

# High Level Expression of Transfected $\beta$ - and $\gamma$ -Actin Genes Differentially Impacts on Myoblast Cytoarchitecture

Galina Schevzov, Catriona Lloyd, and Peter Gunning

Cell Biology Unit, Children's Medical Research Foundation, P.O. Box 61, Camperdown N.S.W. 2050, Australia

**Abstract.** The impact of the human  $\beta$ - and  $\gamma$ -actin genes on myoblast cytoarchitecture was examined by their stable transfection into mouse C2 myoblasts. Transfectant C2 clones expressing high levels of human  $\beta$ -actin displayed increases in cell surface area. In contrast, C2 clones with high levels of human  $\gamma$ -actin expression showed decreases in cell surface area. The changes in cell morphology were accompanied by changes in actin stress-fiber organization. The  $\beta$ -actin transfectants displayed well-defined filamentous organization of actin; whereas the  $\gamma$ -actin transfectants displayed a more diffuse organization of the actin cables. The role of the  $\beta$ -actin protein in generating the enlarged cell phenotype was examined by transfecting a mutant form of the human  $\beta$ -actin gene. Transfectant cells were shown to incorporate the aberrant actin

protein into stress-fiber-like structures. High level expression of the mutant  $\beta$ -actin produced decreases in cell surface area and disruption of the actin microfilament network similar to that seen with transfection of the  $\gamma$ -actin gene. In contrast, transfection of another mutant form of the  $\beta$ -actin gene which encodes an unstable protein had no impact on cell morphology or cytoarchitecture. These results strongly suggest that it is the nature of the encoded protein that determines the morphological response of the cell.

We conclude that the relative gene expression of  $\beta$ - and  $\gamma$ -actin is of relevance to the control of myoblast cytoarchitecture. In particular, we conclude that the  $\beta$ - and  $\gamma$ -actin genes encode functionally distinct cytoarchitectural information.

**A**CTIN is one of the most abundant and highly conserved proteins in nature. Although its function is relatively well defined in the contractile apparatus of muscle cells, the functions of actin in nonmuscle cells has not yet been unequivocally established. Actin is implicated to play a fundamental role in a number of cellular activities such as maintaining cellular architecture, cell motility, and cytokinesis. However, it is only in yeast that a genetic demonstration of a role for actin in the organization, assembly, and function of the yeast cell surface has been provided (Shortle et al., 1984; Novick and Botstein, 1985).

In mammals, at least six different actin isoforms are known, each encoded by a separate gene and they differ by <10% of their amino acid sequence (Erba et al., 1988; Gunning et al., 1983; Hamada et al., 1982; Ng et al., 1985; Vandekerckhove and Weber, 1978). The cytoplasmic  $\beta$ - and  $\gamma$ -actin isoforms predominate in avian and mammalian non-muscle cells. The two smooth muscle actins  $\alpha$ - and  $\gamma$ - are found primarily in smooth muscle, whereas  $\alpha$ -cardiac and  $\alpha$ -skeletal actin are expressed in striated cardiac and skeletal muscle, respectively. Although the biological significance of multiple actin isoforms is not well understood, certain observations suggest functional differences among them (Vandekerckhove and Weber, 1978). Firstly, the expression of actins is tissue-specific and developmentally regulated (Bains et al., 1984; Erba et al., 1988; Garrels and Gibson, 1976;

McHugh and Lessard, 1988; Paterson and Eldridge, 1984; Shani et al., 1981; Sawtell and Lessard, 1989; Eppenberger-Eberhardt et al., 1990). Secondly, the existence of multiple actin isoforms within tissues and even within a single cell suggests functional differences (Bravo et al., 1981). Thirdly, the presence of a strict control of tissue-specific ratios of cytoplasmic  $\beta$ - and  $\gamma$ -actins also suggests that  $\beta$ - and  $\gamma$ -actin may have distinct biological roles (Otey et al., 1987; Erba et al., 1988).

Even though the observations listed provide a compelling argument that different functions exist for these isoforms, there is still a considerable lack of experimental data to prove conclusively that the cell can distinguish between the actin isoforms. Recent experiments have shown subcellular sorting of actin isoforms (Craig and Pardo, 1983; Otey et al., 1988; De Nofrio et al., 1989), which proves that at some level, a cell can discriminate between actin isoforms. However, the observation of preferential subcellular localization of actin isoforms does not necessarily prove that the isoforms are functionally distinct since two isoforms may be differentially localized and yet still be functionally equivalent. Indeed, mouse nonmuscle L cells were unable to discriminate between  $\alpha$ -cardiac actin and their endogenous nonmuscle actins (Gunning et al., 1984). McKenna et al. (1985) similarly observed that both chicken fibroblasts and cardiac myocytes failed to discriminate between muscle and nonmuscle actins.

Thus, if the actin isoforms are functionally distinct, one must conclude that the differences are too subtle to be detected by previously used assays.

The aim of this work was to establish a model system to enable us to evaluate the functional equivalence of nonmuscle actin genes. This system involved the transfection of human  $\beta$ - and  $\gamma$ -actin genes into mouse C2 myoblasts. We succeeded in altering the relative expressions of  $\beta$ - and  $\gamma$ -actin which elicited different changes in myoblast morphology and architecture. Thus, we conclude that these two genes encode different architectural information.

## Materials and Methods

### 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 DMEM medium (Gibco Laboratories, Grand Island, NY) 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 transfected clones was performed as described by Graham and van der Eb (1973). In brief, C2 cells ( $4-6 \times 10^5$  cells) seeded on 100-mm dishes were transfected with 10  $\mu$ g of cesium chloride-purified plasmid DNA by the calcium phosphate precipitation technique. Selection was started 24 h later with 450  $\mu$ g G418/ml medium (G418-geneticin; Sigma Chemical Company, St. Louis, MO). As colonies were formed they were seeded and cultured in 24-well plates followed by 60-mm dishes and finally grown in 100-mm dishes. The constructs used for transfection included (a) the human wild type  $\beta$ -actin gene; (b) the human single mutant form of the  $\beta$ -actin gene; (c) the human triple mutant form of the  $\beta$ -actin gene, all originally isolated as 14 kilobase EcoRI (Leavitt et al., 1984) fragments and subcloned into pSV2-neo (Southern and Berg, 1982) which contain the recombinant gene conferring resistance to the neomycin analogue G418; (d) the human wild type  $\gamma$ -actin gene, a BamHI fragment subcloned into pBR322 (Erba et al., 1988), which was cotransfected with 1  $\mu$ g of pSV2-neo; and (e) the plasmid pUC18 as a control.

### RNA Isolation

Total cellular RNA was isolated from 30% confluent human wild type  $\beta$ , triple mutant and single mutant  $\beta$ -actin transfectants by the method described by Chomczynski and Sacchi (1987). Total RNA from 30% confluent human wild type  $\gamma$ -actin and pUC transfectants was isolated by the phenol-chloroform method described by Palmiter (1974).

### DNA Probes

Probes that hybridize equally well to the human and mouse  $\beta$ -actin mRNA and to the human and mouse  $\gamma$ -actin mRNA were generated by polymerase chain reaction amplification of regions of the  $\beta$ - and  $\gamma$ -actin 3' untranslated regions (3'UTRs) which are identical between mouse and human and unique to each isoform. These regions correspond to bp 178-287 of the human  $\beta$ -actin 3'UTR and to bp 60-183 of the mouse  $\gamma$ -actin 3'UTR. These probes were used to measure the total (human + mouse)  $\beta$ - and  $\gamma$ -actin mRNA levels in the transfectant cells and by comparison with control C2 cells, which have a  $\beta/\gamma$  mRNA ratio of 2.0 (Erba et al., 1988), we calculated the mRNA ratio in the transfectants. Probes specific for the human  $\beta$ - and  $\gamma$ -actin mRNAs and which do not cross-hybridize with the mouse mRNAs, pHF $\beta$ A-3'UT-HH and pHF $\gamma$ A-3'UT-HX, have been previously characterized (Ng et al., 1985; Erba et al., 1986). These DNA fragments were used to measure the level of expression of the transfected human gene in all the mouse C2 clones.

### Northern Blots

Total RNA was denatured and electrophoresed on 1% agarose gels containing 2.2 M formaldehyde and transferred to Hybond N (Amersham Australia) as described in Maniatis et al. (1982). Probes were labeled by the random priming method (Feinberg and Vogelstein, 1983) and hybridized to

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

RNA blots at 10<sup>9</sup> dpm/ $\mu$ g of DNA in a solution containing 4 $\times$  SSC (1 $\times$  SSC is 0.15 M NaCl, 0.015 M sodium citrate), 50 mM NaH<sub>2</sub>PO<sub>4</sub>, 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 at 65°C for 2 h in 0.5 $\times$  SSC, 0.1% SDS. Filters were exposed to Kodak XAR film for 1-3 d. To verify that equivalent amounts of RNA were transferred, the RNA blots were hybridized to an 18S specific ribosomal RNA oligonucleotide probe under conditions of probe excess and washed at 55°C in 4 $\times$  SSC, 0.1% SDS. Levels of mRNAs were quantitated by densitometry as described (Gunning et al., 1990).

