Transfection of Nonmuscle β- and γ-Actin Genes into Myoblasts Elicits Different Feedback Regulatory Responses from Endogenous Actin Genes

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Abstract. We have examined the role of feedback-regulation in the expression of the nonmuscle actin genes. C2 mouse myoblasts were transfected with the human β- and γ-actin genes. In γ-actin transfectants we found that the total actin mRNA and protein pools remained unchanged. Increasing levels of human γ-actin expression resulted in a progressive down-regulation of mouse β- and γ-actin mRNAs. Transfection of the β-actin gene resulted in an increase in the total actin mRNA and protein pools and induced an increase in the levels of mouse β-actin mRNA. In contrast, transfection of a β-actin gene carrying a single-point mutation (βsm) produced a feedback-regulatory response similar to that of the γ-actin gene. Expression of a β-actin gene encoding an unstable actin protein had no impact on the endogenous mouse actin genes. This suggests that the nature of the encoded actin protein determines the feedback-regulatory response of the mouse genes. The role of the actin cytoskeleton in mediating this feedback-regulation was evaluated by disruption of the actin network with Cytochalasin D. We found that treatment with Cytochalasin D abolished the down-regulation of mouse γ-actin in both the γ- and βsm-actin transfectants. In contrast, a similar level of increase was observed for the mouse β-actin mRNA in both control and transfected cells. These experiments suggest that the down-regulation of mouse γ-actin mRNA is dependent on the organization of the actin cytoskeleton. In addition, the mechanism responsible for the down-regulation of β-actin may be distinct from that governing γ-actin. We conclude that actin feedback-regulation provides a biochemical assay for differences between the two nonmuscle actin genes.

MAMMALIAN nonmuscle cell cytoarchitecture is comprised of three major polymeric structures: microtubules, microfilaments, and intermediate filaments. Regulation of the synthesis and assembly of these polymers is likely to have an important role in the determination of cell morphology. In particular, it is to be expected that the regulation of cellular content of each of these polymers may contribute to the determination of cell size and shape. It is therefore important to determine whether the expression of structural protein genes is programmed to produce a fixed level of mRNA output. Such a mechanism could regulate cell morphology via the control of structural protein monomer supply. Alternatively, it might be expected that the function of architectural components is monitored by the cell such that gene expression of a structural protein can be feedback-regulated to satisfy cellular demand. The term autoregulation can be used to describe a system where it has definitely been established that the gene product directly regulates its own expression, however, in all other cases, the phenomenon will be described as feedback-regulation.

The discovery of tubulin autoregulation has demonstrated that the cell can regulate monomer supply of tubulin in accord with cellular demand for microtubules (Ben-Ze'ev et al., 1979). Recent studies have suggested that the pool of unpolymerized αβ-tubulin dimer can directly regulate the stability of β-tubulin mRNA (Pachter et al., 1987). This mechanism allows the cell to set β-tubulin mRNA levels and therefore monomer levels according to the cellular demand for incorporation of monomer into microtubules. While this might be taken to suggest that cellular microtubule levels are not set by tubulin gene expression, the recent work of Katz et al. (1990) suggests otherwise. Transfection studies in yeast suggest that β-tubulin gene expression may determine microtubule levels and that α-tubulin gene expression is feedback-regulated in response to that of β-tubulin.

Intermediate filament gene expression appears to be set in mammalian cells and not subject to feedback-regulation. Forced expression of a number of intermediate filament proteins both in cell culture and in transgenic mice has failed to elicit any feedback-regulatory response from the endogenous intermediate filament genes (Monteiro et al., 1990). These results suggest that intermediate filament levels are largely controlled by programmed gene expression independent of cytoplasmic levels of the protein.

Microfilaments, like microtubules, may be subject to feedback-regulation. It has been suggested that the micro-

filament-associated protein, α-actinin, can regulate its own synthesis (Schulze et al., 1989). Introduction of phalloidin into mouse embryo fibroblasts resulted in an increase in polymerized cytoskeletal-associated actin, a decrease in monomeric actin and also elicited an increase in both actin protein synthesis and mRNA levels suggesting that actin is subject to feedback-regulation (Serpinskaya et al., 1990). Introduction of a mutated human β-actin gene (βsm) into human fibroblasts resulted in a decrease in endogenous β- and γ-actin protein synthesis such that the net level of total actin synthesis remained unchanged (Leavitt et al., 1987*a*), supporting the existence of an actin feedback-regulatory mechanism. However, studies of actin mRNA levels in these cells were inconclusive since it was not possible to discriminate between the transfected and endogenous human genes (Leavitt et al., 1987*a*). Similar transfections of human β- and γ-actin genes into human fibroblasts have been shown to alter the ratio of β- to γ-actin protein in a manner dependent on the introduced gene (Ng et al., 1988). However, complete analysis of actin expression in these cells was not possible since the transfected actin gene could not be distinguished from the endogenous human genes. Thus, while protein analysis indicates that actin synthesis may be subject to feedback-regulation it is unknown if actin mRNA levels are feedback-regulated and also whether β- and γ-actin are equivalent in both their ability to generate and respond to feedback-regulatory stimuli. In the accompanying paper (Schevzov et al., 1992), it is shown that transfection of the β- and γ-actin genes impacts differently on myoblast cytoarchitecture. Is it possible that feedback-regulation contributes to this difference between β- and γ-actin? For example, is there more actin in large cells (β-actin transfectants) and less in small cells (γ-actin transfectants) and therefore does the size of these cells reflect the impact of expression of the actin genes on actin feedback-regulation?

We have investigated the ability of the two human nonmuscle actin genes, β and γ, to elicit a feedback-regulatory response upon transfection into mouse C2 myoblasts. Although the human and mouse isoforms are identical at the protein sequence level, differences between the mRNAs have allowed us to discriminate between transcripts from the endogenous and exogenous genes. We find that actin gene expression is indeed subject to a form of feedback-regulation. However, we observe that the nature of this regulation is determined by the type of actin encoded by the transfected gene and is dependent upon microfilament organization.

Materials and Methods

Cell Culture

Mouse C2 myoblasts isolated in the laboratory of Dr. D. Yaffe (Weizmann Institute, Israel) and subcloned in the laboratory of Dr. H. Blau (Stanford University, Stanford, CA) were grown in DMEM (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). The transfection and selection of cells carrying the human βwt-, βsm-, βtm-, and γ-actin plasmids together with pSV2neo was described (Schevzov et al., 1992). Control cells were generated by transfection with pUC-18 plus pSV2neo. Cells were harvested at 30–40% confluence and the cultures did not contain detectable myotubes. In some experiments, the cells were exposed to the actin depolymerizing drug, Cytochalasin D. Cells at 40% confluence were treated with Cytochalasin D (Sigma Chemical Co., St. Louis, MO) at 2.5 μg/ml (from a stock at 2 mg/ml in DMSO) for 5 h and parallel control cultures were treated with the corresponding amount of DMSO alone.

