Differential Expression of Tropomyosin Forms in the Microfilaments Isolated from Normal and Transformed Rat Cultured Cells*

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Using a newly developed method for microfilament isolation (Matsumura, F., Yamashiro-Matsumura, S. and Lin, J. J.-C. (1983) J. Biol. Chem. 258, 6636–6644), we have analyzed protein composition of microfilaments in “normal” and transformed rat tissue culture cells. They include REF-52 (an established rat embryo cell line) cells, REF-52 transformed by DNA viruses (SV40 or adenvirus type 5), normal rat kidney cells, and normal rat kidney cells transformed by RNA viruses (Kirsten or Rous sarcoma virus). Microfilaments from normal rat culture cells contain three major tropomyosins (apparent Mr = 40,000, 36,500, and 32,400) and two relatively minor tropomyosins (apparent Mr = 35,000 and 32,000). In transformed cells the levels of one or two of the major tropomyosins (Mr = 40,000 and 36,500) are decreased and the levels of one or both of the minor tropomyosins (Mr = 35,000 and 32,000) are increased. These changes in tropomyosin patterns were also observed in temperature shift experiments with rat-1 cells transformed with a Rous sarcoma virus mutant, temperature-sensitive for transformation. Cell-free translation of whole cell mRNA generated similar tropomyosin patterns on two-dimensional gels, suggesting that changes in the pattern of tropomyosin expression were largely effected at the level of RNA rather than by post-translational modification. Such changes in the tropomyosin composition of microfilaments were consistently found to accompany various morphological alterations associated with transformation. We suggest that alterations in the pattern of tropomyosin expression are involved in, or cause, rearrangement of stress fibers and that this may be responsible (in part) for morphological transformation.

There are three major classes of cytoskeletal structures found in cells: microfilaments, microtubules, and intermediate filaments. Microfilaments are at least in part responsible for changes in cell shape and motility (Goldman and Knipe, 1972; Goldman et al. (1976a)). Of the various phenotypic changes that accompany cell transformation, morphological alterations are perhaps the most common (for reviews see Hanafusa, 1977; Erickson et al., 1980; Tooze, 1981). Morphological changes in microfilament patterns upon transformation have been well studied by both immunofluorescent and electron microscopy (Pollack et al., 1975; Edelman and Yahara, 1976; Wang and Goldberg, 1976; Goldman et al., 1976b; McNutt et al., 1973; Vollet et al., 1977). These studies have shown that microfilament bundles (actin cables or stress fibers) in transformed cells exist in a more dispersed state. Moreover, this change in actin cables is tightly correlated both with anchorage-independent growth and with cellular tumorigenicity for many cells (Shin et al., 1975), suggesting a central role for microfilament alteration in oncogenic transformation. However, the biochemical and structural bases for these rearrangements of actin cables are poorly understood.

Transformed cells differ from “normal” cells more by the conspicuous absence of higher ordered structures of microfilaments (i.e. bundles or stress fibers) than by an absence of microfilaments themselves. It seems possible that changes in the composition of microfilaments could be responsible for the change in the organization of stress fibers upon cell transformation, but few experiments have been done in this direction, probably because microfilaments of cultured cells are difficult to isolate. To address this problem, we have recently developed a new method for the rapid isolation of presumably “native” microfilaments from cultured cells using monoclonal antibodies against tropomyosin (Matsumura et al., 1983). This method has allowed us to analyze the protein composition of microfilaments in many cell types, both normal and transformed by viruses. As a major and common difference between normal and transformed cells, we have found changes in the patterns of tropomyosin in the microfilaments.

Hendricks and Weintraub (1981) reported that the total amount of tropomyosin was greatly decreased in RSV-transformed chicken embryo fibroblasts. More recently, Leonardi et al. (1982) also found that not only total tropomyosin but also actin-bound tropomyosin were entirely missing in Kirsten virus-transformed rat kidney cells. However, in both reports only two protein spots on two-dimensional gels were identified as tropomyosins and examined with respect to changes in amount upon cell transformation. Our new method for microfilament isolation, combined with other biochemical and immunological methods, has allowed the detection of five
Changes in Tropomyosin Expression Accompanying Transformation

EXPERIMENTAL PROCEDURES

Cell Culture—The rat embryo-derived cell line REF-52 and four independent SV40 transformants of REF-52 (REF-WT2A, REF-WT4A, REF-WT6A, and REF-WT10A) were isolated as described previously (McCulure et al., 1982). Cell lines of dls84.1A and dls84.4A were isolated by an identical procedure following infection of REF-52 cells with a deletion mutant (dls84) of SV40 incapable of producing small or large mRNAs. Tropomyosin-transformed cell lines (Ad5W-SA, Ad5W-4A, Ad5SD-1A, and Ad5D-4A) were isolated by DNA infection of REF-52 cells with PBR322/Ad5 chimeric plasmids encoding either small-t mRNA. Adenovirus-transformed cell lines (Ad5W.3A, WTIA, REF-WTGA, and REF-WTlOA) were isolated as described previously (McClure et al., 1983). Briefly, microfilaments were isolated from cytochalasin B-treated Rat 1 cells transformed with a ts RSV mutant (ts-1) for transformation were a generous gift from Dr. L. B. Chen (Sidney Farber Cancer Institute, Harvard Medical School). All cell lines were maintained in Dulbecco’s modified Eagle medium containing 10% fetal calf serum in an atmosphere of 5% CO2 and 95% air at 37°C.

