on Arp2 mRNA localization. Under these conditions, only a moderate decrease in the proportion of the cells with Arp2 mRNA localization was observed in BDM-treated cells compared with the no-drug-treatment control cells (not shown). We reasoned that, because we treated the cells with BDM for only 2 hours, such treatment might not affect the mRNA already localized to protrusions even though the transport of newly transcribed mRNA was blocked. Because serum starvation can effectively suppress the Arp2/3-complex mRNA localization and serum stimulates the mRNA localization rapidly (Fig. 6), we serum starved the CEFs for 16 hours and then treated the cells with or without 20 mM BDM in the presence of 10% of FBS for 2 hours. As predicted, BDM treatment of serum-starved cells significantly blocked Arp2 mRNA localization (Fig. 9). Similarly H7, a non-specific myosin inhibitor (Volberg et al., 1994), also showed significant inhibitory effect on Arp2 mRNA localization in the CEFs. Given the recent reports on the non-specificity of BDM on myosins (Ostap, 2002; Straight et al., 2003), we further investigated the role of myosin in the localization of Arp2 mRNA by using more-specific pharmacological reagents such as the myosin-light-chain-kinase inhibitor ML-7 (Saitoh et al., 1987) and the myosin-II-specific inhibitor blebbistatin (Straight et al., 2003). As shown in Fig. 9, both ML-7 and blebbistatin significantly inhibit



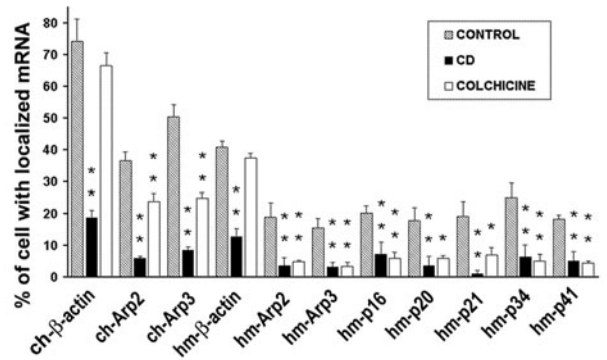


**Fig. 7.** Localization of Arp2/3-complex mRNA is dependent on both actin filaments and microtubules. Cytochalasin D (CD) and colchicine were used to disrupt actin filaments and microtubules in the fibroblasts, respectively. (A,C,E,G) Cells treated with 0.4  $\mu$ M cytochalasin D; (B,D,F,H) cells treated with 5  $\mu$ M colchicine. (A,B) Cells stained for actin filaments with fluorescein-phalloidin. (C,D) Cells stained for microtubules with mouse anti- $\alpha$ -tubulin antibody. (E,F) Localization of  $\beta$ -actin mRNA is dependent on actin filaments but not on microtubules. By contrast, localization of Arp2/3-complex mRNA is dependent on both actin filaments and microtubules (represented by Arp3 mRNA in G,H).

Arp2 mRNA localization. The cells treated with these inhibitors did not show noticeable morphological differences compared with the no-drug controls, suggesting that the inhibition of mRNA localization is unlikely to be due to secondary effects of a different cell morphology. The only exception is the cells treated with blebbistatin, which exhibited a somewhat more elongated morphology. These results suggest that myosin II plays an important role in Arp2 mRNA localization to the protrusions in the CEFs, as it appears to do with  $\beta$ -actin mRNA (Latham et al., 2001).

## Discussion

In this report, we demonstrate that the mRNAs encoding the multiprotein Arp2/3 complex are targeted to the leading