### Immunofluorescence Staining

A total of 5,000 cells were seeded on collagen-coated (Calf Skin Collagen, Calbiochem Austr., Alexandria, N.S.W.) glass slides for 24 h before fixing and permeabilizing in 1% formaldehyde for 20 min followed by 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 with 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 b/w paper.

### Antibodies

The rabbit antibody which specifically recognizes the  $\beta_{sm}$ -actin protein was provided by Dr. U. Aebi (University of Basel, Switzerland) (Leavitt et al., 1987). The  $\gamma$ -actin rabbit antibody was provided by Dr. J. C. Bulinski (Columbia University, New York) (Otey et al., 1986). The C4 monoclonal actin antibody was provided by Dr. J. Lessard (Children's Hospital Research Foundation, Cincinnati, OH) (Lessard, 1988). The goat anti-mouse Igs (G+L) FITC and goat anti-rabbit IgG rhodamine were purchased from Tago Inc. (Medos Co. Pty. Ltd., Burwood, Australia).

### Isoelectric Focusing and Immunoblotting

Transfected cells grown in 100-mm dishes were trypsinized, resuspended in PBS, and centrifuged for 5 min. After removal of the supernatant solution, the cell pellets were stored at -80°C. Before use the cell pellets were thawed and resuspended in IEF sample buffer (9.5 M urea, 5%  $\beta$ -mercaptoethanol, 2% CHAPS (3-[(3-Cholamidopropyl) dimethylammonio]-1-propane-sulfonate, Boehringer Mannheim Australia Pty. Ltd., North Ryde N.S.W.), in a ratio of 150  $\mu$ l of buffer per 10  $\mu$ l cell pellet. IEF electrophoresis and blotting were performed as described by Otey et al. (1987) except for minor alterations. In brief, 0.5-mm-thick gels were poured using the LKB casting gel mould and gelbond film as a support (Pharmacia, Australia Pty. Ltd., North Ryde, N.S.W.). Triton X-100 in the gel mixture was substituted by CHAPS, as this detergent allows adhesion of the gel to the gelbond. Polymerization took place in 2 h followed by a prerun on a flat bed electrophoresis unit (LKB Ultraphor Electrofocusing Unit) at 200 V for 1 h, 10  $\mu$ l samples were loaded and run at 350 V for 16 h. Gels were either protein stained or immunostained as described by Otey et al., (1987). Before the preparation of the sandwich for transfer on a Hoefer Transfer apparatus (Edwards Instruments Co., Narellan, N.S.W.), a dry piece of nitrocellulose was placed on the gel and then carefully peeled off with the gel bound to the nitrocellulose. Transfer was at 20 V for 16 h. Immunostaining of the Western blot was carried out with a C4 mAb, 10  $\mu$ g/ml, (kindly supplied by Dr. J. L. Lessard, Children's Hospital Research Foundation, Cincinnati, OH) which reacts with all actin isoforms. The bands on the immunoblots were quantitated by scanning and integrating the volume of the entire band with a Molecular Dynamics Model 300 series computing densitometer.

### Confocal Microscopic Analysis

Cell surface area and depth were determined with the aid of a Confocal Laser Scanning Microscope (Wild Leitz Instruments, Heidelberg, Germany). Surface area determinations were calculated as follows. (a) Anti- $\gamma$  immunofluorescence stained cells were XY sectioned. (b) The XY images were manipulated so that the pixel intensity of the fluorescence that covered the entire surface of the cells was maximal compared to the 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. (c) The total surface area (in  $\mu$ m<sup>2</sup>)

covered by the cells in a microscopic field was calculated by multiplying the pixel size of  $1.9179 \mu\text{m}^2$  (with  $10\times$  objective 0.45 NA) by the number of pixels of maximal intensity (obtained from the intensity histogram). (d) Finally the surface area per cell was determined by dividing the total surface area covered by the number of cells in the field. A total of six different fields (containing 6–40 cells) for each transfected clone were examined. Cell depth measurements were determined as follows. (a) C4 actin antibody immunofluorescence stained cells were XZ serially sectioned through the nuclei. (b) A total of 15 XZ images per cell were subsequently compiled by using a simulated fluorescence process, previously described in Van der Voort et al. (1989). (c) The point of greatest thickness was thus taken as the cell thickness ( $\mu\text{m}$ ). These means were statistically analyzed by the *t* test and considered to be significantly different when the two-sided *P* value was below 0.05.

## Results

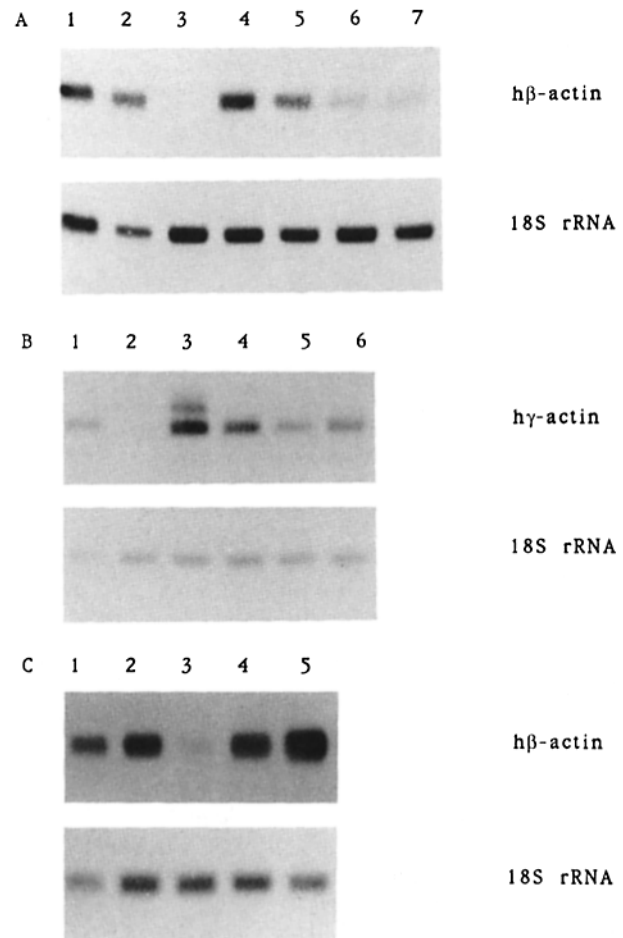
### Strategy

Mouse C2 myoblasts are muscle precursor cells which contain microfilaments composed of nonmuscle isoforms of the contractile proteins. These cells express the two nonmuscle actins,  $\beta$ - and  $\gamma$ -, in the ratio of 2:1, respectively (Erba et al., 1988). The aim of our initial experiment was to perturb this ratio via transfection of the human  $\beta$ - and  $\gamma$ -actin genes into these cells. The human  $\beta$ - and  $\gamma$ -actin proteins differ by only four conservative amino acids and each of the isoforms are identical between human and mouse (Vandekerckhove and Weber, 1978; Erba et al., 1986; Ng et al., 1985). Because the 3'UTRs of the  $\beta$ - and  $\gamma$ -actin mRNAs are completely different (Erba et al., 1986) and not identical between human and mouse for each isoform, we could detect expression of the human genes using human-specific 3'UTR DNA probes (Ng et al., 1985; Erba et al., 1988). The experimental protocol consisted of transfecting the human genes into C2 cells and analyzing all the resulting cell clones for expression of the human genes. All highly expressing clones were then analyzed for the impact on the relative steady state levels of  $\beta$ - and  $\gamma$ -actin mRNA and protein and on cell cytoarchitecture.

### Stable Transfection of Mouse Myogenic C2 Cells

The C2 cell line was initially transfected with the selectable marker pSV2-neo (Southern and Berg, 1982) plus one of the two different human nonmuscle actin genes or with the plasmid pUC18 as a control. In addition to the  $\beta$ - and  $\gamma$ -actin genes, we also transfected a mutant form of  $\beta$ -actin ( $\beta_{\text{m}}$ ) which encodes an unstable protein (Lin et al., 1985). We reasoned that the  $\beta_{\text{m}}$ -actin gene would serve as a control for elevated levels of actin transcription and mRNA content in the absence of production of a functional protein. Transfection of pUC18, the  $\gamma$ -actin gene and the  $\beta_{\text{m}}$ -actin gene each resulted in 20–30 G418-resistant colonies. However, introduction of the normal  $\beta$ -actin gene resulted in a substantial reduction, by  $\sim 50\%$ , in the number of surviving cell clones (13 clones). In addition, we found that the  $\beta_{\text{m}}$  and  $\gamma$ -actin transfected cells displayed a wide range of transfectant gene expression levels; whereas the normal  $\beta$ -actin gene transfectants were skewed to favor low levels of expression (not shown). This correlates with the lower frequency of colony formation with the  $\beta$ -actin gene and suggests that high level expression of this gene in C2 cells may generate a lethal phenotype.