Isolation of Microfilaments—Isolation of microfilaments from cultured cells was accomplished using monolayer antibodies against tropomyosin as described previously (Matsumura et al., 1983). Briefly, monolayer was extracted with Trition X-100, 0.1 M PIPES, 5 mM MgCl2, 0.2 mM EGTA, 6 mM glycerol to stabilize the cytoskeleton. After washing with PBS containing 5 mM MgCl2 and 0.2 mM EGTA, the Triton/glycerol-insoluble residues were homogenized in 20 mM phosphate buffer (pH 6.5) containing 100 mM NaCl, 5 mM MgCl2, 5 mM ATP, 0.2 mM EGTA, and 5 mM phenylmethylsulfonyl fluoride. After centrifugation (12,800 × g for 15 min), the supernatant was incubated with 1% volume of anti-tropomyosin monoclonal antibodies (ascites fluid). Dispersed microfilaments in the supernatant were aggregated by antibodies into ordered bundles (Matsumura and Lin, 1982). The resultant bundles were collected and washed three times with 20 mM phosphate buffer (pH 7.0) containing 100 mM NaCl, 5 mM MgCl2, and 0.2 mM EGTA by low speed centrifugation (12,800 × g for 5 min). The final pellet was analyzed by one- and two-dimensional SDS-polyacrylamide gel electrophoresis.

Microfilaments were also isolated from cytochalasin B-treated cells. When monolayer cells were exposed for 20 min to 10 μg/ml of cytochalasin B, the cells arborized. Because these cells were still attached to culture dishes, microfilaments could be isolated in the same way as described above. In other experiments, we also isolated microfilaments from trypsin-treated cells. Because these cells were detached from dishes, the procedure for Triton/glycerol extraction was modified as described below. REF-52 cells were incubated for 15 min in PBS containing 0.05% trypsin. The resultant “rounded” cells were washed three times with PBS by centrifugation and collected into an Eppendorf centrifuge tube. The cells were extracted for 2 min in the Triton/glycerol solution with gentle agitation and washed quickly with PBS containing 5 mM MgCl2 and 0.2 mM EGTA by brief centrifugation (12,800 × g for 5 s in an Eppendorf centrifuge). The resultant Triton/glycerol-insoluble residues were processed for the isolation of microfilaments in the same way.

Measurement of Ratios of Each Form of Tropomyosin to Actin by Radioactivities of [35S]Methionine Incorporated—Cells (one 100-mm culture dish, 106–107 cells) were first labeled with 250 μCi of [35S]methionine (1110 Ci/mmol) for 16 h. After washing with PBS three times, cells were scraped off in 100 μl of SDS-gel sample buffer (2% SDS, 15% glycerol, 180 mM dithiothreitol, 80 mM Tris-Cl, 0.001% bromphenol blue (pH 6.5)) and incubated at 100°C for 2 min. After homogenization by passage through a No. 26 needle at least six times, cell lysates were again incubated at 100°C for 2 min. Total cell lysates of 10 μl were loaded on each lane of 15% acrylamide gels (12.5% acrylamide, 0.1% bisacrylamide) (Blattler et al., 1981). The ratios of tropomyosin to actin were determined by the high resolution, one-dimensional SDS-polyacrylamide slab gels of the Tris/glycerol buffer system (Laemmli, 1970) with a low concentration of bisacrylamide in separating gels (12.5% acrylamide, 0.1% bisacrylamide) (Blatter et al., 1972; Blose and Meltzer, 1981). The ratios of tropomyosin to actin were calculated from the amount of [35S]methionine incorporated in each protein.

RESULTS

Changes in the Protein Composition of Microfilaments Isolated from Normal and DNA or RNA Virus-transformed Rat Tissue Culture Cells

Using our new method (Matsumura et al., 1983) for microfilament isolation, we have analyzed the protein compositions of microfilaments in normal rat culture cells and in cells transformed by various types of virus.

