Beta and Gamma Actin mRNAs Are Differentially Located within Myoblasts

Mark A. Hill and Peter Gunning
Cell Biology Unit, Children's Medical Research Institute, Wentworthville, New South Wales 2145, Australia

Abstract. Actin is of fundamental importance to all eukaryotic cells. Of the six mammalian actins, beta (β) and gamma (γ) cytoplasmic are the isoforms found in all nonmuscle cells and differ by only four amino acids at the amino-terminal region. Both genes are regulated temporally and spatially, though no differences in protein function have been described. Using fluorescent double in situ hybridization we describe the simultaneous intracellular localization of both β and γ actin mRNA. This study shows that myoblasts differentially segregate the β and γ actin mRNAs. The distribution of γ actin mRNA, only to perinuclear and nearby cytoplasm, suggests a distribution based on diffusion or restriction to nearby cytoplasm. The distribution of β actin mRNA, perinuclear and at the cell periphery, implicates a peripheral localizing signal which is unique to the β isoform. The peripheral β actin mRNA corresponded to cellular morphologies, extending processes, and ruffling edges that reflect cell movement. Total actin and γ actin protein steady-state distributions were identified by specific antibodies. γ actin protein was found in both stress fibers and at the cell plasma membrane and does not correspond to its mRNA distribution. We suggest that localized protein synthesis rather than steady-state distribution functionally differentiates between the actin isoforms.

An emerging mechanism for controlling protein expression is intracellular localization of the translation event. mRNA localization occurs in early embryonic development (Yisraeli et al., 1990), in neurons (for review see Steward and Banker, 1992), and in wound repair (Hoock et al., 1991). Actin mRNAs were among the first shown to be localized within cells (Lawrence and Singer, 1985, 1986) and probes which identify all isoforms show actin mRNA at the motile periphery of chick fibroblasts and myoblasts (Lawrence and Singer, 1985, 1986; Sundell and Singer, 1990), and in association with the cytoskeleton (Sundell and Singer, 1991). Indeed, mRNA association with the cytoskeleton for transportation and positioning is probably the mechanism used both in development (Yisraeli et al., 1990) and in mature cells (Hesketh and Pryme, 1991; Taneja et al., 1992). Mammalian endothelial cells localize β actin mRNA to peripheral regions of motility (Hoock et al., 1991). Transport of mRNA is probably a process involving either the 3′ untranslated region (3′UTR) sequence (Gottlieb, 1992) or its secondary structure (Macdonald and Struhl, 1988; Mowry and Melton, 1992) binding to a carrier. Interestingly, the β and γ actin isoforms share no sequence homology in their 3′UTR when analyzed by sequence comparison routines (Erba et al., 1986). If the 3′UTRs of these two mRNAs have a specific function, then it is possibly different for each of these actin isoforms. One function for the 3′UTR of β actin mRNA is its localization within cells (Singer, R. H., personal communication). One question this raises is whether the actin mRNAs are identically located within cells.

The microfilaments of the cell cytoskeleton are made from the two nonmuscle cytoplasmic β and γ actin isoforms. While both actin isoforms are very similar, differing by only four amino acids near the amino terminus (Vanderkermochove and Weber, 1978), they have different roles in overall cell cytoarchitecture. The β:γ ratio within cells is strictly regulated (Otey et al., 1987), and perturbation of this ratio in myoblasts by transfection with either β or γ actin has significant and opposite effects (Schevzov et al., 1992; Lloyd et al., 1992). Increasing β actin results in larger cells whereas increasing γ actin decreases cell size (Schevzov et al., 1992). This suggests that myoblasts differentiate between isoforms and use the proteins differently. Similarly, there have been shown to be differences between the ability of actin protein isoforms to incorporate into myotubes in vitro (Peng and Fischman, 1991). In contrast, both muscle and nonmuscle isoforms have been shown to incorporate into the cytoskeleton (Gunning et al., 1984; McKenna et al., 1985). It is therefore not yet clear at what level and how these differences between actin isoforms occur and what these differences may mean to actin isoform function within the cell.

A mechanism to generate differences in isoform function...
could be the specific positioning of newly translated actin within the cell. We report in this study that γ and β actin mRNAs are not identically located in myoblasts.

