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Tropomyosin Isoform Switching in Tumorigenic Human Fibroblasts

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We identified six tropomyosin (Tm) isoforms in diploid human fibroblasts. We used computerized microdensitometry of 2-dimensional protein profiles to measure the relative rates of synthesis and abundance of the individual Tm isoforms and actin, the two major structural constituents of microfilaments. In carcinogen-transformed human fibroblasts (HuT-14), the rates of synthesis of three Tm isoforms (Tm1, Tm2, and Tm6) were greatly decreased relative to normal diploid parental fibroblasts and to actin. In contrast, related nontumorigenic HuT fibroblasts which are "immortalized" and anchorage independent exhibited both slight down-regulation of Tm1 and Tm6 and 3.5-fold up-regulation of Tm3. Thus, Tm isoform switching from the predominance of the larger more avid Tm isoforms (Tm1, Tm2, Tm3, and Tm6) to the smaller, less avid Tm isoforms (Tm4 and Tm5) in microfilaments was a transformation-induced change correlated with tumorigenicity in human fibroblasts.

Transformation of diploid KD fibroblasts with chemical carcinogens led to the isolation of stable, focus-derived, neoplastic strains which appear to have arisen from separate clonal transformation events (13, 22). Four of these transformed neoplastic strains, transformed human fibroblasts HuT-11, -12, -13, and -14, have been characterized in great detail with regard to phenotypic differences and changes in gene expression in comparison with parental diploid KD cells (2, 4, 10, 11, 15–19, 21, 22, 33). This family of cell strains exhibits three distinct phenotypic modes that are classified as follows: (i) parental KD cells are phenotypically normal diploid fibroblasts with a finite life span in culture (2, 11, 13, 14, 16, 17, 19, 24, 33); (ii) HuT-11, -12, and -13 are neoplastically transformed, "immortalized" strains that express transformation-specific protein markers (4, 10, 11, 16, 17, 19, 22) and anchorage-independent growth (13, 14), but rarely produce tumors in nude mice (16, 19); and (iii) HuT-14 is a highly tumorigenic strain that exhibits additional differences in cellular morphology and gene expression (4, 16, 17, 19, 22). HuT-14 is further distinguished from the three relatively nontumorigenic strains in that it is diminished in the secretion of fibronectin into the extracellular matrix and fails to organize actin filament bundles into a well-defined cytoskeletal actin cable network (17). HuT-14 cells also express a defective β-actin as the consequence of a point mutation in one of two functional β-actin genes (21, 22, 24, 33).

Matsumura and colleagues (25, 27–29), Hendricks and Weintraub (12), and Cooper et al. (5) have proposed that cytoskeletal tropomyosin down-regulation or isoform "switching" may govern transformation-induced changes in the arrangement of the microfilament system, cytoarchitecture, and tumorigenicity. This hypothesis generally implies that, in transformation, the tropomyosin (Tm) isoforms which strongly bind actin in microfilaments are diminished in synthesis or replaced with Tm isoforms which bind actin weakly in microfilaments (28). Such Tm isoform switching may induce rearrangement of the microfilament system which accompanies development of tumorigenicity by changing the physical-chemical properties of microfilaments. We found that nontumorigenic and tumorigenic HuT cells exhibited two distinctly different patterns of Tm isoform switching in a fashion consistent with the Matsumura hypothesis (28), and we found that the level of Tm synthesis in normal fibroblasts approached the theoretical limit of saturation of actin binding sites in microfilaments.

Identification of the Tm isoforms of human and rat fibroblasts. Autoradiographs of the [^{35}S]methionine-labeled polypeptides of normal KD, transformed nontumorigenic HuT-12, and highly tumorigenic HuT-14 fibroblasts were examined (Fig. 1). To produce these 2-dimensional gel polypeptide patterns, cell monolayers were radiolabeled for 6 h, then rinsed three times with cold phosphate-buffered saline, drained, and lysed directly in O'Farrell lysis buffer A (22). Replicate 2-dimensional polyacrylamide electrophoresis gel patterns were made from each protein sample with the Iso-Dalt system (1) from Electro-Nucleonics, Inc., Fairfield, N.J.) as previously described (4, 10). Kodak XAR-2 film (Eastman Kodak Co., Rochester, N.Y.) 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 six tropomyosin isoforms Tm1 through Tm6, numbered by the convention of Matsumura and colleagues (25, 27–29) and Franza and Garrels (7) were identified, when present, along with two abundant unidentified polypeptides, *a* and *b*, that migrated to the same isoelectric point (pI, ~4.5) and molecular weight range (M_r, 30,000 to 38,000). Polypeptide *b* may have been the antigenic nuclear polypeptide cyclin (3, 7).

