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Expression of Transfected Mutant β-Actin Genes: Alterations of Cell Morphology and Evidence for Autoregulation in Actin Pools

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Two different mutant human β-actin genes have been introduced into normal diploid human (KD) fibroblasts and their immortalized derivative cell line, HuT-12, to assess the impact of an abnormal cytoskeletal protein on cellular phenotypes such as morphology, growth characteristics, and properties relating to the neoplastic phenotype. A mutant β-actin containing a single mutation (Gly-244 → Asp-244) was stable and was incorporated into cytoskeletal stress fibers. Transfected KD cells which expressed the stable mutant β-actin in excess of normal β-actin were morphologically altered. In contrast, a second mutant β-actin gene containing two additional mutations (Gly-36 → Glu-36 and Glu-83 → Asp-83, as well as Gly-244 → Asp-244) did not alter cell morphology when expressed at high levels in transfected cells, but the protein was labile and did not accumulate in stress fibers. In both KD and HuT-12 cells, endogenous β- and γ-actin decreased in response to high-level expression of the stable mutant β-actin, in a manner consistent with autoregulatory feedback of actin concentrations. Since the percent decreases in the endogenous β- and γ-actins were equal, the ratio of net β-actin (mutant plus normal) to γ-actin was significantly increased in the transfected cells. Antisera capable of distinguishing the mutant from the normal epitope revealed that the mutant β-actin accumulated in stress fibers but did not participate in the formation of the actin filament-rich perinuclear network. These observations suggest that different intracellular locations differentially incorporate actin into cytoskeletal microfilaments. The dramatic impact on cell morphology and on β-actin/γ-actin ratios in the transfected diploid KD cells may be related to the acquisition of some of the characteristics of cells that underwent the neoplastic transformation event that originally led to the appearance of the β-actin mutations.

Actin is a ubiquitous, highly abundant protein that is responsible for a variety of cellular activities, including motility and the structural properties of the cytoplasm. This protein, highly conserved in evolution, forms complex structures in all eucaryotic cells by a combination of self-polymerization and interactions with a host of binding proteins (reviewed in reference 37). These complex interactions have been the subject of intense biochemical and structural study but have not yet been amenable to genetic analysis. Few structural mutations in actin are known, as might be expected for a protein whose actions are so central to normal cell morphology and function. The recessive flightless mutants of *Drosophila melanogaster* show a disordered structure of the sarcomeres (18, 31) and induction of stress proteins (15, 18, 31) in the indirect flight muscles of homozygotes in which mutant alleles of a tissue-specific actin are expressed (18, 31).

Human fibroblasts which express mutant β-actins have been generated by the neoplastic transformation of diploid KD fibroblasts in vitro and the isolation of stable focus-derived, neoplastic strains (16, 25). The KD fibroblasts are phenotypically normal diploid cells with a finite life span in culture (2, 16, 20, 22, 25, 26, 40, 42); HuT-11, HuT-12, and HuT-13 are neoplastically transformed "immortalized" strains derived from KD cells that express transformation-specific protein markers (3, 10, 11, 20–22, 25, 26, 40) and anchorage-independent growth (16, 17), but rarely produce tumors in nude mice (20, 22). HuT-14 is a highly tumorigenic strain that exhibits additional differences in cellular morphology and gene expression (19–22, 25, 26, 42). HuT-14 cells are also unique in that they synthesize a defective β-actin as the result of a point mutation (codon 244 Gly to Asp) from one of two functional β-actin genes alleles (24, 25, 29, 42). Subsequently, a substrain of HuT-14 cells was derived, HuT-14T, which displayed an elevated degree of tumorigenicity, a more variant distribution of cytoskeletal actin than that originally observed for HuT-14 cells, and a further change in both the structure and function of the mutant β-actin polypeptide (21). Sequencing of the mutant β-actin gene isolated from HuT-14T cells (29) demonstrated that this gene has acquired two additional point mutations, resulting in additional amino acid exchanges at codons 36 (Gly to Glu) and 83 (Glu to Asp). The resulting triple-mutant β-actin was found to have a short half-life in the cell, a greatly diminished ability to incorporate into the cytoskeleton, and a decrease in affinity for DNase (21). We have cloned both functional (allelic) β-actin genes from HuT-14 and HuT-14T cells (24, 29).

The studies presented here were designated to examine the effect of structural mutation in β-actin on the morphology of the cell and its impact on the synthesis of normal actins. In the accompanying paper (28) we explore the impact of mutant β-actins on cell tumorigenic potential and on total cellular gene expression. We used DNA transfection

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to engender mutant β-actin synthesis in normal diploid human KD fibroblasts and in the immortalized but nontumorigenic derivative HuT-12 cell line. We succeeded in identifying transfectant strains that synthesized mutant β-actin in excess of the combined amount of endogenous β- and γ-actins.

MATERIALS AND METHODS

Cell cultures. The KD strain of diploid human fibroblasts and the HuT cell lines have been described previously (16, 25, 26). All cell types were cultured in Dulbecco modified Eagle medium, supplemented with 20% fetal calf serum.

Transfection of human fibroblasts. Genomic restriction fragments containing mutant and wildtype β-actin genes (24, 29) (14-kilobase *Eco*RI fragments) were subcloned into pSV2-neo (41), which contains the recombinant gene conferring resistance to the neomycin analog G418. Subconfluent KD fibroblasts (1×10^6 to 1.5×10^6 cells per 100-mm-diameter culture dish) were transfected with 10 μg of plasmid DNA by the calcium phosphate precipitation technique of Graham and Van der Eb (12). One to two days after initiation of transfection, the culture medium was replaced with fresh medium supplemented with 600 μg of G418 per ml. Drug treatment was continued for 7 days, after which the drug was omitted. Large colonies developed between 10 and 20 days following transfection at frequencies which varied between 1×10^{-6} and 5×10^{-6} from one transfection trial to another. Transfection of transformed HuT-12 cells with the same plasmids yielded higher frequencies (10^{-4} to 10^{-5}) of G418-resistant colonies. The transfected KD colonies (passage 1) were cultivated for 16 to 28 days until they were approximately 1 cm in diameter. We estimate that colonies of this size contained about 2.5×10^4 cells and thus had achieved 18 population doublings from the time of transfection and 13 to 14 population doublings from the time of removal of G418. The cells of each colony were suspended by trypsinization; 75% of the cells were seeded and cultured in a 60-mm dish, and 25% were put into a 15-mm-diameter culturing well. The next day (passage 2), cells in the small culturing well were labeled with [^{35}S]methionine for 6 h, followed by examination of cellular proteins synthesis by high-resolution two-dimensional (2-D) gel electrophoresis (26). The passage 2 cells in the 60-mm dish were cultured continuously with serial dilution at a split ratio of 4:1 at each subsequent passaging.