DNA Virus-transformed Cells—Fig. 1 shows a SDS-polyacrylamide gel electrophoretic analysis of microfilaments isolated from normal REF-52 and four independent SV40-transformed REF-52 (REF-WT2A, WT4A, WT6A, and WT10A) cell lines. The yields of microfilaments were similar for both normal and SV40-transformed cells when measured by the amount of actin recovered in the microfilament fraction (approximately 90% of the total cell actin). The major components of these isolates of each Form of Each Tropomyosin

[The rest of the document continues with detailed scientific analysis and experimental results, which are not included here due to space constraints.]
Changes in Tropomyosin Expression Accompanying Transformation

The five forms of tropomyosin are indicated by numbers (1-5). Note the changes in the levels of these tropomyosin species. α-Antin and 37,000- and 83,000-dalton protein are indicated by arrows. An 83,000-dalton protein appears to be a new microfilament-associated protein (Matsumura et al., 1983); however, we cannot find any correlation of the level of this protein with transformation.

anti-tropomyosin monoclonal antibody (LCK16) used for the isolation of microfilaments.

In a previous paper (Matsumura et al., 1983), we have identified five forms of tropomyosin in rat cultured cells, including REF-52 cells, NRK cells, rat-1 cells, and L6 myoblasts, based on the criteria described below. These five proteins, with apparent Mr of 40,000 (TM-1), 36,500 (TM-2), 35,000 (TM-3), 32,400 (TM-4) and 32,000 (TM-5), are immunoprecipitated with conventional rabbit anti-tropomyosin antiserum in the presence of SDS. They exhibit isoelectric points of pH 4.5 on two-dimensional gels; they lack both proline and tryptophan; they are heat-stable proteins; and they possess the ability to bind to F-actin. However, it should be noted that the molecular weights of these multiple forms of tropomyosin are apparent because tropomyosin molecules are known to show different migration on SDS gels even though they have the same chain length (Mak et al., 1980).

The patterns of these five forms of tropomyosin in isolated microfilaments appear to be different between normal and SV40-transformed REF-52 cells, although the protein compositions were otherwise very similar. As described before (Matsumura et al., 1983), microfilaments of normal REF-52 cells contain three major (TM-1, TM-2, and TM-4) and two minor (TM-3 and TM-5) tropomyosins. One of the minor tropomyosins with apparent Mr = 35,000 (TM-3) was particularly increased in its level in microfilaments from SV40-transformed cells when relative intensities of these tropomyosin bands were compared (quantitative data are described later). When lower amounts of samples were loaded on gels (see lane 5 of Fig. 1), the bands of TM-4 and TM-5 could be separated as a doublet. These experiments show that the 32,000-dalton (TM-5) tropomyosin is also increased in the microfilaments from SV40-transformed cells. In addition, the levels of protein bands with Mr = 250,000 and 37,000 were also increased in microfilaments from SV40 transformants (lanes 3-5) except WT2A cells (lane 2). The increases in the levels of these bands upon SV40 transformation were confirmed by measuring radioactivities of [35S]methionine incorporated in these bands relative to the radioactivity of actin band. On the other hand, there appeared no correlation between the level of 83-kDa protein and SV40 transformation, although 83-kDa protein is one of major component of microfilaments.

We examined whether these changes in the protein composition of microfilaments were associated with the morphological alterations in transformed cells. For this purpose, microfilaments were prepared from dl884.1A cells, which were isolated by infection of REF-52 cells with a deletion mutant (dl884) of SV40 that is incapable of maturing small-t mRNA. Unlike the above SV40-transformed cells, dl884.1A cells show a well spread morphology similar to that of normal cells, do not express the small-t protein, but do express SV40 large-T protein (Graessmann et al., 1980; Topp and Rüfikin, 1980). As lane 6 in Fig. 1 shows, the protein composition of microfilaments from the dl884.1A cells was very similar to that of normal cells (lane 1). The levels of 250,000-, 37,000-, 35,000 (TM-3)-, and 32,000 (TM-5)-dalton proteins were not observed to be increased. Identical results were obtained with another independent isolate, dl884.4A. Therefore, the changes in tropomyosin patterns as well as increases in the levels of 250,000- and 37,000-dalton proteins appeared to be associated with SV40 transformation.

The changes in tropomyosin patterns described above are more clearly seen by two-dimensional gel analysis (Fig. 2). In order to compare tropomyosin patterns with the morphologies of normal and virus-transformed rat cells, phase-contrast micrographs of these cells are included in Fig. 2.

Fig. 2D shows the “less spread” morphology of one (REF-WT4A) of the SV40-transformed REF-52 cells as compared with normal REF-52 cells (Fig. 2B). As Fig. 2A shows, three (TM-1, TM-2, and TM-4) are major and two (TM-3 and TM-5) are minor tropomyosins in microfilaments of normal REF-52 cells. In the microfilaments isolated from SV40-transformed cells (Fig. 2C), the levels of both minor tropomyosins (TM-3 and TM-5) were increased and the level of one major tropomyosin (TM-1) appeared to be decreased. Each of the four independent SV40 transformants (REF-WT2A, -WT4A, -WT6A, and -WT10A) exhibited similar changes in both tropomyosin patterns and cell morphology.