To identify the Tm isoforms of human and rat fibroblasts, cytoskeletal proteins were isolated by extraction of the Triton X-100-soluble proteins from cells that attached to the plastic substratum of a culture dish (17, 22). The cytoskeletal monolayer was eluted from the plastic substratum by scraping with a rubber policeman. The cytoskeletons were suspended in 1 ml of 1% Triton X-100 solution (22) and pelleted by a 2-min centrifugation in a microfuge. The pelleted cytoskeletons were suspended in 100 μl of Triton X-100 solution and heated for 10 min in a boiling water bath (25). The insoluble proteins were removed by pelleting by 10 min of centrifugation in a microfuge. Cytoskeletal Tm isoforms were then further purified from the heat-solublized proteins by precipitation with anti-Tm monoclonal antibody. The heat-solublized Tm solution (90 μl) was incubated with 9 μl

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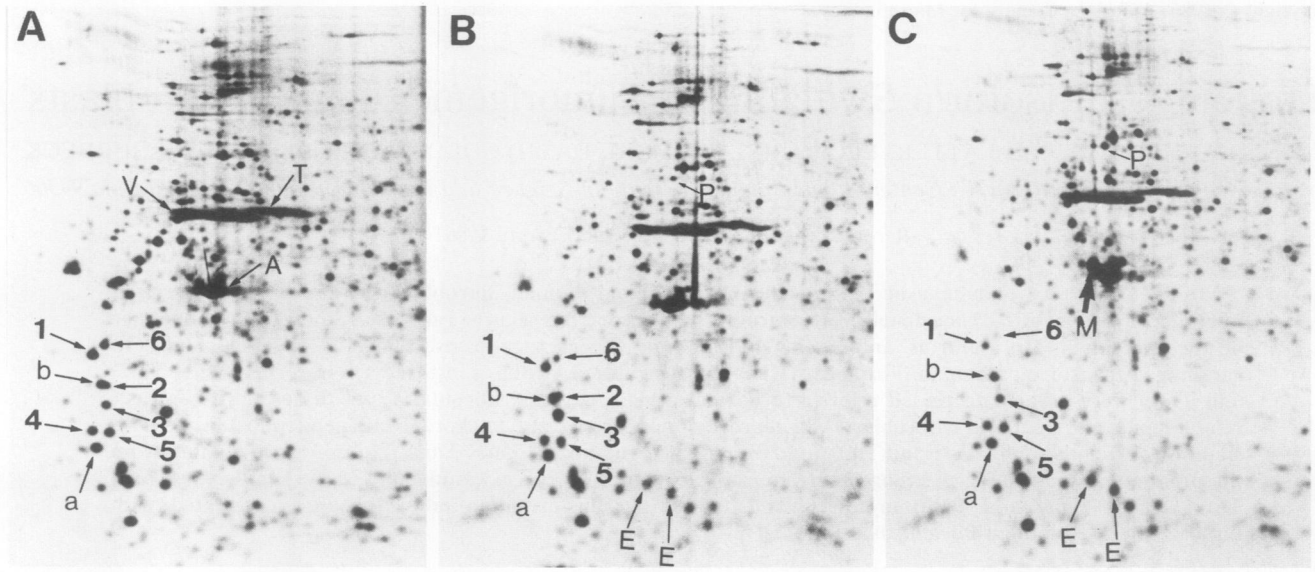