RNA Isolation and Analysis

Total cellular RNA was isolated from the cells by the method of Chomczynski and Sacchi (1989). RNA was size-fractionated by formaldehyde gel electrophoresis and transferred to nylon membranes as described (Gunning et al., 1990). DNA probes were radiolabeled to 10^9 dpm/μg by the random priming method (Feinberg and Vogelstein, 1983) and hybridized to RNA blots at 10^6 dpm/ml in a solution containing 4× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate), 50 mM NaH_2PO_4, pH 7.0, 5× Denhardt's solution (Denhardt, 1966), and 10% (wt/vol) dextran sulfate at 65°C for 16 h. After hybridization the blots were washed in 0.5× SSC, 0.1% SDS at 65°C except for the total actin probe which was washed at 50°C. Membranes 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 rRNA-specific oligonucleotide probe under conditions of probe excess and washed in 4× SSC, 0.1% SDS at 55°C. Levels of mRNAs were quantitated by densitometry as described (Gunning et al., 1990).

DNA Probes

The human βwt-, βsm-, and βtm-actin mRNAs were detected using a human-specific β-actin-specific 3′ untranslated region (3′UTR)[1] probe, pHFβA-3′UT-HH, as described (Ng et al., 1985). The human nonmuscle γ-actin mRNA was detected using a human-specific γ-actin-specific 3′UTR probe, pHFγA-3′UT-HX (Erba et al., 1986). The level of human β- and γ-actin mRNA was measured relative to that in myoblasts cultured from adult human skeletal muscle as described (Gunning et al., 1987). The mouse β-actin mRNA was detected using a mouse-specific β-actin–specific DNA fragment containing bp 278–480 of the mouse β-actin 3′UTR (Mβ-3′UT-202). The mouse β-actin fragment was generated by PCR amplification from mouse genomic DNA using appropriate oligonucleotides as described (Gunning et al., 1990). The resulting fragment was cloned between the BamHI and SmaI sites of pGEM-3. The mouse γ-actin mRNA was measured using a mouse-specific γ-actin-specific DNA fragment containing bp 199–465 of the mouse γ-actin 3′UTR (Mγ-3′UT-HA). The mouse γ-actin probe was isolated as a HpaI–AvaII DNA fragment from a mouse cDNA supplied by Dr. D. Leader (University of Glasgow, Scotland) and was subcloned into the SmaI site of pGEM-3. Total actin mRNA levels were measured directly using a chicken β-actin coding region probe under low stringency conditions (Cleveland et al., 1980).

Actin Protein Analysis

Total protein from cells at 30-40% confluence was size-fractionated by SDS-PAGE (Laemmli, 1970) and immunoblotted using the method of French and Jeffrey (1986). Actin protein was detected using a C4 mAb (kindly supplied by Dr. J. Lessard, Children's Hospital Research Foundation, Cincinatti, OH; Lessard, 1988) which reacts with all actin isoforms. Tubulin protein was detected using an α-tubulin monoclonal antibody (Product No. T-9026; Sigma Chemical Co., St. Louis, MO). The Western blots were scanned using a Molecular Dynamics Model 300 series computing densitometer which allowed volumetric determinations (3 dimensional integrations). The levels of actin and α-tubulin protein were expressed as a percentage of that detected in control cells which was set at 100%. Identical gels were run concurrently and stained with Coomassie blue. The Coomassie staining and the level of α-tubulin for each clone was used to correct any differences in the total actin levels that were due to unequal loading.

Results

γ-Actin Gene Elicits a Feedback-Regulatory Response

Mouse C2 myoblasts were transfected with the human γ-actin gene and all highly expressing cloned cells as determined by Northern blot analysis, were analyzed in detail (Table I). Cells were harvested for RNA and protein at 30% confluence

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

Table I. Actin Protein Pool Size in Actin Gene Transfectants

Gene	Clone	Human actin mRNA level*	Total actin protein levels‡
pUC-18			100
γ	γ_{58}-A2	0.55	107
γ	γ_{58}-B1	0.59	111
γ	γ_{33}-B5	1.02	98
γ	γ_{58}-B2	1.49	103
γ	γ_{33}-B1	2.52	84
βwt	βwt-25	0.10	102
βwt	βwt-12	0.16	96
βwt	βwt-13	0.38	179
βwt	βwt-11	0.61	233
βsm	βsm-62	0.45	78
βsm	βsm-72	0.69	61
βsm	βsm-22	1.73	89
βtm	βtm-45	0.12	132
βtm	βtm-22	0.17	99
βtm	βtm-43	0.36	102
βtm	βtm-33	0.77	85

* Values are expressed relative to the level found in human myoblasts which was set at 1.
‡ Values are expressed as a percentage of that found in control cells (transfected with plasmid pUC-18) which was set at 100%.

to ensure that cells were in logarithmic growth. Fig. 1 shows that increasing levels of the human γ-actin mRNA had no impact on the total level of actin protein. The Western blot shown in Fig. 1 was reacted with an α-tubulin antibody in parallel with the actin antibody to confirm equal transfer of samples to the nitrocellulose. A gel run in parallel with this Western blot was stained with Coomassie blue and also used to confirm equal loading of samples. In lane 4 the actin level is decreased, however, both the Coomassie blue and α-tubulin were also decreased from the control and when this was taken into account the actin level was comparable to the other clones and the control (Table I). Additionally, the Coomassie-stained gel supported the results since the 42-kD actin band was essentially identical in all samples (not shown). Increasing expression of the human γ-actin mRNA was associated with an altered proportion of β- to γ-actin protein such that in the highest expressing clone, γ_{33}-B1, the β/γ ratio was 0.3 compared to 2.0 in control cells (Schevzov et al., 1992). We conclude that expression of the human γ-actin gene alters the relative levels of β- and γ-actin protein in favour of γ-actin. Since the total actin protein level is essentially unchanged (Table I), this suggests that the human γ-actin gene elicited a feedback-regulatory response from the mouse actin genes.

The impact of human γ-actin expression on the levels of mouse β- and γ- actin mRNAs is shown in Fig. 2. There was a progressive decrease in the level of the mouse β- and γ-ac-

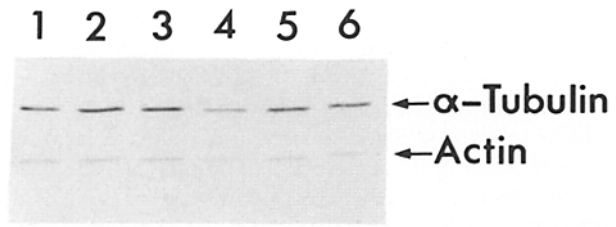

Figure 1. Total actin protein levels in human γ-actin transfectants. The levels of actin and α-tubulin protein in human γ-actin transfectant cells. 10 μg of total protein from five γ-actin transfectants and control cells (transfected with the plasmid pUC-18), all at low confluence, was size fractionated by electrophoresis on a denaturing polyacrylamide gel. The protein was transferred to nitrocellulose and immunostained with antibodies to total actin (*C4*) and α-tubulin. The level of α-tubulin was used to normalize the actin levels for loading. Loading: control (lane *1*), γ_{33}-B1 (lane *2*), γ_{58}-B2 (lane *3*), γ_{58}-A2 (lane *4*), γ_{33}-B5 (lane *5*), and γ_{58}-B1 (lane *6*).