In order to examine quantitatively these changes in tropomyosin patterns upon transformation, we estimated ratios between tropomyosins and actin by measuring radioactivities of [35S]methionine incorporated in each form of tropomyosins and actin in isolated microfilaments as described under “Experimental Procedures.” The ratios between each form of tropomyosin are expressed per 100 parts of actin. Since TM-4 and TM-5 could not be separated well on one-dimensional gels, these forms were treated as one. As Table I shows, normal REF-52 cell microfilaments have 7.0 parts of TM-1, 4.5 parts of TM-2, 0.8 parts of TM-3, and 3.5 parts of doublet tropomyosin (TM-4 plus TM-5) to 100 parts of actin. Upon trans-
Changes in Tropomyosin Expression Accompanying Transformation

FIG. 2. Changes in tropomyosin patterns of microfilaments and morphologies of normal and DNA virus-transformed REF-52 cells. A, C, and E, two-dimensional gel analysis of the microfilament fractions from normal and transformed REF-52 cells (pH 5–7 ampholytes for the first dimension (left, acidic; right, basic) and 12.5% polyacrylamide gel for the second dimension). After electrophoresis, gels were processed for fluorography. Only those parts of the two-dimensional gels containing tropomyosin and actin are shown here. B, D, and F, phase-contrast micrographs. A and B, normal REF-52 cells; C and D, SV40-transformed REF-52 cells (REF-WT4A); E and F, adenovirus type 5-transformed REF-52 cells (Ad5D.1A). Numbers (1–5) indicate the multiple forms of tropomyosin (TM-1 to TM-5). Note the decrease in the level of TM-1 and the increase of both TM-3 and TM-5 in the microfilaments from SV40 transformants (C). TM-1 is entirely missing and TM-3 is greatly increased in the microfilaments from adenovirus transformants (E).

| Ratio of radioactivities of [35S]methionine incorporated in tropomyosins of microfilaments isolated from normal and transformed rat cultured cells |
|---------------------------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
|                                | REF-52 | WT2A | WT4A | WT6A | WT10A | d1884.1A | Ad5W.3A | Ad5W.4A | Ad5D.1A | Ad5D.4A | NRK 1570* | NRK 1569* | 4/435* |
|--------------------------------|--------|------|------|------|------|--------|--------|--------|--------|--------|--------|----------|----------|--------|
| TM-1                           | 7.0    | 4.3  | 4.3  | 3.6  | 4.2  | 7.2    | ND     | ND     | ND     | ND     | 6.2     | 2.9      | 2.8    |
| TM-2                           | 4.5    | 2.7  | 2.0  | 2.6  | 2.0  | 2.6    | 4.8    | 4.5    | 5.2    | 1.3    | 5.0     | ND       | ND     |
| TM-3                           | 0.8    | 2.4  | 2.3  | 2.2  | 2.4  | 1.0    | 2.9    | 4.6    | 12.0   | 9.1    | 1.2     | ND       | ND     |
| TM-4 + 5                       | 3.6    | 5.1  | 5.4  | 5.8  | 5.4  | 3.8    | 9.0    | 6.1    | 9.0    | 2.0    | 5.4     | 19.0     | 23.9   |

* Fibroblastic NRK cells (American Type Culture Collection, ATCC CRL 1570).
* Kirsten virus-transformed NRK cells (ATCC CRL 1569).
* Rous sarcoma virus-transformed NRK cells.
* ND, not detectable.

formation by SV40, the ratios of tropomyosins to actin altered in a manner that was conserved between individual transformants. The levels of both TM-1 and TM-2 were decreased by half, but the level of TM-3 was increased 3-fold in the microfilaments of SV40 transformants. The combined levels of doublet tropomyosin (TM-4 plus TM-5) were increased by 50%. On the other hand, the ratios of the tropomyosins to actin in the microfilaments from d1884.1A were very similar to those found in microfilaments from normal REF-52 cells, with the exception that the amount of TM-2 was decreased by almost half.

The relation between changes in tropomyosin expression and cell transformation by DNA viruses was extended to human adenovirus type 5-transformed REF-52 cells, which have a yet more rounded morphology (Fig. 2F) than do SV40 transformants (Fig. 2D). Microfilaments were isolated from four independent transformants (Ad5W.3A, Ad5W.4A, Ad5D.1A, and Ad5D.4A) as above. However, the recovery of
Changes in Tropomyosin Expression Accompanying Transformation

FIG. 3. One-dimensional SDS-polyacrylamide gel analysis of microfilaments isolated from adenovirus type 5-transformed REF-52 cells. Microfilaments were isolated from normal REF-52 cells (lane 1) and from four independent adenovirus transformants (Ad5W.3A (lane 2), Ad5W.4A (lane 3), AD5D.1A (lane 4), and Ad5D.4A (lane 5)). Microfilaments from one of the SV40 transformants, REF-WT4A (lane 6), were included for comparison. Asterisks indicate the heavy and light chains of IgM monoclonal antibody (LCK16). Numbers (1-5) indicate the five forms of tropomyosin. No TM-1 was detected in the microfilaments isolated from these adenovirus-transformed REF-52 cells. Lane std, molecular mass standards are the same as described in the legend to Fig. 1.