FIG. 1. Polypeptides ([^{35}S]methionine labeled) of normal and transformed human fibroblasts electrophoretically separated by high-resolution 2-dimensional gel electrophoresis. (A) Normal KD fibroblast (13, 22) polypeptides; (B) transformed HuT-12 (13, 22) polypeptides; (C) HuT-14 (13, 22) polypeptides. The autoradiograms show the more acidic polypeptides from pI 4 on the left to pI ~6 on the right in each panel, and M_r ranges from 200,000 (top) to 20,000 (bottom) in each panel. The major cytoskeletal polypeptides identified in the electrophoretic pattern of panel A only are normal β- and γ-actins (A) (pI 5.3, M_r 42,000) (22, 35), vimentin (V) (pI 5.1, M_r 56,000), or intermediate filament protein (17, 22), and α-, β-tubulin (T) (22) which formed a poorly resolved horizontal streak. The transformation-induced polypeptides plastin (P) (pI 5.3, M_r 68,000) (10, 11, 19) and the two epidermal growth factor-related polypeptides (E) (pI 5.2 to 5.3, M_r 26,000 to 27,000) (4, 11, 19) are identified in panels B and C. The Tm isoforms (pI 4.5, M_r 28,000 to 37,000) are identified by numbers corresponding to Tm1 through Tm6 by the convention of Matsumura and colleagues (25, 29). a and b, Additional polypeptides that are discussed further in the text. The mutant β-actin (M) present only in HuT-14 cells (17, 19, 22) is identified in panel C.

of hybridoma ascites fluid containing mouse anti-Tm monoclonal antibody IV-15 (supplied by Fumio Matsumura; 27) for 1 h at 4°C. In parallel, a sample of heat-soluble Tm solution was also incubated with hybridoma ascites fluid containing monoclonal antibody 5 (18) as a nonspecific control. Then 15 μl of a conjugate antibody, rabbit anti-mouse Ig (Miles-Yeda, Ltd.), was added, and incubation was extended for 2 h at 4°C. The antibody aggregates were collected in a pellet by centrifugation for 10 min in the microfuge. The pellet was suspended in Triton X-100 solution and repelleted twice. The washed immunoprecipitated pellet was then dissolved in 50 μl of lysis A solution and 10-μl samples were analyzed by 2-dimensional gel electrophoresis.

Comparison of the human and rat Tm isoforms (Fig. 2) established for the first time that Tm isoforms were electrophoretically identical between these two species. The following observations summarize the full set of criteria used to establish the identity of the Tm isoforms: (i) they resolved in 2-dimensional gel electrophoretic positions which corresponded to electrophoretic positions previously reported by others (Fig. 1) (7, 25, 29); (ii) the amino acid composition of the polypeptides designated Tm1, Tm4, and Tm5 in HuT-14 cells previously provided evidence that they were closely related to nonmuscle tropomyosin (15); (iii) they were major constituents of the nonionic detergent-resistant cytoskeleton fraction along with actin and vimentin (Fig. 2A and E) (25, 27, 29); (iv) all six Tm isoforms shared the unusual property of absence of proline and tryptophan and thus were not labeled with these [^{14}C]amino acids (data not shown) in 2-dimensional gel patterns (7, 9, 29); (v) they were solublized from insoluble cytoskeletal microfilaments and vimentin by heating in a boiling water bath (Fig. 2B and F) (25, 29); (vi) they were precipitated with anti-Tm monoclonal antibody (Fig. 2C and G) (7, 25); and (vii) they migrated at a higher M_r when 3.8 M urea was included in the sodium dodecyl sulfate gel dimension of the 2-dimensional gel (data not shown) (9).

Levels of Tm isoforms in normal fibroblasts. Measurements of the relative rates of synthesis of the Tm isoforms, actins, vimentin, and the transformation-induced epidermal growth factor-related proteins and plastin (p788/p789 and p219/p220, respectively; 4, 10, 11, 17, 19) in normal KD and transformed HuT cells were made (Table 1). The rates reflected the relative abundance of Tm, actin, and vimentin, since the half-lives of these proteins are similar (>30 h) when the average cell division cycle is less than 24 h (our unpublished observation). To calculate these relative rates, about 1,000 to 1,200 polypeptide species were measured by automated computerized microdensitometry (8), the 820 most radioactive species were used as the total, and the percentages of [^{35}S]methionine incorporation were based on the 820 measurements. In KD cells, β- and γ-actin (combined) amounted to 17% of the total incorporation, and vimentin, the third most abundant polypeptide, was 6% of the total. The amount of actin did not vary significantly in KD, HuT-12, and HuT-14 (19), ranging between 17.2 and 18.5% (Table 1). About 2.5% of the [^{35}S]methionine was incorporated into the six Tm isoforms (Table 1).