All clones were analyzed as follows. Cells were seeded at low confluence and allowed to grow to only 30% confluence,



**Figure 1.** Expression of human cytoplasmic  $\beta$ - and  $\gamma$ -nonmuscle actin gene stably transfected into mouse myogenic C2 cells. Northern blot analysis of total RNA isolated from (A) lane 1,  $\beta_{\text{m}}-33$ ; lane 2, human myoblast; lane 3, pUC-A transfected clone; lanes 4–7,  $\beta_{\text{wt}}-11$ ,  $\beta_{\text{wt}}-13$ ,  $\beta_{\text{wt}}-12$ ,  $\beta_{\text{wt}}-25$ . (B) lane 1, human myoblast; lane 2, pUC-pool transfected clone; lanes 3–6,  $\gamma_{33}-B1$ ,  $\gamma_{58}-B2$ ,  $\gamma_{33}-A5$ , and  $\gamma_{33}-B5$ . (C) lane 1, human myoblast; lane 2,  $\beta_{\text{m}}-33$ ; lanes 3–5,  $\beta_{\text{sm}}-47$ ,  $\beta_{\text{sm}}-72$ ,  $\beta_{\text{sm}}-22$ . RNA electrophoresed through an agarose-formaldehyde gel and transferred to a nylon membrane was hybridized to human specific DNA probes to (A and C)  $\beta$ -actin (pHF $\beta$ A-3'UT-HH) and (B)  $\gamma$ -actin (pHF $\gamma$ A-3'UT-HX). The Northern blots were also hybridized to an 18S specific ribosomal RNA probe in order to verify the amount of RNA present.

this was to ensure that the cells were in log phase growth. RNA was isolated and hybridized to the human-specific  $\beta$ - and  $\gamma$ -actin probes. Fig. 1 shows the result of such hybridization to the highest expressing clones. The highest levels of human  $\beta$ -actin mRNA were observed in clones  $\beta_{\text{wt}}-11$  and  $\beta_{\text{wt}}-13$  which expressed at 61 and 38%, respectively, of the level observed in human myoblasts (Table I). The remaining clones either did not express the exogenous gene or were very low expressers like clones  $\beta_{\text{wt}}-12$  and  $\beta_{\text{wt}}-25$  which expressed at 16 and 10%, respectively. The  $\beta_{\text{m}}$  transfectant, clone  $\beta_{\text{m}}-33$ , expressed the introduced construct at 77% of the level of expression of the endogenous  $\beta$ -actin gene in human myoblasts (Table I), comparable to that of the highest expressing  $\beta$ -actin clone  $\beta_{\text{wt}}-11$ , and was therefore selected as a suitable control clone. The highest expressing  $\gamma$ -actin transfectants  $\gamma_{33}-B1$ ,  $\gamma_{33}-B5$ , and  $\gamma_{33}-A5$  accumulated the human  $\gamma$ -actin mRNA at 252, 102, and 79%, respectively, of

**Table I. Comparing the  $\beta/\gamma$  Ratio of the Actin Transfectants**

Clone	Human actin mRNA level*	Direct $\beta/\gamma$ mRNA <sup>‡</sup>	Indirect $\beta/\gamma$ mRNA <sup>§</sup>	$\beta/\gamma$ protein
C2	—	2.0	2.0	2.0
pUC-pool	—	2.0	2.0	2.0
$\beta_{\text{m}}-33$	77	3.0	2.7	2.0
$\beta_{\text{wt}}-25$	10	2.2	2.8	2.2
$\beta_{\text{wt}}-12$	16	1.9	2.9	2.3
$\beta_{\text{wt}}-13$	38	3.6	4.6	2.6
$\beta_{\text{wt}}-11$	61	4.4	5.2	2.8
$\gamma_{33}-A5$	79	ND	0.8	1.1
$\gamma_{33}-B5$	102	0.7	0.8	0.8
$\gamma_{58}-B2$	149	0.6	0.5	0.7
$\gamma_{33}-B1$	252	0.3	0.3	0.3

\* Values expressed as a percentage of the level of expression of the relevant isoform in human myoblast, which was set to 100%.

<sup>‡</sup> The direct  $\beta/\gamma$  mRNA ratios were determined using the total  $\beta$  and total  $\gamma$ -actin DNA probes (see Materials and Methods).

<sup>§</sup> The indirect  $\beta/\gamma$  mRNA ratios were determined by the addition of the independently determined  $\beta$ - and  $\gamma$ -actin transcript levels from both human and mouse genes (Lloyd et al., 1992). Values expressed as arbitrary units relative to the level found in control cells where  $\beta$ - and  $\gamma$ -actin mRNA levels were set to 2 and 1, respectively, after converting them to mouse equivalents. Since actin in human myoblast is 70% the level of actin found in mouse C2 myoblast the human values were multiplied by a factor of 0.7.

that in human myoblasts (Table I). Clone  $\gamma_{33}-B1$  also displayed a minor RNA species migrating at 2.5 kb (Fig. 1 B). Hybridization of parallel panels with  $\gamma$ -actin intron probes demonstrated that this is an incompletely processed  $\gamma$ -actin transcript containing intron 3 (data not shown). Since intron 3 contains inframe stop codons, no actin protein is produced from this transcript.

We also transfected the C2 cells with a form of the  $\gamma$ -actin gene containing 3 kb of the 3' flanking sequence ( $\gamma_{58}$ ) instead of the 5 kb present in the original form of the gene ( $\gamma_{33}$ ). The highest expressing clone from this transfection, clone  $\gamma_{58}-B2$ , expressing the  $\gamma$ -actin mRNA at 149% of the level found in human myoblast (Fig. 1 B lane 4 and Table I) was chosen for further detail analysis.

### Expression of Transfected Nonmuscle Actin Genes Alters the $\beta/\gamma$ Ratio

The total  $\beta/\gamma$  actin steady state mRNA ratio was determined in transfectants which expressed the human genes at a significant level. We measured the total  $\beta$ -actin mRNA level in transfected cells relative to that in pooled control pUC-transfected cells using a  $\beta$ -actin 3'UTR probe which hybridizes equally well to the human and mouse mRNAs. Total  $\gamma$ -actin mRNA was measured similarly using a  $\gamma$ -actin 3'UTR probe which hybridizes equally to the human and mouse mRNAs. Since the  $\beta/\gamma$  mRNA ratio is 2:1 in both C2 cells (Erba et al., 1988) and the pooled pUC-transfectants (Table I), we could calculate the absolute mRNA ratio in the various transfectants. To ensure the accuracy of these values, we measured the same ratio by a second method. Individual levels of mouse  $\beta$ - and  $\gamma$ -actin mRNA relative to pooled pUC-transfectants and human  $\beta$ - and human  $\gamma$ -actin mRNA relative to a human myoblast standard were determined using isoform and species-specific cDNA probes, (see Lloyd et al., 1992 for more detail). We determined that the level of actin

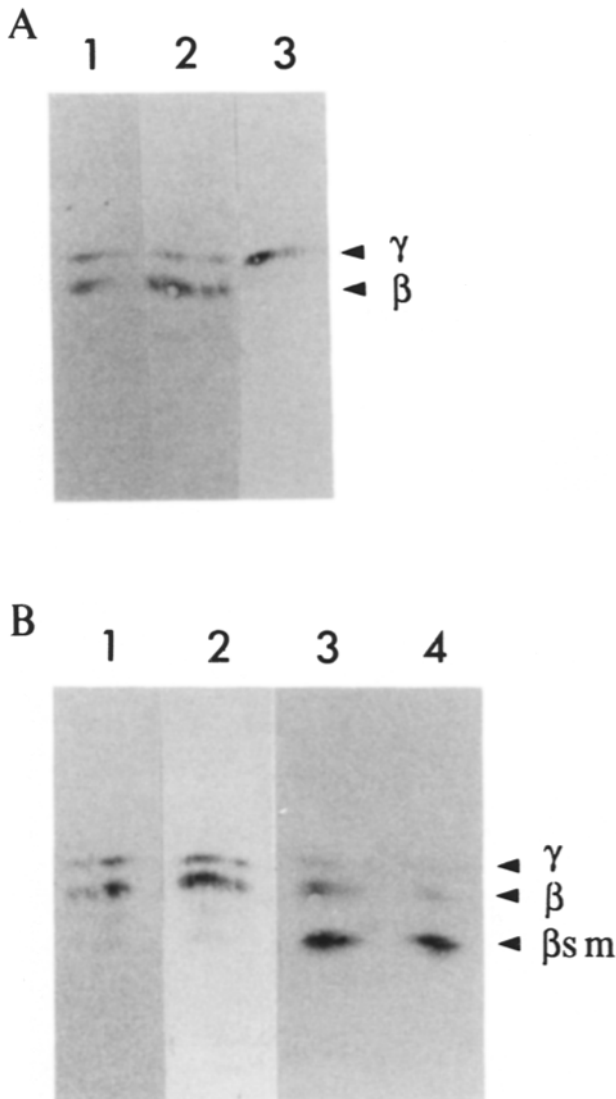
mRNA in our human myoblast standard was 70% of that present in the mouse C2 pooled pUC transfectants. This value was utilized to convert the human transcript levels into "mouse equivalents" which then provided us with a second independent measurement of the  $\beta/\gamma$  mRNA ratio in our transfectant clones. There was good agreement between  $\beta/\gamma$  values when measured by the two independent methods.