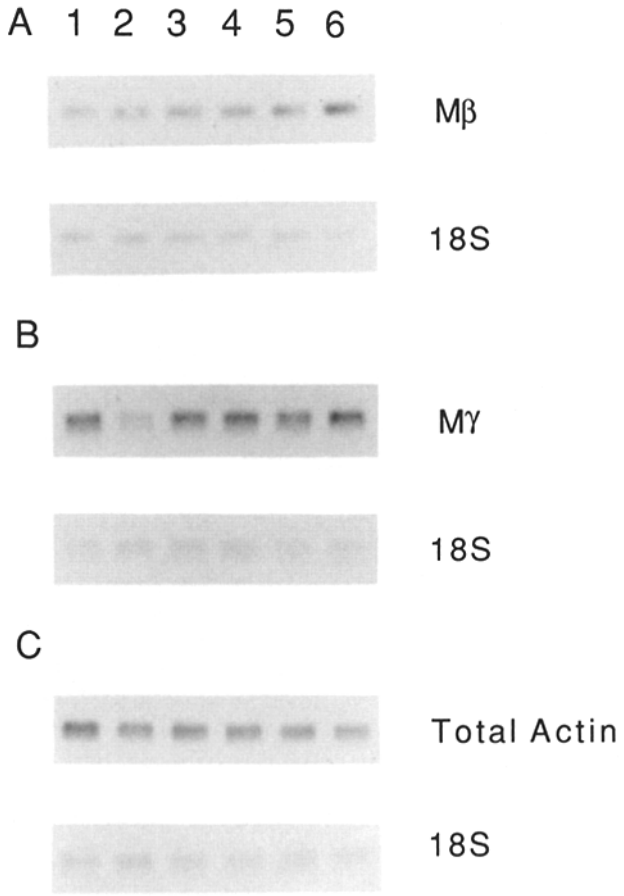

Figure 2. Actin gene expression in human γ-actin gene transfectants. Total RNA was isolated from five γ-actin transfectant clonal lines with different levels of expression of the human gene. The RNA was size fractionated by electrophoresis on denaturing agarose gels and transferred to nylon membranes as described in Materials and Methods. The filters were hybridized with mouse-specific β- and γ-actin probes and a chicken β-actin coding region probe, used to detect total actin levels, then washed and autoradiographed as described in Materials and Methods. RNA from C2 pUC-18 transfectants was used as the control. To correct for loading discrepancies, the filters were subsequently hybridized with a probe specific for 18S ribosomal RNA. 3 μg of RNA was loaded for each sample. (*A*) Lane 1, γ_{33}-B1; lane 2, γ_{58}-B2; lane 3, γ_{58}-A2; lane 4, γ_{33}-B5; lane 5, γ_{58}-B1; lane 6, control (pUC-18 transfectant). (*B* and *C*) Lane 1, control; lane 2, γ_{33}-B1; lane 3, γ_{58}-B2; lane 4, γ_{33}-B5; lane 5, γ_{58}-A2; lane 6, γ_{58}-B1.

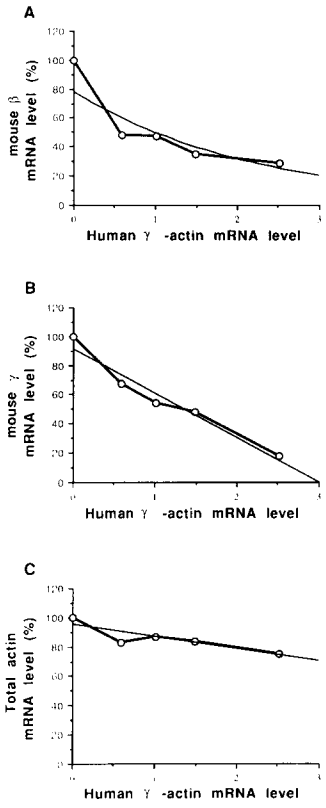

Figure 3. Actin mRNA levels in human γ-actin gene transfectants. Endogenous β (*A*), γ (*B*), and total (*C*) actin mRNA levels were measured in a number of clones with different levels of expression of the transfected human gene. Panels were also probed for 18S ribosomal RNA and corrected for loading discrepancies. The values for each of the mRNA levels were expressed as a percentage of those found in control cells (those transfected with the plasmid pUC-18) which were set at 100%. The expression values for the transfected gene are shown relative to that observed in a human myoblast RNA sample. Linear or logarithmic regression analysis was performed on the data points and the resulting line is shown on each graph.

tin mRNAs with increasing expression of human γ-actin gene. The comparison of the highest expressing clone, γ_{33}-B1, and the control, pUC-18 transfectants, for the mRNA levels of mouse β-actin (*A*, lanes 1 and 6, respectively) and mouse γ-actin (*B*, lanes 2 and 1, respectively) highlight the down-regulation of the mouse actin mRNA levels. The total actin mRNA levels in the γ-actin gene transfectants appeared slightly decreased compared to control cells (Fig. 2 C, lanes *1* and *2*). The actin mRNA levels of the transfectants are depicted graphically in Fig. 3. Regression analysis was performed on the data points to determine if significant relationships existed between the level of expression of the transfected gene and that of the endogenous genes or that of the sum of the actin genes. The curves of best fit, either linear or logarithmic, are shown. Linear relationships were found to exist between the level of human γ-actin gene expression and total actin ($r = 0.87$, $P < 0.025$) and mouse γ-actin ($r = 0.98$, $P < 0.005$) gene expression and a logarithmic relationship existed between human γ-actin and mouse β-actin ($r = 0.91$, $P < 0.025$) gene expression. Therefore, increasing human γ-actin gene expression affected the levels of mouse β-, mouse γ-, and total actin expression progressively such that mouse β- and γ-actin mRNA levels decreased markedly to compensate for human γ-actin gene expression resulting in a slight decrease in total actin mRNA levels. Subsequent analysis of the highest expressing clone in independent experiments yielded similar results to that depicted in Fig. 3 (Table II). The decrease measured in the mouse β- and γ-actin mRNA levels was statistically significant (Table II). The total actin mRNA level in the highest expressing clone, however, was not found to be significantly different to the control level. This result is due to the presence of one determination

Table II. Actin Expression Levels in the Highest Expressing Clones of the Actin Transfectants

Gene	Clone	Mγ	Mβ	Total actin
γ	γ_{33}-B1	29 ± 13*	27 ± 12*	93 ± 32
β	β_{wt}-11	108 ± 23‡	182 ± 34*‡	238 ± 31*
βsm	β_{sm}-22	28 ± 10*‡	38 ± 10*‡	112 ± 19

Values are expressed as a percentage of that found in control cells (transfected with plasmid pUC-18) which was set at 100%. The results are the mean and standard deviations of three to seven independent determinations. The values were compared using the *t* test to determine whether they were significantly different from the control levels (* $P < 0.05$) or whether the γ- and β-actin levels were significantly different from each other (‡ $P < 0.05$).