Microfilaments was less than that from normal or SV40-transformed cells (approximately 20% of the total cell actin was recovered in the microfilament fractions from adenovirus-transformed cells). Although the protein compositions of microfilaments isolated from adenovirus-transformed REF-52 cells (lanes 2–5 in Fig. 3) were more complex than those isolated from normal (lane 1 in Fig. 3) and SV40-transformed REF-52 cells (lane 6 in Fig. 3), one-dimensional SDS-polyacrylamide gel analysis revealed similar but more drastic changes in tropomyosin patterns. TM-1 was completely missing and TM-3 was greatly increased in amount. The added amount of TM-4 and TM-5 was also increased in all of the adenovirus transformants except Ad5D.4A cells (the most refractile and poorly attached of all the REF-52 cells studied). The levels of both the 37,000- and 250,000-dalton proteins were also increased, as observed in SV40 transformants.

Two-dimensional gel analysis of microfilaments isolated from Ad5D.1A cells (Fig. 2E) confirmed the above results. The same result was obtained with the microfilaments of the other adenovirus type 5-transformed cell line (Ad5D.4A) which expresses the 57,000-dalton E1b protein. However, in cell lines lacking the 57,000-dalton protein (Ad5W.3A and Ad5W.4A), trace amounts of TM-1 were detectable in two-dimensional gels upon prolonged exposure. An increase in the level of TM-5 was also observed in these adenovirus-transformed REF-52 cells, except in Ad5D.4A cells.

Quantitative data on the changes in tropomyosin patterns upon adenovirus transformation are also shown in Table I. By this method, TM-1 was not detected in any of the adenovirus-transformed cells. The ratios of TM-3 to actin in the microfilaments of Ad5W and Ad5D cell lines were increased 3–6-fold and 10–15-fold, respectively, above that found in
Changes in Tropomyosin Expression Accompanying Transformation

normal cell microfilaments. The level of doublet tropomyosin (TM-4 plus TM-5) was increased 2-3-fold (except in the Ad5D.4A cell line). Unlike the SV40 transformants, decreases in the level of TM-2 were not observed in the microfilaments of adenovirus-transformed cells (except again the Ad5D.4A cell line).

These changes in tropomyosin expression upon DNA virus transformation were also apparent when total cell lysates were examined. Fig. 4A shows the two-dimensional gel pattern of total cell lysates from REF-52 cells. The three major and two minor tropomyosin spots were found in a pattern similar to that in isolated microfilaments (see Fig. 2A). Total cell lysates of SV40 (Fig. 4B) or adenovirus-transformed cells (Fig. 4C) showed changes in tropomyosin patterns similar to those observed in the isolated microfilaments (compare Fig. 2).

RNA Virus Transformation—Because REF-52 cell lines transformed by RNA viruses are not available at the present time, we have used NRK cells (ATCC CRL 1570) and their RNA virus transformants (ATCC CRL 1569 for a Kirsten virus transformant; 6/55C and 4/435 for RSV transformants) to determine whether similar changes in tropomyosin expression accompany transformation by RNA viruses. The amounts of actin recovered in the microfilament fraction from normal NRK cells and from RNA virus-transformed cells were about 20 and 4% of total cell actin, respectively.

Fig. 5 shows two-dimensional gel analysis of tropomyosin patterns in microfilaments isolated from NRK cells (Fig. 5A) and Kirsten virus (Fig. 5C) or RSV (Fig. 5E)-transformed NRK cells. Judged by co-migration on two-dimensional gels and other biochemical studies including immunoprecipitation with rabbit anti-tropomyosin antiserum, these NRK cells also contain the same five forms of tropomyosin as observed in REF-52 cells. The tropomyosin pattern in microfilaments isolated from normal NRK cells (Fig. 5A) was very similar to that of “normal” REF-52 cells (Fig. 2A) except that TM-5 was more prominent in NRK cells. Microfilaments isolated from either Kirsten virus (ATCC CRL 1569; Fig. 5C) or RSV (4/435; Figure 5E)-transformed NRK cells show similar changes in tropomyosin patterns. The level of TM-1 was decreased and TM-2 was entirely missing. Although the level of TM-3 was not increased (unlike with DNA virus transformation), the level of the other minor tropomyosin (TM-5) was found to be increased and became higher than the level of TM-4. These changes in tropomyosin patterns were also observed in total cell lysates when examined on two-dimensional gels (data not shown). NRK cell microfilaments also contained protein bands which co-migrated with the 37,000-
and 250,000-dalton proteins found in REF-52 cell microfilaments. However, these proteins appeared largely unaffected by RNA virus transformation.