Labeling of cellular proteins and 2-D PAGE. All cell types in high-density subconfluent monolayers were labeled for 2 to 6 h with [^{35}S]methionine (New England Nuclear Corp.) as previously described (19, 25, 26). Radiolabeled cell monolayers were rinsed three times with cold phosphate-buffered saline, drained, and then lysed directly in O'Farrell lysis buffer A (25). Protein samples were stored at −70°C prior to electrophoresis. Replicate 2-D polyacrylamide gel electrophoresis (PAGE) gels were made from each protein sample with the Iso-Dalt system from Electro-Nucleonics (Rockville, Md.). However, the second-dimension gels were thinner (1 mm versus 1.5 mm). Kodak XAR-2 film was used for autoradiography. Exposure time was 1 to 2 h for measurement of actin synthesis and 2 to 10 days for measurement of total protein synthesis. The film was developed in a Kodak X-Omat model 5 processor and digitized on an Optronics P-1000 film scanner at a pixel size of 200 μm (26).

Computerized microdensitometry of 2-D PAGE autoradiogram images. Standard calibration strips with 18 doublings were produced as described by Garrels (8) and exposed with each film. After exposure, the autoradiograms of both the gels and the calibration strips were digitized with a P-1000 Optronics scanner. Data points derived from the exposed strips were used to generate calibration curves, and the digitized images were converted to counts. The calibration curves are designed to correct for background radiation and counts contributed by the film base. High-frequency noise is removed from the significant portions of the image by convolution with the 7×7 template described in Vo et al. (43). Regions near background are smoothed instead with a smaller, 3×3 template, both to improve efficiency and to prepare for subsequent streak removal. Streaks and unfocused areas of radioactivity not assignable to any given protein spot were removed with an algorithm similar to that used by Anderson et al. (1).

Manual method. The amount of polypeptide in a resolved "spot" was measured after being displayed on a color graphics system (Chromatics CGC 7900). The operator designated the polypeptide spot to be quantified by specifying the upper left and lower right corners of a rectangle surrounding the protein spot. The counts per minute value of the spot was computed by integrating the area within the rectangle.

Automated method. After streak removal, peaks were detected by finding those areas of the gel above a set threshold that had negative second derivatives in both coordinate axes. The location of the highest pixel in the area, its height, and the approximate standard deviation in each axis were stored in a candidate peak list. These parameters were then refined by iteratively fitting 2-D Gaussians to each candidate peak by a least-squares method similar to that described in Lutin et al. (30). At this stage, peaks swamped by larger neighbors and at the threshold of resolution were removed from the list. The total number of counts in each remaining peak was then integrated from the area within 2 standard deviations of the peak's center. When two or more peaks competed for the counts from a single pixel, the value of the pixel was apportioned on the basis of the value predicted by each competing peak's Gaussian parameters.

Indirect immunofluorescence microscopy. Cells were seeded onto sterile microscope slides 20 h prior to fixing. For fixing of cytoskeletons, slides were flicked to remove culture medium, rinsed briefly in cation-free phosphate-buffered saline, incubated for 1 min at room temperature in aqueous 1% Triton X-100 containing 0.01 M Tris hydrochloride (pH 7.9), 0.03 M KCl, and 0.01 M MgCl, and then fixed with acetone for 10 min at room temperature. After the slides were air dried, the fixed cytoskeletons were incubated for 15 min at room temperature with purified rabbit anti-actin immunoglobulin G (IgG). The antibody-reacted slides were then rinsed for 20 min in phosphate-buffered saline with gentle agitation and three changes of phosphate-buffered saline and then drained and air dried. The antibody-reacted slides were then incubated for 15 min with fluorescein-conjugated anti-rabbit IgG antibody and rinsed and dried as before. Fluorescence reactions were examined and photographed with the Zeiss photomicroscope, a 20× objective, and Kodak 35-mm film (Tri-X 5063).

RESULTS

Mutant β-actin expression in transfected KD fibroblasts. KD and HuT-12 cells cotransfected with a β-actin gene plus the neomycin resistance gene were selected by resistance to G418. Twenty-three subclonal isolates of KD cells and more than 40 isolates of HuT-12 cells were picked and screened by

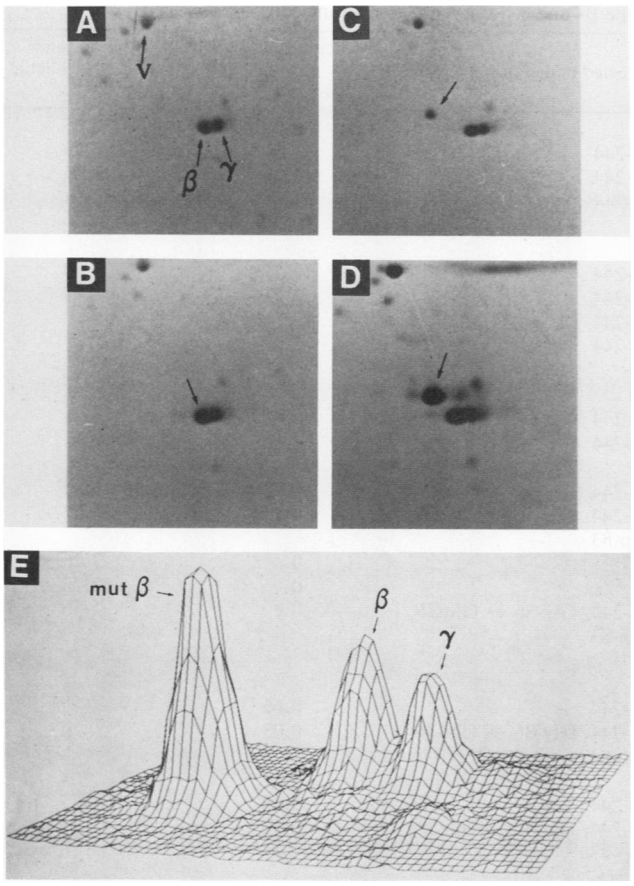

FIG. 1. Autoradiograms of [^{35}S]methionine-labeled actin polypeptides in total unfractionated polypeptides of normal and transfected KD fibroblasts separated by 2-D PAGE (21, 25, 26). (A) Untransfected KD cells; (B) KDwt-3 cells transfected with the wild-type β-actin gene 14Tβ17 (24, 28); (C) KDtm-2 cells transfected with the triply mutated β-actin gene 14Tβ21 (24, 28); (D) KDsm-5 cells transfected with the singly mutant β-actin gene 14Tβ29 (24, 28); (E) disintegrations per minute per pixel in actin polypeptides in an unsaturated autoradiogram pattern from the 2-D gel shown in panel D. The electrophoretic positions of vimentin (v), β-actin, and γ-actin from endogenous genes are indicated in panel A. The electrophoretic position of wild-type and mutant β-actin from exogenous genes is indicated by the arrows in panels B, C, and D and by mutβ in panel E.