(of 138%) which was substantially higher than the other four determinations made on RNA from this clone. This one value increased the average mRNA level and the standard deviation. At this stage it cannot be concluded as to whether there is no change in the total actin mRNA level in γ-actin transfectants or a slight decrease, although the latter is more likely.

Addition of the mRNA levels of each of the actin species, mouse β- and γ-actin and human γ-actin, present in the γ-actin transfectants suggested that the total actin mRNA pool was slightly decreased in these cells. The calculation for each transfectant clone was carried out in the following manner. Firstly, the levels of mouse β- and γ-actin mRNA in the γ-actin transfectants were determined relative to those in control cells and expressed in arbitrary units. The units were set such that in control cells the mRNA levels of mouse β- and γ-actin were 2 and 1, respectively, since these cells have a β/γ of 2 (Erba et al., 1988). Secondly, the human γ-actin mRNA contribution was determined using the following method. The level of human γ-actin in the transfectants was determined relative to that in a human myoblast standard. Next, the actin mRNA level in the human myoblast standard was compared to that in the mouse C2 control cells and found to be 70% (data not shown). The level of human γ-actin mRNA in the transfectants was then converted to mouse equivalents by multiplication by the factor 0.7. The levels of all actin mRNA, human and mouse, in the transfectants were added and then divided by the total actin mRNA level found in control cells to obtain the calculated percentage of total actin compared to the control level (Table III). The calculated percentages of total actin mRNA were compared with direct measurements of the total actin mRNA levels which were achieved by using an actin coding region probe at reduced stringency (Figs. 2 *C* and 3). The values for total actin mRNA levels determined by these two methods are similar (Table III). The down-regulation of the mouse actin mRNAs compensated for the increasing human γ-actin mRNA levels such that the total actin mRNA level in the cells decreased only slightly (Fig. 3, Table III).

We conclude that expression of the human γ-actin gene is capable of eliciting a feedback-regulatory response from the myoblast actin genes. Total actin levels in the transfectants were similar to those for control cells at the protein level (measured by immunoblotting of Westerns and Coomassie blue staining of protein gels) and slightly lower than control cells at the mRNA level (directly measured with a total actin-specific probe and indirectly by addition of the levels of all

Table III. Total Actin mRNA Pool Size in Actin Gene Transfectants

Clone	Mβ*	Mγ*	Hβ‡	Hγ‡	Total	Calculated percentage of total actin§	Measured percentage of total actin§
pUC-18	2.0	1.0	–	–	3.0	100	100
γ_{58}-B1	1.0	0.7	–	0.4	2.1	70	83
γ_{33}-B5	0.9	0.5	–	0.7	2.1	70	87
γ_{58}-B2	0.7	0.5	–	1.0	2.2	73	84
γ_{33}-B1	0.6	0.2	–	1.8	2.6	87	75
βwt-25	2.5	0.9	0.1	–	3.5	117	132
βwt-12	3.3	1.2	0.2	–	4.7	157	122
βwt-13	3.6	0.9	0.5	–	5.0	167	156
βwt-11	3.6	0.9	0.9	–	5.4	180	241
βsm-62	2.0	0.6	0.6	–	3.2	107	71
βsm-72	1.3	0.4	1.0	–	2.7	90	80
βsm-22	1.1	0.2	2.4	–	3.7	123	93
βtm-45	2.0	1.1	0.2	–	3.3	110	114
βtm-22	2.1	0.9	0.2	–	3.2	107	127
βtm-43	1.9	0.9	0.5	–	3.3	110	122
βtm-33	1.9	1.1	1.1	–	4.1	137	137

* Values are expressed as arbitrary units relative to the level found in control cells where β- and γ-actin levels were set to 2 and 1, respectively.
‡ Values were initially determined relative to the level found in human myoblasts (expressed as arbitrary units as for *) and then adjusted to the levels found in mouse control cells. This was achieved by multiplication of the relative human level by 0.7 since human myoblasts contain 70% the level of actin as in mouse C2 myoblasts (data not shown).
§ Values are expressed as a percentage of that found in control cells (transfected with plasmid pUC-18) which was set at 100%.
M and *H* refer to mouse and human, respectively.

the actin mRNAs). The endogenous β- and γ-actin mRNA levels decreased in a logarithmic and linear fashion, respectively, with increasing human γ-actin gene expression and this change in the relative expression of β- to γ-actin was reflected in the protein β/γ ratio measured by IEF determinations (Schevzov et al., 1992).

β-Actin Gene Increases Actin Pool Size

Transfection of the human βwt-actin gene into C2 cells led to an increase in the total actin protein pool size of up to 230% in the highest expressing clone (Table I). An increase in the actin pool size was also observed by visualization of the 42-kD actin band using Coomassie staining of the gel loaded with total protein from the βwt-actin transfectants (data not shown). The increase in pool size was paralleled by an increase in the ratio of β- to γ-actin protein, up to 2.8 in the highest expressing clone (Schevzov et al., 1992). This suggests that, unlike the γ-actin gene, expression of the βwt-actin gene does not elicit a feedback-regulatory response from the endogenous mouse actin genes.

To test directly that there had been no feedback-regulatory response from the mouse actin genes in the βwt-actin transfectants, the mouse β- and γ-actin mRNA levels were measured. Fig. 4 shows that the mouse γ-actin mRNA level was unchanged; however, the mouse β-actin mRNA level increased in parallel with expression of the human gene. Regression analysis was performed on the data points and a linear relationship was found to exist between the mRNA levels of human βwt-actin and total actin ($r = 0.89$, $P < 0.01$) and mouse β-actin ($r = 0.85$, $P < 0.025$) suggesting that expression of the human βwt-actin gene affected endogenous β-actin expression. However, regression analysis of the mouse γ-actin results indicated that there was no relationship with human βwt-actin gene expression, but rather that mouse γ-actin mRNA levels were constant ($r = 0.51$, $P > 0.1$). The levels of the mouse actin mRNAs in the highest expressing clone were analyzed in subsequent independent experiments and similar results were obtained (Table II).

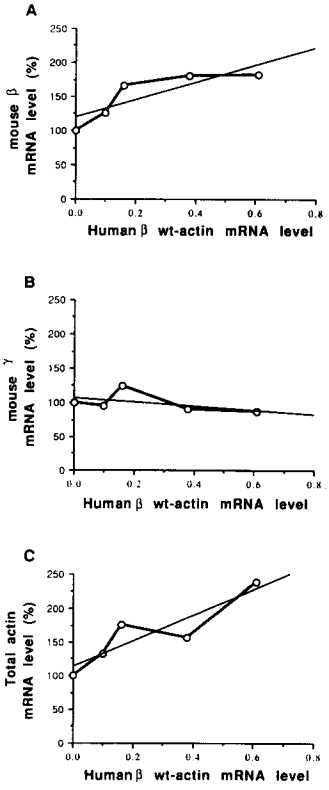

Figure 4. Actin mRNA levels in human βwt-actin gene transfectants. Endogenous β (*A*), γ (*B*), and total (*C*) actin mRNA levels were measured in a number of clones with different levels of expression of the transfected human gene. Determination and analysis of mRNA levels was as described in the legend to Fig. 3. Linear regression of the data points for γ-actin (*B*) revealed that the line which fits the points does not have a slope significantly different from zero, indicating that mouse γ-actin levels were constant for varying βwt-actin actin levels.

