

Noncoding Regions of the γ -actin Gene Influence the Impact of the Gene on Myoblast Morphology

Catriona Lloyd and Peter Gunning

Cell Biology Unit, Children's Medical Research Institute, Locked Bag 23, Wentworthville, N.S.W. 2145, Australia

Abstract. We have addressed the question of whether two highly conserved noncoding regions of the γ -actin gene are of functional importance. Human γ -actin gene constructs deleted for either the entire 3' untranslated region (UTR) and 3' flank or intron III sequences were transfected into mouse myoblasts and the resulting clones were analyzed for cell morphology and actin protein expression. Transfectants carrying the γ -actin gene deleted for the 3' end (γ 22) exhibited numerous long pseudopods and increased surface area. In contrast, transfectants expressing the γ -actin gene deleted for intron III (γ 156) were rounded with blebs over the cell surface and showed decreased surface area. The relative expression of β - to γ -actin protein

decreased for both transfectant types. The total actin protein levels remained constant for the γ 22 cells but decreased for the γ 156 cells. The results indicate that alterations to transfectant cell morphology can be influenced by the presence or absence of different noncoding regions in the transfected γ -actin gene. The mechanisms by which noncoding regions of the γ -actin gene influence the impact of the gene are unknown. Nevertheless, these noncoding regions are isoform specific and highly conserved in evolution. We propose that the functional significance of the different actin isoforms may involve the properties of these noncoding regions in addition to the differences in protein sequence.

ACTIN is a major structural protein in most cell types. It is a member of a multigene family and in the mammalian system there are six isoforms: β and γ in nonmuscle cells, α -skeletal and α -cardiac in striated muscle cells, and α -aortic and γ -enteric in smooth muscle cells (Vandekerckhove and Weber, 1978). The existence of this multigene family raises the question of the functional importance (if any) of the individual isoforms. There are three levels at which specific isoforms could be of importance: (a) the proteins may have specific biological functions; (b) the mRNAs may be functionally distinct allowing different regulation and localization of the mRNAs; and (c) the different gene regulatory sequences may allow cells to use different programs of expression during development.

Several observations have been made which strongly suggest that actin isoforms are functionally different. First, muscle and nonmuscle actins have been found to localize to different areas of cells (Pardo et al., 1983; DeNofrio et al., 1989; Otey et al., 1988) suggesting they may perform different functions within cells. Second, the relative expression of β - to γ -actin, both at the protein and mRNA levels, has been found to differ between different tissues (Vandekerckhove and Weber, 1981; Otey et al., 1987; Erba et al., 1988) suggesting that the ratio of β to γ is of functional importance. Third, regulation of β and γ has been found to differ through

the cell cycle (McCairns et al., 1984; Masibay et al., 1988) compatible with the idea that β and γ perform different functions or that their regulatory sequences respond to different cues. However, such studies do not provide proof of a functional difference between the β - and γ -actin genes.

Recently, we have used transfection studies to show that the β - and γ -actin genes are biologically distinct. High level expression of β - and γ -actin genes in mouse myoblasts resulted in different cytoarchitecture and cell morphology (Schevzov et al., 1992) demonstrating that β and γ gene expression has distinct functional impact in C2 myoblasts. The β -actin transfectants were well spread and showed well defined actin stress fibers, whereas γ -actin transfectants were small, elongated, and showed poorly defined actin stress fibers. Additionally, the endogenous actin genes were feedback regulated differently in the β - and γ -actin transfectants indicating that the two isoforms can be distinguished with respect to actin regulation (Lloyd et al., 1992). However, this work falls short of demonstrating the level at which these genes are functionally distinct. The entire β - and γ -actin genes were transfected in these experiments and so the different resultant morphologies and actin feedback regulation of the transfectants could be due to the different proteins encoded by the genes, or to properties of the different mRNAs and/or regulatory regions of the two genes. For example, as the two genes are regulated differently through the cell cycle (McCairns et al., 1984; Masibay et al., 1988) a change in the relative expression of β to γ may result in

Catriona Lloyd's present address is Howard Hughes Medical Institute Research Laboratories; University of Chicago, Chicago, IL.

changes to total actin levels through the cell cycle leading to morphological changes.

The nonmuscle actins, β and γ , differ by only four amino acids out of a possible 374. These changes, all at the amino terminus, are highly conservative making it unlikely that β and γ proteins differ in function. However, the amino acid sequence of γ -actin from human, rat, mouse, and *Xenopus* is identical (Erba et al., 1986) as is the β -actin sequence from human, rat, and chicken (Ng et al., 1985). This conservation of the nonmuscle actins through different species does suggest that there has been a strong selective pressure to maintain their protein sequences and therefore their binding sites. This may reflect the existence of subtle specific functions for the two nonmuscle actin isoforms.

Both β - and γ -actin have unique 3' untranslated regions (UTRs)¹ which have been conserved between mammals and chickens in the upstream half, but conserved only between mammals in the downstream halves (Erba et al., 1986; Yaffe et al., 1985). These observations suggest that regions of the 3'UTRs have been under strict evolutionary constraint and that they may endow the mRNAs with isoform-specific functions such as localization of mRNA. Indeed, Kislauskis, E., and R. Singer (personal communication) have found that sequences in the 3'UTR of β -actin are responsible for the peripheral localization of β -actin mRNA in chicken embryonic fibroblasts and myoblasts.

The regulatory sequences of the β - and γ -actin genes have some regions of similarity and some regions which differ. There is significant nucleotide sequence similarity between human γ -actin 5' flank and human, rat, and chicken β -actin and *Xenopus borealis* type 1 actin 5' flank (Erba et al., 1988). The similarity of this region may reflect the conservation of sequences involved in the co-expression of these genes. There are, however, some differences between the 5' flanks of β and γ and in fact γ has more similarity to *Xenopus* type 1 actin than β -actin in this regard (Erba et al., 1988). These sequences of the 5' flank in the β - and γ -actin genes may allow flexibility of expression of nonmuscle actin in the cell. In addition, the β - and γ -actin genes show some similarity in their intron III sequences. Interestingly, a segment of the γ -actin intron III sequence which is not found in the β -actin sequence has been conserved between human and *Xenopus borealis* type 1 actin gene with 82% similarity (Erba et al., 1988). The conservation of this intronic region strongly suggests that it is of functional importance and that its function may differ from that of the β -actin intron region.

The existence of the specific and conserved regions of the genes for the two nonmuscle actin isoforms suggest they have functional importance. It is possible that the properties of the β - and γ -actin genes that affected cell architecture, cell morphology, and actin feedback regulation after transfection into myoblasts (Schevzov et al., 1992; Lloyd et al., 1992) may be partially attributed to the noncoding regions as well as to the protein itself. This work addresses the question of whether the two noncoding regions, 3'UTR/3' flank and intron III, of the human γ -actin gene are of functional importance. Human γ -actin gene constructs deleted for one or other of these regions were transfected into mouse myoblasts

and the resulting clones were analyzed for cell morphology and actin protein expression. The results indicate that these noncoding regions can influence the impact of γ -actin gene expression on myoblast morphology.

Materials and Methods

γ -actin Gene Constructs

The full length γ -actin gene construct (γ 33) was prepared from the 9-kb Bam HI fragment cloned into pBR322 by Erba et al. (1988). The γ -actin gene was released by cutting out the gene as a Sall-EcoRI fragment (see Fig. 1). The vector fragment was prepared from the Okayama-Berg vector, pCDV1 (Okayama and Berg, 1983). The XhoI-Sall region of pCDV1, containing the ampicillin resistance gene and the SV-40 polyadenylation and termination signals, was ligated to the γ -actin gene fragment such that the SV-40 sequence was cloned next to the 3' end of the γ -actin gene (see Fig. 1). The γ 22 construct, lacking the 3'UTR and flank, was prepared in a similar manner to γ 33 except that only the Sall-XbaI fragment of the γ -actin gene was used (see Fig. 1). The XbaI restriction site contains the termination codon (TAG) therefore, this fragment contains the entire 5' flanking sequence, 5'UTR, all codons and exons and codes for the normal γ -actin protein. The γ -actin gene construct deleted for intron III (γ 156) was prepared by removal of the BglII-BstEII region of the γ 33 construct and the introduction of the BglIII-BstEII region of the γ -actin cDNA (Erba et al., 1986). In this way intron III was precisely eliminated from the γ -actin gene construct. The DNA sequences either side of the XbaI site in the γ 22 construct and the BglIII-BstEII region of the γ 156 construct were confirmed by DNA sequencing.