The relative rates of expression were converted to molar (monomer) ratios between Tm and actin (Table 2). These ratios took into account the relative number of methionine residues in each Tm isoform and in β- and γ-actin. The combined sum of Tm monomers amounted to 41% of total actin monomers, or a ratio of 1 Tm subunit per 2.4 actin subunits. Franza and Garrels (7) demonstrated that Tm1 and Tm6 are precipitated by Tm antibody that fails to precipitate Tm4 and Tm5. Therefore, we tentatively concluded that Tm6 was more closely related to the class of Tm isoforms

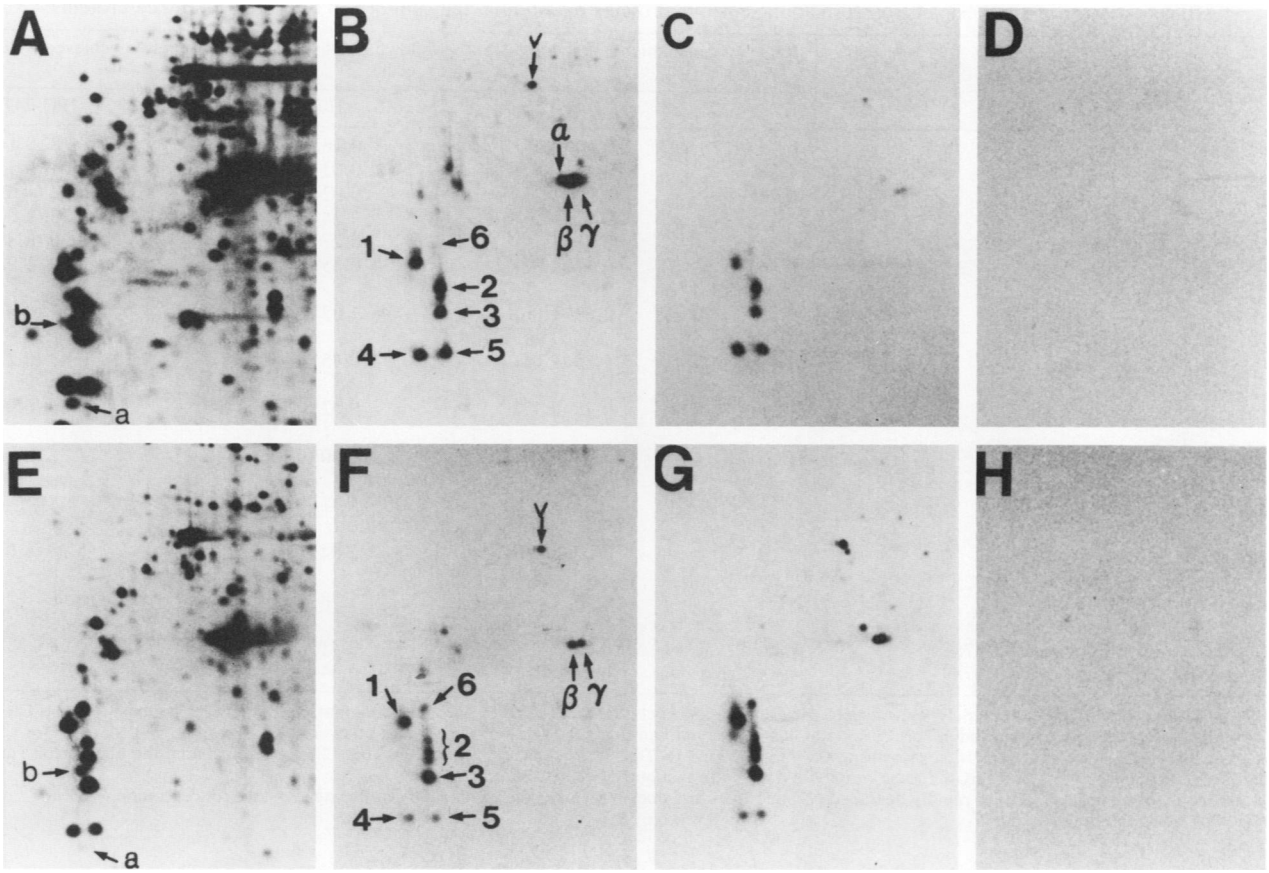

FIG. 2. Identification of the Tm isoforms of Rat-2 (20, 32) (A through D) and human KD (E through H) fibroblasts by immunoprecipitation. (A) and (E), Autoradiographs of nonionic detergent-resistant cytoskeletal ([^{35}S]methionine labeled) polypeptides (16, 17, 22) separated in 2-dimensional gels; (B) and (F), Tm isoforms solubilized by heating the cytoskeletal fractions at 100°C for 10 min (25); (C) and (G), Tm isoforms precipitated with anti-Tm antibody; IV-15 (F. Matsumura and S. Yamashiro-Matsumura, J. Cell Biol. **101**:403a, 1985); and (D) and (H), polypeptides precipitated by the nonspecific monoclonal antibody 5. Indicated in panels A and E are polypeptides *a* and *b* (Fig. 1); indicated in panels B and F are the Tm isoforms (Tm1 through Tm6), the actin isoforms (α, β, γ), and vimentin (V) (Fig. 1).

(Tm1, Tm2, and Tm3) that bind more avidly in microfilaments (28). The tightly binding Tm isoforms (Tm1, Tm2, Tm3, and Tm6) were in slight molar excess of the weaker-binding Tm isoforms (Tm4 and Tm5), and these ratios were conserved between KD and HuT-12 cells (Table 2). The corrected measurements of synthesis of the six Tm isoforms of rat REF-52 cells (taken from Franza and Garrels [7]) were examined (Table 2). Although individual Tm isoforms were expressed at different rates than in KD cells, total Tm synthesis led to a similar ratio of one Tm monomer to 2.8 actin monomers, and the distribution between the weaker-binding and stronger-binding Tm isoforms was virtually identical.

Tm isoform modulation in the KD-HuT cell system. Comparison of the rates of synthesis of the Tm isoforms between KD cells (Fig. 3A) and HuT-12 cells (Fig. 3B) revealed that Tm1 and Tm6 were reduced 47 and 58%, respectively, whereas Tm3 was elevated to 355% (Table 1). The rates of synthesis of Tm2, Tm4, and Tm5 did not appear to be affected. There was no net reduction in the sum of Tm1, Tm2, Tm3, and Tm6, nor significant change in the balance of these Tm isoforms versus Tm4 and Tm5 (Tables 1 and 2).

Comparison of rates of synthesis of the Tm isoforms between KD cells (Fig. 3A; Table 1) and HuT-14 (Fig. 3C; Table 1) revealed that Tm1, Tm2, and Tm6 declined 83, >90, and 90%, respectively, whereas Tm3, Tm4, and Tm5 were relatively unmodulated (Table 1). Tm2 was not detectable in whole cell samples because of interference from the more abundant polypeptide *b* (Fig. 3C and D), but a trace of Tm2 was found in the detergent-resistant cytoskeleton fraction which lacked polypeptide *b* (data not shown). Therefore we estimated the amount of Tm2 in HuT-14 and a more tumorigenic subcolonial strain, HuT-14T (17), by using the ratio between Tm2 and Tm3 from cytoskeletal preparations. Tm isoform expression in HuT-14T was the same as that expressed by HuT-14 cells (Fig. 3D; Table 1). Thus, HuT-14 and HuT-14T, which are distinguished from HuT-12 by their elevated tumorigenicity in athymic mice, exhibited a stable decrease in rates of synthesis and abundance of Tm1, Tm2, and Tm6 isoforms (Table 1).