Again, to calculate the percentage of total actin mRNA, the mouse and human actin mRNA levels were added and as expected, the total actin mRNA pool size increased in parallel with the total protein pool (Tables I and III). Independent determination of total actin mRNA levels using a total actin-specific probe yielded similar results (Fig. 4 and Table III). We conclude that increasing expression of the human βwt-actin gene induces a response from the mouse β-actin gene which promotes an increase in both actin mRNA (Table III) and protein (Table I) pool size. This is in contrast to the response to expression of the human γ-actin gene indicating that C2 cells can discriminate between the nonmuscle actin genes at least with respect to feedback-regulation. In addition, human βwt-actin gene expression invokes statistically significant different responses from the endogenous actin genes (Table II). Beta-actin expression increases; whereas γ-actin expression remains constant. This also indicates feedback-regulatory differences between the two nonmuscle actin genes.

βsm-Actin Gene Elicits a Feedback-Regulatory Response

We have previously observed that the highest expressing βwt-actin transfectants show a substantial increase in cell volume (over twofold), whereas the γ-actin transfectants have a reduced cell volume (Schevzov et al., 1992). In the case of βwt-actin gene transfectants, there is a clear correlation between the increase in actin pool size and cell morphology. In the same experiment, we observed that transfection of a β-actin gene (βsm) carrying a single point mutation produced a morphological phenotype similar to that of γ-actin (Schevzov et al., 1992). This suggested to us that the morphological impact of these different genes was dependent on the encoded protein. By analogy, we would predict that the βwt- and βsm-actin genes may have different feedback-regulatory impact on the endogenous actin genes.

Analysis of C2 cells transfected with the βsm-actin gene revealed that the total actin protein pool was slightly decreased (Table I). Coomassie staining of a gel loaded with total protein from the transfectants revealed that there was a progressive replacement of the 42-kD actin band by the 44-kD mutant actin band with increasing expression of the βsm-actin gene (data not shown). Direct determination of the total actin mRNA pool using a total actin-specific probe and addition of the mouse and human actin mRNAs both showed that total actin mRNA levels were essentially unchanged (Fig. 5 and Table III). Regression analysis was performed on the data points and demonstrated that there was no relationship between βsm-actin and total actin mRNA expression; instead total actin mRNA levels were constant ($r = 0.04$, $P > 0.1$). Thus, expression of the βsm-actin gene invoked a feedback-regulatory response such that endogenous actin expression decreased to compensate for the human actin expression in order to maintain a relatively constant total actin protein and mRNA pool size.

Increasing βsm-actin gene expression is compensated by a progressive decrease in the levels of both mouse β- and γ-actin mRNAs (Fig. 5). Regression analysis was performed on the data points and a linear relationship was found to exist between the level of human βsm-actin and mouse β-actin ($r = 0.88$, $P < 0.05$) mRNA expression and a logarithmic relationship was found between βsm-actin and γ-actin ($r = 0.98$, $P < 0.025$) mRNA expression indicating that βsm-actin gene expression resulted in altered endogenous actin expression. In addition, it is apparent that mouse γ-actin mRNA is down-regulated to a greater extent than β-actin mRNA in all transfectants (Fig. 5). Independent experiments with the highest expressing clone also exhibited a statistically significant difference in the response of the mouse β- and γ-actin genes (Table II).

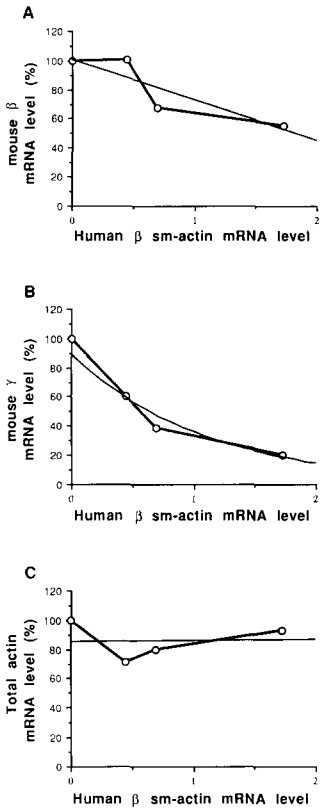

Figure 5. Actin mRNA levels in human βsm-actin gene transfectants. Endogenous β (*A*), γ (*B*), and total (*C*) actin mRNA levels were measured in a number of clones with different levels of expression of the transfected human gene. Determination of mRNA levels was as described in the legend to Fig. 3. Linear regression of the data points for total actin (*C*) revealed that the line which fits the points does not have a slope significantly different from zero, indicating that total actin levels were constant for varying βsm-actin actin levels.

The feedback-regulatory response elicited by the introduction of the βsm-actin gene into C2 myoblasts contrasted with that elicited by the βwt-actin gene. Expression of the βsm-actin gene resulted in a decrease in both endogenous β- and γ-actin mRNA levels (Fig. 5) while the total actin pool, both mRNA and protein, was relatively constant (Tables I and III, Fig. 5). In contrast, βwt-actin gene expression induced expression of the endogenous β-actin gene while not significantly altering γ-actin gene expression resulting in an increased total actin pool size (Tables I and III, Fig. 4). The different regulatory responses elicited by the introduction of βwt- and βsm-actin genes into C2 myoblasts suggests that it is the nature of the protein encoded by the transfectant gene that determines the feedback-regulatory response of the mouse actin genes.

Promoter competition is eliminated as a possible mechanism for the feedback-regulatory responses elicited because the βwt- and βsm-actin genes have identical promoters. To further rule out promoter competition as a feedback-regulatory mechanism, analysis of βtm-actin gene transfectants was performed. This form of the β-actin gene has three point mutations and results in an unstable protein product (Lin et al., 1985). The βtm-actin gene transfectants were found to exhibit normal C2 cell morphology and had a normal β to

γ protein ratio (Schevzov et al., 1992). Increasing βtm-actin gene expression resulted in no change in total actin protein levels (Table I), contrasting with the increase observed in βwt-actin transfectants. The actin mRNA levels were then examined in these transfectants (Fig. 6). There was no significant change in either mouse β- or γ-actin levels in response to increasing levels of βtm-actin gene expression. Regression analysis was performed on this data and confirmed these findings (for mouse γ-actin $r = 0.24$, $P > 0.1$ and for mouse β-actin $r = 0.70$, $P < 0.05$). However, total actin mRNA levels increased in a linear fashion with increasing βtm-actin gene expression ($r = 0.85$, $P < 0.025$). This increase was confirmed by calculation of the percentage of total actin mRNA from the levels of mouse β- and γ-actin and human βtm-actin mRNA (Table III). Therefore, expression of the βtm-actin gene did not affect expression of the mouse actin genes but simply added to the total actin mRNA pool. However, the actin protein pool was not altered and no βtm-actin gene protein product was detectable (Schevzov et al., 1992). The βtm-actin gene results are also not compatible with a promoter competition mechanism operating for actin feedback-regulation since expression of an additional β-actin promoter did not decrease endogenous β- or γ-actin expression. Additionally, the difference in the responses by the mouse β-actin gene to expression of the βwt- and βtm-actin genes strongly suggests that the form of the protein encoded by the transfectant gene influences the feedback-regulatory responses of the mouse actin genes.