examination of their relative rates of wild-type and mutant β-actin synthesis. Figure 1 shows autoradiograms of ^{35}S-labeled unfractionated proteins from untransfected KD cells (Fig. 1A), clonal cells transfected with a wild-type β-actin gene clone (Fig. 1B), clonal cells transfected with the triply mutated β-actin gene (Fig. 1C), and clonal cells transfected with a singly mutated β-actin gene (Fig. 1D). The relative rates of actin synthesis, determined by microdensitometry, are listed in Table 1. Untransfected KD and HuT cells expressed a ratio of 1.6 to 1.7 parts β-actin to 1 part γ-actin (also see reference 22). Two strains, out of a total of eight colonies transfected with the wildtype β-actin gene, exhibited a significant increase in the relative ratio of β-actin to γ-actin synthesis in KD cells from 1.65 to 2.25 (Fig. 1B, Table 1). There was no way to verify whether these altered strains were expressing the transfected gene as well as the endogenous gene, but similar experiments with rodent cells suggested that the KD clones were, in fact, expressing the exogenous wildtype β-actin gene. When we transfected Rat-2 cells (29) with the wild-type β-actin gene, we isolated a number of clones that also exhibited elevated expression of β-actin protein. In these colonies we were able to determine that the exogenous, transfected gene was being expressed because we could distinguish the human mRNA from the rat mRNA with the human-specific β-actin 3′ untranslated region DNA probe (35, 39) (data not shown).

Following transfection of KD cells with the triply mutated β-actin gene, one transfectant strain (KDtm-2) out of five assayed exhibited synthesis of the triply mutated β-actin (Fig. 1C). Following transfection with the singly mutated β-actin gene, 6 of 10 transfectant strains exhibited mutant β-actin synthesis. The actin region of a 2-D autoradiogram derived from labeling a strain that demonstrated the highest rate of singly mutated β-actin synthesis (KDsm-5, Table 1) is shown in Fig. 1D. The relative amount of exogenous mutant β-actin to endogenous β- and γ-actin polypeptides in this cell strain is depicted graphically in Fig. 1E. From the results tabulated in Table 1 we calculated that the ratio of mutant β-actin to endogenous wild-type β-actin and to total β-actin plus γ-actin in KDsm-5 cells was about 1.65:1 and 1.03:1, respectively. Similar ratios were obtained for actins of passages 11 to 13 of KDsm-9 cells, a second transfected strain expressing singly mutant β-actin. Thus, mutant β-actin from the exogenous gene had become the most abundant polypeptide in these transfectant strains.

Since all transfectant clones were initially selected by acquired resistance to G418, we also examined the possibility that expression of the mutant β-actins might vary over time in the absence of selective conditions. Accordingly, we tested the stability of the pattern of increased mutant β-actin expression in KDsm-5 and KDtm-2 cells in successive passages (two population doublings per passage) by microdensitometry of labeled total cellular proteins in 2-D gels. The rates of expression of the two mutant β-actins did not diminish after continuous subculturing (>20 population doublings) of these cell strains in the absence of G418 (data not shown).

Abnormal metabolic characteristics are an intrinsic property of the mutant β-actins. Having determined that the transfected actin gene products could become major components of the complement of cellular proteins, we were in a position to determine whether the characteristics of mutant β-actin metabolism in the parental KD cell strain matched those of the mutant β-actins in the mutagenized, immortalized cell lines from which they had been derived (Hut-14 and Hut-14T [21, 29]). Since the triply mutant β-actin in HuT-14T cells is degraded rapidly (21), we first tested the half-lives of these mutant β-actins in the two transfectant KD strains KDsm-5 (Fig. 1D) and KDtm-2 (Fig. 1C). The half-life properties of these two mutant β-actins were similar if not identical to their half-lives in the HuT-14 and HuT-14T cells lines (Fig. 2) (21). The singly-mutant β-actin of HuT-14 cells had a stability (≥30 h) nearly equal to that of the wild-type β- and γ-actins, whereas the triply-mutant β-actin of HuT-14T cells was highly unstable, exhibiting a half-life of about 2 h (Fig. 2) (21). This also means that the level of expression of the triply mutated β-actin detected by 6 h of metabolic labeling of the transfectant strain KDtm-2 (Fig. 1C, and Table 1) underestimated the true rate of expression of the mutant β-actin for that strain. We estimate from short pulse labeling of actins that the relative rate of expression of triply mutated β-actin to and β-,γ-actin in this transfected cell strain was about 1:1 (Fig. 2).

Since the singly and triply mutated β-actins of HuT-14 and

TABLE 1. Relative amounts of mutant and wild-type β- and γ-actins in transfectant cell strains

Cells	Transfectant strain	Transfected mutation[a]	Ratio, mutant β-actin/ β-+ γ-actin[b]	Ratio, total wild-type β-actin/ γ-actin[b]
Whole KD	KD	None		1.65
	KDsm-5	Gly-244 → Asp-244	1.03	1.65
	KDsm-6	Gly-244 → Asp 244	0.02	1.79
	KDsm-7	Gly-244 → Asp-244	0.18	1.68
Trial 1				
Passage 3	KDsm-9	Gly-244 → Asp-244	0.07	1.50
Passage 8	KDsm-9	Gly-244 → Asp-244	0.43	—[c]
Passage 11	KDsm-9	Gly-244 → Asp-244	0.70	—
Passage 13	KDsm-9	Gly-244 → Asp-244	0.75	—
Trial 2				
Passage 4	KDsm-9	Gly-244 → Asp-244	0.04	—
Passage 11	KDsm-9	Gly-244 → Asp-244	1.12	—
	KDsm-10	Gly-244 → Asp-244	0.12	1.85
	KDtm-2	Gly-244 → Asp-244, Gly-36 → Glu-36, Glu-83 → Asp-83	0.17	1.52
	KDwt-2	Wild type	0	2.26
	KDwt-3	Wild type	0	2.25
	KDtm-1	Gly-244 → Asp-244, Gly-36 → Glu-36, Glu-83 → Asp-83	0	1.56
Fractionated KD				
Detergent-resistant cytoskeleton	KDsm-5	Gly-244 → Asp-244	0.66	—
	KDtm-2	Gly-244 → Asp-244, Gly-36 → Glu-36, Glu-83 → Asp-83	0.03	—
Detergent-soluble cytosol	KDsm-5	Gly-244 → Asp-244	1.18	—
	KDtm-2	Gly-244 → Asp-244, Gly-36 → Glu-36, Glu-83 → Asp-83	0.38	—
Whole HuT-12	HuT-12	None		1.65
	HuT-12sm2	Gly-244 → Asp-244	0.38	—
	HuT-12sm4	Gly-244 → Asp-244	0.90	1.60

[a] Mutations present in the exogenous genes that were expressed in the transfectant strain (29).
[b] Determined by computerized microdensitometry of 2-D gel autoradiograms (26).
[c] —, Not determined.

