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Patterns of Organization of Actin and Myosin in Normal and Transformed Cultured Cells

(immunofluorescence/contraction/simian virus 40/anchorage/microfilaments)

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ABSTRACT **The patterns of distribution of intracellular actin and myosin were examined by specific immunofluorescence in a series of normal, simian-virus-40-transformed, and revertant cell lines of rat and mouse origin. A consistent correlation was found between sensitivity to anchorage-dependent growth control and the presence of large, thick sheaths of actin-containing material. The presence of these sheaths was temperature-dependent in a rat line transformed by a temperature-sensitive mutant in the complementation group *A* of the oncogenic virus simian virus 40.**

Many changes in cell structure, such as cell movement, ruffling, and cytokinesis, can be traced to contractions. These changes very likely occur by the contraction of intracellular actomyosin (1–3), in which the actin is very similar or identical to muscle actin (2, 4–6) and the myosin is similar to the myosin in smooth muscle (7, 8) and platelets (9, 10).

Microfilaments (6 nm), but not other intracellular fibers, bind heavy meromyosin (4, 11, 12), which demonstrates that they contain actin. Microfilaments are arrayed in at least two distinct configurations in the normal migrating fibroblastic cell: as a 3-dimensional matrix about 100 nm thick, just under the cell membrane, and in subcortical sheaths (11, 13–17). In flattened cells, the sheaths (also called stress fibers) run along the bottom and out into the edges of a cell (13, 18–20).

Recently, the preparation of an antibody directed specifically against actin permitted an examination by immunofluorescence of the localization of actin in normal cultured 3T3 cells (21). Actin was found to occur predominantly in the form of long and thick sheaths in a variety of non-transformed cells of different species (21). In the case of well-spread mouse 3T3 cells, these sheaths correspond to "stress fibers" seen in phase microscopy and are related to the thick bundles of microfilaments seen in electron microscopy (22). Myosin also has been visualized by immunofluorescence, using antibody to chick smooth muscle myosin (23). These studies have revealed that myosin in well-spread mouse 3T3 cells is arranged in striated structures, which seem to be related to, if not identical to, the thick actin-containing fibers. However, presence of myosin in a less structured organization, possibly outside the thick phase dense fibers, was also indicated (23).

Previously several electron microscopic studies have indicated that the transformed state is accompanied by a less pronounced expression of microfilament sheaths (19, 20, 38). Since immunofluorescence can reveal the position of actin and myosin within a large number of cells very quickly and easily, we have used this procedure to study the effects of viral transformation on the intracellular organization of actin and myosin in a series of normal, simian virus 40 (SV40)-transformed and revertant cells of rat and mouse origin. The cell lines chosen for this study have been previously well characterized with respect to at least three conditions that signal the inhibition of cell proliferation: serum deprivation, cell–cell contact, and absence of a solid substrate, signals to which the untransformed cell is responsive *in vitro* (24). Previous studies have shown that cells respond to each of these signals separately, since transformation by oncogenic viruses, such as SV40, can cause a cell to hereditably lose sensitivity to one, two, or all three of these signals (25), and since revertants regaining sensitivity to any one, or any combination of these signals can be isolated from fully transformed cells. Cells in each of these categories have been assayed. In addition we have used transformants of rat embryo cells isolated after exposure to SV40 mutants in the complementation group *A* (28). In these cells sensitivity to many of the above signals appears to be temperature sensitive. A preliminary report of this work appears elsewhere (30).

RESULTS

We have examined the immunofluorescent patterns of actin and myosin in cell lines well characterized for their sensitivity to different growth controls. The cells include the mouse cell line 3T3, primary rat embryo cells, and lines derived by transformation and reversion from these two growth-controlled cell populations. All cells were examined under conditions where sparse cell density and excess (10%) serum assured that neither serum-dependent nor contact-dependent inhibition of cell growth were in effect. Cell culture protocols are given elsewhere (26). Antibody preparations, indirect immunofluorescent staining, and photography have been described previously (21, 23).