Actin Cytoskeletal Organization Is Involved in Actin Feedback-Regulation

The feedback-regulatory responses of the mouse actin genes may have been related to the change in cell morphology resulting from expression of the human genes. Certainly, the observation that the nature of the encoded protein dictates the mouse actin feedback-regulatory response is most easily reconciled with a mechanism that relates actin function to gene regulation. For example, it may be that feedback-regulation of actin mRNA levels is mediated in some manner by the morphological demands of the cell such as tension maintained by actin cables in the cell. Assuming this to be the case, alterations in the organization of the actin cytoskeleton may lead to changes in actin gene expression. Therefore, we tested whether the down-regulation of mouse β- and γ-actin mRNA levels in the γ- and βsm-actin transfectants was determined by the actin organization in these cells.

The actin cytoskeleton of transfectant cells was disrupted by exposing the cells to Cytochalasin D. Treatment of cells with Cytochalasin D has been found to severely disrupt network organization, increase the number of actin filament ends, and lead to the formation of filamentous aggregates composed mainly of actin filaments (Schliwa, 1982). After a 5-h treatment with Cytochalasin D, actin stress fibers, as evaluated by immunofluorescent staining with an actin antibody, were essentially eliminated (Fig. 7). After disorganization of the control cell actin cytoskeleton a 1.5- and 2-fold increase was observed in β- and γ-actin mRNA levels, respectively (Fig. 8, Table IV). Similar increases in actin mRNA levels (Sympson and Geoghegan, 1990) and protein synthesis (Tannenbaum, 1986) after Cytochalasin D treatment have been described. Interestingly, the βsm- and γ-actin gene transfectant cells also exhibited an increase in the mouse actin mRNA levels. Mouse β-actin mRNA levels increased twofold while γ-actin mRNA levels increased sixfold (Fig. 8, Table IV). In particular, while the mouse β-actin mRNA level increased after Cytochalasin D treatment, it did not return to control cell values (Fig. 8). The mouse γ-actin mRNA level, however, increased to a level that was not significantly different to that seen in the treated control cells (Fig. 8). Thus, the compensating down-regulation of mouse γ-actin expression in the βsm- and γ-actin transfectants was abolished after disruption of the actin cytoskeleton. This suggests that the organization of the actin cytoskeleton is involved in the feedback-regulation of γ-actin gene expression in the transfectants.

The down-regulation of mouse β-actin in the γ- and βsm-actin transfectants was relieved partially after disorganization of the cytoskeleton. Interestingly, the fold increase in mouse β-actin mRNA levels after Cytochalasin D treatment was statistically equivalent in all transfectant cell lines tested (Table IV). This suggests that there was no specific relief of β-actin mRNA down-regulation in the γ- and βsm-actin

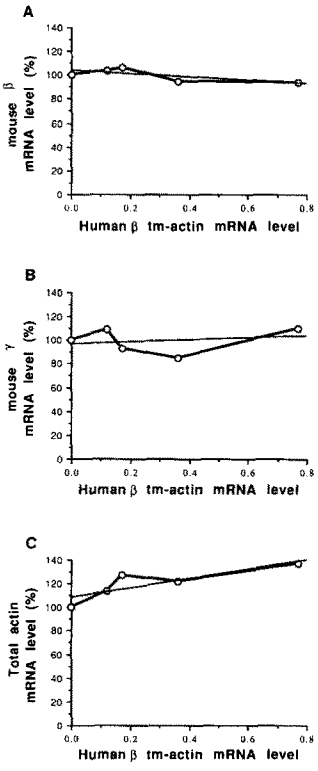

Figure 6. Actin mRNA levels in human βtm-actin gene transfectants. Endogenous β (*A*), γ (*B*), and total (*C*) actin mRNA levels were measured in a number of clones with different levels of expression of the transfected human gene. Determination of mRNA levels was as described in the legend to Fig. 3. Linear regression of the data points for β- and γ-actin (*A* and *B*) revealed that the lines which fit the points do not have a slope significantly different from zero, indicating that mouse β- and γ-actin levels were constant for varying βtm-actin levels.

Table IV. Increase in Endogenous Actin Expression after Cytochalasin D Treatment

Clone	Mγ	Mβ
pUC-18	196 ± 36	147 ± 39
γ_{33}-B1	550 ± 177*	220 ± 45
β_{sm}-22	623 ± 173*	182 ± 36

The values are expressed as a percentage of the actin expression level in the untreated cells. The results are the mean and standard deviations of three independent experiments.
* This symbol indicates that the values are significantly different ($P < 0.05$ using the t test) from the increase measured for the pUC-18 cells.