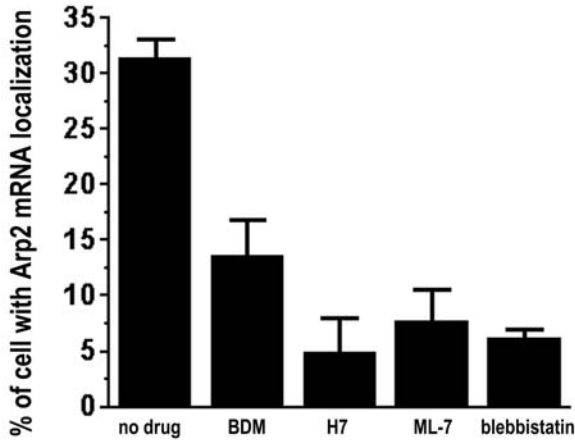


**Fig. 8.** Quantification of Arp2/3-complex and actin mRNA localization in fibroblasts treated with cytochalasin D or colchicine. Cytochalasin D (CD) and colchicine were used to disrupt actin filaments and microtubules in the fibroblasts, respectively. The proportion of the cells with localized mRNA was quantified by counting all the cells in randomly selected fields in the fluorescence microscope. 300-500 cells were counted for each type of mRNA from at least three independent experiments. Prefixes: ch, chicken; hm, human. Error bar indicates s.e.m. \*, statistically significant ( $P < 0.05$ ) differences from control cells treated with no drug. \*\*, statistically significant differences from control cells treated with no drug ( $P < 0.01$ ).

protrusions of polarized fibroblasts. This is the first evidence that mRNAs for all the subunits of a protein complex are localized to its site of function, supporting the hypothesis that multiprotein complexes are localized in part by mRNA targeting. The Arp2/3-complex mRNA localization is dynamic and dependent on extracellular signals. The dependence on both actin filaments and microtubules for the Arp2/3-complex mRNA targeting suggests a more-complex mechanism than that for the localization of  $\beta$ -actin mRNA, which is independent of microtubules. Furthermore, we demonstrate that myosin motors, probably myosin II, play an important role in Arp2 mRNA localization.

## Advantage of targeting a stable protein complex via mRNA localization

One of the predicted advantages of local protein synthesis via mRNA localization versus protein transport is energy efficiency: transporting one molecule of mRNA requires less energy than transporting thousands of protein molecules. More importantly, mRNA localization might prevent unwanted protein interactions by segregating proteins into different cellular compartments. Additionally, synthesis of the components of a protein complex in proximity would facilitate complex assembly, perhaps even co-translational assembly. In this report, we present evidence supporting this rationale: all the mRNAs for the actin-polymerization-nucleator Arp2/3 complex are localized to protrusions in motile fibroblasts. Considering that there are seven members of the Arp2/3 complex, localized synthesis of all the components would certainly increase the chances of subunits finding each other for assembly, owing to higher local concentrations. Furthermore, it helps to overcome the diffusion constraint



**Fig. 9.** Myosin inhibitors decrease Arp2 mRNA localization in the CEFs. CEFs were serum starved for 16 hours and then stimulated with 10% FBS alone (no drug) or 10% FBS plus 20 mM BDM, 100  $\mu$ M H7, 40  $\mu$ M ML-7 or 100  $\mu$ M blebbistatin for 2 hours before fixation for FISH-TSA for Arp2 mRNA. Data are from at least 300 cells that derived from at least three independent experiments with error bar indicating s.e.m. All the data from drug-treated samples are significantly different from the no drug control ( $P < 0.01$ ).

imposed by the large size of the complex and its possibility of high-affinity binding to actin filaments in regions outside protrusions.

#### Anchorage and transport of Arp2/3-complex mRNAs

How the Arp2/3-complex mRNAs are transported and anchored is not clear. One of the questions raised by this study is whether all the mRNAs for the Arp2/3 complex are transported and anchored together in a complex or whether each of them is transported and anchored separately. It is reasonable to suppose that they are transported together in a larger complex because they share the same destination. There are examples in which many mRNA molecules of different species are incorporated into a complex and co-transported to the same site of function (Barbarese et al., 1995; Simmonds et al., 2001; Wilkie and Davis, 2001). However, there is also evidence suggesting mRNAs might move as single molecules in the cell (Fusco et al., 2003). Although the Arp2/3-complex mRNAs are not precisely colocalized with each other, this does not rule out the possibility that they are transported as a complex, because the transport step may be rapid and transporting mRNA complexes might be rare and therefore difficult to detect. Further work to resolve this will be required using imaging of mRNA transport in live cells.