3T3 and its derivatives

Untransformed 3T3 cells showed sheaths, or stress fibers, which stained very brightly with antibody to actin (Fig. 1a). The most pronounced of these fibers appear to be about 1 × 50 μm in size. Myosin antibody stained a set of striated fibers (Fig. 1b). Both antibodies, in addition, stained the cytoplasm diffusely (Fig. 1a and b).

SV101 is a clone derived from 3T3 by transformation with the oncogenic virus SV40 (26). SV101 has lost sensitivity to

Abbreviations: SV40, simian virus 40; RE, rat embryo; WT, wild type.

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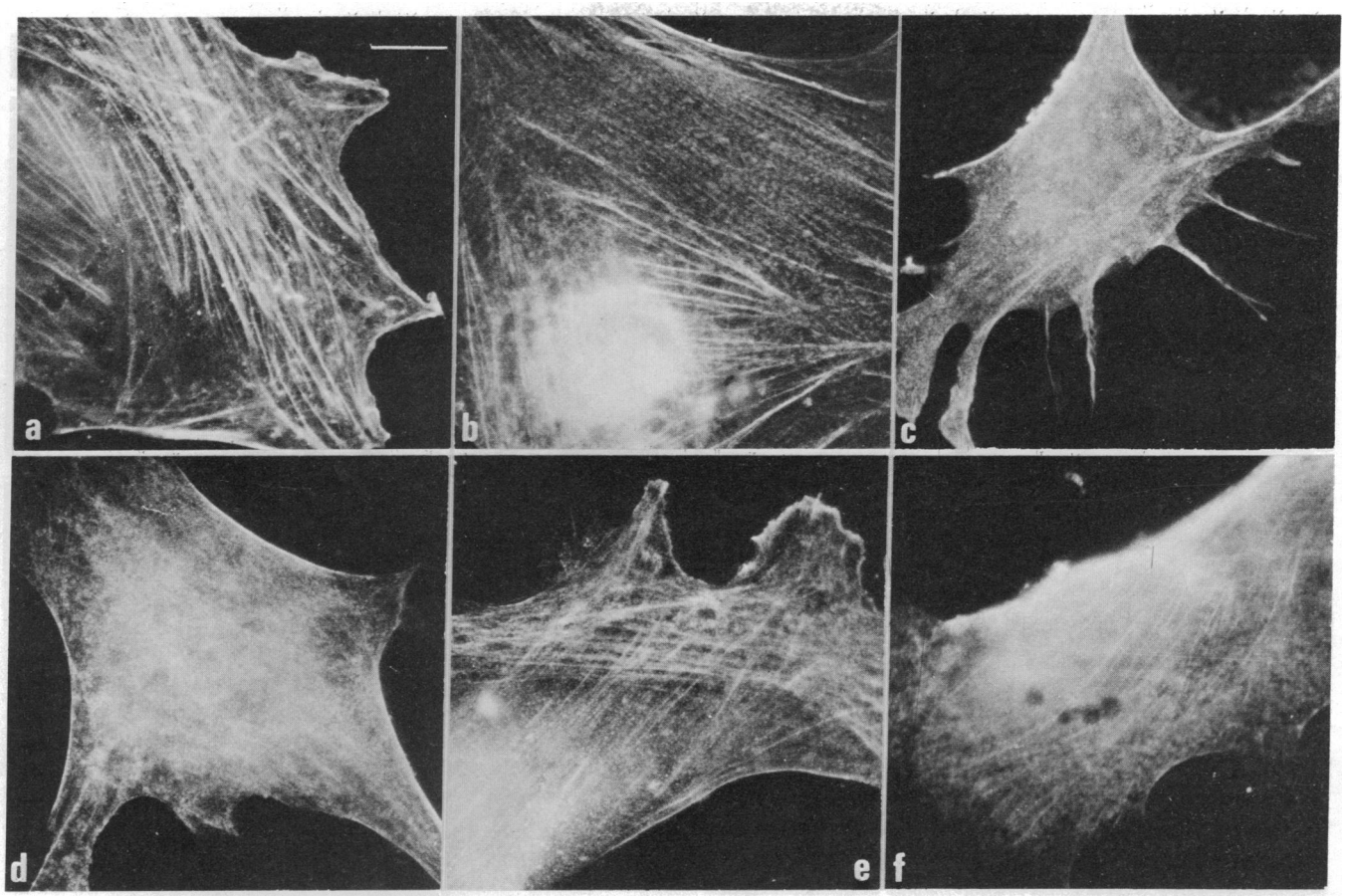