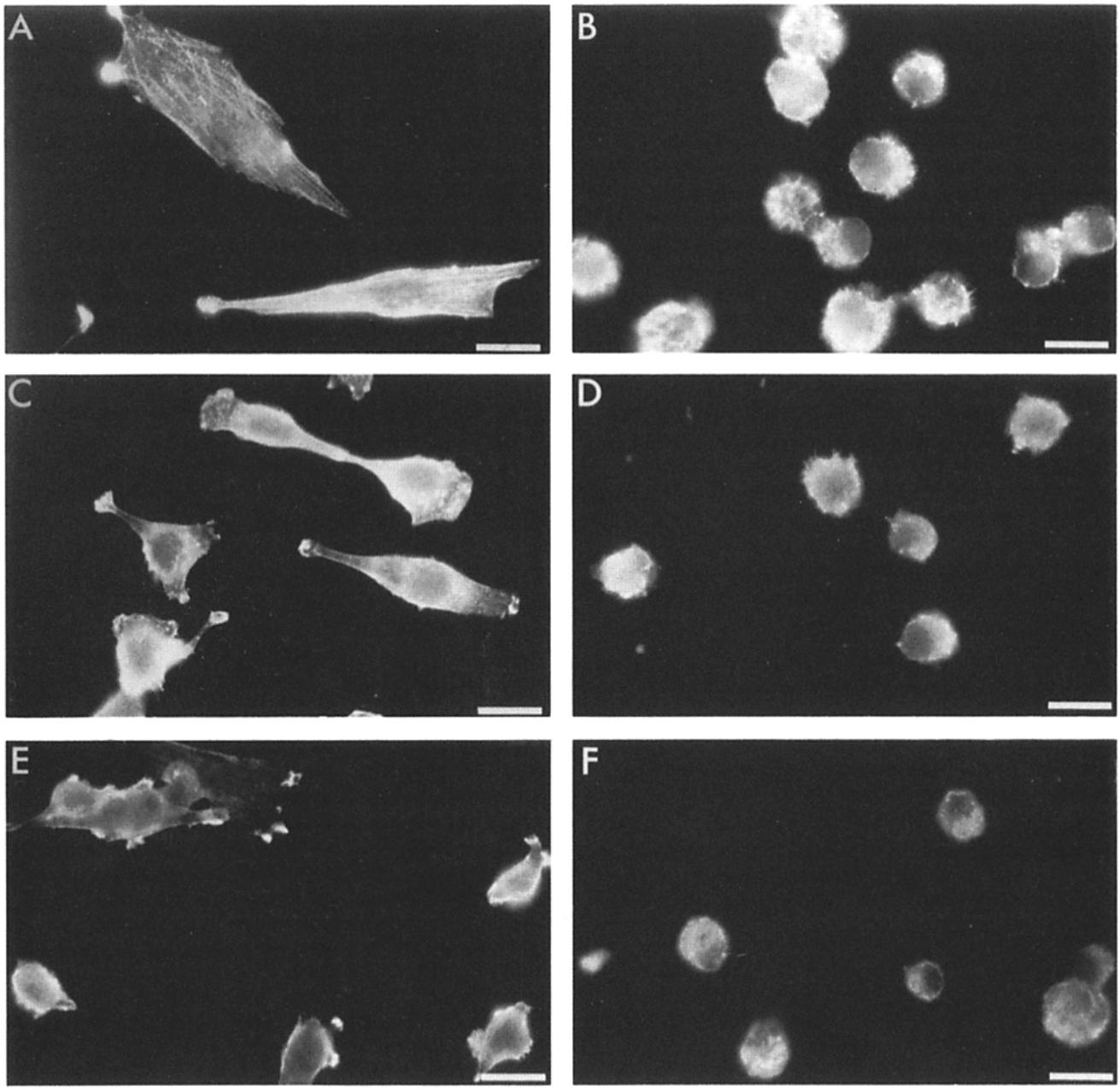

Figure 7. Depolymerization of actin filaments after Cytochalasin D treatment. Control (transfected with the plasmid pUC-18) and clonal derived cells were treated with Cytochalasin D at 2.5 μg/ml (stock 2 mg/ml in DMSO) for 5 h or with an appropriate amount of DMSO (samples without Cytochalasin D treatment). The cells were grown on collagen-coated glass slides, treated with Cytochalasin D or with DMSO, and then fixed and permeabilized using 1% formaldehyde followed by methanol. Visualization of actin filaments was achieved by indirect immunofluorescence staining of the cells with a general actin antibody (*C4*) followed by fluorescein-conjugated goat anti-mouse IgG. Photos (*A* and *B*) control cells, (*C* and *D*) γ_{33}-B1 and (*E* and *F*) βsm-22. Cells without Cytochalasin D treatment (*A*, *C*, and *E*) and cells with Cytochalasin D treatment (*B*, *D*, and *F*). Bar, 10 μm.

transfectants. Instead β-actin expression was stimulated after disorganization of the actin cytoskeleton in all transfectants perhaps resulting from activation of an enhancer region in the β-actin promoter. The difference in the magnitude of the increase in β- versus γ-actin mRNA levels in the transfectants also indicates that the two actin isoforms are regulated differently. In particular, the results suggest that the mechanism responsible for the down-regulation of β-actin in γ- and βsm-actin transfectant cells may be distinct from that governing γ-actin.

Discussion

Actin Gene Expression Is Subject to Feedback Regulation

Our data shows that the size of both the actin protein and mRNA pools is not absolutely fixed in C2 myoblasts. Previous studies in human fibroblastic cell lines have suggested that transfection of the four genes used in this study does not alter total actin protein synthesis (Leavitt et al., 1987*a*; Ng

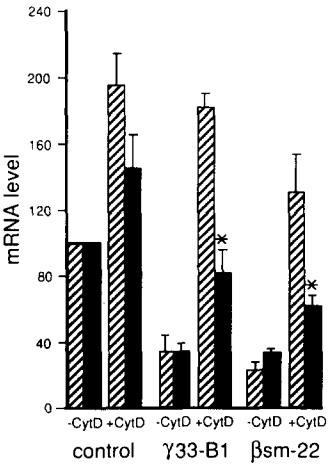

Figure 8. Effect of Cytochalasin D treatment on the actin mRNA levels in actin gene transfectants. Endogenous γ (▨) and β (■) actin mRNA levels were measured in human actin gene transfectants with and without Cytochalasin D treatment. Pooled clones were used for the control cells (pUC-18 transfectants) and the clones with the highest levels of expression of the transfected genes were used from each actin gene transfection: human γ-actin transfectant, γ_{33}-B1 and human β-single mutant actin transfectant, βsm-22. Cells were treated with Cytochalasin D at 2.5 μg/ml (stock 2 mg/ml in DMSO) for 5 h or with an appropriate amount of DMSO (samples without Cytochalasin D treatment). Panels were probed for 18S ribosomal RNA and corrected for loading discrepancies. The values for endogenous actin mRNA levels were expressed relative to those detected in untreated control cells. The error bars represent the standard deviations of the means. The * denotes that the mRNA level after Cytochalasin D treatment was significantly different from that of the treated control mRNA level (*t* test, $P < 0.05$).

et al., 1988). The difference between these experiments may reflect the extent of expression achieved with the βwt-actin gene in these two systems. High level expression of the transfected βwt-actin gene was required to observe an increase in the myoblast actin pool size. Because Leavitt and co-workers could not discriminate between the transfected and endogenous βwt-actin mRNAs, it is uncertain that they attained levels of exogenous gene expression comparable to those at which we observed an increased pool size (Leavitt et al., 1987*a*).

C2 myoblasts transfected with the βsm-actin gene exhibited differential down-regulation of the endogenous β- and γ-actin mRNA levels. In contrast, Leavitt et al. (1987*a*) found that in human fibroblasts expressing the βsm-actin gene the protein synthesis for both endogenous β- and γ-actin decreased to the same extent. Interestingly, the γ-actin mRNA level did not decrease in Hut-12 cells transfected with the βsm-actin gene (Leavitt et al., 1987*b*). The expression of endogenous β-actin in the human transfectants could not be determined since it was impossible to discriminate between the transfected and endogenous β-actin mRNAs. However, there are clearly differences in γ-actin expression between the fibroblasts and C2 myoblasts expressing the βsm-actin gene. This suggests that the actin feedback-regulation found in C2 myoblasts may not be extrapolated to all other cell types.

Feedback-regulation of actin isoform expression in response to expression of an exogenous actin gene occurs at the level of mRNA accumulation. The simplest mechanism which might account for this is promoter competition. For example, both the β- and γ-actin genes contain serum response elements in their promoter regions (Erba et al., 1988). However, the observation that expression of the βwt-, βsm-, and βtm-actin genes elicit such divergent feedback-regulatory responses is not compatible with a promoter competition model. Indeed, the finding that expression of the human βwt-actin gene results in elevated mouse β-actin expression is totally incompatible with promoter competition.