High level expression of both the human  $\beta$ - and  $\gamma$ -actin genes in C2 cell transfectants resulted in changes in the relative expression of  $\beta$ - to  $\gamma$ -actin compared with that in control cells. In the  $\beta_{\text{wt}}-11$  cells, the  $\beta/\gamma$  mRNA ratio was increased from 2.0 in C2 cells to 4.4 and 5.2 as determined with the two independent measurements; whereas the  $\gamma_{33}-B1$  cells showed a marked decrease in the ratio to 0.3 (Table I). Examination of other clones revealed that increasing expression of the human  $\beta$ -actin gene resulted in an increase in the  $\beta/\gamma$  mRNA ratio. Conversely, the  $\gamma$ -transfectants showed a progressive decrease in the  $\beta/\gamma$  mRNA ratio with increasing expression of the exogenous  $\gamma$ -actin gene. Since the determination of the  $\beta/\gamma$  mRNA ratios by two independent methods results in similar trends, we conclude that transfection of the human  $\beta$ - and  $\gamma$ -actin genes has allowed us to generate cells with altered relative expression of  $\beta$ - and  $\gamma$ -actin.

The changes in the relative expression of  $\beta$ - and  $\gamma$ -actin genes also lead to changes in the relative accumulation of  $\beta$ - and  $\gamma$ -actin proteins. The total  $\beta:\gamma$  protein ratio was estimated from IEF immunoblots probed with a total actin antibody. Fig. 2 (A) shows an immunoblot of actin isoforms present in the highest expressing transfected clones  $\beta_{\text{m}}-33$ ,  $\beta_{\text{wt}}-11$ , and  $\gamma_{33}-B1$ . Although the IEF immunoblots showed high level resolution of the actin bands, uneven distribution of the protein occurred. Due to this, quantitation of the immunoblot was performed by integrating the volume of the entire band using a laser computing densitometer (Molecular Dynamic Model 300). The estimated  $\beta/\gamma$  protein ratios parallel the changes seen in the mRNA ratios (Table I). Thus, increasing expression of the human  $\beta$ -actin gene led to an increase in the  $\beta/\gamma$  ratio from 2.0 to 2.8. In contrast, an increase in human  $\gamma$ -actin gene expression resulted in a decrease in the  $\beta/\gamma$  protein ratio to 0.3 (Table I). The levels of  $\beta$ - and  $\gamma$ -actin in the control  $\beta_{\text{m}}-33$  were identical to that seen in normal C2 cells and in pUC18 transfected C2 cells (Table I). Thus, we have established a model system of C2 myoblasts with altered relative expression of the nonmuscle actin genes.

### Elevated Expression of the Nonmuscle Actins Results in Differential Changes in Cell Morphology and Cytoarchitecture

The availability of C2 cells which express the human  $\beta$ - and  $\gamma$ -actin genes at similar levels allows us to evaluate the impact of these two genes on both the morphology and cytoarchitecture of these transfectants. Firstly, the morphological changes in these transfectants were assayed by determining the areas of the cell in contact with the substratum and the thickness of the cells. Measurements of C2 cells and pooled and individual clones cotransfected with pUC18 plus SV2neo are shown in Table II. The surface areas of these control cells do show variations and range from  $1,146 \pm 95$  to  $1,818 \pm 143 \mu\text{m}^2$ . These observed surface area variations may possibly be due to intrinsic clonal variation. The surface area of the  $\beta_{\text{m}}-33$  cells was very similar to that observed for the



**Figure 2.** Relative amounts of the cytoplasmic actins in transfected clones. IEF gels were immunoblotted with an actin-specific antibody, C4, as described in Materials and Methods. (A) Lane 1 corresponds to control cells  $\beta_{im-33}$ ; lane 2,  $\beta_{wt-11}$ ; and lane 3,  $\gamma_{33-B1}$ . (B) lane 1,  $\beta_{im-33}$ ; lane 2,  $\beta_{sm-47}$ ; lane 3,  $\beta_{sm-72}$ ; and lane 4,  $\beta_{sm-22}$ . The positions of  $\beta$ - and  $\gamma$ -actin are indicated. Note the trace level of staining in the  $\beta_{sm}$  position in control cells reflects low level expression of muscle  $\alpha$ -actin which has a similar isoelectric point to that of the  $\beta_{sm}$  protein.

C2 and pUC-pooled cells (Table II). Thus, high level expression of the  $\beta$ -actin gene in the absence of production of a functional protein does not impact on cell surface area.

The expression of the human  $\beta$ - and  $\gamma$ -actin genes had opposite effects on C2 cell surface area. High level expression of the human  $\beta$ -actin gene resulted in a twofold increase in the average surface area as seen in both highly expressing clones  $\beta_{wt-13}$  and  $\beta_{wt-11}$ ,  $3,650 \pm 676$  and  $3,545 \pm 180 \mu m^2$ , respectively (Table II). In contrast, high level expression of the human  $\gamma$ -actin gene resulted in a significant decrease in the surface area to  $763 \pm 119 \mu m^2$  in clone  $\gamma_{33-B1}$  (Table II). Analysis of low expressing  $\beta$ -actin transfectants revealed a more heterogeneous response. The  $\beta_{wt-25}$  clone

**Table II. Determination of Surface Area and Cell Thickness of C2 Transfectants**

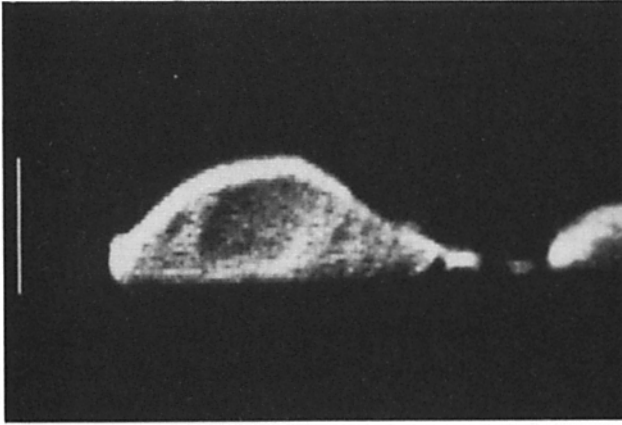
Clone	Human actin mRNA level <sup>  </sup>	Surface area	Cell thickness
		$\mu m^2$	$\mu m$
C2	—	$1,327 \pm 41$	$7.0 \pm 0.8$
pUC-pool	—	$1,332 \pm 205$	$7.4 \pm 1.0$
pUC-A	—	$1,146 \pm 95^\ddagger$	$7.5 \pm 0.7$
pUC-B	—	$1,587 \pm 171^\ddagger$	$7.3 \pm 0.9$
pUC-C	—	$1,818 \pm 143^*$	$7.7 \pm 0.9$
$\beta_{im-33}$	77	$1,332 \pm 106$	$7.6 \pm 1.5$
$\beta_{wt-25}$	10	$1,938 \pm 129^*$	$8.7 \pm 1.2^*$
$\beta_{wt-12}$	16	$1,338 \pm 353$	$10 \pm 1.0^*$
$\beta_{wt-13}$	38	$3,650 \pm 676^*$	$10.3 \pm 1.5^*$
$\beta_{wt-11}$	61	$3,545 \pm 180^*$	$9.9 \pm 1.3^*$
$\gamma_{33-A5}$	79	$1,028 \pm 205^\ddagger$	$7.9 \pm 1.2$
$\gamma_{33-B5}$	102	$1,083 \pm 209^\ddagger$	$8.1 \pm 1.3^\ddagger$
$\gamma_{38-B2}$	149	$1,109 \pm 180^\ddagger$	$8.8 \pm 1.8^\ddagger$
$\gamma_{33-B1}$	252	$763 \pm 119^*$	$7.9 \pm 1.3$
$\beta_{sm-47}$	5	$1,361 \pm 205$	$9.1 \pm 1.7^*$
$\beta_{sm-62}$	45	$1,224 \pm 155$	$10.5 \pm 1.5^*$
$\beta_{sm-72}$	69	$1,037 \pm 132^\ddagger$	$7.6 \pm 0.9$
$\beta_{sm-22}$	173	$752 \pm 63^*$	$11.3 \pm 2.2^*$

Values for surface area and cell thickness are expressed as means  $\pm$  SD. The significance of differences between the different clones in comparison with the  $\beta_{im-33}$  cells was estimated using the *t* test. The symbol \* denotes  $P < 0.0005$ ,  $^\ddagger P < 0.005$ , and  $^\S P < 0.025$ . Absence of a symbol indicates that the value is not significantly different to  $\beta_{im-33}$ . Note that the same results were obtained when either the C2 cells or pUC-pool were used as the control for comparison.

<sup>||</sup> Values expressed relative to the level of expression of the appropriate isoform in human myoblast.

showed a significant increase in the surface area whereas  $\beta_{wt-12}$  was indistinguishable from the controls. In the case of the  $\gamma$ -transfectants, the other high expressors,  $\gamma_{33-B5}$ ,  $\gamma_{33-A5}$ , and  $\gamma_{38-B2}$ , showed a significant decrease in the surface area but the effect was less severe than that seen in  $\gamma_{33-B1}$ . It is apparent that there is not a simple linear relationship between the expression level of the transfected gene and cell surface area (Table II). Thus, while actin expression can influence the determination of cell surface area, it is most likely that other cell components such as actin-binding proteins also contribute to this process. Finally, it is important to note that although variation in the surface area of the control cells does exist, the high expressing  $\beta$ -actin transfectants fall into a subset which does not overlap the surface areas of all the high expressing  $\gamma$ -actin transfectants. The consistency of these observations rules out the possibility that the results represent random clonal variation.