FIG. 1. Actin and myosin visualized in 3T3 cells and their SV40-transformed derivatives: (a) 3T3, actin; (b) 3T3, myosin; (c) SV101, actin; (d) SV101, myosin; (e) FlSV101, actin; (f) FlSV101, myosin. All cultures were grown at 37° for 48 hr before fixation. All figures are taken at the same final magnification. The bar indicates 10 μm. Note that transformed cells are, in general, somewhat smaller than their normal and revertant counterparts.

serum deprivation and contact inhibition and loss of anchorage (Table 1). SV101 cells and 3T3 cells have about the same complement of chromosomes (31). The long and thick actin-containing sheaths common in 3T3 are missing in SV101 cells (Fig. 1c). However, the diffusely staining actin-containing matrix is well represented in these cells. This is a result of a redistribution, rather than of a lower concentration of actin, in SV101, since both lines have about the same amount of actin per mg of cell protein (30). The myosin antibody also stains SV101 diffusely, while often displaying characteristic striations. The large fibers typical of 3T3 cells are rarely observed (Fig. 1d).

FlSV101 cells are partial revertants isolated from SV101 by negative selection with fluorodeoxyuridine (26). They have wholly regained their anchorage requirement (27) and partially regained their sensitivity to contact inhibition (32), while remaining fully transformed with regard to serum-sensitivity (27). FlSV101 cells have more chromosomes than either SV101 cells or 3T3 cells (31). In FlSV101, the thick, long actin-containing sheaths typical for 3T3 cells have reappeared in many of the cells (Fig. 1e), as have the fibers of striated myosin-containing material (Fig. 1f). Two serum-sensitive partial revertants derived from SV101, LS and Aγ, are partially sensitive to density inhibition, and have only slightly regained any anchorage requirement (27). The cytoplasms of both LS and Aγ cells appeared similar to that of the parental SV101 cells when stained with actin or myosin antibody.

Thus, in the absence of full reversion, only reacquisition of sensitivity of cell proliferation to the deprivation of anchorage is accompanied by reacquisition of the actin-containing sheaths characteristic of 3T3 cells.

Rat embryo (RE) cells and their SV40 transformants

Primary rat (RE) cells, obtained from 16-day embryos, do not grow well in the absence of extensive cell–cell contact, even in 10% serum (33). After 48 hr, the standard time of subculture used in these studies, some RE cells flatten and enlarge, becoming as much as 100 μm in diameter. The microfilament distribution in such primary rat cells has been extensively studied previously by electron microscopy (13).

Actin and myosin are distributed in RE cells in very much the same pattern as in 3T3 cells. Actin antibody reveals that such cells express a multitude of long actin-containing sheaths. These often run parallel for many micrometers, and can be as much as 1 μm in diameter (Fig. 2a). Myosin is distributed in striated sheaths (Fig. 2d). Both antigens are also present in a diffuse matrix as in 3T3 and SV101 (Fig. 2a and d).