Cell Culture and Transfection

C2 cells originally isolated by Dr. D. Yaffe (Weizmann Institute, Israel) and subcloned in the laboratory of Dr. H. Blau (Stanford University, Stanford, CA) were grown in DME medium (GIBCO BRL, Gaithersburg, MD) supplemented with 20% FCS (Commonwealth Serum Labs, Melbourne, Australia) and 0.5% chicken embryo extract (Flow Laboratories, Australasia Pty. Ltd, North Ryde). DNA transfection and isolation of transfectant clones was performed as described by Schevzov et al. (1992). The constructs used for transfection included (a) the human wild type γ -actin gene [γ 33], (b) a truncated form of the γ -actin gene lacking the 3'UTR and 3' flank [γ 22], (c) a deleted form of the γ -actin gene missing the intron III sequence [γ 156], and (d) the plasmid pUC19 as a control. These constructs were all cotransfected with 1 μ g of pSV2-neo.

RNA Isolation and Analysis

Total cellular RNA was isolated from 30% confluent transfectants by the method of Chomczynski and Sacchi (1989). RNA was size fractionated by formaldehyde gel electrophoresis and transferred to Hybond N⁺ (Amersham Australia, North Ryde, N.S.W., Australia) as per manufacturer's instructions. DNA probes were radiolabeled to 10⁸ dpm/ μ g by the random priming method (Feinberg and Vogelstein, 1983) and hybridized to RNA blots at 10⁶ dpm/ml in a solution containing 4 \times SSC (1 \times SSC is 0.15 M NaCl, 0.015 M sodium citrate), 50 mM NaH₂PO₄, pH 7.0, 5 \times Denhardt's solution (Denhardt, 1966), and 10% (wt/vol) dextran sulfate at 65°C for 16 h. After hybridization the blots were washed in 1 \times SSC, 0.1% SDS at 65°C. Membranes were exposed to Kodak XAR film (Eastman Kodak Co., Rochester, NY) for 1 d. To verify that equivalent amounts of RNA were transferred, the RNA blots were hybridized to an 18-S rRNA-specific oligonucleotide probe under conditions of probe excess and washed in 4 \times SSC, 0.1% SDS at 55°C. Levels of mRNAs were quantitated by densitometry as described (Lloyd et al., 1992). All measurements were made within the linear range of the film as described (Gunning et al., 1987).

DNA Probes

The DNA probe used to detect human γ -actin mRNAs was a human γ -specific 5'UTR sequence. It was generated by PCR amplification as previously described (Gunning et al., 1990) using the oligonucleotides 5'-GCGGATCCGCTTAAATAACGGCGGG-3' and 5'-TGCGACGTCAGCTCAGGCC-3' and the cloned gene (Erba et al., 1988) as the template. The resulting DNA fragment, corresponding to base pairs -33 to +105 of the gene, was cloned

1. Abbreviation used in this paper: UTR, untranslated region.

between the BamHI and SmaI sites of pGEM-3 (Promega Corp., Roselle, NSW, Australia).

Immunofluorescence Staining

A total of 5,000 cells were seeded on collagen-coated (Calf Skin Collagen; Calbiochem-Novabiochem Pty., Alexandria, N.S.W., Australia) glass slides for 24 h before fixing and permeabilizing in 1% formaldehyde for 20 min followed by a 20-min incubation in -20°C methanol. Slides were stored at 4°C containing 0.02% sodium azide until ready for use. Nonspecific binding on slides was blocked by incubation at 37°C in PBS containing 10% FCS. Antibody incubations were carried out for 2 h at 37°C and PBS containing 10% FCS was used for all subsequent washes. Cells were examined and photographed on a Zeiss epifluorescence microscope (Carl Zeiss, Inc., Thornwood, NY) with either a $16\times$ objective 0.40 NA, or a $40\times$ objective 0.75 NA. Photographs were taken with Kodak T-max 400 ASA film, developed with D-76 (1:2) and printed on Agfa black and white paper. Some cells were examined using a confocal microscope as described below.

Antibodies

The γ -actin rabbit antibody was provided by Dr. J. C. Bulinski (Columbia University, New York). The C4 monoclonal actin antibody was provided by Dr. J. Lessard (Children's Hospital Research Foundation, Cincinnati, OH) (Lessard, 1988). The goat anti-mouse IgG FITC and goat anti-rabbit IgG rhodamine were purchased from Tago, Inc. (Medos Co. Pty. Ltd., Burwood, N.S.W., Australia).

Actin Protein Analysis

Transfected cells were collected at 30% confluence by trypsinization, washing once with PBS and then the cell pellets were stored at -80°C . The cell pellets were then used for either isoelectric focusing gels or SDS-PAGE. Isoelectric focusing electrophoresis and blotting was performed as described by Schevzov et al. (1992). Immunostaining of the Western blot was carried out with a C4 mAb, $10\ \mu\text{g/ml}$, which reacts with all actin isoforms. This was followed by incubation with a ^{35}S -labeled anti-mouse IgG (Amersham Australia). The membrane was then exposed to Kodak XAR film for 1-3 d. The bands on the immunoblots were quantitated by scanning and integrating the volume of the entire bands with a Molecular Dynamics computing densitometer (model 300 series; Molecular Dynamics Australia Ltd., Pty., Victoria, Australia). The ratio of the values for the bands corresponding to β - and γ -actin were then calculated. The SDS-PAGE was carried out as described (Lloyd et al., 1992) except that the protein was detected using a ^{35}S -labeled anti-mouse IgG (Amersham Australia). The membrane was then exposed to Kodak XAR film for 1-3 d. The bands on the immunoblots were quantitated as described previously. Identical gels were run concurrently, stained with Coomassie blue and used to check for equal loading. The level of actin was expressed as a percentage of that detected in control cells which was set at 100%.

Confocal Microscope Analysis

Some images of immunostained cells were taken using a Confocal Laser Scanning Microscope (Wild Leitz Instruments, Heidelberg, Germany) with either a $10\times$ objective 0.45 NA or a $40\times$ objective 1.3 NA. The images of sections through two cells (see Fig. 7) was achieved by taking the images in the xy plane (parallel to substratum) at fixed steps of $1.8\ \mu\text{m}$ in the z direction and then manipulating them such that they were presented along side each other, where left to right corresponded to top to bottom of the cells.

Cell surface area was determined as follows. Anti- γ -actin-stained cells were XY sectioned. The XY images were manipulated so that the pixel intensity of the fluorescence that covered the entire surface of the cells was maximal compared with background. This was achieved by shifting the digits of each intensity byte to the specified bit positions in the direction of maximal intensity. This was repeated until the cells' pixel intensity was maximal. The total surface area (in μm^2) of cells in contact with the substratum in a microscopic field was calculated by multiplying the pixel size of $1.9179\ \mu\text{m}^2$ (for $10\times$ objective, 0.45 NA) by the number of pixels of maximal intensity (obtained from the intensity histogram). Finally, the average surface area per cell was determined by dividing the total surface area by the number of cells in the field (determined manually). A total of six different fields (containing 15-35 cells) were examined for each transfected clone. To obtain accurate cell numbers for these calculations, fields were chosen which had evenly plated cells with minimal cell contact. The means were statistically analyzed by the *t* test.

Results

Transfection of γ -actin Gene Constructs with Deletions from Two Noncoding Regions

To investigate the potential role of the conserved 3'UTR region and intron III of the γ -actin gene, different forms of the human γ -actin gene (Fig. 1) were transfected into C2 myoblasts. The protein encoded by all these constructs should be identical. The transfection frequency for the $\gamma 22$ construct (0.14×10^{-4} clones/cell) was lower than that for both the entire gene, $\gamma 33$ (0.53×10^{-4}) (Schevzov et al., 1992) and the control, pUC-19 DNA (0.54×10^{-4}) whereas $\gamma 156$ had the same frequency (0.50×10^{-4}) as the controls. The difficulty in obtaining transfectants suggests that the $\gamma 22$ construct conveys some information which is detrimental to cell viability.