Both RNA virus-transformed cell lines exhibited significant morphological changes. In contrast to the well spread morphology of NRK cells (Fig. 5B) (although a little less spread than normal REF-52 cells (Fig. 2B), Kirsten virus (Fig. 5D) and RSV (Fig. 5F)-transformed NRK cells had a much more rounded morphology.

The ratios of tropomyosin to actin in these NRK cells are shown in Table I. In the microfilaments of RNA virus (either Kirsten virus or Rous sarcoma virus)-transformed NRK cells, the level of TM-1 was decreased to one-third that in the normal NRK cell microfilaments. TM-2 and TM-3 could not be detected. However, the level of doublet tropomyosin (TM-4 plus TM-5) was increased by about 4-fold.

*Rat-1 Cells Transformed with a Temperature-sensitive Mutant of RSV*—The correlation between the changes in tropomyosin patterns and cell transformation was further examined in rat-1 cells transformed with a RSV mutant, temperature-sensitive for transformation. These cells had a well spread morphology at 39 °C, similar to that of normal rat-1 cells, but at 34 °C they displayed a transformed phenotype, namely a rounded morphology (compare Fig. 6, C and D). As with the other rat cultured cells, rat-1 cells also contained the five forms of tropomyosin. Cells were cultured at 39 °C, and microfilaments were isolated from these cells (Fig. 6A). A second culture was shifted down to 34 °C and cultured for 16 h to allow these cells to transform. Microfilaments were isolated from these cells and analyzed on two-dimensional gels (Fig. 6B). Changes in tropomyosin patterns similar to those observed in RNA virus transformation (see Fig. 5) were observed in these two isolated microfilaments. The level of TM-1 was decreased; TM-2 and TM-3 were entirely missing; and the level of TM-5 increased over the level of TM-4 upon transformation by temperature shift-down. Likewise, the changes in tropomyosin patterns were reversed when the temperature was shifted up from 34 to 39 °C (data not shown).

![Fig. 6. Changes in tropomyosin patterns and morphologies in ts Rat-1 cells by temperature shift. A and B, two-dimensional gel analysis of the isolated microfilaments (pH 5–7) ampholytes for the first dimension (left, acidic; right, basic) and 12.5% polyacrylamide for the second dimension). After electrophoresis, gels were processed for fluorography. C and D, phase-contrast micrographs of cell morphologies. Cells were first cultured at 37 °C (C) for 48 h, then labeled for 2 h with 250 µCi of [35S]methionine (1110 Ci/mmol) in methionine-free Dulbecco’s modified Eagle’s medium containing 2.5% fetal calf serum, and microfilaments were analyzed on two-dimensional gels (A). Then, temperature was shifted down to 34 °C. After 16 h, a cell morphology of these cells was changed to a transformed phenotype (D) and the cells were labeled in the same way as described above. Microfilaments from these cells were analyzed on two-dimensional gels (B). Note that tropomyosin patterns change by temperature shift in a way similar to those found in microfilaments between normal and RNA virus-transformed NRK cells (see Fig. 5).
We have also found similar changes in tropomyosin patterns in the microfilaments isolated from NRK cells transformed with another temperature-sensitive mutant (LA29) of RSV. These results indicate that the changes in tropomyosin patterns are associated with morphological transformation.

**Cell-free Translation of RNA from Transformed and Normal Cells**

To explore the possibility that the changes in tropomyosin patterns resulted from post-translational modification of some tropomyosin species, the products of cell-free translation of mRNA from these cell lines were examined on two-dimensional gels (Fig. 7). Proteins which co-migrated with the five forms of tropomyosin were detected in the translation products directed by RNA from either normal REF-52 cells (Fig. 7A), SV40-transformed REF-52 cells (Fig. 7B), or normal NRK cells (Fig. 7D). However, TM-3 in translation products from both normal cells could only be detected after prolonged exposure of gels. Cell-free translation products largely reproduced the tropomyosin patterns found in total cell lysates (Fig. 4).