SVRE 9 and SVRE 12 are two transformed clones isolated from rat embryo cells after infection with SV40 (33, 34). Like the 3T3-derived revertants, these transformants show separate responses to the three signals of growth control described

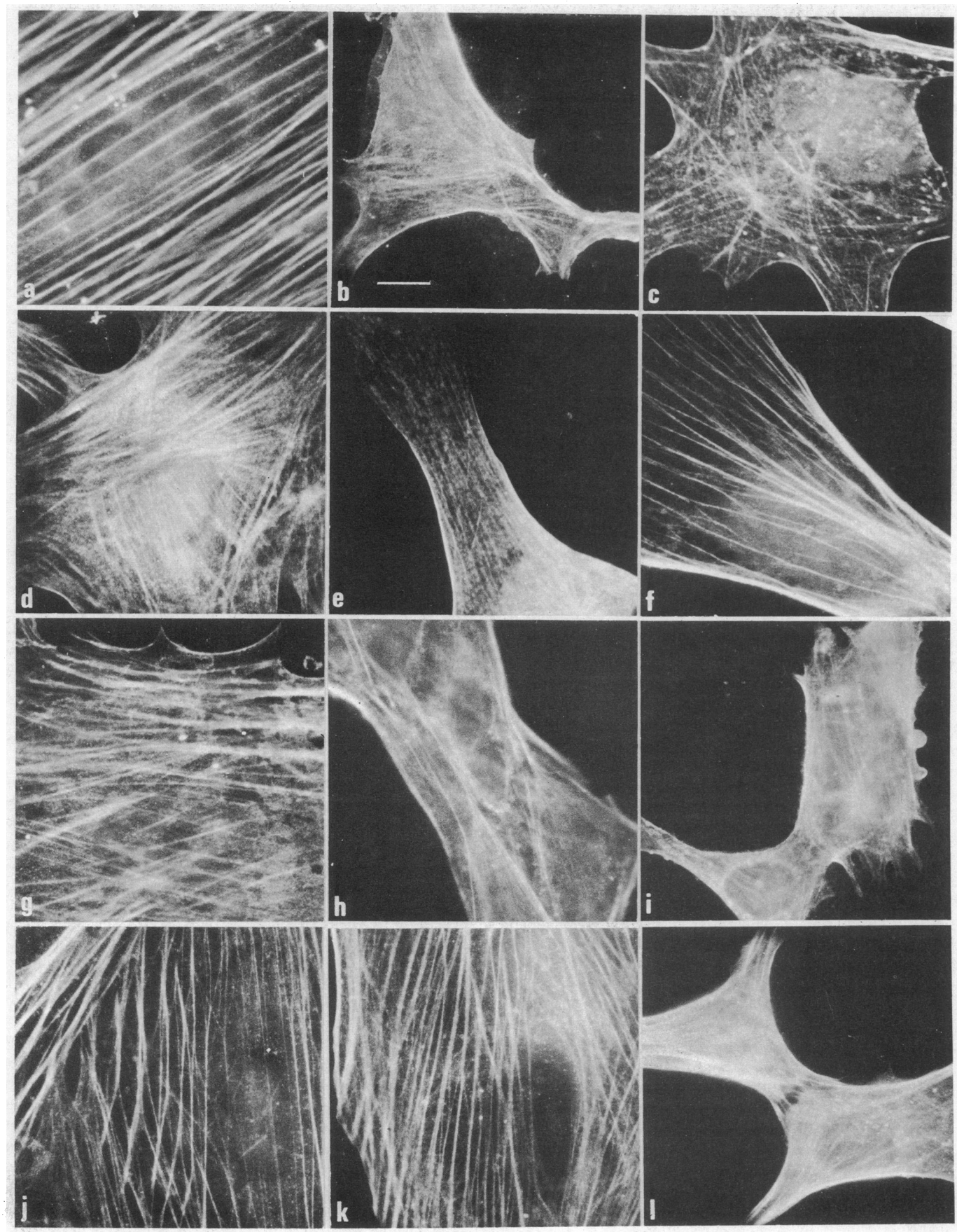

FIG. 2 (*Legend appears at bottom of the next page.*)