Hut-14T cells also differed in their ability to incorporate into the cytoskeltons of these mutagenized and immortalized cell lines (21), we next examined the ability of the products of these mutant β-actin genes to incorporate into the cytoskeleton of transfected KD cell strains. We measured the amounts of exogenous mutant β-actin relative to the amounts of endogenous wild-type β-actin found in the Triton X-100-soluble cytosol and Triton X-100-insoluble cytoskeleton (21) by microdensitometry of polypeptides from these two fractions in 2-D gel autoradiograms. The partitioning of these two types of mutant β-actin was about the same as that previously reported for the HuT-14 and HuT-14T cell lines (Table 1) (21). The singly mutated β-actin synthesized in KDsm-5 cells and in KDsm-9 cells was incorporated into the cytoskeleton in significant amounts (≅36%), so that the ratio between mutant β-actin and wild-type β- plus γ-actin was 0.7:1 in the insoluble cytoskeleton fraction (Table 1). In contrast, less than 4% of the triply mutated β-actin in KDtm-2 cells was incorporated into the cytoskeleton (Table 1). The relative abundance of these two mutant β-actins was concomitantly increased in the cytosolic fractions of these transfected cell strains (Table 1). Thus, the characteristics of turnover and cytoskeletal partitioning of the mutant β-actins in transfected KD cells matched those of the immortalized cell lines from which the mutant β-actin genes were derived. We conclude that the differences in these metabolic properties must be intrinsic to the actin proteins themselves and not a property of the transformed status of the cell line.

Morphological effects. High-level synthesis of the singly mutated β-actin gene elicited a striking change in the cellular appearance of transfectant KD cells. The appearances of normal uncloned KD cells and several strains expressing high levels of singly or triply mutated β-actin are shown in Fig. 3. The most obvious difference between singly-mutant β-actin transfectants (Fig. 3C and 3D) and either normal KD (Fig. 3A) or the triply mutant β-actin transfectant cells (Fig. 3B) was their refractile appearance and their long cytoplasmic processes. Characteristically, these altered cells exhibited long dendritelike processes which were packed with vimentin-containing intermediate filaments (unpublished results). Also, fragments of cytoplasm were released from these cells that remained attached to the substratum. These cytoplasmic "minicells" eventually became round, detached from the substratum, and floated in the culture medium, accumulating in number with time. This difference in appearance allowed us to use normal light microscopy to distinguish the transfectant cells in a mixed culture containing an excess of cells that did not synthesize abundant levels of mutant β-actin. These morphological changes were not detectable in transfectants synthesizing either the triply mutant β-actin or low levels of the singly mutant β-actin or in the KD cell strains suspected of high-level expression of

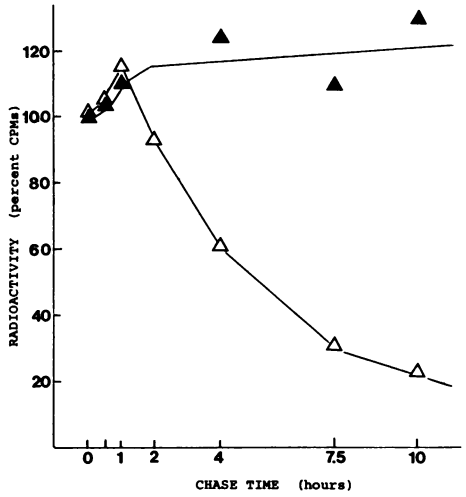

FIG. 2. Stability of mutant β-actins synthesized after transfection. Transfectant strains KDsm-5 (▲) and KDtm-2 (△) with singly- and triply-mutated β-actin, respectively, were labeled with [^{35}S]methionine for 1 h. Labeled cells were then washed free of unincorporated label and incubated further (chase time) with culture medium containing nonradioactive methionine. Ratios of the two mutant β-actins to stable, wild-type β- and γ-actin were determined by microdensitometry of 2-D gel autoradiograms produced from total cellular proteins taken after increasing chase times. One hundred percent equals the ratio of mutant β-actin to total wild-type β- and γ-actin after the 1-h pulse (see Table 1).

transfected copies of the wild-type β-actin gene. Thus, it appears that the morphological changes in cell phenotype are specifically correlated with the amount of singly mutant β-actin accumulated in the cells.

Cytoarchitectural location of mutant β-actin. The pronounced morphological change associated with high-level expression of the singly mutant β-actin implied that this protein may interfere with the finer structure of the cytoskeleton. One of us (U.A.) has developed two polyclonal antisera (anti-Asp-244 and anti-Gly-244) that can distinguish proteins bearing the single actin mutation (Asp-244) from the wild-type β- and γ-actins (Gly-244). The specificity of these antibodies was established by using Western blot analysis performed on high-resolution 2-D gel patterns in which mutant β-actin and wild-type β- and γ-actin were electrophoretically resolved (U. Aebi, manuscript in preparation). The availability of these antisera allowed us to answer several questions about the impact of singly-mutant β-actin on the cytoarchitecture of normal KD cells. First, is the mutant β-actin intermingled with the wild-type actin in microfilamentous structures? Second, does the presence of the mutant β-actin prevent the formation of normal in vitro cytoskeletal structures such as stress fibers? Third, is mutant β-actin distributed coincidently with wild-type (β- plus γ-) actin?

Figure 4 shows photomicrographs of cells examined by indirect immunofluorescence with the two rabbit antiactin antibodies, anti-Asp-244 and anti-Gly-244. The antibody which detected both wild-type β- and γ-actin (Gly-244) revealed a similar pattern in all cell types shown: it heavily stained both the perinuclear region of the cytoskeleton and the stress fibers and did not distinguish normal KD (not shown) and KDsm-5 (Fig. 4E) cells. The perinuclear localization of significant quantities of filamentous cytoskeletal actin was verified by results obtained with two independently isolated antiactin monoclonal antibodies (data not shown). The specificity of the anti-Asp-244 antiserum was demonstrated by its ability to detect stress fibers in KDsm-5 cytoskeletons (Fig. 4A and B) and in KDsm-9 cytoskeletons (Fig. 4D) and by its inability to bind significantly to the cytoskeletons of cells which lacked mutant β-actin (Fig. 4C and D). Consistently, the mutation-specific antiactin antibody gave greater resolution of stress fibers in transfectant KDsm-5 and KDsm-9 cells (Fig. 4A and D) than antibodies against wild-type β- and γ-actins (Fig. 4E). This result established that mutant β-actin is efficiently incorporated into stress fibers and that its presence does not block the formation of actin cables.