Two general concomitant effects were found in measuring Tm synthesis rates (Table 1). First, the modulations found in HuT-14 cells resulted in a 56% reduction in the combined amount of Tm subunits relative to actin subunits, whereas the ratio of Tm to actin was unchanged in HuT-12 (Table 2). This change resulted in one Tm monomer per 5 actin monomers in HuT-14 cells, versus one Tm monomer per 2.4 actin monomers in KD and HuT-12 cells. In HuT-14T cells which lost about 29% of their actin because of two additional

TABLE 1. Rates of synthesis of transformation-sensitive proteins based upon the sum of 820 abundant reference proteins[a]

Polypeptide	Rate (% [35S]methione) of synthesis of the following cell strains (fraction of rate achieved by KD cells):			
	KD	HuT-12	HuT-14	HuT-14T
Tm1	0.94	0.50 (0.53)	0.16 (0.17)	0.13 (0.14)
Tm6	0.35	0.15 (0.42)	0.03 (0.10)	0.05 (0.14)
Tm2[b]	0.21	0.23 (1.11)	<0.02 (<0.10)	<0.02 (<0.10)
Tm3	0.27	0.97 (3.55)	0.24 (0.87)	0.28 (1.04)
Tm4	0.34	0.41 (1.19)	0.27 (0.80)	0.30 (0.88)
Tm5	0.36	0.37 (1.02)	0.35 (0.99)	0.39 (1.08)
Total Tm	2.47	2.63 (1.06)	1.07 (0.43)	1.17 (0.47)
Tm1 + Tm2 + Tm3 + Tm6	1.77	1.85 (1.05)	0.45 (0.25)	0.48 (0.27)
Tm4 + Tm5	0.70	0.78 (1.12)	0.62 (0.89)	0.69 (0.99)
β-, γ-actin	17.31	18.45 (1.07)	11.80 (0.68)	12.36 (0.71)
Mutant β-actin (stable form)			5.41 (0.31)	—[c]
Vimentin	5.03	2.47 (0.49)	2.84 (0.56)	5.28 (1.05)
Plastin (p219 + p220)	ND[d]	0.15	1.78	0.58
Epidermal growth factor-related p788	trace	0.29	0.41	0.29
Epidermal growth factor-related p789	trace	0.22	0.34	0.29

[a] Distintegrations per minute in each polypeptide species in 2-dimensional gels were measured by automated computerized microdensitometry (4, 11). 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.
[b] Amount of Tm2 was estimated by first measuring the ratio of Tm2 to Tm3 in cytskeletal preparations (Fig. 2), then calculating the amount of Tm2 in unfractionated cellular proteins by using the same ratio with Tm3 in unfractionated cellular proteins.
[c] Mutant β-actin in HuT-14T cells is rapidly degraded (17) and therefore does not contribute significantly to the steady-state concentration of actin.
[d] ND, Not detectable.

labilizing mutations in the mutant β-actin (24), the ratio of Tm to actin monomer increased (Table 2), but the net synthesis of Tm isoforms remained as low as in HuT-14 cells (Table 1). Based on these calculations, HuT-14 cells must be about 30% deficient in microfilaments, although the ratio of functional Tm to actin monomer was reduced only 17%, or one Tm monomer per 3.3 actin monomers (Table 2).

The second effect is the synthesis of the more avid Tm isoforms (Tm1, Tm2, Tm3, and Tm6) relative to the less avid Tm isoforms (Tm4 and Tm5) (28). There was no net change relative to actin in the more avid versus less avid Tm isoforms in HuT-12. However, in HuT-14 cells, tightly binding Tm monomers were diminished from one Tm in 4 actin monomers to one Tm in 16.2 actin monomers, with only a slight reduction for the weakly binding Tm monomer (Table 2). Since Tm molecules bind to filamentous actin in the form of isoform homodimers (28), the maximum periodicity of tightly binding homodimers possible in a microfil-

TABLE 2. Monomer ratios of Tm isoforms to total β- and γ-actins[a] in whole cells

Tm isoform	No. of methionine residues per molecule[b]	Ratio of Tm isoforms to total β- and γ-actins[c] in whole cells from strain:				
		KD	HuT-12	HuT-14	HuT-14T	REF-52[d]
Tm1	7	0.124	0.062	0.021	0.024	0.132
Tm6	6	0.054	0.022	0.005	0.011	0.010
Tm2	5	0.039	0.040	<0.004	<0.005	0.076
Tm3	7	0.039	0.120	0.032	0.052	0.009
Tm4	3	0.105	0.118	0.084	0.130	0.103
Tm5	6	0.056	0.054	0.054	0.084	0.021
Total Tm (actin molecules/Tm molecules)		0.412 (2.4)	0.416 (2.4)	0.199 (5.0)	0.306 (3.3)	0.351 (2.8)
Tm1 + Tm2 + Tm3 + Tm6 (actin molecules/Tm molecules)		0.252 (4.0)	0.244 (4.1)	0.061 (16.2)	0.092 (10.9)	0.227 (4.4)
Tm4 + Tm5 (actin molecules/Tm molecules)		0.160 (6.3)	0.172 (5.8)	0.138 (7.3)	0.214 (4.7)	0.124 (6.5)