**Materials and Methods**

**Cell Culture and Protein Analysis**

The intracellular distribution of γ and β actin mRNA and protein was analyzed in C2 myoblasts (Yaffe and Saxel, 1977) and C2 clones transfected with the human β actin gene (Schevzon et al., 1992). Cells were grown on collagen-coated (calf-skin collagen, Calbiochem, Sydney, Australia) glass multwell slides (Nunc Inc., Naperville, IL), in MEM plus 20% FCS (CytoSystems, Sydney, Australia), and 0.5% chick embryo extract (Flow, Sydney, Australia). The next day cells were fixed in 1% formaldehyde, and then in cold methanol for 20 min.

For protein studies cells were fixed and incubated for 1 h at 37°C with primary antibody, washed three times with PBS, and identified with a fluorescently conjugated secondary antibody (Iago Inc., Burlingame, CA). Confocal microscopic analysis on double-labeled cells was carried out as described for in situ analysis. The C4 monoclonal total actin antibody was provided by Dr. J. Lessard (Children's Hospital Research Foundation, Cincinnati, OH). The γ actin rabbit antiserum was provided by Dr. J. C. Bulinski (Columbia University, New York).

**RNA Probes**

Isoform specific 3' UTR probes were derived from the mouse genes, with complementary strands used as the controls. The β actin probe was a 198 bp fragment from 1,468-1,665 (accession no. x03672). The γ actin probe was a 264 bp fragment from 1,310-1,574 (accession no. x13055). These fragments were cloned into pGEM-3Z (Promega Corp., Madison, WI) and orientation determined by Northern and slot blot analysis (data not shown). All chemicals were of RNA grade, solutions diethylpyrocarbonate treated and/or autoclaved with sterile plasticware used throughout. Plasmids were linearized appropriately for either SP6 or T7 polymerase directed RNA synthesis. Transcribed RNA was labeled by incorporating either digoxigenin-conjugated UTP or fluorescein-conjugated UTP as recommended by the manufacturer (Boehringer Mannheim, Sydney, Australia). Briefly, to 1 μl of linearized template was added 2 μl of a 10 mmol/l nucleotide mix (including 6.5 mmol/l labeled UTP and 3.5 mmol/l unlabeled UTP, 2 μl of 10× transcription buffer, 2 μl of either polymerase, 12 μl H2O, and 1 μl of RNase inhibitor. After 2 h at 37°C, the template was destroyed by DNase treatment, RNA precipitated and resuspended in 98 μl of H2O and 2 μl of RNase inhibitor. Digoxigenin-labeled RNA was then quantified by comparison with known standards on slot blot analysis with alkaline phosphatase (AP)-conjugated antibodies and staining as described for slide staining below. Typically 1/10 of the labeled product was used for each hybridization series.

**In Situ Hybridization**

Hybridization procedures were based on those described in Lawrence and Singer (1985, 1986). All chemicals were of RNA grade. All solutions diethylpyrocarbonate treated and/or autoclaved. Glassware and instruments were baked overnight at 200°C. Hybridization solution was made fresh each time and contained: 50% formamide, 5× SSC, 0.1% N-lauroylsarcosine, 0.1% SDS, 100 μg/ml RNA, 5% blocking solution (Boehringer Mannheim, Sydney, Australia), 20% dextran sulfate. Slide prehybridization was in 500 μl of hybridization solution without probe at 45°C for 1 h with a coverslip. The prehybridization solution was removed and probe or control RNA was added in 100 μl of hybridization solution, coveredslipped, and then incubated at 45°C overnight in a humidified container. In double-labeling experiments both probes were added together. Slides were washed with 2× SSC at 45°C, and then 1× SSC/0.1% SDS at 65°C. High stringency wash conditions were three washes in 0.1× SSC at 65°C each for 30 min with agitation. Lower temperature washes at 40°C in 0.5× SSC with addition of 50% formamide and posttreatment with RNase gave similar results. Slides were then equilibrated in PBS. Experiments using fluorescein-conjugated RNA alone were then mounted and analyzed. Experiments using digoxigenin-labeled RNA alone or double-labeled with fluorescein-conjugated RNA were further processed. Rhodamine- and fluorescein-conjugated antibodies (Boehringer Mannheim, Sydney, Australia) were diluted 1:10 in PBS with 0.5% BSA, reacted with blocked slides (10% inactivated FCS in PBS 1 h at 37°C) for 1-2 h at 37°C, and then washed in PBS at pH 7.5, or at pH 8 to reduce nonspecific binding. Slides were mounted in 1,4-diazabicyclo[2.2.2]octane (Sigma Chem. Co., St. Louis, MO) in glycerol.