However, it appears that the KD cells did discriminate between the endogenous, wild-type actins and the singly-mutant β-actin: the mutation-specific anti-Asp-244 antibody consistently detected mutant β-actin in stress fibers but did

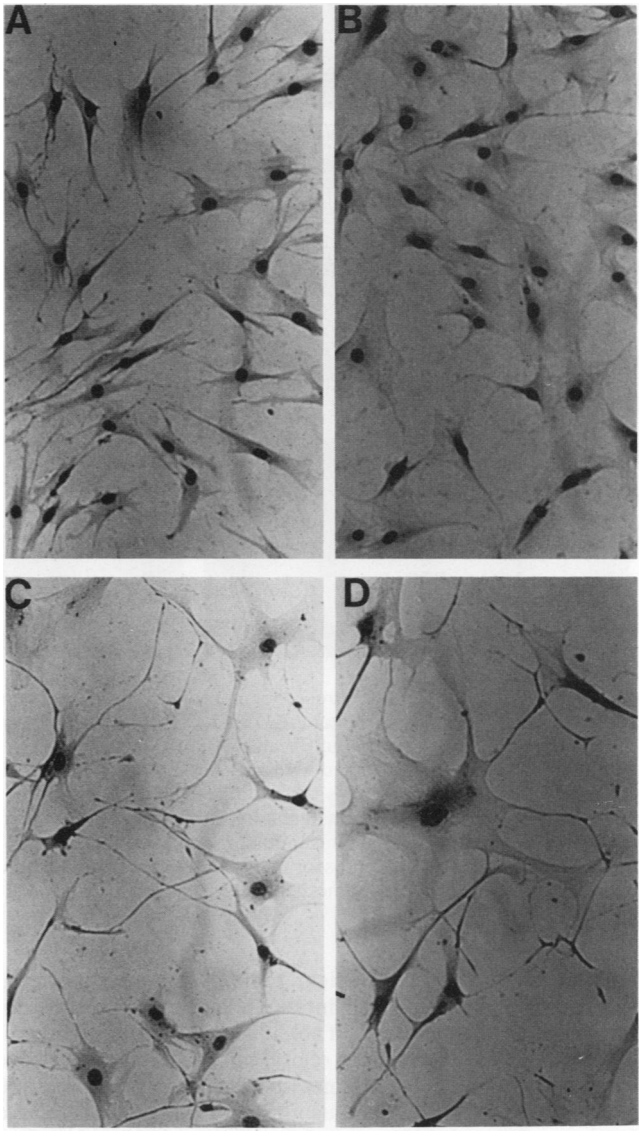

FIG. 3. Transfectant cells of strains (A) KD, (B) KDtm-2, (C) KDsm-5 passage 8, (D) KDsm-9 passage 12 (see Table 1) seeded into a plastic culture dish for 48 h were fixed with methanol, stained with Geimsa, and photographed by regular light microscopy (20 × objective, Kodak Tri-X 5063).