TABLE 1. *Properties of cell lines examined by immunofluorescence*

Line	Type	Temperature, C°	Actin cables*	Sensitivity to growth control of†			SV40 T-antigen	Chromosome no.
				Anchorage	Density	Serum		
3T3	Mouse cell line	37	+	+	+	+	−	70 ± 5
SV101	3T3 transformed by SV40	37	−	−	−	−	+	70 ± 5
FlSV101	Density-sensitive revertant of SV101	37	+	+	+	−	+	110 ± 20
A_γ	Serum-sensitive revertant of SV101	37	−	−	+	+	+	100 ± 10
LS	Serum-sensitive revertant of SV101	37	−	−	+	+	+	100 ± 10
RE	Primary rat embryo	37	+	+	+	+	−	41 ± 1
SVRE 9	RE transformed by SV40	37	−	−	−	−	+	70 ± 5
SVRE 12	RE transformed by SV40	37	+	+	−	−	+	70 ± 5
WT4	RE transformed by SV40	33	−	(−)‡	−	−	+	ND
		41	−	(−)‡	−	−	+	ND
*tsA*28-3	RE transformed by *tsA* SV40	33	−	(−)‡	−	−	+	ND
		41	+	(+)‡	+	+	−	ND

* See *text.*

† Anchorage-dependent cells will not form colonies in 1.2% methyl cellulose (34). Density-sensitive cells will maintain a constant low cell density despite frequent changes of medium (26). Serum-sensitive cells will not grow in 1% serum (27). In each case + means the line has a given sensitivity, while − means the line will grow under the restrictive condition.

‡ Temperature sensitivity of anchorage dependence has not yet been determined for WT4 and *tsA*28-3. However, transformants of other cell lines isolated after exposure to *tsA*28 are known to be temperature sensitive for anchorage dependence (35).

above. Both clones are fully transformed with regard to serum and density but they differ in their anchorage requirement (Table 1). SVRE 9 can grow well in semi-solid medium. SVRE 12 cells, like RE cells and FlSV101 cells, require anchorage for growth (34). The diffuse matrices of actin and myosin-containing material are present in all RE-derived cells (Fig. 2b, c, e, and f). However, SVRE 9 cells have lost the thickest actin sheaths (Fig. 2b), while SVRE 12 cells have not (Fig. 2c). The distribution of myosin in both transformed lines resembles that found for actin (Fig. 2d–f).

tsA transformants

*tsA*28-3 and WT are transformed lines isolated after infection of rat embryo cells at 33° with the SV40 temperature-sensitive gene *A* mutant *tsA*28 and with the parental SV40 wild-type stock, respectively (28). At 33°, *tsA*28-3 and WT4 both appear fully transformed. At the nonpermissive temperature, however, WT4 remains transformed, while *tsA*28-3 (28), and some other lines transformed by *tsA* mutants (29, 35, 37), regain the growth control characteristic of untransformed cells (Table 1). We examined RE cells, *tsA*28-3, and WT4, at 33° and 41°, with actin and myosin antibodies (Fig. 2g–l).

The pattern of actin organization showed a marked temperature dependence in *tsA*28-3 (Fig. 2 h and k). At 33° and 41°, RE cells were seen showing the characteristic heavy sheaths (Fig. 2g and j), while WT4 transformed cells lacked the sheaths at both temperatures (Fig. 2i and l). Sheaths were small and rare in *tsA*28-3 cells at 33° (Fig. 2h), but were long, thick, and well developed in *tsA*28-3 cells at 41° (Fig. 2k). Like some other properties already described for the *tsA* transformants (28), acquisition of actin-containing sheaths was not instantaneous after shift to the nonpermissive temperature; after 2–4 days at 41°, almost all cells contained sheaths. Myosin-containing fibers were clearly visible in *tsA*28-3 at 41°. However, *tsA*28-3 cells at 33°, and the WT4 cells at both temperatures, were too small (Fig. 2h, i and l) to permit us to determine whether the striations characteristic of other cells were present in this system after staining with myosin antibody. The results with actin antibody on the *tsA* transformants suggest that expression of the SV40 *A* gene product may be required for the loss of actin sheaths upon transformation.