The substantial alteration in surface area of these cells raised the question of whether the thickness of the cells had been compromised to compensate for changes in the cell surface area. The cells were examined using the XZ scanning mode on the confocal microscope. Serial XZ optical sections through the nucleus of a single cell were made and a simulated fluorescence projection was generated. Fig. 3 shows a typical XZ scan. Measurements of the point of greatest thickness were made on cells from each clone. We found that the  $\beta$ -actin transfectant cells were consistently thicker than the  $\beta_{im-33}$  and pUC controls whereas the  $\gamma$ -transfectants



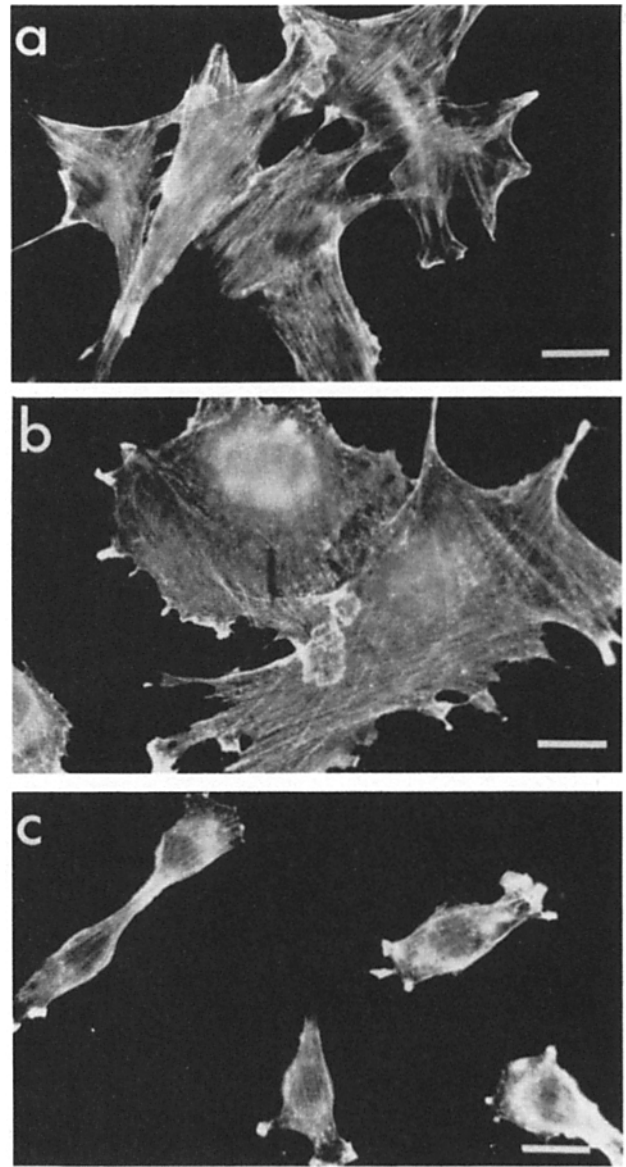
**Figure 3.** Cross sectional scanning of a C2 transfectant. C4 actin antibody-stained C2 transfectant was serially scanned on the XZ axis with a confocal laser scanning microscope through the nucleus and the thickness of the cell was determined. Photo shows a simulated fluorescence projection. Bar, 10  $\mu\text{m}$ .

were similar to the controls with the exception of clone  $\gamma_{58}$ -B2 (Table II). We conclude that the volume of the cells expressing the human  $\beta$ -actin gene has increased; whereas that of the  $\gamma$ -actin transfectants has decreased. This clearly demonstrates that C2 cell volume is not fixed in an absolute manner.

To determine whether any cytoarchitectural changes had occurred in the actin transfectants we initially visualized the actin microfilament network of only the highest expressing clones. Fig. 4 shows typical fields of  $\beta_{\text{wt}}-33$ ,  $\beta_{\text{wt}}-11$ , and  $\gamma_{33}$ -B1 cells stained with a total actin antibody. The  $\beta_{\text{wt}}-11$  cells contained well-defined stress-fibers (Fig. 4 *b*), whereas in the case of the  $\gamma_{33}$ -B1 cells, a diffuse organization of the actin microfilament network was observed (Fig. 4 *c*). In addition, the  $\gamma_{33}$ -B1 cells displayed intense deposits of actin at their periphery (Fig. 4 *c*). This suggested that actin organization is altered by the transfection of  $\beta$ - and  $\gamma$ -actin genes. This alteration in actin organization is not a function of the use of a particular actin antibody since the same results were obtained using a  $\gamma$ -actin-specific antibody (Fig. 5).

The availability of a  $\gamma$ -actin-specific antibody, previously characterized (Otey et al., 1986), allowed us to specifically visualize this isoform in the transfected cells. Fig. 5 (*a-c*) shows pUC pool, pUC-A, and  $\beta_{\text{wt}}-33$  cells stained with the  $\gamma$ -actin antibody. All control clones showed well-defined actin cables. The organization of the actin microfilament network was then visualized in the  $\beta$ -actin transfectants. Fig. 5 (*d-f*) shows  $\beta_{\text{wt}}-12$ ,  $\beta_{\text{wt}}-13$ , and  $\beta_{\text{wt}}-11$  stained with the  $\gamma$ -actin antibody. All  $\beta$ -transfectants had well organized actin cables with the two highest expressing clones  $\beta_{\text{wt}}-13$  and  $\beta_{\text{wt}}-11$  showing a dense meshwork of actin cables (Fig. 5, *e* and *f*). In contrast, it was difficult to see actin stress fibers in the  $\gamma$ -actin transfectants, possibly due to the high background of disorganized actin in these cells (Fig. 5, *g-i*). We also observed that clones  $\gamma_{33}$ -B5 and  $\gamma_{58}$ -B2 also showed extensive granular staining which is rarely seen in any of the control cells (Fig. 5, *g* and *i* compared to *a-c*).

In summary, it is apparent that manipulating the levels of expression of  $\beta$ - and  $\gamma$ -actin genes in C2 cells has a profound impact on both cell morphology and cytoarchitecture. Ele-