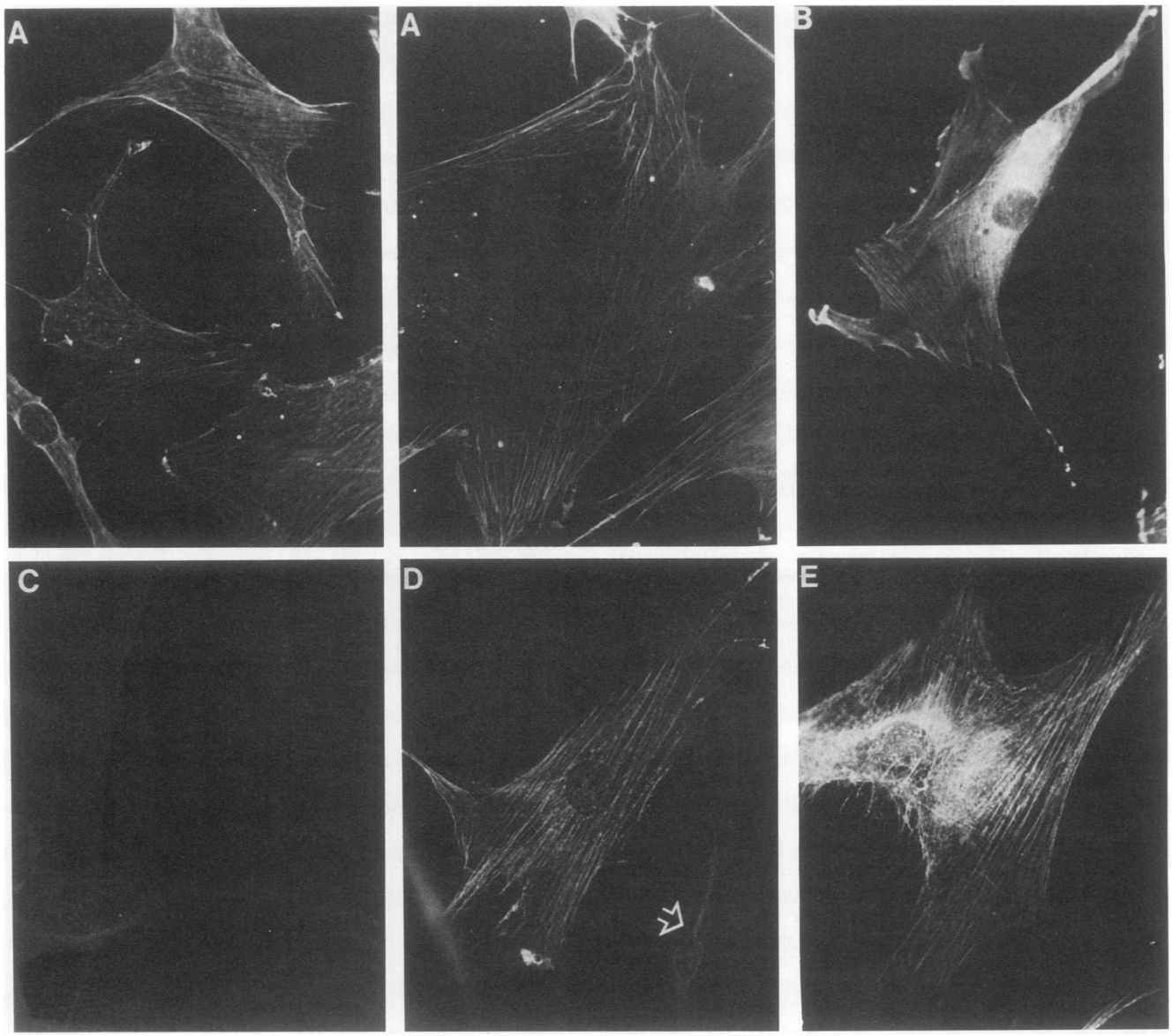

FIG. 4. Indirect immunofluorescence microscopy of transfectant KD cells (20, 21) with peptide antibodies specific for either Asp-244 or Gly-244 in actin. (A) Transfectant KDsm-5 cells (Triton X-100-resistant cytoskeletons fixed with acetone [19, 21]), with a 1:1 ratio of exogenous mutant β-actin to endogenous β- plus γ-actin (Table 1) stained with anti-Asp-244 antibody. (B) Same cells as in panel A (whole cells fixed with methanol) stained with anti-Asp-244 antibody. (C) Transfectant KDtm-1 cells (Triton X-100-resistant cytoskeletons fixed with acetone) which failed to express the exogenous mutant β-actin but did express normal amounts of endogenous β-plus γ-actin, stained with anti-Asp-244 antibody. (D) Transfectant KDsm-9 passage 5 cells (Triton X-100-resistant cytoskeletons fixed with acetone); 9% of the cells expressed ~1:1 ratio of exogenous mutant β-actin to endogenous β- plus γ-actin, and the remaining cells lacked detectable amounts of mutant β-actin. A mutant β-actin expressor cell is in the foreground, and a nonexpressor is indicated with the white arrow. Cells were stained with anti-Asp-244 antibody. (E) Same cells as in panel D (Triton X-100-resistant cytoskeletons fixed with acetone) stained with anti-Gly-244 antibody.

not detect it in the perinuclear region of the cytoskeleton (Fig. 4A and D). Thus, all intracellular actin pools do not appear to be equivalent. The absence of singly mutant β-actin from the perinuclear region of cells fixed to preserve the cytoskeleton may explain the results of Triton X-100 extractability of mutant versus normal β- plus γ-actin (Table 1) since perinuclear mutant β-actin was detectable when anti-Asp-244 antibody was used with whole cells fixed so as to preserve both soluble and cytoskeletal actin structures (Fig. 4B). The immunofluorescence pattern suggests that at least half of the normal actin in structures was perinuclear in location (Fig. 4E). The singly mutant β-actin appeared to be transported to this area because of its perinuclear location in whole fixed cells (Fig. 4B) but it was not incorporated into the perinuclear detergent-insoluble cytoskeleton (Fig. 4A and D). One would therefore expect that about twice as much wild-type actin as singly-mutant β-actin would be detergent insoluble assuming that both forms are equally likely to be incorporated into the cytoplasmic stress fibers. Indeed, the ratio of singly-mutant β-actin to normal β- plus γ-actin was twice as high in soluble cytoplasm as in the detergent-resistant cytoskeleton of KDsm-5 cells (Table 1).

TABLE 2. Rates of synthesis of stable mutant β-actin and β- plus γ-actin in transfected human fibroblasts

Expt no. and cells	Total actin %	Endogenous β- plus γ-actin (%)	Exogenous singly-mutant β-actin (%)	Total no. of polypeptides measured
1				
KD	40.4	40.4		160[a]
KDsm-5	42.2	21.7	20.5	160
KDtm-2	40.6	40.6		160
KDwt-3	36.6	36.6		160
KD	27.1	27.1		600[b]
KDsm-5	31.4	16.5	14.9	600
KDtm-2	24.8	24.8		600
KDwt-3	24.3	24.3		600
KDtm-1	24.8	24.8		600
2				
KDsm-9 (passage 4)	42.9	41.2	1.7	200[c]
KDsm-9 (passage 11)	37.8	17.8	20.0	200
3				
HuT-12	31.2	31.2		600[c]
HuT-12sm-2	31.6	24.5	7.1	600
HuT-12sm-8	26.8	19.0	7.8	600
HuT-12sm-4	27.5	14.4	13.1	600

[a] Polypeptides (20 to 250 kilodaltons, pI 4.0 to 6.5) quantified in Table 1 and Figure 3 of reference 32 measured by manual microdensitometry (see text).
[b] Polypeptides (20 to 250 kilodaltons, pI 4.0 to 6.5) quantified in Table 2 of reference 32 measured by automated microdensitometry (see text).
[c] Most abundant polypeptides (15 to 120 kilodaltons, pI 4.0 to 6.0) measured by automated microdensitometry (see text).

Growth characteristics of a mutant β-actin transfectant subpopulation of cells in a mixed culture. In one transfection experiment with the singly-mutant β-actin gene, we obtained a cluster of G418-resistant KD cell colonies which became confluent in a single culture dish and could not be separated as pure clonal strains. This culture, KDsm-9 (Table 1), was propagated collectively as a single strain and monitored for mutant β-actin expression by 2-D gel analysis and by indirect immunofluorescence (Fig. 4D) with the anti-Asp-244 antiserum during serial passaging until cells of this culture stopped dividing at passage 17. In the earliest passage tested by 2-D PAGE (passage 3), this culture synthesized a low amount of amount β-actin (about 7% of wild-type β- plus γ-actin) (Table 1). By passages 8, 11, and 13 the level of synthesis of mutant β-actin in the mixed culture had risen to 43, 70, and then 75% of the endogenous wild-type actins, respectively (Table 1). This phenomenon was reexamined by reculturing a stock of KDsm-9 cells that had been frozen in liquid nitrogen at passage 2. Again, this KDsm-9 culture exhibited low-level synthesis of mutant β-actin at passage 4 (Table 1, trial 2). However, by passage 11, mutant β-actin synthesis had increased to a level in excess of wild-type β- and γ-actins. We attribute the slight differences in levels of mutant β-actin expression between trials 1 and 2 to minor differences in the way in which these two cultures were propagated.