The cell morphology of pooled stable transfectants, $\gamma 33$ (>100 clones), $\gamma 22$ (>30), $\gamma 156$ (>100), and control (>100), was examined by immunofluorescent staining of the cells using an actin antibody, C4, (kindly supplied by Dr. J. Lessard). Any random field of cells will contain cells at different points in the cell cycle. Thus, we expect to see cells which have just divided and are rounded-up, those just beginning to spread and those which have fully flattened and extended ruffling edges. The morphology of control, $\gamma 33$ and $\gamma 22$ pooled transfectants was indistinguishable (Fig. 2, a, b, and c). The majority of the cells had spread to some extent and there were no obvious features which distinguished one pool of clones from another. However, the morphology of the $\gamma 156$ transfectants differed markedly from the other transfectants (Fig. 2 d). Whereas only a small fraction of cells in the other transfectants appeared rounded in shape, almost half of the cells in the pooled $\gamma 156$ clones displayed this phenotype. The existence of a morphological phenotype in the pooled $\gamma 156$ transfectants suggests that the cells are sensitive to the expression of this deletion construct and that the gene acts to inhibit spreading of the cell.

To further examine the effects of expression of these γ -actin gene constructs, further transfections were conducted. All the clones arising from one $\gamma 22$ transfection (13 total) and one $\gamma 156$ transfection (24 total) were isolated and screened

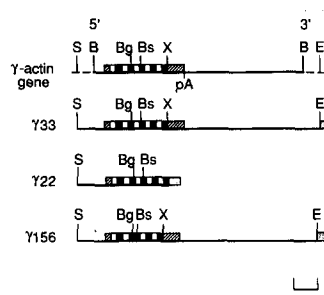


Figure 1. Deletion constructs of the human γ -actin gene. The human γ -actin gene is shown as a 9-kb BamHI fragment cloned into the plasmid pBR322. (■) Exons; (□) introns, and (▨) the 5' and 3'UTRs. pA, polyadenylation signal. (▩) The SV-40 3'UTR (polyadenylation and termination sequence). The restriction enzyme sites are shown. The lines below the map represent the portions of the gene cloned in the different γ -actin gene constructs; $\gamma 33$ has the SalI-EcoRI fragment, $\gamma 22$ has the SalI-XbaI fragment and $\gamma 156$ has the SalI-EcoRI fragment with the intron III region deleted by replacement of the BglIII-BstEII region of the gene with the corresponding region of the γ -actin cDNA. The restriction enzyme sites are abbreviated as follows: B, BamHI; Bg, BglIII; Bs, BstEII; E, EcoRI; S, SalI; and X, XbaI. Bar, 1 kb.

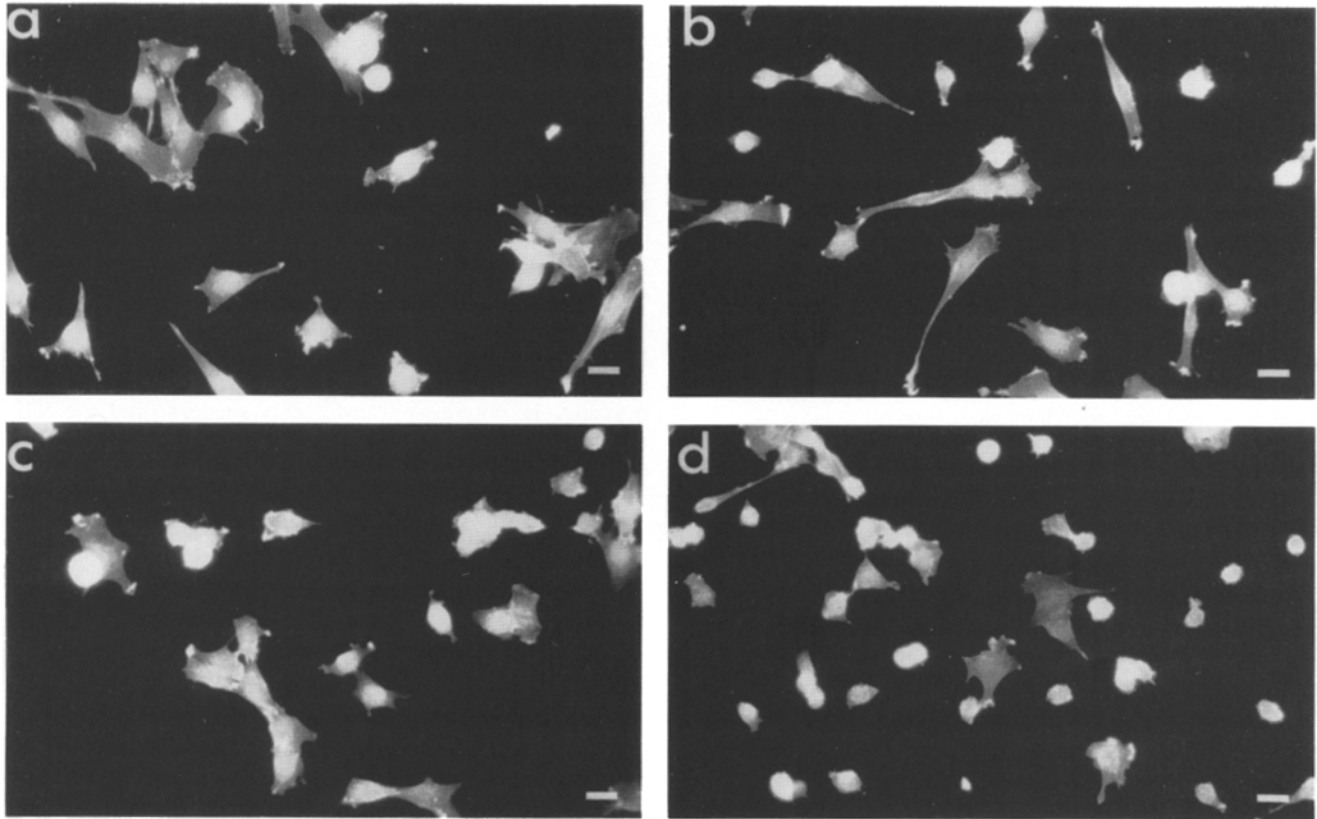


Figure 2. Cell morphology of pooled γ -actin gene transfectants. Immunofluorescent staining of pooled transfectants with an antibody for actin, C4. Each pool consisted of at least 100 clones except for γ 22 where there was at least 30 clones. Staining with the C4 antibody was followed by fluorescein-conjugated goat anti-mouse IgG. (a) Control transfectants (pUC-19 plasmid), (b) full length γ -actin gene transfectants (γ 33), (c) γ -actin gene lacking the 3'UTR and flanking region transfectants (γ 22), and (d) γ -actin gene lacking intron III transfectants (γ 156). Cells were grown on collagen-coated glass slides 24 h before fixing and permeabilizing with 1% formaldehyde followed by methanol. Bar, 20 μ m.

for human γ -actin gene expression levels. This was achieved using a 5'UTR human γ -actin-specific probe which hybridizes to the human but not the mouse γ -actin mRNA (Fig. 3 A). The three highest expressing clones from each transfection were selected to be analyzed in detail and a Northern blot showing their expression levels is shown in Fig. 3 A. The transcripts detected from the γ 22 cells were shorter than those of the γ 156 cells due to loss of the γ -actin 3'UTR. The level of expression in the highest γ 22 clone was lower than that found for the highest γ 156 clone. The levels of expression for each clone were quantitated and are shown in Table I. The transfectant, γ 156-A1, has levels of human γ -actin expression similar to the two lower γ 22 clones. The breadth of the mRNA bands observed in Fig. 3 A was an artefact of the individual gel as suggested by the parallel size of the 18-S rRNA bands. This was confirmed by analysis of a number of γ 156 clones on a separate gel and also revealed the mRNAs to be identical in size to that produced by the intact γ 33 gene (Fig. 3 B).