[a] Actin has 16 methionine residues (33).
[b] Matsumura and Yamashiro-Matsumura (28). The number of methionine residues for Tm6 was assumed to be 6, the average of methionine residues in Tm1 through Tm5.
[c] The ratios of each Tm isoform to total β- and γ-actins based upon relative incorporation of [35S]methionine was corrected to reflect the molar ratios of Tm to actin.
[d] Corrected microdensitometry measurements for REF-52 rat embryo fibroblasts of Franza and Garrels (7).

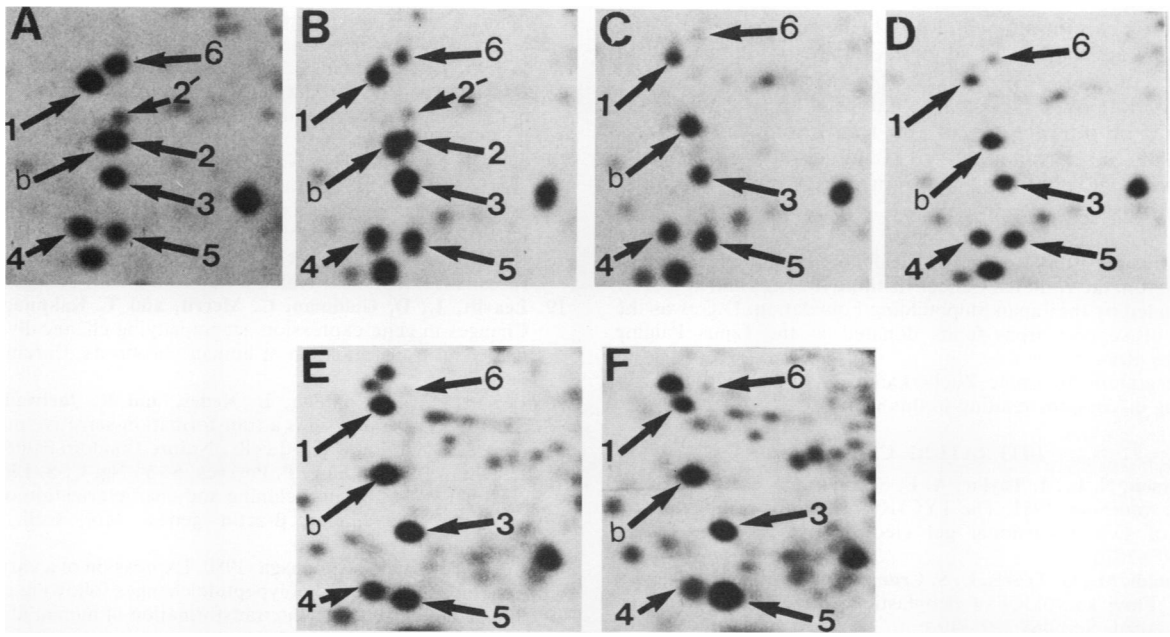

FIG. 3. Comparison of Tm isoform synthesis in normal and transformed human fibroblasts. (A) Normal KD fibroblasts; (B) transformed HuT-12 fibroblasts; (C) tumorigenic HuT-14 fibroblasts; (D) tumorigenic HuT-14T fibroblasts; (E) tumor-derived HT1080 fibrosarcoma cells (10); and (F) tumor-derived HOS cells (10). Tm2′ is most likely an electrophoretic variant of Tm2.

ament is likewise reduced from one Tm dimer per 8 actin monomers to one per 32 actin monomers.