To determine the percentage of cells in the culture that were synthesizing mutant β-actin, we stained fixed cells with anti-Asp-244 antiserum at passages 6 (Fig. 4D), 8, 13, and 15. We found that the culture contained a subpopulation of cells that abundantly expressed the mutant β-actin in amounts similar to KDsm-5 cells (Fig. 4A and D) as judged by fluorescence intensity. This subpopulation of cells increased progressively from 9% (passage 6) to 23% (passage 8) to a maximum of 90% (passage 13) and 88% (passage 15). This progressive outgrowth of a subpopulation of KD cells that expressed high levels of singly-mutant β-actin demonstrated that this mutant β-actin did not inhibit the growth of KD cells. As the mutant β-actin transfectant subpopulation increased in the culture, the morphological appearance of the KDsm-9 cell culture (Fig. 3D) became more and more like that of KDsm-5, the other transfectant strain that expressed high levels of singly-mutant β-actin.

Autoregulation of endogenous actin synthesis. The ability to introduce and express redundant copies of the wild-type, singly- and triply-mutant β-actin genes into normal diploid KD cells allowed us to assess the impact of exogenous β-actin expression on the levels of endogenous β- and γ-actin. To determine whether the total amount of actin increased per cell as the result of expression of additional exogenous β-actin genes, we examined the relative levels of as many as 600 individual polypeptide species resolved in 2-D gels, comparing transfectant substrains that exhibited the highest levels of expression of each exogenous mutant β-actin gene (KDsm-5, KDtm-2, and KDtm-1 as a control strain) with the nontransfectant parental KD strain (Fig. 1).

We calculated the relative rates of synthesis of exogenous mutant β-actin and endogenous β- and γ-actins in transfectant KD subclones (KDsm-5 and early- and late-passage KDsm-9) and also in three transfectant HuT-12 subclones. Table 2 (experiment 1) presents the relative rates of synthesis of the actin isoforms calculated as the percentage of the rate of synthesis of 160 highly resolved, abundant polypeptides by both a manual method of microdensitometry and an automated method in which 600 polypeptide species in the 2-D gel were measured (26). The assumption in these calculations is that the sum of synthetic contributions to a large set of polypeptides is relatively fixed in all cell types examined (22, 26). Thus, comparisons can be made of the behavior of specific polypeptides in different cell strains. The strains transfected with a neomycin resistance gene but not expressing an exogenous β-actin gene had protein synthesis patterns indistinguishable from those of wild-type KD cells (28). Thus, the transfection and selection procedures did not themselves affect the patterns of protein synthesis.

Among the 160 polypeptides measured manually, endog-

enous β- plus γ-actin represented about 40% of the ^{35}S-labeled protein in KD cells and in two strains transfected with the triply-mutant β-actin gene KDtm-2 and with the wild-type gene KDwt3 (Table 2). In contrast, the rate of synthesis of endogenous β-plus γ-actin was dramatically reduced, by nearly one-half (to 22% in the singly-mutant β-actin transfectant (KDsm-5), but this was compensated for by synthesis of nearly equal amounts of exogenous mutant β-actin. Virtually identical results were obtained for the relative rates of actin expression by automated measurement of 600 polypeptides (randomly picked), among which endogenous β-plus γ-actins amounted to about 27% (Table 2). Thus, the amount of total actin synthesis remained almost the same as in wild-type KD cells in cells expressing abundant levels of singly-mutant β-actin (26).

We also examined of actin synthesis in KDsm-9 cells. The passage 4 culture which expressed a low amount of mutant β-actin was compared with the cells of the passage 11 culture, in which the transfectant subpopulation expressing abundant amounts of mutant β-actin represented 90% of the cell culture (Table 1, KDsm-9 trial 2). The total amount of actin in each culture was approximately 40% of the total amount of 200 reference polypeptides measured (Table 2, experiment 2). When the relative amount of mutant β-actin increased about 11-fold to 20%, the levels of endogenous β- and γ-actin synthesis decreased by a little more than one-half, autocorrecting for excessive expression from the transfected mutant β-actin gene.

A similar result was obtained for three transfectant HuT-12 strains which synthesized mutant β-actin at similarly high levels (Table 2, experiment 3). In these three transfectant strains, the decrease in wild-type β- and γ-actin synthesis was essentially equivalent to the level of exogenous mutant β-actin synthesis. Shifting in the relative amounts of mutant β-actin, β- and γ-actin, and neighboring abundant polypeptides in the 2-D gel is depicted graphically in Fig. 5, which compares actin synthesis in parental HuT-12 cells and in HuT-12sm2 and HuT-12sm4 cells (Tables 1 and 2). In the polypeptide pattern of HuT-12sm4 cells, the endogenous β- and γ-actin (peak volume equals total disintegrations per minute for each polypeptide) were reduced by one-half compared with the neighboring proteins in the pattern. It is notable that the synthesis of both normal β- plus normal γ-actin in both KD and HuT-12 transfectant strains was reduced equally and proportionally, since their ratio did not change in these cells (Fig. 1 and 5, Table 1). This result suggests that total actin synthesis is regulated in these cells so that expression of an exogenous actin inhibits endogenous actin synthesis. Furthermore, it indicates that the relative synthesis of endogenous β- and γ-actin is fixed so that the decreased synthesis maintains the same wild-type β- to γ-actin ratio (Fig. 1, Tables 1 and 2).

DISCUSSION

High-level expression of stable mutant β-actin elicits a morphological change in the cytoskeleton of diploid KD fibroblasts. What is the basis of this morphological transformation? It was not due to the presence of a transcribed exogenous β-actin gene per se, because cells which expressed the triply-mutant gene at high levels did not display any alteration in cell morphology. Neither was it due to altered β-/γ-actin ratios because wild-type β-actin transfectants with altered β-/γ-actin ratios did not exhibit altered morphology. Instead, our results suggest that morphological transformation of KD cells is directly a function of the stable accumulation of the singly-mutant β-actin in these cells. Furthermore, the morphological response appeared to depend on the level of mutant β-actin synthesized, since cells which accumulated low levels of this protein did not show any morphological abberations.