Altered Cell Morphology in Transfectant Clones Expressing the γ -actin Gene Missing the 3'UTR and Flanking Region

Expression of the human γ -actin gene deleted for the entire 3'UTR and 3' flanking region resulted in aberrant cell morphology in transfectant clones. The transfectant cells ex-

hibited long pseudopods originating from all around the cell periphery (Fig. 4, b, c, and d). In control cells (Fig. 4 a), some projections are observed. However, control cell pseudopods are much shorter than those observed in γ 22 transfectants. In addition, control cells show only one or two polar pseudopods per cell whereas γ 22 cells frequently show more than two projections. These extensions were attached to other extensions and cells, resulting in an apparent network of cells. Time-lapse photography of these cells (data not shown) revealed that these pseudopods actively extend and touch other cells and then pull away. While the role of these pseudopods is unknown, it is clear that they are not remnants of the trailing edge of the cells nor are they always extended in the direction of movement. The average surface areas of the γ 22 transfectant clones were all increased relative to control cells (Table I), however, only one (γ 22-A1) is elevated beyond the range previously observed with individual control cells (Schevzov et al., 1992).

The cytoarchitecture of the γ 22 transfectant clones was examined (Fig. 5). Immunostaining for γ -actin in the γ 22 cells, revealed that actin stress fibers could be detected almost to the same degree as those in the control cells. There was γ -actin staining present in the long pseudopods but it could not be determined whether it was filamentous or diffuse staining (Fig. 5). In addition, γ -actin deposits were observed at the cell periphery similar to that seen previously in the γ 33 transfectants (Schevzov et al., 1992).

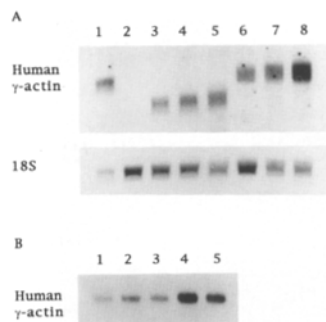


Figure 3. Human γ -actin gene expression in transfectant clones. (A) Total RNA was isolated from the three highest expressing clonal lines at low confluence, size fractionated by gel electrophoresis on a denaturing gel and transferred to a nylon membrane as described in Materials and Methods. The filter was hybridized with the human γ -actin specific 5'UTR

probe, washed and autoradiographed. A human myoblast RNA sample was also applied to the gel to allow the determination of relative γ -actin expression in the transfectant clones. To correct for loading discrepancies the filter was subsequently hybridized with a probe specific for 18-S ribosomal RNA. 5 μ g of RNA was loaded for each of the transfectants and 2.5 μ g for the human sample. Lane 1, human myoblast sample; lane 2, pUC-19 transfectants; lane 3, γ 22-A2; lane 4, γ 22-A1; lane 5, γ 22-A3; lane 6, γ 156-A1; lane 7, γ 156-D1; and lane 8, γ 156-A6. (B) 5 μ g of RNA isolated from four γ 156 transfectants and from a γ 33 transfectant was analyzed for γ -actin expression as in A. Lane 1, γ 156-B5; lane 2, γ 156-D1; lane 3, γ 156-A1; lane 4, γ 156-A6, and lane 5, γ 33-B5.

Altered Cell Morphology in Transfectants Expressing the γ -actin Gene Missing Intron III

Individual clones transfected with the γ -actin gene deleted for intron III display the phenotype observed in pooled

Table I. Surface Area of the γ -actin Gene Transfectant Clones

Clone	Human actin mRNA level*	Surface area (μm^2)†
pUC-19	-	1332 \pm 106
γ 22-A2	0.25	1506 \pm 108§
γ 22-A1	0.34	2988 \pm 747
γ 22-A3	0.57	1749 \pm 154†
γ 156-A1	0.28	725 \pm 255
γ 156-D1	0.68	327 \pm 29†
γ 156-A6	1.18	240 \pm 53†

* Values are expressed relative to the level found in human myoblasts.

† Surface area corresponds to the area of the substratum covered by the cell. Values are shown as means \pm SD. The significance of differences between the clones compared with the pUC-19 transfected pooled clones was estimated using the *t* test.

‡ $p < 0.0005$

§ $p < 0.005$

|| $p < 0.025$

clones (Fig. 2). The cell morphology of these cells was determined by immunofluorescent staining for γ -actin (Fig. 6). The γ 156 transfectants exhibited a different cell morphology to the control cells. They were smaller in surface area and rounded in shape. The morphology of the γ 156 transfectants became more extreme with increasing expression of the human γ -actin gene (Fig. 6). The γ 156-A1 cells were smaller than control cells and slightly elongated. The γ 156-D1 cells were very small and rounded. The highest expressing transfectants, γ 156-A6, were more extreme than γ 156-D1 and the

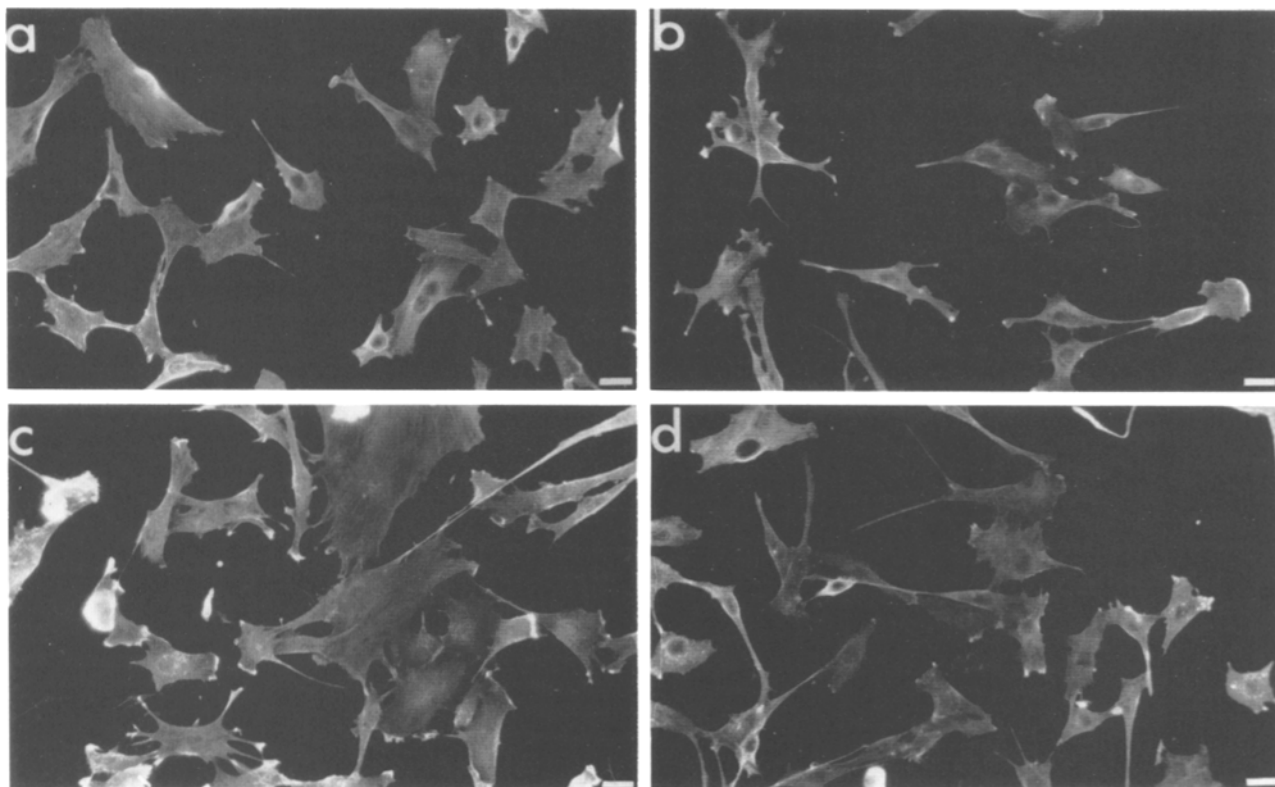


Figure 4. Transfectants expressing the γ -actin gene lacking the 3' end exhibit long pseudopods. Immunofluorescent staining of pooled control cells and clonal γ 22 transfectants with a γ -actin specific antibody. Staining with the γ -actin antibody was followed by rhodamine-conjugated goat anti-rabbit IgG. (a) Control transfectants (pUC-19 plasmid), (b) γ 22-A2, (c) γ 22-A1, and (d) γ 22-A3. Cells were prepared and immunostained as in Fig. 2. Bar, 20 μ m.