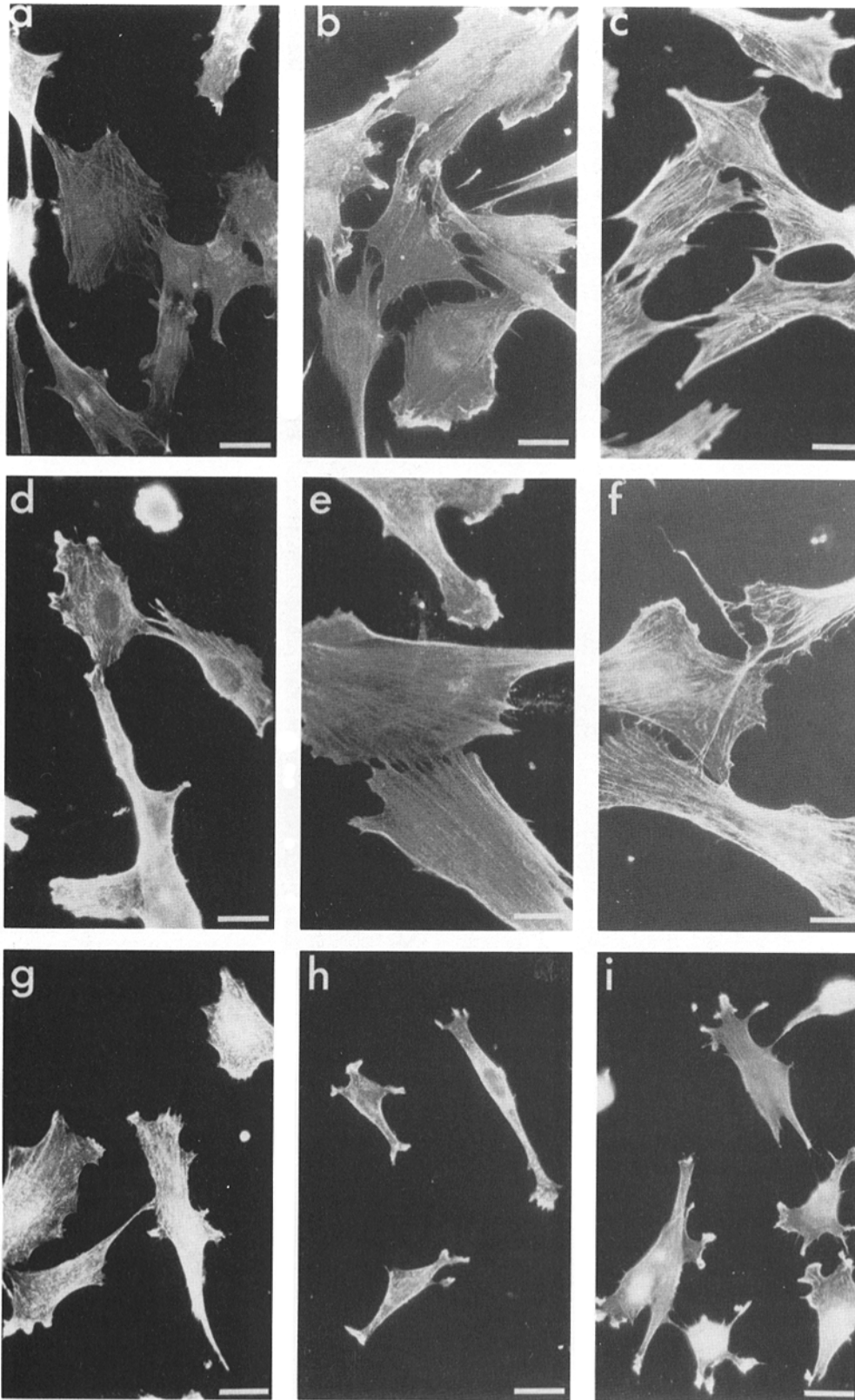


**Figure 4.** Elevated expression of the human nonmuscle actins in C2 cells results in different cytoarchitectural changes. Immunofluorescence staining of C2 transfectants with 1:50 dilution of the C4 total actin antibody followed by fluorescein-conjugated goat anti-mouse IgG. Cells were grown on collagen-coated glass slides for 24 h before fixing and permeabilizing with 1% formaldehyde followed by methanol. (*a*) Control cells,  $\beta_{\text{wt}}-33$ ; (*b*)  $\beta_{\text{wt}}-11$ ; and (*c*)  $\gamma_{33}$ -B1. Bar, 10  $\mu\text{m}$ .

vated expression of  $\beta$ - and  $\gamma$ -actin genes have reciprocal effects on cell morphology and distinct effects on the cytoarchitectural organization of the cell. This suggests that the two genes encode different structural information of significance to the cell.

#### **Impact of a Mutant Form of $\beta$ -Actin on Myoblast Cytoarchitecture**

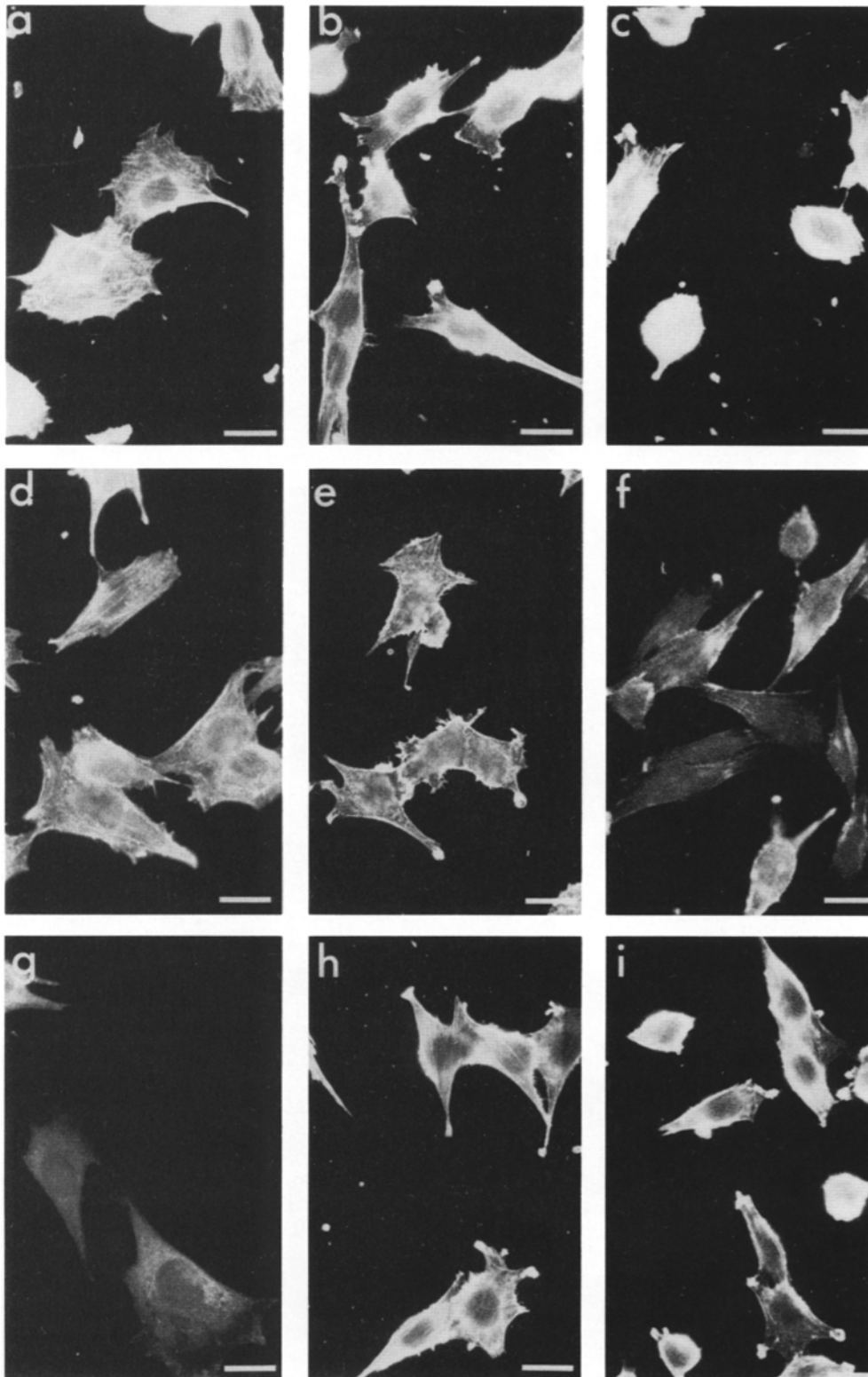
The role of the  $\beta$ -actin protein in generating the enlarged cell phenotype was examined by transfecting a mutant form of the human  $\beta$ -actin gene. The encoded mutant protein,  $\beta_{\text{m}}$ , car-



**Figure 5.** Organization of the actin microfilament network in control and the actin transfectants. Immunofluorescence staining of control C2 clones,  $\beta_{wt}$  and  $\gamma$ -actin transfectants stained with a 1:5,000 dilution of a  $\gamma$ -actin-specific antibody followed by rhodamine-conjugated goat anti-rabbit IgG. Cells were prepared and treated as described in Fig. 4. (a) pUC-pool; (b) pUC-A; (c)  $\beta_{m-33}$ ; (d)  $\beta_{wt-12}$ ; (e)  $\beta_{wt-13}$ ; (f)  $\beta_{wt-11}$ ; (g)  $\gamma_{33-B5}$ ; (h)  $\gamma_{33-B1}$ ; and (i)  $\gamma_{58-B2}$ . Bar, 10  $\mu\text{m}$ .

ries a single point mutation at codon 244 which changes a glycine to an aspartic acid residue (Lin et al., 1985). Previously reported studies have demonstrated that elevated expression of the  $\beta_{sm}$  gene in human KD fibroblasts gives rise to cells with an altered cell morphology (Leavitt et al.,

1987). In addition, *in vitro* studies have also shown that this mutant actin protein does not assemble into proper filaments under filament forming conditions, but it does form filament bundle-like structures that resemble stress-fibers (Millonig et al., 1988). Therefore, this mutant actin potentially pro-



**Figure 6.** Elevated expression of the human single mutant  $\beta$ -actin gene in C2 cells results in altered cell morphology and cytoarchitecture. Immunofluorescence staining of the  $\beta_{sm}$  transfectants with the C4,  $\gamma$ -actin and with an antibody specific to the single mutant  $\beta$ -actin (anti-asp, 1:50 dilution) antibody. Cells were prepared and treated as described in Fig. 4. (a-c)  $\beta_{sm-47}$ ,  $\beta_{sm-72}$ , and  $\beta_{sm-22}$  stained with the C4 antibody. (d-f)  $\beta_{sm-47}$ ,  $\beta_{sm-72}$ , and  $\beta_{sm-22}$  stained with the  $\gamma$ -actin antibody. (g-i)  $\beta_{sm-47}$ ,  $\beta_{sm-72}$ , and  $\beta_{sm-22}$  stained with the anti-asp antibody. Bar, 10  $\mu$ m.

vides us with a model system in which to investigate the significance of an aberrant protein in regulating cell cytoarchitecture.

Transfection of the  $\beta_{sm}$  gene into C2 cells resulted in a similar number of G418-resistant colonies to that observed with pUC18 and they displayed a broad range of expression

levels. All clones were assayed for expression of the human gene and Fig. 1 (C) shows a Northern blot of the human mRNA in the highest expressing clones. The three transfectants with the greatest level of human transcript,  $\beta_{sm-22}$ ,  $\beta_{sm-72}$ , and  $\beta_{sm-62}$  accumulated the  $\beta_{sm}$ -actin mRNA at 173, 69, and 45%, respectively, of the  $\beta$ -actin mRNA level in hu-



man myoblasts (Table II). As illustrated in Fig. 2 (B), increasing levels of the  $\beta_{sm}$  protein in these cells, which migrates as a more acidic protein (Leavitt et al., 1987), parallel the increasing levels of expression of the  $\beta_{sm}$  mRNA transcript. Since protein from the  $\beta_{sm}$ -72 clone (Fig. 2 B, lane 3) was overloaded only a relative comparison between the  $\beta_{sm}$  protein and the other actins can be made.