As for anchorage, our observation that the Arp2/3-complex mRNAs do not precisely colocalize with each other in the cell suggests that each of the Arp2/3-complex mRNAs might be anchored separately. It is of interest to notice de Hoog's recent report (de Hoog et al., 2004) that RNA-binding proteins and RNA are localized to a cell-adhesion structure called the spreading initiation centre (SIC) at the edge of spreading cells. These proteins include the hnRNP family proteins and translation elongation factor 1A, whose homologues have been shown to bind to  $\beta$ -actin mRNA at the leading protrusions of CEFs (Gu et al., 2002; Liu et al., 2002). Although specific

mRNAs were not detected in the SIC, there might be mRNAs in the SIC that can be detected only if a more sensitive method such as FISH-TSA is used. Because cell spreading might mimic the process of leading-edge extension and adhesion, further investigation of the SIC might provide insights into how mRNA is maintained at the leading lamellae of migrating cells.

#### Serum stimulation is required for targeting of mRNAs encoding the Arp2/3 complex

Using serum depletion and stimulation, we demonstrate that extracellular factors in serum influence intracellular Arp2/3-complex mRNA localization. It appears that sustained Arp2/3-complex mRNA localization requires the presence of these factors, and that the distribution of Arp2/3-complex mRNA is a very dynamic process. Although yet to be elucidated, it is very likely that growth factors in serum play a crucial role in mediating the signalling pathways that influence Arp2/3-complex mRNA localization. The Rho-dependent signalling pathway (through myosin II) is responsible for growth-factor induction of  $\beta$ -actin mRNA localization in CEFs (Latham et al., 2001). Our preliminary data suggest that growth factors and Rho GTPases are also involved in Arp2/3-complex mRNA localization (L.A.M. and G.L., unpublished). Data presented in Fig. 9 indicate that myosin II might play a major role in Arp2 mRNA transport on the actin cytoskeleton in the cell.

#### Arp2/3-complex mRNAs and $\beta$ -actin mRNA might be targeted to the protrusions by different mechanisms

The Arp2/3-complex mRNAs and  $\beta$ -actin mRNA are localized to the protrusions of fibroblasts. These mRNAs are also dependent on actin cytoskeleton and myosin motors (probably myosin II) for localization. These observations suggest that these mRNAs are targeted to the protrusions by the same mechanism. However, other evidence indicates that the mechanism for the localization of Arp2/3-complex mRNAs and  $\beta$ -actin mRNA might be different. For instance, Arp2/3-complex mRNA localization is dependent on microtubules, whereas  $\beta$ -actin mRNA localization is not (Figs 7, 8) (Sundell and Singer, 1991). Localization of mRNA depends on a localization sequence (the 'zipcode') in the mRNA. In CEFs, the  $\beta$ -actin zipcode has been well characterized, at the 3' UTR of the transcript (Kislauskis et al., 1994). Recent sequence analysis and systematic evolution of ligands by exponential enrichment (SELEX) studies have revealed a motif of GACU- $X_n$ -ACACC as a consensus motif for  $\beta$ -actin mRNA (Farina et al., 2003; Shestakova et al., 2001). Our sequence analysis indicates that such a motif exists only in Arp3 mRNA, not in the other mRNAs of the Arp2/3 complex. Unlike the  $\beta$ -actin mRNA, Arp3 mRNA localization could not be significantly inhibited by using an antisense oligonucleotide against the motif (G.L., N.N.O. and L.A.M., unpublished). Many lines of evidence demonstrate that RNA localization is dependent on RNA secondary structure, which usually involves relatively long sequences (Chartrand et al., 1999; Hesketh, 1996; Ross et al., 1997; Simmonds et al., 2001; St Johnston, 1995; Wilhelm and Vale, 1993). Current investigations in our laboratories are identifying the zipcodes for the Arp2/3-complex mRNAs.



Supported by the Albany Medical College Start Up Fund and US NIH grants GM70560 (to G.L.), T32-HL-07194 (to L.A.M.), CA100324 (to J.C.) and AR41480 (to R.H.S.).

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