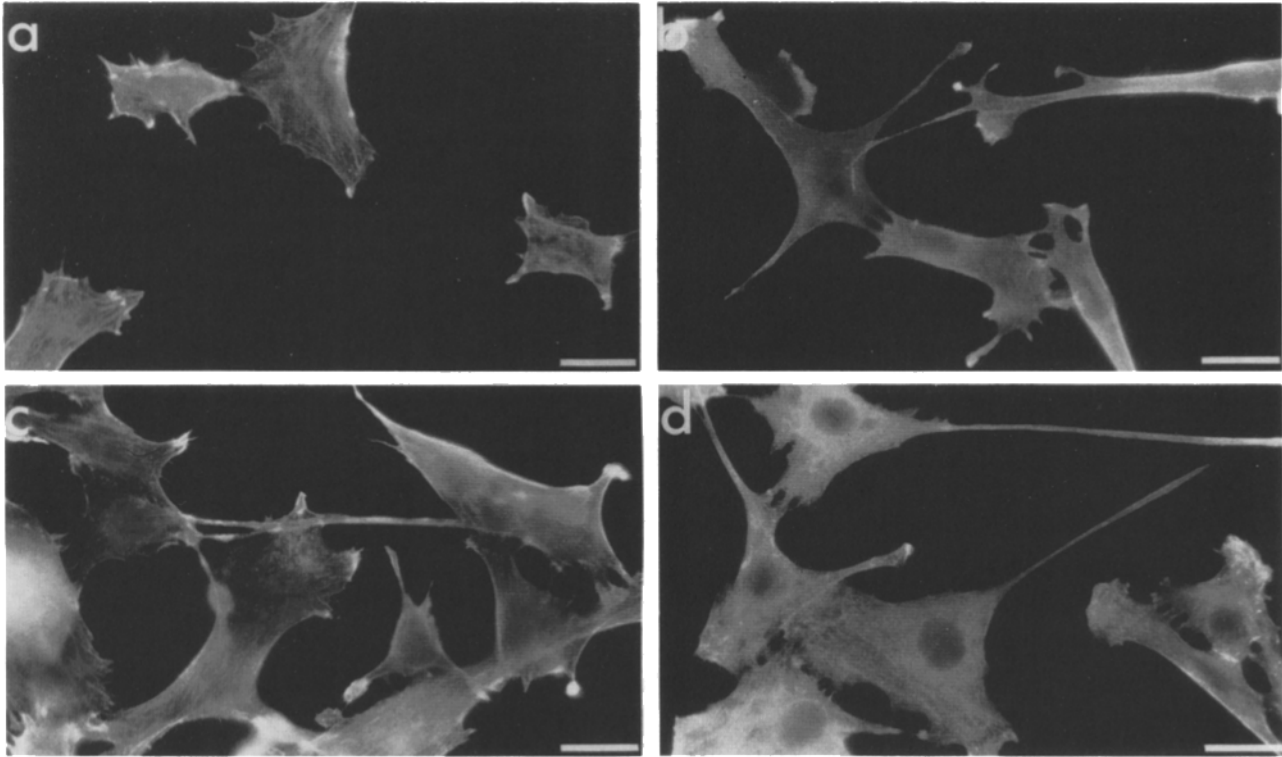


Figure 5. Cell morphology of clones expressing the γ -actin gene lacking the 3' end. Immunofluorescent staining of pooled control cells and clonal γ 22 transfectants with a γ -actin-specific antibody. Staining with the γ -actin antibody was followed by rhodamine-conjugated goat anti-rabbit IgG. (a) Control transfectants (pUC-19 plasmid), (b) γ 22-A2, (c) γ 22-A1, and (d) γ 22-A3. Cells were prepared and immunostained as in Fig. 2. Bar, 20 μ m.

majority of cells appeared to have essentially no cytoplasm. The average cell surface area was determined for the different transfectants (Table I) and it can be seen that the highest expressing clone, γ 156-A6, showed a sixfold reduction in surface area compared with the control cells.

The cytoarchitecture of the γ 156 transfectants was highly disorganized (Fig. 6). There were very few γ -actin cables visible in γ 156-A1 cells and no actin cables were detected in the higher expressing clones, γ 156-D1 and γ 156-A6 (Fig. 6). Additionally, the small rounded cells often showed numerous blebs all over the cell surface. A typical example of these blebs is shown in Fig. 7. The blebs were examined by optically sectioning two γ 156-A6 cells, from top to bottom, using a confocal microscope (Fig. 7). Blebs appear to exist over the entire surface of the cells and individual blebs can be followed through the different sections. These blebs suggest that there has been an aberration in the membrane integrity of these cells which, although in a different form, was also observed in the γ 22 cells with the long extensions from the cells. Confocal sectioning of the γ 156-A6 transfectant cells (Fig. 7) also did not reveal any actin cables.

The noncoding regions of the human γ -actin gene, 3'UTR and flank, and intron III, can influence the impact of the transfected γ -actin gene upon cell morphology. The morphology of the γ 22 and γ 156 transfectants each differed to that of the control cells. Additionally, there was an extreme difference in cell morphology between the γ 22 and γ 156 cells even though the expression levels of the human γ -actin gene were similar for the transfectants (Table I). Therefore,

the expression level of the transfected γ -actin gene is not the sole determinant of cell morphology. We conclude that the noncoding regions of the γ -actin gene can affect the impact of expression of the human γ -actin gene on C2 myoblast morphology.

Relative Expression of β - to γ -actin Protein in γ -actin Gene Transfectants

The actin expression in the γ 22 and γ 156 transfectants was determined to gain insight into the mechanisms behind the altered cell morphology. Exogenous expression of the γ -actin gene in the transfectants was expected to result in a decrease of the β/γ actin protein ratio, as found in Schevzov et al. (1992). Isoelectric focusing gels were used to separate the two isoforms, β and γ , using total protein isolated from these transfectant cells. The protein was transferred to nitrocellulose and actin was detected by use of the C4 antibody with a 35 S-labeled secondary antibody. The resulting autoradiographs (Fig. 8) were scanned using densitometry and the β/γ ratios were determined (Table II). The β/γ ratio was decreased in all the transfectants compared to that of control cells. A graduated response was found in the γ 156 transfectants where the β/γ ratio decreased with increasing expression of the γ -actin gene. Additionally, the ratios found were similar for all the γ 22 cells and the two lowest expressing γ 156 transfectants. Therefore, the relative amount of β - to γ -actin protein in the myoblast does not determine cell morphology since the γ 22 and, γ 156-A1 and γ 156-D1 cells showed very different morphologies (Figs. 5 and 6).