The morphological changes of the  $\beta_{sm}$ -transfectants shown by determination of the surface area revealed that increasing expression of the  $\beta_{sm}$  gene produced a progressive decrease in cell surface area (Table II). The thickness of these cells was examined using the XZ scan mode on the confocal microscope. Expression of the  $\beta_{sm}$  gene does not produce a simple progressive alteration to cell thickness (Table II). In all clones but  $\beta_{sm}$ -72, the cell thickness balances the decrease in surface area to maintain a volume similar to that of control cells (Table II). Thus, the  $\beta_{sm}$  and  $\gamma$ -actin genes have similar impact on cell surface area but differ in their effect on cell thickness (Table II).

All highly expressing  $\beta_{sm}$  transfectant cells were stained with the C4 and the  $\gamma$ -actin-specific antibodies to visualize the organization of actin filaments. Typical fields of each are shown in Fig. 6 (a-c) C4 stained cells and (d-f)  $\gamma$ -actin stained cells. Elevated expression of the  $\beta_{sm}$  gene resulted in poor organization of the actin cables (Fig. 6, b and c). Interestingly, a comparison of the C4 staining between the highest expressing  $\gamma$ -actin transfectant,  $\gamma_{33}$ -B1, and the highest expressing  $\beta_{sm}$  transfectant,  $\beta_{sm}$ -22, revealed that both cell types ultimately give rise to poor cable organization (Fig. 4 c compared to Fig. 6 c).

The localization of the mutant  $\beta$ -actin protein in the  $\beta_{sm}$  transfectants was visualized using an antibody directed against the mutation site in the  $\beta_{sm}$  protein. This antibody was characterized by Dr. U. Aebi and found to be specific for the mutated form of the  $\beta$ -actin (Leavitt et al., 1987). Fig. 6 (h and i) shows that the  $\beta_{sm}$ -actin protein does incorporate into stress-fiber-like structures, the lowest expressing  $\beta_{sm}$  transfectant,  $\beta_{sm}$ -47, showing background staining (Fig. 6 g).

The above experiments clearly demonstrate that incorporation of an aberrant actin protein into stress fiber-like structures leads to morphological and cytoarchitectural changes. Interestingly, high level expression of the  $\beta_{sm}$  gene results in a phenotype similar to that seen with the introduced  $\gamma$ -actin gene (Fig. 5 h). Since the difference between the  $\beta_w$  and the  $\beta_{sm}$  gene is only one base change, it is likely that the protein encoded by this gene must be responsible for the observed cytoskeletal alterations. These observations also suggest that native actin can regulate cell morphology and cytoarchitecture, an observation only previously genetically demonstrated in yeast (Novick and Botstein, 1985).

## Discussion

### The Nonmuscle $\beta$ - and $\gamma$ -Actin Genes Are Functionally Distinct

The essential premise of our experiments is that if the human  $\beta$ - and  $\gamma$ -actin genes are functionally equivalent, then expression of these genes at similar levels in C2 cells should result in similar phenotypic consequences. Comparison of the data obtained from  $\beta$ - and  $\gamma$ -actin transfections clearly demonstrates that these two genes are not equivalent.

Elevated expression of the  $\beta$ -actin gene promotes an increase in cell surface area and cell thickness; whereas  $\gamma$ -actin gene expression produces an opposite result. This suggests that the relative gene expression of  $\beta$ - and  $\gamma$ -actin reflects a competition between opposing influences and that the balance may regulate myoblast morphology and cytoarchitecture. This leads us to conclude that the  $\beta$ - and  $\gamma$ -actin genes encode functionally distinct cytoarchitectural information.

The observation of different  $\beta/\gamma$  protein and mRNA ratios in a variety of cell types (Otey et al., 1987; Erba et al., 1988) may be of functional significance. The work by Otey et al. (1987) has shown that rat tissues have  $\beta/\gamma$  ratios ranging from 1 in testis to 3.4 in liver. In addition, neoplastic transformation often leads to changes in the  $\beta/\gamma$  ratio and disruption and/or reorganization of the microfilament system resulting in cells with an altered phenotype. For example, previous studies of spontaneously and virally transformed cells have shown a consistent elevation in  $\gamma$ -actin expression and thus an overall decrease in the  $\beta/\gamma$  ratio. These include transformed Rat-2 cells and a human T-cell leukemia, Molt-4 cell line, (Leavitt et al., 1985; Leavitt et al., 1980) with the exception of RSV transformation of CEF cells which show no such decrease (Witt et al., 1983). Interestingly, the majority of these transformed cells have a reduced ability to spread on the substratum and thus show a highly rounded morphology. In light of our transfection results, we suggest that the elevation of  $\gamma$ -actin expression in transformed cells may contribute to the observed disruption of the cytoskeleton in these cells.

Most intriguing is the finding that  $\beta$ -actin expression reduces the viability of the cells, as opposed to  $\gamma$ -actin expression. As previously mentioned, the number of clones arising due to the  $\beta$ -actin gene transfection was reduced by 50%. A possible explanation could be that an overall increase in expression of  $\beta$ -actin leads to extremely stable stress fibers that could interfere with the process of cytokinesis. In fact, the  $\beta_w$ -13 clone displayed a number of multinucleated cells suggesting an uncoupling of karyokinesis from cytokinesis. In contrast, expression of the  $\gamma$ - and  $\beta_{sm}$ -actin genes caused severe morphological and cytoarchitectural changes, presumably due to instability of the stress fibers, but had no effect on the viability of the cells. This leads us to suggest that the increase in cell size, seen only with the  $\beta$ -actin gene, compromises cell viability. Karlsson et al. (1991) have reported that expression of chicken  $\beta$ -actin in a yeast strain lacking endogenous actin results in increased cell size and lethality at 37°. Because  $\beta$ -actin is a foreign protein to the yeast cell, one cannot extrapolate from such studies to normal  $\beta$ -actin function within a vertebrate cell. However, the striking similarity between the findings in yeast and in C2 cells suggests that some of the phenotypic consequences of  $\beta$ -actin transfection may be intrinsic to the protein.

### Does the $\beta/\gamma$ Protein Ratio Alone Determine Morphology?

The impact of the  $\beta$ - and  $\gamma$ -actin genes on cell morphology and architecture may not be entirely attributed to alterations in the  $\beta/\gamma$  protein ratios. Preliminary observations indicate that the 3'UTR and 3'flanking regions of the  $\beta$ - and  $\gamma$ -actin genes influence the outcome of these transfection experi-

ments. Removal of the 3'UTR and flank of the  $\beta$ -actin gene eliminates the lethality observed with the complete gene. In contrast, removal of the same area from the  $\gamma$ -actin gene results in more severe lethality than that observed with the  $\beta$ -actin gene (C. Lloyd and P. Gunning, unpublished observations). Therefore, care must be taken in attributing all the functional differences between these two genes to the encoded proteins. A possible explanation for the changes observed could be due to alterations in the absolute levels of each isoform either at the protein or mRNA level. We found that the  $\beta$ -actin transfectants displayed an increase in the total actin mRNA and protein pool; whereas, the  $\beta_{sm}$ - and  $\gamma$ -actin transfectants show a decrease or no change in the total actin pool (Lloyd et al., 1992). While this change in total actin pool size is at least partially related to the encoded protein ( $\beta$  vs  $\beta_{sm}$ ), it is also possible that the  $\beta$ - and  $\gamma$ -actin genes differ in their response to feedback regulation.

It is unlikely that all nonmuscle cell types will interpret the relative expression of  $\beta$ - and  $\gamma$ -actin in the same way. Such an example are mouse L cells and human erythrocytes (Otey et al., 1986; Bennett, 1985). Although their  $\beta/\gamma$  ratios are high, they have quite different morphologies and cytoarchitecture. Neither of these cell types resemble our highest expressing  $\beta$ -actin transfectants. The L cells spread well on a substratum but their actin filament network is highly disorganized (Otey et al., 1986). In contrast, human erythrocytes have a rounded morphology and their actin is organized in shorter filaments (Bennett, 1985). Leavitt et al. (1987) have previously reported that elevation of the  $\beta/\gamma$  protein ratio from 1.65 to 2.26 in diploid human fibroblasts did not result in any obvious impact on cell morphology. In the same experiment, it was found that  $\beta_{sm}$ -actin expression resulted in the appearance of long cytoplasmic processes. This suggests that fibroblasts may interpret the expression of the  $\beta$ - and  $\beta_{sm}$ -actin genes differently to myoblasts. Furthermore, we have observed that transfection of the  $\beta_{sm}$ -actin gene into human epithelial cells results in localization of the mutant protein to a perinuclear region and exclusion from stress fibers (R. Reddel, G. Schevzov, and P. Gunning, unpublished observations). This strongly argues that the introduction of these genes into other cell types may result in different morphological alterations. This could be explained by differences in the composition of the cytoskeleton in varying cell types such as differences in the actin-binding proteins present and in particular tropomyosin isoform composition. Furthermore, since myogenic cells undergo extreme changes in cell size and shape during development, mechanisms may be in place to accommodate these alterations which may not be necessary for fibroblasts or epithelial cells.