For AP-conjugated antibodies, the slides were blocked for 30 min, washed three times in TBS, pH 7.5, for 10 min, incubated with antidigoxigenin-AP (Boehringer Mannheim, Sydney, Australia) diluted 1:100 with TBS, reacted in staining solution for 1 h, color development stopped with water, and then were dehydrated, mounted, photographed, and analyzed (Leitz Fluorovert FS). Staining solution (Boehringer Mannheim, Sydney, Australia) consisted of: 10 ml Tris buffer, pH 9.5; 50 μl nitroblue tetrazolium chloride solution (100 mg in 1.3 ml dimethyformamide, 70% vol/vol); 37.5 μl β-4-bromo-4-chloro-3-indolyl-phosphate solution (50 mg in 1 ml dimethyformamide).

**Laser Scanning Confocal Microscope Analysis**

A Confocal Laser Scanning Microscope (CLSM) (Wild Leitz Instruments, Heidelberg, Germany) with a 40× objective (1.3 NA) allowing a minimum slice thickness of 0.75 μm was used to analyze label distribution within cells. Cells with a fully spread morphology, previously determined by time-lapse video analysis (data not shown), were analyzed for β and γ actin mRNA distribution. Cells were optically sectioned in the xy and xz planes (parallel and perpendicular to substrate) with multiple scan averaging and all images stored on optical disc. Extended focus is an algorithm which compiles individual sections of the whole cell (Wilson and Carlini, 1988). In double-labeling experiments, identical confocal slices were taken for both probes allowing direct comparison of both mRNAs within each cell. Labeling intensity was analyzed directly on the CLSM with supplied software or on images transferred to Macintosh computers (Apple, Australia) and examined with NIH Image 1.41.

**3'UTR Sequence Comparison**

Primary sequence comparisons for both mouse β and γ actin full length 3'UTR sequences were compared using Macvector (Version 3.5, IBI, New Haven, CT) routines. Secondary structures of the 3'UTR sequences were determined by the minimal energy folding method of Zuker (1989).

**Results**

**Folding of β and γ Actin mRNA 3'UTRs**

Many studies to date point to the 3'UTR as the region of localization signals either as part of the primary sequence (Gottlieb, 1992) or the secondary structures they form (Macdonald and Struhl, 1988; Mowry and Melton, 1992). We compared β and γ actin 3'UTR primary sequences and found no similarities between them. In contrast, the 3'UTR of each has been highly conserved between species (Erba et al., 1988; Tokunaga et al., 1988), suggesting an evolutionary selection to maintain some specific function. Minimal energy folding of both 3'UTRs by the method of Zuker (1989) showed quite different overall secondary structures (Fig. 1). The complex patterns of stem loop structures does not exclude the possibility that a specific smaller subsequence could be involved with a common mRNA localizing mechanism. However, the lack of similarity between the two 3'UTRs suggested to us the possibility of different handling of the actin isoform mRNAs by the cell.

**β and γ Actin mRNA Localization**

C2 myoblasts (Yaffe and Saxel, 1977) were the cell type used in this study. Clonal C2 cell lines transfected with human β actin (Schevzon et al., 1992) were also used as they were substantially larger, allowing more accurate mRNA localization, and gave identical results to C2 cells in this study. Initial experiments used AP color development to locate mRNA within cells (Fig. 2). Both actin mRNAs were concentrated...
Myoblasts Sort β and γ Actin mRNA

Quantification of β and γ Actin mRNA Localization

Quantification of β and γ actin mRNA distribution was carried out for individual single-labeled cells along a line from the center of the nucleus to the cell periphery (Fig. 5 A). Both β and γ actin concentration fall with increasing distance from the nucleus, while β actin then increases to high levels at the cell periphery. Interestingly, the volume ratio β:γ under the actin plot lines (2.2:1) approximates the 2:1

Downloaded from jcb.rupress.org on July 8, 2017
Figure 2. γ and β actin mRNA localization in C2 myoblasts by in situ hybridization with alkaline phosphatase detection. (a) Digoxigenin-labeled γ probe, (b) β probe, and (c) γ complementary strand control hybridization under identical staining conditions. Both actin controls gave staining identical to that shown in c. Actin probes show strong nuclear and perinuclear staining. Shorter periods of color development show nuclear regional hotspots (data not shown).