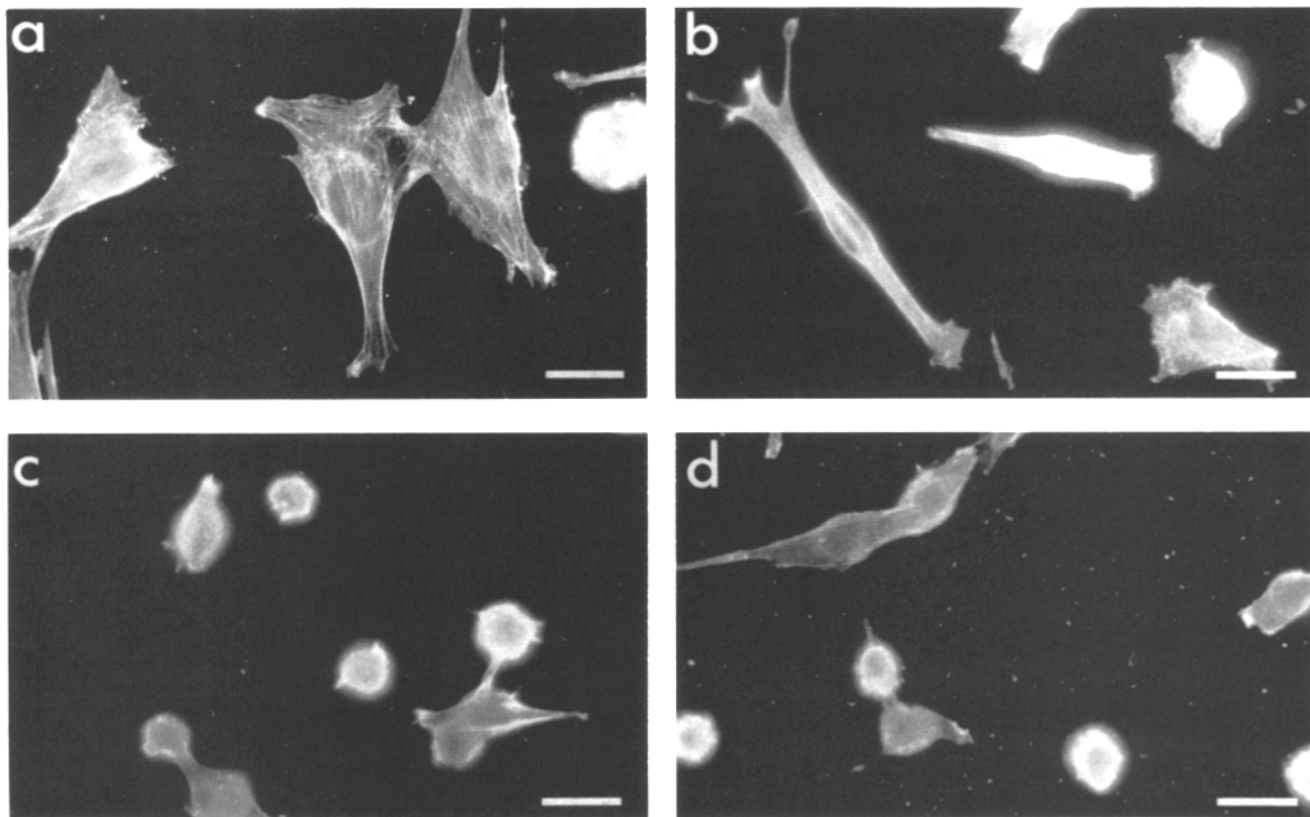


Figure 6. Cell morphology of clones expressing the γ -actin gene lacking intron III. Immunofluorescent staining of pooled control cells and clonal γ 156 transfectants with a γ -actin specific antibody. Staining with the γ -actin antibody was followed by rhodamine-conjugated goat anti-rabbit IgG. (a) Control transfectants (pUC-19 plasmid), (b) γ 156-A1, (c) γ 156-D1, and (d) γ 156-A6. Cells were prepared and immunostained as in Fig. 2. Bar, 20 μ m.

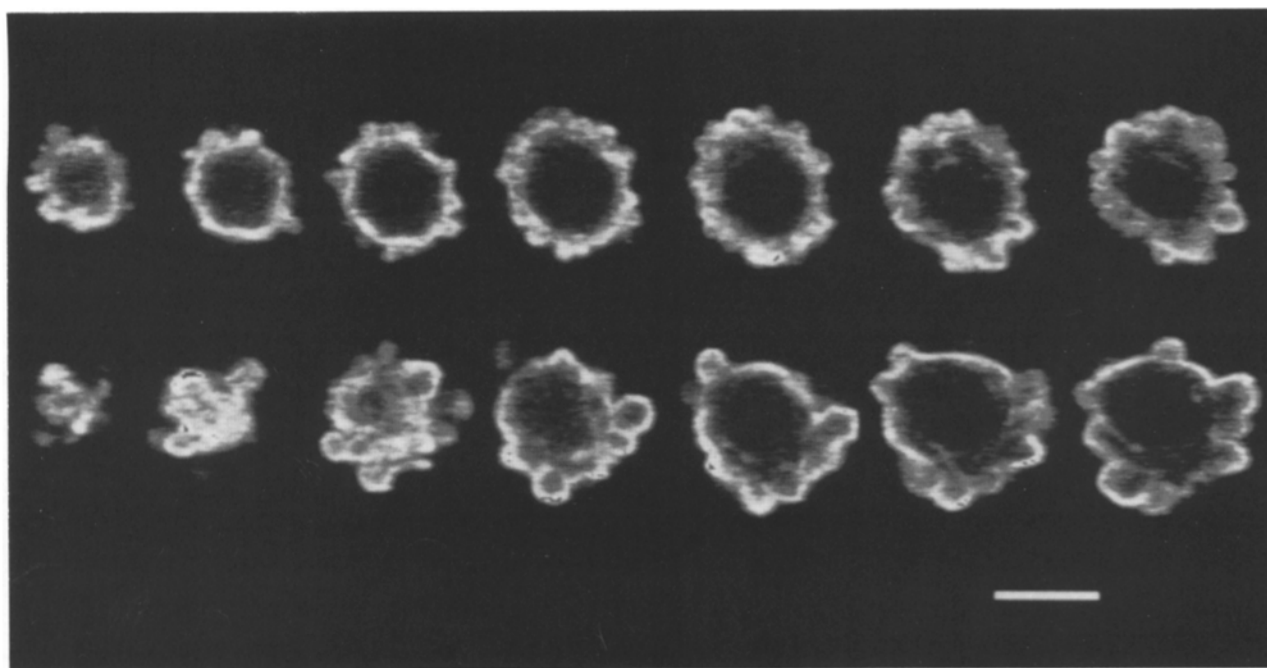


Figure 7. Sections through two transfectants expressing the γ -actin gene lacking intron III. Sections through two γ 156-A6 cells from the top of the cells to the bottom, (left to right in the figure). The cells were immunofluorescently stained for γ -actin followed by rhodamine-conjugated goat anti-rabbit IgG. Cells were prepared and immunostained as in Fig. 2. These images were prepared using a confocal microscope. Images were taken of these cells in the xy plane (parallel to substratum) at fixed steps of 1.8 μ m in the z direction. Bar, 10 μ m.

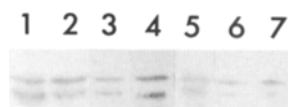


Figure 8. Relative amounts of the cytoplasmic actins in transfected clones. An IEF gel was immunoblotted with an actin-specific antibody, C4, as

described in Materials and Methods. The positions of the β - and γ -actin protein isoforms are indicated. Lane 1 corresponds to control pUC-19 transfected cells; lane 2, γ 22-A2; lane 3, γ 22-A1; lane 4, γ 22-A3; lane 5, γ 156-A1; lane 6, γ 156-D1; and lane 7, γ 156-A6.

Total Actin Expression in the γ -actin Gene Transfectants

Total actin protein levels were measured in the transfectants to determine whether the total pool was differently altered by these two γ -actin constructs. The total actin protein levels were determined by Western blotting total protein from the transfectants using an actin antibody, C4, with a ^{35}S -labeled secondary antibody. The resulting autoradiograph is shown in Fig. 9. A gel was run in parallel and stained with Coomassie blue to show any loading differences between the lanes (Fig. 9). An obvious decrease in total actin protein levels can be seen in the γ 156 transfectants. The total actin protein levels were quantitated by scanning the autoradiograph (Table II). Total actin protein levels for the γ 22 cells were the same as that found for the control cells, compatible with that found by Lloyd et al. (1992). Therefore, the increased surface area of the γ 22 cells was not a result of an increase in total actin protein. The level of actin protein, however, decreased two-fold with increasing expression of the γ -actin gene in the γ 156 cells. The decrease in the total actin pool of the γ 156 cells differed to the response for the entire γ -actin gene where total actin levels remained essentially constant (Lloyd et al., 1992). Therefore, lack of the human γ -actin intron III sequence appears to have altered regulation of actin expression with respect to the actin pool. This suggests that intron III may play a role in actin pool regulation.