DISCUSSION

The data obtained with actin antibody show that different cells display markedly different distributions of actin-containing sheaths. Two arguments support the conclusion that the actin-containing sheaths observed by immunofluorescence resemble the organization of microfilament sheaths detectable by electron microscopy. First, actin is a major structural protein of the microfilament; its presence in this structure as well as in microfilament sheaths has been well established by numerous electron microscopy studies using heavy meromyosin decoration (see, for instance, ref. 3). Second, sheaths of microfilaments have an organization inside the cell which is rather similar to the one found by immunofluorescence for the actin-containing fibers. This is certainly so for the two cell lines which have been examined both by immunofluorescence

FIG. 2 (*on preceding page*). Actin and myosin visualized in primary rat embryo (RE) cells and their SV40-transformed derivatives. Figures (a–f) are of cultures grown at 37° for 48 hr before fixation: (a) RE, actin; (b) SVRE 9, actin; (c) SVRE 12, actin; (d) RE, myosin; (e) SVRE 9, myosin; (f) SVRE 12, myosin. Figures (g–l) are stained with antibody to actin. Figures (g–i) are of cultures grown at 33° for 48 hr before fixation: (g) uninfected RE; (h) *tsA*28-3; (i) WT4. Figures (j–l) are of cultures grown at 33° for 24 hr, then shifted up to 41° for 4 days before fixation: (j) uninfected RE; (k) *tsA*28-3; (l) WT4. All figures are taken at the same final magnification. The bar indicates 10 μm. Note that transformed cells are, in general, somewhat smaller than their normal and revertant counterparts.

and by electron microscopy, 3T3 (22) and primary rat embryo cells (13). Our data on changes in expression of actin-containing sheaths observed by immunofluorescence are in close agreement with the earlier work by McNutt *et al.* on changes in the organization of microfilament sheaths detectable by electron microscopy (19, 20). We have shown by immunofluorescence that SV40 transformation of 3T3 cells is accompanied by a decreased expression of actin-containing sheaths, and that reversion of the SV101 line to a flat revertant line (FlSV101) is accompanied by an increased expression of the same structures. McNutt *et al.* reached a similar conclusion by electron microscopy for a closely related set of three cell lines derived from Balb/c 3T3 (19, 20).

The results with myosin antibody on the different cell lines appear to give results qualitatively similar to those obtained with the actin antibody. However, a full interpretation of the behavior of the myosin containing structures on transformation must await confirmation by electron microscopy of the association with the microfilament bundles suggested by the immunofluorescent results (23).

The availability of a well-characterized set of SV40-transformed and revertant cells allowed us to ask the question as to whether the loss of actin-containing sheaths on transformation was correlated with any of the properties usually associated with the transformed state, i.e., anchorage dependence, cell density, or growth in low serum.

Serum concentration had no marked effect on the expression of actin sheaths (30). Although cells generally flatten out in low (0.1–1.0%) serum, this flattening was not accompanied by any change in the fraction of cells containing actin sheaths (30).

The results presented here (Table 1) show that only anchorage dependence is correlated with the loss of actin-containing sheaths (Table 1), and thus only anchorage dependence appears to require a change in the internal architecture of the cell of a magnitude observable with the light microscope. It is perhaps significant that of the three parameters of transformation described, the loss of an anchorage requirement seems best correlated with the production of plasminogen activator (34) and with tumorigenicity in the immunodeficient *nude* mouse (36).

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