The autoregulatory mechanism described by Pachter et al. (1987) for β-tubulin involves the αβ-tubulin dimer directly regulating tubulin mRNA levels such that the level of β-tubulin mRNA appears to be fixed. If a similar mechanism was operating for actin feedback-regulation it would be expected that exogenous actin expression would be compensated for by a decrease in endogenous actin expression in order to maintain a constant actin mRNA pool size. The elevated mouse β-actin expression in the βwt-actin transfectants is incompatible with a simple actin monomer-driven feedback mechanism. If the level of monomer was to control expression of the actin genes, one would predict that as a transfected gene began to be expressed, the monomer pool would start to rise which would then lead to reduced expression of both the endogenous and transfected genes. In this way, a monomer control mechanism would essentially act to prevent any increase in actin monomer and polymer pools. Thus, an increase in actin pool size and especially an increase in endogenous β-actin expression should not be possible under a simple monomer control mechanism.

The feedback-regulation of expression of the three major structural polymer systems in mammalian cells appears to be unique for each structural element. Cleveland and coworkers (Pachter et al., 1987; Yen et al., 1988) have found that the αβ-tubulin dimer levels regulate β-tubulin mRNA stability. This serves to regulate β-tubulin mRNA levels in accordance with the ability of the protein product to incorporate into microtubules. Thus, tubulin represents a true autoregulatory system in which the product directly controls its own synthesis. However, the autoregulation of tubulin expression may not simply be controlled by the subunit levels since Katz et al. (1990) found that expression of an extra tubulin gene in yeast resulted in an increase in tubulin mRNA levels and a slight increase in tubulin protein levels. Although similar to that observed with the β-actin transfectants, the protein levels did not parallel the mRNA levels contrasting with that found for the actin transfectants. The intermediate filament system appears to have no feedback-regulation of pool size such that expression of an exogenous gene can elevate the pool without having any impact on endogenous gene expression (Monteiro et al., 1990). Actin may therefore present us with a third mechanism in which actin filament organization may play a role in the feedback-regulation of the levels of actin mRNA. Thus, actin gene expression appears to be subject to flexible feedback-regulation, and not true autoregulation, in which the actin cytoskeletal organization may be a key component of the regulatory mechanism.

β- and γ-Actin Genes Are Functionally Different

In this study, we have found that transfection of βwt- and γ-actin genes are not equivalent in their ability to elicit feedback-regulatory responses from the mouse actin genes. Furthermore, the mouse actin genes respond differently to the same human actin gene; at least for the βwt- and βsm-actin genes. Finally, the different responses of the mouse β- and γ-actin genes in both the γ- and βsm-actin transfectants after disruption of the cytoskeleton indicates that regulation of β- and γ-actin may be mediated differently. This suggests that the actin feedback-regulation mechanism can

discriminate between the β- and γ-actin genes at some level. Therefore, we have identified a biochemical difference between the nonmuscle actin genes. The availability of this biochemical assay now provides us with a crucial tool necessary to define the level at which the genes encoding actin isoforms are functionally different. Experiments are presently underway to determine whether the protein product of the actin genes, the gene sequences themselves or a combination of the two determine the actin feedback-regulatory response. It should be noted, however, that the differing responses elicited in the βwt-, βsm-, and βtm-actin transfectants suggest involvement of the protein in the actin feedback-regulatory response.

Actin feedback-regulation differs to tubulin autoregulation with respect to the regulatory responses of different isoforms. Both of the mouse nonmuscle actin isoforms responded to the expression of the human γ-actin gene in C2 myoblasts demonstrating that the levels of β- and γ-actin are not fixed in these cells. In contrast, the expression of a chicken class IV β-tubulin gene in CHO cells precipitated a decrease in expression of the endogenous class IV isotype so that the total level of class IV isotypes remained unchanged. However, the class I isotype level was not affected (Sisodia et al., 1990). Expression of β-tubulin appears to involve a true autoregulatory system in that the levels of specific isotypes are fixed. The CHO cells were able to maintain these fixed expression levels by reducing endogenous expression of the tubulin isotype corresponding to that which was transfected into the cells. In contrast, C2 myoblasts allow the levels of β- and γ-actin to change and so transfectant cells with skewed β/γ ratios can be obtained. There are two possible explanations for the differences between the β-tubulin and actin feedback-regulatory systems: actin and tubulin are regulated differently, or C2 myoblasts and CHO cells have different feedback-regulatory mechanisms. At this time, neither possibility can be eliminated.

Relationship of Actin Gene Expression and Cell Morphology

There is a close correlation between cell morphology and actin gene expression in human actin gene transfectants. The contrasting effects of transfection of the βwt-, βsm-, βtm-, and γ-actin genes on cell morphology and surface area (Schevzov et al., 1992) closely parallel their impact on actin pool sizes and mouse actin gene expression. The question arises as to whether actin gene regulation reflects the morphological impact of the human gene or the morphological effects observed in the transfectants result from altered mouse actin gene expression in response to human actin gene expression. In a multicomponent system, such as transfected cells, it is almost impossible to answer such a question. However, our results would support the first possibility. The ability of Cytochalasin D treatment to abolish the down-regulation of mouse γ-actin mRNA in the βsm- and γ-actin transfectants emphasizes the role that the actin cytoskeleton plays in the gene regulatory process. Additionally, the elevation of mouse β-actin expression in the βwt-actin transfectants is hard to explain by promoter competition or other described feedback-regulatory mechanism. It may be explained by the morphological demands of the βwt-actin transfectants. Immediately after transfection, high levels of βwt-actin may have been expressed resulting in an increase in β-actin protein which was then incorporated into the cytoskeleton. After integration of the human βwt-actin gene, the exogenous gene expression may have decreased and in order to maintain the resultant morphology, large cells with well-defined stress fibers (Schevzov et al., 1992), there was an increase in the endogenous β-actin gene expression.

The role of cell morphology in the feedback-regulation of cytoskeletal protein gene expression has been the subject of considerable debate (Ben-Ze'ev, 1986). Previous experiments by Ben-Ze'ev and co-workers have suggested that cell morphology can feedback-regulate the expression of genes involved in the maintenance of cell cytoarchitecture (Ben-Ze'ev, 1986). In such studies, changes in cell growth conditions and interactions with extracellular matrix components have been found to alter actin protein synthesis and mRNA levels (Farmer et al., 1983; Ben Ze'ev and Amsterdam, 1986). Because such studies may involve changes in the activity of signal transduction pathways in the cells, it is unclear if the observed actin regulation is truly due to feedback control via the cytoskeleton. Indeed, both actin and vinculin gene expression are very sensitive to growth factor stimulation (Ben-Ze'ev et al., 1990; Greenberg and Ziff, 1984). While our experiments support the argument that actin gene expression can be feedback-regulated via changes in the cytoskeleton, it is likely that other mechanisms also operate to regulate actin genes.

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