The finding that Tm3 synthesis is elevated 3.5-fold in HuT-12 cells suggests that more than one Tm regulatory process may control Tm isoform expression in the KD-HuT system. Two opposing regulatory effects resulting in Tm3 up-regulation in one instance and Tm3 down-regulation in another instance may have combined to produce only a slight apparent reduction of Tm3 synthesis (by 11%) in HuT-14. The occurrence of these two opposing regulatory events is supported by the following observation: we isolated a stable tumorigenic subclone of HuT-12 cells after transfection with the mutant β-actin gene from HuT-14 cells and observed Tm3 down-regulation to the same synthesis rate found in HuT-14 cells; moreover, Tm1, Tm2, and Tm6 synthesis also diminished to the level found in HuT-14 cells (J. Leavitt, S.-Y. Ng, U. Aebi, M. Varma, G. Latter, L. Lutomski, S. Burbeck, P. Gunning, and L. Kedes, manuscript in preparation).

Tm isoform expression in human tumor-derived fibroblasts. Since the HuT strains were derived by in vitro transformation, we examined Tm isoform expression in two human tumor-derived cell lines, HT1080 (11) fibrosarcoma cells (Fig. 3E) and human osteosarcoma (HOS) cells (10) (Fig. 3F), to assess the mode of Tm isoform expression in human tumor cells which developed by natural tumorigenesis. In HT1080 cells (Fig. 3E), Tm1 and Tm6 were reduced in rate of expression 49 and 94%, respectively, Tm2 was undetectable, and Tm3, Tm4, and Tm5 were synthesized at nearly the same rate in comparison with KD cells (Fig. 3A). In HOS cells (Fig. 3F), Tm1 and Tm6 were both reduced 80 to 95%, Tm2 was undetectable, Tm5 was elevated about fourfold, and Tm3 and Tm4 were unmodulated in comparison with KD cells (Fig. 3A). The ratios of Tm isoform expression in HT1080 and HOS tumor cells were similar to those of HuT-14 cells and therefore may have reflected significant quantitative down-regulation of Tm1, Tm2, and Tm6 at the time of transformation.

Possible relevance of Tm isoform modulation to the neoplastic phenotype. A surprising finding of our study was the high rate of synthesis of total Tm relative to actin. Measurements of the rates of synthesis and abundance of Tm isoforms based on [^{35}S]methionine labeling are underestimated in 2-dimensional gels if not corrected for the differences in methionine residues in the two proteins (28, 33). Our measurements indicated that the ratio of Tm to actin monomer synthesis is about 1:2.5 (Tm to actin) in KD cells. Thus, normal fibroblasts synthesize enough Tm to saturate actin-binding sites in microfilaments (28). This ratio is significantly diminished in tumorigenic cells derived from KD fibroblasts. This diminished rate of synthesis may lead to alterations in the physical-chemical nature of the microfilament system (12, 25, 28, 29). The consequences of this change were presumably compounded in HuT-14 cells by the presence of the mutant β-actin (17). Rodent and chick fibroblasts exhibit Tm isoform switching (5, 7, 12, 27) and concomitant down-regulation of α-actin (7, 21, 37) in tumorigenic transformation. Loss of α-actin may also contribute to changes in properties of microfilaments, especially if α-actin has different Tm-binding properties than do β- and γ-actin (6). The differences in amino acid sequences that exist between α-actin and β- and γ-actin (31, 34) may contribute further to alterations in the physical-chemical nature of the microfilament system that accompany coordinant modulation of Tm and actin expression in transformation.

There are two additional striking examples of linkage between changes of components of the microfilament system, morphologic transformation, and tumorigenicity. First, the conversion of the HOS strain, which is not tumorigenic in nude mice, to the tumorigenic substrain MNNG-HOS is associated with a dramatic change in cell morphology and 50% shutdown of β-actin synthesis without Tm isoform modulation (10). Leukemic Molt-4 cells also exhibit a similar down-regulation of β-actin (23). Second, the acute transforming oncogenes of the *fgr*-feline sarcoma virus and a

human colon carcinoma code for hybrid proteins with a large portion of the amino acid sequence corresponding to actin (30) and Tm (26), respectively. These examples and the others cited here demonstrate that regulatory changes in expression or interactions of the two principal structural components of microfilaments, Tm and actin, are intimately coupled with morphologic and tumorigenic transformation of human, rodent, and avian cells.

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