Discussion

Noncoding Regions of the Human γ -actin Gene Can Influence the Impact of a Transfected γ -actin Gene in Myoblasts

The cell morphology of γ 22 and γ 156 transfectants was profoundly different demonstrating that noncoding regions of

Table II. Actin Protein Expression in the γ -actin Gene Transfectant Clones

Clones	Total actin*	β/γ
pUC-19	100	1.7
γ 22-A2	84	0.8
γ 22-A1	99	0.9
γ 22-A3	95	1.0
γ 156-A1	60	0.9
γ 156-D1	49	0.8
γ 156-A6	44	0.3

* Values are expressed as a percentage of that found in control cells (transfected with the plasmid pUC-19) which was set at 100%.

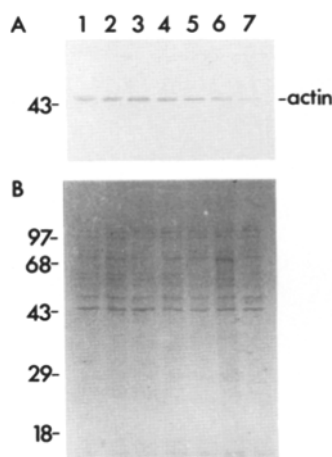


Figure 9. Total actin protein levels in γ -actin transfectants. 10 μg of total protein from γ 22, γ 156 and control transfected cells (transfected with the plasmid pUC-19) all at low confluence, was size fractionated by electrophoresis on a denaturing polyacrylamide gel. The protein was transferred to nitrocellulose and immunostained with an antibody to total actin (C4). The antibody was detected by ^{35}S -labeled IgG anti-mouse and subsequent autoradiography of the filter. A gel was run in parallel and stained with Coomassie blue. This was used to normalize the actin levels in case of loading errors. (A) Western blot immunostained with an antibody to actin, and (B) Coomassie blue stained gel. Lane 1, control; lane 2, γ 22-A2; lane 3, γ 22-A1; lane 4, γ 22-A3; lane 5, γ 156-A1; lane 6, γ 156-D1; and lane 7, γ 156-A6. Size markers (kD) were run with the gels and are indicated.

the γ -actin gene do influence the impact of the gene on cell morphology. The difference in impact of the transfected γ -actin gene constructs cannot be attributed to a difference in the expression levels of the genes in the different transfectant clones (Table I) since the second and third highest expressing γ 156 clones showed similar expression levels to the three highest expressing γ 22 clones. Therefore, the different cell morphologies observed are influenced by the presence or absence of different noncoding regions of the transfected γ -actin gene. Previous studies have highlighted the unusually high level of nucleotide sequence conservation present in both intron III and the 3'UTR of the γ -actin gene (Erba et al., 1986, 1988). That these conserved sequences are also unique to the γ -actin gene has suggested that they may contribute to isoform-specific properties of this gene (Erba et al., 1986, 1988). Our experiments indicate that, at least in a myoblast, these gene sequences are likely to convey functionally significant information to the cell.

The difference between γ 22 and γ 156 cells with respect to cell size (10-fold difference) and phenotype (long pseudopods versus none) could be considered as significant as that observed between β - and γ -actin gene transfectants. Schevzov et al. (1992) found only a sevenfold difference in surface area between the largest β -actin transfectant clone, β wt-13, and the smallest γ -actin transfectant clone, γ 33-B1. Thus, the role of noncoding gene regions may be at least as important as the differences between β - and γ -actin proteins in the manipulation of myoblast morphology by β - and γ -actin gene expression. Indeed, it is a formal possibility that all functional differences between the β - and γ -actin genes could be due to noncoding region sequences.

The 3' end of the γ -actin gene is informative with respect to the cell morphology of C2 myoblasts. First, the cell morphology of the γ 22 transfectants differed markedly to that of both the full length γ -actin gene (γ 33) and the γ 156 transfectants. In particular, γ 33 and γ 156 clones are smaller in surface area than control cells whereas γ 22 cells appear to be larger. Second, actin cables are poorly organized in γ 33 and

γ 156 clones in contrast to the clear actin cables in γ 22 cells. Finally, neither γ 33 nor γ 156 clones display the long pseudopods characteristic of the γ 22 cells. Since both γ 33 and γ 156 differ from γ 22 by the removal of the 3' end, we conclude that it is this region which determines the overall morphological impact of the γ -actin gene in these experiments.

The effects of the γ 156 gene on myoblast morphology were more extreme than observed for the γ 33 construct. The clone γ 156-A1 exhibited a very similar cell morphology to that of the highest expressing γ 33 clone γ 33- β 1 (Schevzov et al., 1992). Both these transfectants consisted of small (725 ± 255 and $763 \pm 119 \mu\text{m}^2$ respectively), elongated cells (Figure 6 and Schevzov et al., 1992) yet the expression levels of the transgene were 0.28 and 2.52, respectively. The γ 156 clones with expression levels of 0.68 and 1.18 (Table I) exhibited a much more severe phenotype with the cells showing almost no cytoplasm and surface areas of 327 ± 29 and $240 \pm 53 \mu\text{m}^2$ (Fig. 6). Although the overall cell morphology of transfectants was similar between the full length and intron III deletion constructs, the γ 156 cells showed a more extreme phenotype than γ 33 transfectants with the same human γ -actin gene expression levels. This suggests that intron III does not play a direct role in the definition of the resultant cell morphology but may act to amplify the effect of exogenous γ -actin gene expression by altering actin regulation. The total actin protein pool size was decreased in the γ 156 transfectants, differing from other γ -actin gene transfectants, γ 22 and γ 33, where the pool remained relatively constant (Table II and Lloyd et al., 1992). This suggests that intron III may play a role in the control of regulation of actin expression levels and in particular pool size. At this time, we cannot discriminate between the impact of γ 156 directly altering the actin pool or directly altering cell morphology which secondarily alters the actin pool via feedback regulation (Lloyd et al., 1992).

Possible Mechanisms for Influence of Noncoding Regions on Cell Morphology

A transfected gene may act at a number of different levels and in the absence of an understanding of the detailed mechanism, all possibilities must be considered. For example, the transfected copies of the gene may potentially bind factors which are normally regulating the endogenous copies of the actin genes. This is not easily reconciled with the observation that it is the removal of DNA sequences from the transfected gene that results in an altered phenotype. While complex scenarios involving the interaction of factors binding to different gene regions (some of which are deleted in the exogenous gene) cannot be ruled out, we argue that such a mechanism is not the most likely explanation of our results. Similarly, it may be that the mechanisms operating in these experiments involve processes about which we have no knowledge. There is enough known about actin gene regulation, however, to suggest mechanisms which are both reasonable and amenable to experimental evaluation.

We consider it most likely that our two transfected genes (γ 22 and γ 156) differ from each other and from the endogenous γ -actin gene in terms of the temporal and/or spatial metabolism of the γ -actin produced by these two genes. In essence, we argue that the mechanism of regulation of a gene product may influence the phenotypic impact of that gene product. This proposal first requires that the transfected gene pro-

duces a protein product and we cannot prove this because the human and mouse γ -actins are identical. Nevertheless, high level expression of the transfected gene's mRNA does correlate with a decrease in the β/γ protein ratio which suggests that the human gene does contribute γ -actin protein to the cell's actin pool. Second, this proposal requires that the γ -actin gene is capable of temporal and/or spatial regulation. Analysis of actin mRNA intracellular localization and accumulation through the cell cycle suggest that nonmuscle actin genes are indeed subject to such regulation.

Lawrence and Singer (1986) have proposed that actin mRNA is segregated to sites where actin filaments are rapidly polymerizing, the leading lamellae. Additionally, β -actin mRNA has been found to be preferentially localized in the leading edge of wounded fibroblasts (Hooek et al., 1991). This may indicate a specific subcellular localization of β -actin compared with γ -actin but as yet the comparable experiment with γ -actin mRNA has not been completed. Nevertheless, the nucleic acid sequence responsible for the peripheral localization of the β -actin mRNA has been located in the 3'UTR (Kislauskis and Singer, personal communication). It is possible that γ -actin has a sequence with similar function residing in its own 3'UTR. Therefore, in the γ 22 transfectants the exogenous γ -actin mRNA may no longer confer the correct localization information and the mRNA would be localized incorrectly. The subsequent synthesis of γ -actin protein could result in abnormal amounts of the protein in some regions of the cell.