We are very grateful to Dr. U. Aebi for providing us with the mutant  $\beta$ -actin-specific antibody, to Dr. J. C. Bulinski for the  $\gamma$ -actin-specific antibody, and Dr. J. L. Lessard for the C4 antibody. We are indebted to Christine Smyth for her help with the confocal microscope. We thank Dr. R. Weinberger for frequent invaluable advice and help with the IEF focusing gels. We would also like to thank Dr. J. Leavitt for the human  $\beta$ -actin gene constructs. Finally, we would like to thank Drs. E. Hardeman, K. Esser and P. B. Rowe for the valuable advice and critical reading of the manuscript.

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

Received for publication 22 October 1991 and in revised form 29 January 1992.

## References

- Bains, W., P. Ponte, H. Blau, and L. Kedes. 1984. Cardiac actin is the major actin gene product in skeletal muscle cell differentiation in vitro. *Mol. Cell. Biol.* 4:1449-1453.
- Bennett, V. 1985. The membrane skeleton of human erythrocytes and its implications for more complex cells. *Annu. Rev. Biochem.* 54:273-304.
- Bravo, R., S. J. Fey, J. V. Small, P. M. Larsen, and J. E. Celis. 1981. Coexistence of three major isoactins in a single sarcoma 180 cell. *Cell.* 25:195-202.
- Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156-159.
- Craig, S. W., and J. V. Pardo. 1983. Gamma actin, spectrin, and intermediate filament proteins colocalize with vinculin at costameres, myofibril-tarsolemma attachment sites. *Cell Motil.* 3:449-462.
- 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.
- Eppenberger-Eberhardt, M., I. Flamme, V. Kurer, and H. M. Eppenberger. 1990. Reexpression of  $\alpha$ -smooth muscle actin isoform in cultured adult rat cardiomyocytes. *Dev. Biol.* 139:269-278.
- Erba, H. P., P. Gunning, and L. Kedes. 1986. Nucleotide sequences of the human  $\gamma$  cytoskeletal actin mRNA: anomalous evolution of vertebrate nonmuscle 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  $\gamma$ -actin gene: differential evolution, location, and expression of the cytoskeletal  $\beta$ - and  $\gamma$ -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.
- Garrels, J. I., and W. Gibson. 1976. Identification and characterization of multiple forms of actin. *Cell.* 9:793-805.
- Graham, F. L., and A. J. van der Eb. 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology.* 52:456-467.
- Gunning, P., P. Ponte, H. Okayama, J. Engel, H. Blau, and L. Kedes. 1983. Isolation and characterization of full-length cDNA clones for human  $\alpha$ -,  $\beta$ - and  $\gamma$ -actin mRNA's: skeletal but not cytoplasmic actins have an amino terminal cysteine that is subsequently removed. *Mol. Cell. Biol.* 3:787-795.
- Gunning, P., P. Ponte, L. Kedes, R. J. Hickey, and A. I. Skoultschi. 1984. Expression of human cardiac actin in mouse L cells: a sarcomeric actin associates with a nonmuscle cytoskeleton. *Cell.* 36:709-715.
- 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.
- Hamada, H., M. Petrino, and T. Kakunaga. 1982. Molecular structure and evolutionary origin of human cardiac muscle actin gene. *Proc. Natl. Acad. Sci. USA.* 79:5901-5905.
- Karlsson, R., P. Aspenstrom, and A. S. Bystrom. 1991. A chicken  $\beta$ -actin gene can complement a disruption of the Saccharomyces cerevisiae ACT1 gene. *Mol. Cell. Biol.* 11:213-217.
- Leavitt, J., A. Leavitt, and A. M. Attallah. 1980. Dissimilar modes of expression of  $\beta$ - and  $\gamma$ -actin in normal and leukemic human T lymphocytes. *J. Biol. Chem.* 255:4984-4987.
- Leavitt, J., P. Gunning, P. Porreca, S.-Y. Ng, C.-S. Lin, and L. Kedes. 1984. Molecular cloning and characterization of mutant and wild type human  $\beta$ -actin genes. *Mol. Cell. Biol.* 4:1961-1969.
- Leavitt, J., P. Gunning, L. Kedes, and R. Jariwalla. 1985. Smooth muscle  $\alpha$ -actin is a transformation-sensitive marker for mouse NIH 3T3 and Rat-2 cells. *Nature (Lond.)* 316:840-842.
- Leavitt, J., S.-Y. Ng, U. Aebi, M. Varma, G. Latter, S. Burbeck, L. Kedes, and P. Gunning. 1987. Expression of transfected mutant  $\beta$ -actin genes: alterations of cell morphology and evidence for autoregulation in actin pools. *Mol. Cell. Biol.* 7:2457-2466.
- Lessard, J. L. (1988). Two monoclonal antibodies to actin: one muscle selective and one generally reactive. *Cell Motil. Cytoskeleton.* 10:349-362.
- Lin, C.-S., S.-Y. Ng, P. Gunning, L. Kedes, and J. Leavitt. 1985. Identification and order of sequential mutations in  $\beta$ -actin genes isolated from increasingly tumorigenic human fibroblast strains. *Proc. Natl. Acad. Sci. USA.* 82:6995-6999.
- Lloyd, C., G. Schevzov, and P. Gunning. 1992. Transfection of nonmuscle  $\beta$ - and  $\gamma$ -actin genes into myoblasts elicits different feedback regulatory responses from endogenous actin genes. *J. Cell Biol.* 117:787-797.
- Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory, New York. 545 pp.
- Mc Hugh, K. M., and J. L. Lessard. 1988. The developmental expression of the rat alpha-vascular and gamma-enteric smooth muscle isoactins: isolation and characterization of a rat gamma-enteric actin cDNA. *Mol. Cell. Biol.* 8:5224-5231.
- McKenna, N., J. B. Meigs, and Y. L. Wang. 1985. Identical distribution of fluorescently labelled brain and muscle actins in living cardiac fibroblast and myocytes. *J. Cell Biol.* 100:292-296.
- Milington, R., H. Salvo, and U. Aebi. 1988. Probing actin polymerization by

- intermolecular cross-linking. *J. Cell Biol.* 106:785-796.
- Ng, S.-Y., P. Gunning, R. Eddy, P. Ponte, J. Leavitt, T. Shows, and L. Kedes. 1985. Evolution of the functional human  $\beta$ -actin gene and its multi-pseudogene family: conservation of noncoding regions and chromosomal dispersion of pseudogenes. *Mol. Cell. Biol.* 5:2720-2732.
- Novick, P., and D. Botstein. 1985. Phenotypic analysis of temperature-sensitive yeast actin mutants. *Cell.* 40:405-416.
- Otey, C. A., M. H. Kalnoski, J. L. Lessard, and J. C. Bulinski. 1986. Immunolocalization of the gamma isoforms of nonmuscle actin in cultured cells. *J. Cell Biol.* 102:1726-1737.
- 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.
- Palmiter, R. D. 1974. Magnesium precipitation of ribonucleoprotein complexes: expedient techniques for the isolation of undegraded polysomes and messenger ribonucleic acid. *Biochemistry.* 13:3606-3615.
- Paterson, B. M., and J. D. Eldridge. 1984. Alpha-cardiac actin is the major sarcomeric isoform expressed in embryonic avian skeletal muscle. *Science (Wash. DC).* 224:1436-1438.
- Sawtell, N. M., and J. L. Lessard. 1989. Cellular distribution of smooth muscle actins during mammalian embryogenesis: expression of the  $\alpha$ -vascular but not the  $\gamma$ -enteric isoform in differentiating striated myocytes. *J. Cell Biol.* 109:2929-2937.
- Shani, M., D. Zevin-Sonkin, O. Saxel, Y. Carmon, D. Katcoff, U. Nudel, and D. Yaffe. 1981. The correlation between the synthesis of skeletal muscle actin, myosin heavy chain and myosin light chain and the accumulation of corresponding mRNA sequences during myogenesis. *Dev. Biol.* 86:483-492.
- Shortle, D., P. Novick, and D. Botstein. 1984. Construction and genetic characterization of temperature-sensitive alleles of the yeast actin gene. *Proc. Natl. Acad. Sci. USA.* 81:4889-4893.
- Southern, P. J., and P. Berg. 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. *J. Mol. Appl. Genet.* 1:327-341.
- 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.
- Van der Voort, H. T. M., G. J. Brakenhoff, and M. W. Baarslag. 1989. Three-dimensional visualization methods for confocal microscopy. *J. Microsc.* 153:123-132.
- Witt, D. P., D. J. Brown, and J. A. Gordon. 1983. Transformation sensitive isoactin in passaged chick embryo fibroblasts transformed by Rous Sarcoma Virus. *J. Cell Biol.* 96:1766-1771.