In b, black arrows show peripheral localization of β actin mRNA and white arrows show strongly labeled fine filipodial extensions. Similar processes in a are unlabeled by the γ actin probe.

Figure 3. γ and β actin mRNA localization in C2 myoblasts by in situ hybridization with direct detection by fluorescein-conjugated probe. (A, C, E, and G) Transmitted image and corresponding (B, D, F, H, and I) CLSM extended focus image of 1-μm slices parallel to substratum (xy) through whole cells. (B) γ actin mRNA nuclear hotspot and perinuclear localization in flattened cells with arrow indicating the absence of peripheral labeling. (D) β actin mRNA also shows the perinuclear signal with additional localization at the cell periphery with arrows indicating labeled extended process and at edge of cell. Individual xy and xz sections (data not shown) confirm that labeling is primarily perinuclear rather than intranuclear. (F) γ complementary control hybridization, both γ and β controls gave either no detectable or low nuclear labeling as shown. Color panel in F shows scale for increasing label intensity (blue, highest intensity). In G, two boxes show recently divided cells on top of flattened cells with arrows indicating cleavage plane. Corresponding pairs of cells (H and I) show high γ actin mRNA signal in this cleavage region and in cytoplasm on opposite side of nucleus.
Figure 4. γ and β actin mRNA localization in a recently divided and spreading C2 myoblast by double in situ hybridization. Serial 2-μm slices from the top cell surface (a and b) to the substrate contact (i and j) with a transmitted image (k) of the cells. (a, c, e, g, and i) β actin mRNA slices (digoxigenin detection) with corresponding (b, d, f, h, and j) γ actin mRNA slices (fluorescein detection). The lowest slice (i and j) shows that β and not γ actin mRNA is found at the extending edge of the cell (white arrow). A fluorescence labeling intensity scan of the lowest cell slice (i and j) is shown (l), running from the center of the nucleus to the periphery along the line shown in k. The scans are equalized in intensity to show the difference in labeling at the cell periphery, increasing γ actin intensity does not show peripheral labeling.

overall ratio of actin protein (Otey et al., 1987) and mRNA (Schevzov et al., 1992) within the cells.

The same arbitrary divisions (nuclear, perinuclear, cytoplasmic, peripheral) of the cell were used for analysis of whole cell populations. In the AP study, peripheral localization occurred in 80 and 85% of cells (in random samples C2 and β actin transfected clones, respectively, n > 80). Quantitation of all the cells in the fluorescence studies produced very similar results (Fig. 5B). In contrast, high density cultures show less overall peripheral distribution of label (data not shown). Cells in high density cultures are in close contact and less motile, supporting the concept of an association of peripheral β actin mRNA with cell motility (Hoock et al., 1991).

Total and γ Actin Protein Localization

The asymmetric distribution of actin isofrom mRNA would perhaps predict a restricted γ actin protein distribution. The distribution of total actin and γ actin protein was found to be essentially the same, in both cytoplasmic bundles and at the cell periphery (Fig. 6). No differences in structures labeled were seen, although some intensity differences occurred. This suggests that the β and γ proteins are not differentially localized overall in structures within myoblasts (Otey et al., 1986) though differences have been described for muscle and non-muscle isoforms in myotubes (Pardo et al., 1983; Peng and Fischman, 1991) and pericytes (DeNofrio et al., 1989). In addition, localization differences for two actin isoforms which apparently incorporate into similar structures may be more subtle or transient than can be detected by this form of analysis. This result does however show that γ actin is not restricted by the distribution of its mRNA.