How does the mutant β-actin protein precipitate these morphological alterations? This was not due to extensive disruption of actin cables, since cells transfected with the singly-mutant β-actin gene displayed well-defined cytoplasmic stress fibers. Furthermore, the singly-mutant β-actin was present in these stress fibers. These observations dem-

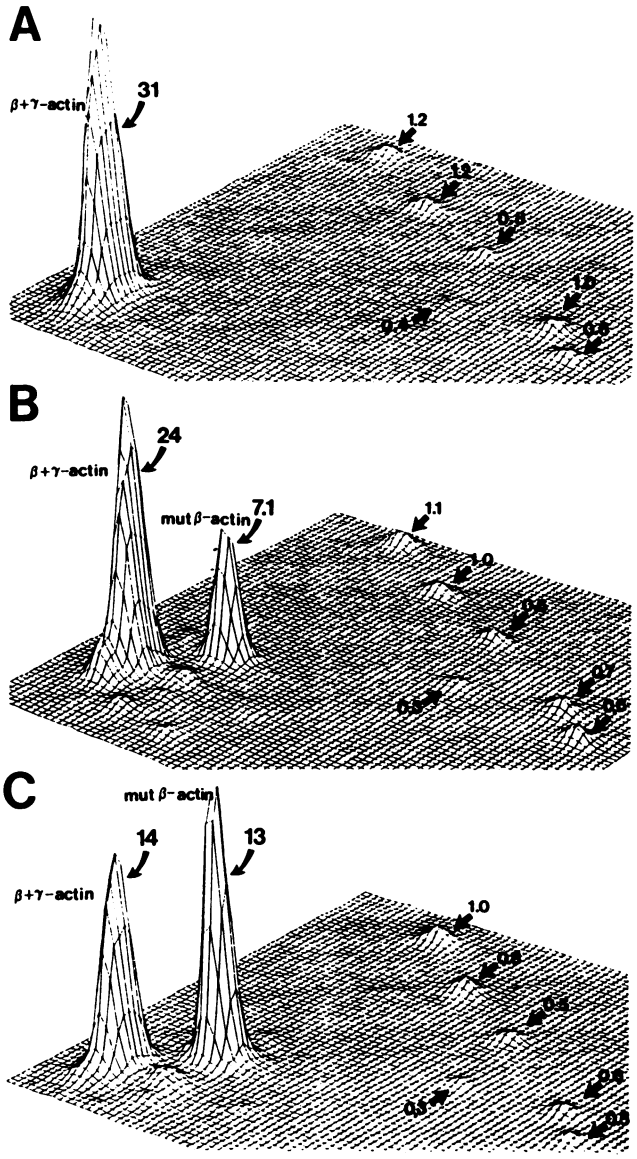

FIG. 5. Relative abundances of actin and surrounding polypeptides ([^{35}S]methionine labeled) in total unfractionated polypeptides of HuT-12 (A), HuT-12sm-2 (B), and HuT-12sm-4 (C) cells separated by 2-D PAGE. The patterns are depicted by plotting disintegrations per minute per pixel in actin and other surrounding polypeptides from an autoradiogram in which neither wild-type β-plus γ-actin (A) nor mutant β-actin (B) had saturated the film. The numbers next to each peak represent the total volume in each polypeptide in percent disintegrations per minute from measurement of 600 species per cell line.

onstrate that the mutant β-actin is proficient at polymerization into filaments, which form bundles and then coalesce into stress fibers. Thus, if these stress fibers are responsible for the morphological alteration, then they must be defective in their cytoarchitectural role. Alternatively, the soluble pool of mutant β-actin may mediate the morphological response. Until the biological relationships between soluble and insoluble actin and cell morphology are better understood, it is not possible to unambiguously resolve these points.

Actin expression is autoregulated by steady-state actin levels. Our results strongly suggest that the steady-state level of actin autoregulates net actin synthesis to maintain what one may presume to be an optimal homeostatic cellular concentration of actin. Furthermore, our observations rule out models such as competition for rate-limiting *trans*-acting factors which determine net actin transcript levels, since it was not actin synthesis per se which autoregulated but rather steady-state actin protein levels. In the transfectant strain that expressed the triply-mutant β-actin, total actin synthesis was substantially elevated over that in normal and singly-mutant transfected KD cells and yet there was no detectable effect on endogenous β- and γ-actin accumulation.

The ratio of β- to γ-actin synthesis does not appear to be autoregulated because the relative synthesis rates of endogenous β- and endogenous γ-actin appear to be fixed. Thus, introduction of additional functional β-actin genes decreases endogenous γ-actin and β-actin expression to the same extent. The inability of cells to autocorrect to maintain the same ratio of β- and γ-actin following expression of the exogenous β-actin gene has been repeatedly reproduced in Rat-2 and mouse NIH 3T3 cells (unpublished results). This indicates that in the presence of high-level accumulations of exogenous β-actin, the mechanism responsible for decreasing the synthesis of the actins encoded by the endogenous genes does not discriminate between β- and γ-actin but rather affects both to the same extent.

Autoregulation of actin synthesis is apparently not capable of responding to elimination of actin alleles. In the HuT-14T cells, which expressed one normal β-actin and one allele encoding the triply-mutant β-actin, there was a substantial decrease in actin steady-state level (26). This decrease is explained by the failure of HuT-14T cells to accumulate stable actin encoded by the mutant allele. Thus, we conclude that autoregulation can operate to decrease actin expression in response to exogenous gene activity but cannot increase in response to an effectively null allele. These observations imply that expression of actin genes is not regulated by a rate-limiting pool of transcription factors. This inability to increase expression to compensate for a null actin allele appears to be demonstrated in the MNNG-HOS osteosarcoma cell line (11) and the T leukemic cell line Molt-4 (11, 27). Studies of *D. melanogaster* indirect flight muscle mutants also suggest that actin genes may not be able to compensate for decreased numbers of functional genes. Heterozygous flies which carry a null allele for the indirect flight muscle actin gene 88F synthesize insufficient levels of actin to form functional sarcomeres (15, 31). This suggest that if the autoregulatory mechanism we have detected also operates in the indirect flight muscles of *D. melanogaster*, then it too is incapable of promoting substantially increased levels of expression of a single gene but instead can only decrease expression in response to elevated actin levels.

Actin mutations and pleiotropic responses. The results presented in this paper indicate that actin mutations can have broad effects on cell behavior. Indeed, in the accompanying paper (28), we show that high-level expression of the singly-mutant β-actin in tranfectant HuT-12 cells leads to acquisition and elevation of tumorigenic potential and accompanying changes in tropomyosin isoform synthesis. By virtue of the biological role of actin filaments in regulating cell shape and intracellular movement, it is not unexpected that mutations in actin could affect many cellular processes, either directly or indirectly. Indeed, a similar variety of responses has also been seen with actin gene mutations in *D. melanogaster*. Others (18, 31) have observed that actin mutations may be responsible for a reduction in the synthesis of four myofibrillar proteins and an induction of heat shock proteins in *D. melanogaster* indirect flight muscle. Similarly, Hiromi et al. (15) have demonstrated that two actin mutations are responsible for the constitutive induction of heat shock proteins in flight muscle. In the case of the mutant β-actin genes, we have not observed any effect on heat shock protein synthesis in the KD transfectant cells (unpublished results). It therefore appears that different tissues or organisms may respond differently to different actin mutations. Evaluation of the effects of different actin mutations in different systems is likely to increase our appeciation of the integration of cellular processes and the pivotal role that actin plays in them.

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