![Fig. 7. Two-dimensional gel analysis of cell-free translation products of total RNA isolated from normal and transformed rat cultured cells.](http://www.jbc.org/)

RNA was added to nuclease-treated reticulocyte lysate (Pelham and Jackson, 1976) at around half-saturating RNA levels. Translation products were labeled with $[^{35}S]$methionine. Isoelectric focusing was from left (acidic) to right (basic) (pH 5-7 gradient), and SDS-gel electrophoresis was from top to bottom (12.5% polyacrylamide gel). Numbers (1-5) indicate the five forms of tropomyosin. A, REF-52; B, SV40-transformed REF-52 (REF-WT4A); C, adenovirus transformed-REF-52 (Ad5D.1A); D, NRK cells; E, Kirsten virus-transformed NRK cells (ATCC 1569); F, Rous sarcoma virus-transformed NRK cells (4/435). Note that the changes in tropomyosin patterns are similar to those found both in isolated microfilaments (Figs. 2 and 5) and in total cell lysates (Fig. 4).
cell lysates or in isolated microfilaments following metabolic labeling. While mRNA from both REF-52 and NRK cells directed synthesis of three major and two minor forms of tropomyosin as observed in isolated microfilaments, the levels of both TM-1 and TM-2 were decreased and the levels of both TM-3 and TM-5 were increased in SV40-transformed cells (Fig. 7B). No TM-1 could be detected in the cell-free translation products directed by mRNA from one (Ad5D.1A) of adenovirus-transformed cells (Fig. 7C). The level of TM-1 was decreased, and the level of TM-5 increased in both Kirsten virus (Fig. 7E)- and RSV (Fig. 7F)-transformed NRK cells. Neither TM-2 nor TM-3 were detected in the translation products of mRNA from these RNA virus-transformed cells. Thus, the changes in tropomyosin patterns were maintained when examined in total cell lysates, in isolated microfilaments, and by cell-free translation of mRNA.

**Effect of Cytochalasin B or Trypsin Treatment on the Pattern of Tropomyosin in Microfilaments**

Finally, we asked whether agents which induce rounding up of the cells produce similar changes in tropomyosin patterns. To address this question, the morphology of REF-52 cells was altered by treatment with either trypsin or cytochalasin B (Weber et al., 1976; Heuser and Kirschner, 1980). Microfilaments isolated from such rounded cells were compared with those from untreated cells. In both cases, the patterns of tropomyosins as well as other microfilament components appeared the same in treated and in untreated cells.

**DISCUSSION**

In this paper, we have shown that changes in tropomyosin patterns are major and common changes in the protein composition of microfilaments of rat culture cells after transformation by various tumor viruses. Microfilaments of normal rat cell cultures with a well spread morphology contain three major (TM-1, TM-2, and TM-4) and two relatively minor (TM-3 and TM-5) tropomyosins. Upon transformation by either DNA or RNA viruses, the levels of one or both of the major tropomyosins (TM-1 and TM-2) are decreased and the levels of one or both of the minor tropomyosins (TM-3 and TM-5) are increased. These changes in tropomyosin patterns also occur in rat-1 cells or NRK cells transformed with a ts RSV mutant by shift to permissive temperature (Fig. 6). These results suggest that there is some correlation between the changes in tropomyosin and transformation. Furthermore, preliminary experiments with chemically transformed BALB/3T3 cells (in collaboration with Dr. T. Kukunaga at National Institutes of Health) and NIH/3T3 cells transformed with cancer tissue DNA (in collaboration with Dr. M. Wigler at Cold Spring Harbor Laboratory) have shown similar changes in tropomyosin patterns. Although the tropomyosin forms of mouse cells are of different molecular weights and charges than those seen in rat cells, the level of one tropomyosin form with a higher molecular weight is decreased and the other tropomyosin with a lower molecular weight is increased in amount. Therefore, changes in tropomyosin patterns appear to be a general phenomenon associated with oncogenic transformation.

The changes in tropomyosin patterns appear to correlate with morphological transformation. Cells transformed by small-t defective SV40 (di884) show a well spread morphology and well developed actin cables (Graessmann et al., 1980; Topp and Rifkin, 1980). These cells do not show changes in tropomyosin patterns (Fig. 1 and Table 1). Moreover, preliminary time course experiments with ts Rat-1 cells show that the changes in tropomyosin patterns occur at approximately the same time (about 8 h after temperature shift-down) as the morphological change from a well spread to rounded shape becomes recognizable by phase-contrast microscopy. Whether these changes play a primary or secondary role in morphological transformation requires further studies including detailed kinetics of the changes in tropomyosin patterns during the process of cell transformation.

The changes in tropomyosin patterns are not simply the result of morphological alteration because cell shape changes by trypsin or cytochalasin B treatment do not cause changes in tropomyosin patterns. This might mean that the mechanisms by which cell shape is changed by these agents differ from those caused by transformation by viruses.

Our newly developed method for the isolation of microfilaments (Matsumura et al., 1983) gave good yields (20–30% of total cell actin) of microfilaments for the cell lines studied in this paper except RNA virus-transformed NRK cells. It should be noted, however, that this method detects only tropomyosin-containing microfilaments. The lower yield (4% of total cell actin) of actin in the microfilaments from the RNA virus-transformed cells does not appear to mean that the transformed cells have more nonpolymerizable actin. It resulted rather from poor extractability of actin from Triton/glycerol-insoluble residues (see "Isolation of microfilaments") in these transformed cells. While approximately 80% of actin was extracted from Triton/glycerol-insoluble residues of normal and DNA virus-transformed cells by homogenization in the presence of Mg-ATP, only 20% of actin is extracted from the same fraction of RNA virus-transformed cells by the same procedure. The same procedure extracted most of the tropomyosin, and furthermore, most of tropomyosin-containing microfilaments in the extracts were recovered in the microfilament fractions, regardless of the cell line. These results would suggest that RNA virus-transformed cells contain lesser amounts of tropomyosin-containing microfilaments. Further studies are required to determine whether or not actin in Triton/glycerol-insoluble residues is present as microfilaments in these RNA virus-transformed cells.