The 3' end or intron III sequences may alternatively be involved in gene regulation of γ -actin through the cell cycle. Masibay et al. (1988) found that γ -actin mRNA and protein levels increased transiently during the G1 period of the cell cycle and therefore the relative expression of β to γ decreased during this time. This decrease in the β/γ ratio of mRNA levels during the G1 stage of the cell cycle was also observed by McCairns et al. (1984) after activation of human peripheral blood lymphocytes. If either of the noncoding regions of the γ -actin gene are involved in the regulation of the relative expression of β to γ during the cell cycle then the loss of these sequences would result in altered actin expression during the cell cycle. The altered relative expression of β to γ and/or total actin levels at different stages of the cell cycle may result in an altered impact of the gene on cell morphology.

A common feature of the cell phenotypes of the γ -actin gene transfectants, lacking either the 3' end or intron III, is the apparent perturbation of the plasma membrane organization resulting in aberrant cell morphology. The localization of β -actin mRNA and possible enrichment of β -actin protein at peripheral regions of motile cells may suggest a specific function for β -actin perhaps in the organization, remodeling and spreading of the cell membrane (Lawrence and Singer, 1986; Hooek et al., 1991). It is possible that the reduction of β - compared with γ -actin protein in the γ -actin transfectants (Table II) results to perturbations of the membrane organization due to reduced availability of β -actin at the periphery or replacement of β by γ in this location.

The functional importance of the actin multigene family may involve more than differences between the actin proteins. Previous studies have revealed that some intron and 3'UTR sequences in the human β - and γ -actin genes are isoform specific and highly conserved during evolution (Ng et

al., 1985; Yaffe et al., 1985; Erba et al., 1986, 1988). Our observations suggest that these gene regions may be of functional significance. This result raises the possibility that the different actin genes have been maintained during evolution not only on the basis of encoding protein variants but also for their unique properties of gene regulation.

We greatly appreciate the help given by Christine Smythe for the confocal microscope work and the generation and photography of these images. We wish to thank Dr. J. Lessard for the C4 actin antibody and Dr. J. C. Bulinski for the γ -actin antibody. Also thanks to Dr. R. Weinberger for technical assistance with the protein analysis. We would like to thank Professor P. Rowe, Drs. K. Esser, M. Hill, and Galina Schevzov for valuable advice and critical reading of the manuscript. We thank Dr. G. Schevzov for assistance in preparing the revised figures and Professor R. Singer for communicating his results before publication.

Peter Gunning is a National Health and Medical Research Council Senior Research Fellow. This work funded in part by a grant to Peter Gunning from the National Health and Medical Research Council.

Received for publication 30 June 1992 and in revised form 15 December 1992.

References

Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156-159.

Denhardt, D. T. 1966. A membrane-filter technique for the detection of complementary DNA. *Biochem. Biophys. Res. Commun.* 23:641-646.

DeNofrio, D., T. C. Hoock, and I. M. Herman. 1989. Functional sorting of actin isoforms in microvascular pericytes. *J. Cell Biol.* 109:191-202.

Erba, H. P., P. Gunning, and L. Kedes. 1986. Nucleotide sequences of the human γ cytoskeletal actin mRNA: anomalous evolution of vertebrate non-muscle actin genes. *Nucleic Acids Res.* 14:5275-5294.

Erba, H. P., R. Eddy, T. Shows, L. Kedes, and P. Gunning. 1988. Structure, chromosome location, and expression of the human γ -actin gene: differential evolution, location, and expression of the cytoskeletal β - and γ -actin genes. *Mol. Cell. Biol.* 8:1775-1789.

Feinberg, A. P., and B. Vogelstein. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* 132:6-13.

Gunning, P., E. Hardeman, R. Wade, P. Ponte, W. Bains, H. M. Blau, and L. Kedes. 1987. Differential patterns of transcript accumulation during hu-

man myogenesis. *Mol. Cell Biol.* 7:4100-4114.

Gunning, P., M. Gordon, R. Wade, R. Gahlmann, C.-S. Lin, and E. Hardeman. 1990. Differential control of tropomyosin mRNA levels during myogenesis suggests the existence of an isoform competition-autoregulatory compensation control mechanism. *Dev. Biol.* 138:443-453.

Hoock, T. C., P. M. Newcomb, and I. M. Herman. 1991. β actin and its mRNA are localized at the plasma membrane and the regions of moving cytoplasm during the cellular response to injury. *J. Cell Biol.* 112:653-664.

Lawrence, J. B., and R. H. Singer. 1986. Intracellular localization of messenger RNAs for cytoskeletal proteins. *Cell.* 45:407-415.

Lessard, J. L. 1988. Two monoclonal antibodies to actin: one muscle selective and one generally reactive. *Cell Motil. Cytoskeleton.* 10:349-362.

Lloyd, C., G. Schevzov, and P. Gunning. 1992. Transfection of nonmuscle β - and γ -actin genes into myoblasts elicits different feedback regulatory responses from endogenous genes. *J. Cell Biol.* 117:787-797.

Masibay, A. S., P. K. Qasba, D. N. Sengupta, G. P. Damewood, and T. Sreevalsan. 1988. Cell-cycle specific and serum-dependent expression of gamma-actin mRNA in swiss mouse 3T3 cells. *Mol. Cell. Biol.* 8:2288-2294.

McCairns, E., D. Fahey, G. E. O. Muscat, M. Murray, and P. B. Rowe. 1984. Changes in levels of actin and tubulin mRNAs upon the lectin activation of lymphocytes. *Mol. Cell Biol.* 4:1754-1760.

Ng, S.-Y., P. Gunning, R. Eddy, P. Ponte, J. Leavitt, T. Shows, and L. Kedes. 1985. Evolution of the functional human β -actin gene and its multipseudogene family: conservation of non-coding regions and chromosomal dispersion of pseudogenes. *Mol. Cell Biol.* 5:2720-2732.

Okayama, H., and P. Berg. 1983. A cDNA cloning vector that permits expression of cDNA inserts in mammalian cells. *Mol. Cell Biol.* 3:280-289.

Otey, C. A., M. H. Kalnoski, and J. C. Bulinski. 1987. Identification and quantification of actin isoforms in vertebrate cells and tissues. *J. Cell. Biochem.* 13:113-124.

Otey, C. A., M. H. Kalnoski, and J. C. Bulinski. 1988. Immunolocalization of muscle and nonmuscle isoforms of actin in myogenic cells and adult skeletal muscle. *Cell Motil. Cytoskeleton.* 9:337-348.

Pardo, J. V., M. F. Pittenger, and S. W. Craig. 1983. Subcellular sorting of isoactins: selective association of γ -actin with skeletal muscle mitochondria. *Cell.* 32:1093-1103.

Shevzov, G., C. Lloyd, and P. Gunning. 1992. High level expression of transfected β - and γ -actin genes differentially impacts on myoblast cytoarchitecture. *J. Cell Biol.* 117:775-785.

Vandekerckhove, J., and K. Weber. 1978. At least six different actins are expressed in a higher mammal: an analysis based on the amino acid sequence of the amino-terminal tryptic peptide. *J. Mol. Biol.* 126:783-802.

Vandekerckhove, J., and K. Weber. 1981. Actin typing on total cellular extracts: a highly sensitive protein-chemical procedure able to distinguish different actins. *Eur. J. Biochem.* 113:595-603.

Wilson, T., and A. R. Carlini. 1988. Three-dimensional imaging in confocal imaging systems with finite sized detectors. *J. Microsc.* 149:51-66.

Yaffe, D., U. Nudel, Y. Mayer, and S. Neuman. 1985. Highly conserved sequences in the 3' untranslated region of mRNAs coding for homologous proteins in distantly related species. *Nucleic Acids Res.* 13:3723-3737.