Discussion

Intracellular Sorting of mRNAs from a Multigene Family

Localization of mRNA potentially allows intracellular sorting of protein isoforms from a multigene family that are very similar in primary sequence. Such a mechanism has not been previously described. We have shown that β and γ actin isoforms, which differ by only four amino acids (Vandekerckhove and Weber, 1978), have different intracellular distribu-
A

Figure 5. (A) An analysis of γ and β actin mRNA distribution in the nuclear, perinuclear, cytoplasmic, or peripheral regions of cells. The normalized intensity of fluorescence along an arbitrary line from the nucleus center to the periphery for two individual cells (upper line β, lower line γ) showing the decrease in γ actin away from the perinuclear region and the high peripheral β actin level. (B) The percentage of cells showing localized labeling was scored for the total number of cells studied by CLSM: 69 C2 cells for β actin (closed squares); 117 C2 cells for γ actin (closed diamonds); 42 human β actin transfected cells for β actin (open squares); 33 human β actin transfected cells for γ actin (open diamonds). Both C2 and human β actin transfected C2 cells show similar distributions.

B

Figure 6. Double labeling of (a) γ actin and (b) total actin protein in C2 myoblasts. CLSM extended focus visualization show similar staining patterns near the nucleus, in stress fibers and at the periphery of the cell. γ actin protein does not appear to be restricted by its mRNA distribution. Inset shows very high actin labeling in a pair of recently divided cells. No differences in overall structures labeled were seen, although some intensity differences occurred.

The Function of mRNA Localization

Historically, localizing mRNA within the cell appears functionally important when the synthesis of the protein is regionally important. In the egg, polarity determining maternal mRNAs are specifically targeted to different regions by their 3'UTR (Macdonald and Struhl, 1988; Mowry and Melton, 1992; Gavis and Lehmann, 1992). In other cells, specifying the location of translation may also regulate the positive (Merlie and Sanes, 1985; Simon et al., 1992) or negative
postsynaptically concentrated calcium/calmodulin-dependent protein kinase II (Burgin et al., 1990), are specifically transported into dendrites. Thus morphologically and cytoskeletonally specialized neuronal cells, as well as simpler epithelial cells, use mRNA localization to control protein function.

Actin mRNA localization does not easily fit such a model. Actin is found throughout the cell in microfilaments and directly under the plasma membrane. The peripheral localization of actin mRNA (Lawrence and Singer, 1985, 1986; Sundell and Singer, 1990, Hoock et al., 1991) does not restrict the distribution of the actin protein isoforms. Similarly, endothelial cells which do appear to concentrate β actin at motile regions also have β actin throughout the entire cell (Hoock et al., 1991). We show that in myoblasts γ actin is also found wherever any actin is found. While we cannot exclude that temporal or subtle differences in protein distribution or concentration may occur, it is apparent that γ actin mRNA distribution does not act to determine the steady-state location of the protein. Furthermore, it would now be informative to look at the distribution of both β and γ actin isoforms in the same cell.

We suggest an alternative model for the function of the localization of actin isoform mRNA; that of a single gene used to regulate availability of new actin monomer at the cell periphery. To form new actin filaments for cytoskeletal remodeling, free actin monomer is required at concentrations that allow polymerization to occur. Free actin monomer levels are probably very low, with most cellular actin associated with binding proteins (for review see Pollard and Cooper, 1986) and incorporated into microfilament polymer. The availability of newly synthesized free β actin monomer at the periphery would provide a ready pool to allow rapid changes to the cytoskeleton. Perinuclear mRNA may then act as a reservoir which could be drawn upon to supply specific motile regions, transported peripherally by microfilaments (Sundell and Singer, 1991). Alternatively the perinuclear pool of actin mRNA may provide a functionally distinct source of actin monomer within the cell.

Localization may allow specific regional changes in the cell cytoskeleton to occur in association with cell motility. A model of rapid cellular movement is that which occurs during wound healing. Epithelial cells in vivo localize actin intracellularly at a wound edge as part of the process to close the wound (Martin and Lewis, 1992). Endothelial cells in vitro localize β mRNA, and indeed may enrich for β actin protein, at wound edges where rapid movement is occurring (Hoock et al., 1991). Another system which points to β actin as the dynamically responsive isoform is in neural regeneration after lesion. During neural regeneration, when processes are extending, β actin mRNA increases (Phillips and Steward, 1990) while γ actin is downregulated (Poirier et al., 1992).

Function of Actin Isoforms

The differential localization of β and γ actin mRNAs may contribute to the functional differences between these two genes. We have previously observed that transfection of the β actin gene can produce larger cells whereas transfection of the γ actin gene results in reduced cell size (Schervoz et al., 1992). In the case of the β actin transfection, the total β actin mRNA pool more than doubles. In contrast, the γ actin transfection reduces the β actin mRNA pool (Lloyd et al., 1992).