Cell-free translation of mRNA (Fig. 7) has revealed changes in tropomyosin patterns similar to those found in either isolated microfilaments or total cell lysates. These results would suggest that at least five mRNAs are present for these multiple forms of tropomyosin and that the changes in tropomyosin patterns are regulated at the level of RNA rather than by post-translational modification. Because there are some known examples that specific proteases are synthesized in active form in cell-free translations (Pelham, 1978; Shih et al., 1979), it remains possible that these multiple forms of tropomyosin have a precursor-product relationship. However, in pulse-chase experiments, we have found that the rates of synthesis of the multiple forms of tropomyosin are very similar (data not shown). Moreover, preliminary one-dimensional peptide mapping using the method of Cleveland et al. (1977) has shown that each form of tropomyosin has a considerably different spectrum of peptides. These results would argue against the possibility that precursor-product relationships exist between these multiple forms of tropomyosin.

It is tempting to speculate that the substitution of one set of tropomyosin for another in microfilaments alters the physiological properties of the microfilaments. Although tropomyosin is one of the major components of microfilaments in cultured cells, the biological role of tropomyosin in non-muscle cells is not clearly understood. The only known biological function of tropomyosin is the regulation of skeletal muscle contraction. In association with the troponin complex, tropomyosin regulates actomyosin interaction in a calcium-
dependent manner (Hanson and Lowry, 1963; Ebashi et al., 1969). However, because nonmuscle cells do not contain tropomyosin, tropomyosin may not function in this way.

**In vitro** studies on the interaction of skeletal muscle tropomyosin with muscle F-actin filaments have shown that tropomyosin appears to stabilize the structure of F-actin filaments. By electron microscopy, for example, tropomyosin-containing F-actin appears “straighter” (Kawamura and Maruyama, 1970; Takebayashi et al., 1977) and is stabilized during fixation (Maupin-Samzamier and Pollard, 1978).

Immunofluorescent studies on cultured fibroblasts have shown that tropomyosin is localized in the microfilament bundles (stress fibers or actin cables) but is absent in the highly motile area of cells such as ruffling membranes (Lazarides, 1976). This observation has been further supported by microinjection of fluorescently labeled tropomyosin into living cells (Wehland and Weber, 1980). These observations, coupled with the ability of tropomyosin to stabilize F-actin filaments, suggest that tropomyosin may play structural and/or regulatory roles in the organization of the microfilaments into bundles as stress fibers.

Nonmuscle tropomyosin is known to be different in actin-binding properties from muscle tropomyosin. Côte and Smillie (1981) reported that nonmuscle tropomyosin from horse platelets binds less strongly to F-actin and needs magnesium ions for binding. Judged by the apparent molecular weight on SDS-polyacrylamide gels, this nonmuscle tropomyosin possibly corresponds to TM-4 and TM-5 whose levels are increased upon cell transformation. Preliminary experiments showed that the actin binding of these tropomyosins was weaker than that of the other forms of tropomyosin. Thus, it is possible that the weak binding of these tropomyosins to actin may change the structure of the microfilaments containing these forms of tropomyosin (such as destabilization of microfilaments). Such altered properties of the microfilaments may be reflected in the instability of the microfilaments in transformed cells to aggregate into stress fibers or to undergo morphological changes.

Recently, Bernstein and Bamburg (1982) have reported that tropomyosin binding to F-actin protects the F-actin from severing by severs F-actin into short filaments (Yin and Stossel, 1979), is inhibited by the presence of tropomyosin. It was reported that gelsolin is present both in normal and transformed fibroblasts (Yin et al., 1981) and localized in stress fibers in normal fibroblasts. Therefore, it is possible that the action of gelsolin is prevented by tropomyosin in normal cells. If the tropomyosins (TM-3 and TM-5) whose levels are increased in transformed cells cannot counteract the action of gelsolin or actin-depolymerizing factor, the disorganization of stress fibers in transformed cells can be explained.

The differential expression of tropomyosin forms may be one of the causes for changes in organization of actin bundles which in turn determines cell shape in transformed cells. However, biochemical and functional characterizations of each form of tropomyosin as well as their interactions with microfilament-associated proteins such as gelsolin or actin-depolymerizing factor are necessary to understand the relationship between the changes in tropomyosin patterns and the disruption of actin cables.

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