It is not unreasonable to expect that these changes in the β actin mRNA level will produce parallel changes in the peripheral pool of β actin mRNA. If this peripheral pool is used to control membrane remodeling, then changes in this pool may control the ability of the cell to spread. This presents a provocative hypothesis. The difference between the impact of the β and γ actin transfections may not simply be a function of different properties of the proteins themselves. Indeed, it is possible that whereas β actin rather than γ actin mRNA is used to supply new monomer at the leading edge, the two proteins may be functionally equivalent at this intracellular site. Alternatively, β actin may be the functionally preferred protein to be used at this site. To test these two alternatives, it will be necessary to carry out a transfection with the β actin mRNA localizing signal inserted into the γ actin gene.

Three other results support this concept of actin isoform function. First, the effects of γ actin gene transfection on cell size correlates with effects seen in neural regeneration models. After neural damage, the regrowth of neuron processes is inhibited by increasing γ actin mRNA (Poirier et al., 1992). Second, during the cell cycle γ actin isoform expression changes (McCairns et al., 1984; Maisbey et al., 1988). Increasing γ actin may be involved with cell rounding before cell division and potentially with cell cleavage after division. Our current experiments do not address the relationship between the cell cycle morphological changes and actin mRNA expression, though γ actin mRNA is increased and highly localized after karyokinesis. Third, we have shown that whereas myoblasts transfected with the entire γ actin gene are small, removal of the 3'UTR results in myoblast morphologies which exhibit extended processes (Lloyd and Gunning, 1993). It is possible that this result may reflect a failure to control localization of the γ actin mRNA. We are currently studying the possible specific functions encoded by the γ actin gene 3'UTR.

All mRNA within a cell is associated with the cytoskeleton either directly or indirectly (Hesketh and Pryme, 1991; Taneja et al., 1992). The distribution of mRNA is therefore related to the cell architecture, which itself responds to extracellular and intracellular signals. Actin is intriguing as it is both a major component of the cytoskeleton and provides its own mRNA distribution. The localization of mRNA may well be a signaling mechanism used by the cell to make morphological decisions. Alternatively, redistributing mRNA within the cell may occur in response to morphological decisions made by other factors. Thus the crucial issue is now to define the biological significance of differential actin isoform mRNA localization. This promises to add a new dimension to our understanding of the function of the actin multigene family.

We thank C. Smyth for CLSM assistance and Professor. P. B. Rowe and Professor J. Bamburg for helpful comments.

This work was supported by a National Health and Medical Research Council (NHMRC) Postdoctoral Fellowship to M. A. Hill and funded in part by a NHMRC project grant to P. Gunning. P. Gunning is a NHMRC Senior Research Fellow.

Received for publication 22 March 1993 and in revised form 24 May 1993.
Gunning, P., P. Ponte, L. Kedes, R. J. Hickey, and A. I. Skoultehi. 1984. Ex-
McKenna, N., J. B. Meigs, and Y. L. Wang. 1985. Identical distribution of
Gottlieb, E. 1992. The 3' untranslated region of localized maternal messages
Sreevalsan. 1988. Cell-cycle specific and serum-dependent expression of
Wilson, T., and A. R. Carlini. 1988. Three-dimensional imaging in confocal
Yisraeli, J. K., S. Melton, and D. A. Melton. 1990. A two-step model for the
Vanderkerckhove, J., and K. Weber. 1978. At least six different actins are ex-
Sakiyama. 1988. Isolation of cDNA clones for mouse cytoskeletal 3,-actin
Otey, C., A., M. H. Kalnosi, J. L. Lessard, and J. C. Bulinski. 1986. Im-
morpholization of muscle and nonmuscle isoforms of actin in myogenic cells and adult skel-
Pardo, J.-V., M.-F. Pittenger, and S. W. Craig. 1983. Subcellular sorting of
Phillips, L. L., O. S. Stewart. 1990. Increases in mRNA for cytoskeletal proteins in the denervated neuropil of the dentate gyrus: an in situ hybridization study using riboprobes for 3-actin and 4-tubulin. Mol. Brain Res. 8:249-