

Localization and Distribution of Actin Fibers in Normal, Transformed and Revertant Cells

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Actin is not restricted to muscle tissue but is a major protein component of most, if not all, eukaryotic cells (see review of Pollard and Weihing 1973). Electron microscopy of various cells grown in culture has shown that there are at least three major fibrous structures found in a wide variety of cells: microfilaments, 100-Å filaments and microtubules (Goldman and Knipe 1972). Of these three structures, only microfilaments can be decorated with heavy meromyosin, the proteolytic fragment of skeletal muscle myosin still capable of binding to actin (Ishikawa et al. 1969). Although this result establishes that microfilaments contain actin, it cannot be taken to suggest that microfilaments are the only form of organization of actin in eukaryotic cells. Indeed, electron microscopy studies of different cell types containing approximately the same amount of actin show a great range of microfilament expression. A striking example of nonequivalence of microfilaments and actin occurs in an Ad5-transformed hamster cell (Goldman et al., this volume), which shows very few microfilaments although it has a normal complement of actin. Similarly, platelets from a retracted clot have abundant microfilaments, whereas normal circulatory platelets have none, even though both preparations of platelets most likely have the same amount of actin (Behnke et al. 1971).

Recently an antibody against electrophoretically purified SDS-denatured actin from mouse 3T3 cells (SV40-transformed clone SV101) has been obtained (Lazarides and Weber 1974). Here we summarize some recent results obtained with this antibody preparation. When this antibody was used in indirect immunofluorescence studies on several fibroblastic cells grown in culture, it revealed the presence of an elaborate and complex array of long and thick (>1 μ diameter) actin-containing fibers (Lazarides and Weber 1974).

Encouraged by the convenience of immunofluorescence in rapidly screening a large number of cells, we have used the antibody to examine normal rat embryo (RE) cells, the growth-controlled mouse cell lines 3T3 and Balb 3T3, a variety of virus-transformed clones of these lines and revertants derived from the transformants. As judged by immunofluorescence studies, the number and size of thick actin-containing fibers seems to be diminished in transformed clones and is partially recovered in

some revertant clones (Pollack et al., unpubl.). The implication of these results for the organization of actin in different clones and for the possible role of this organization in the maintenance of growth control is discussed.

Actin Antibody

Preparing antibodies against the different major structural proteins of the cell provides a relatively simple way of selectively visualizing intracellular structures containing these structural proteins. The problem of purifying each potential antigen separately was circumvented by using sodium dodecyl sulfate gel electrophoresis as the major purification step. The denatured protein obtained in this step is often antigenic, and if the antibody obtained against it shows cross-reactivity with the native protein, then the antiserum obtained can be used in indirect immunofluorescence to visualize the structure in the cell with which the antigen is associated. In our work with actin, the protein was purified from a high-speed supernatant of SV101 cells (a clone of SV40-transformed 3T3 cells) by preparative SDS gel electrophoresis and was used to obtain an antibody in rabbits (Lazarides and Weber 1974). Based on the following results, we believe that the major protein species electrophoresing with a molecular weight of 45,000 is indeed actin: (1) co-electrophoresis with homogeneous muscle actin; (2) precipitation of this protein from a supernatant fraction with vinblastine and/or Mg^{++} ; (3) typical monomer to polymer transformation; and (4) binding of ATP. The antibody obtained against the SDS-denatured actin prepared by preparative gel electrophoresis proved to react with native actin from SV101 cells as well as with homogeneous chicken muscle actin both in immunodiffusion and complement fixation analyses (Lazarides and Weber 1974). While the possibility of obtaining antibodies against other minor proteins with a polypeptide chain molecular weight of 45,000 is likely, the possibility of antibodies to proteins with different molecular weight is excluded by the principle of protein separation upon SDS gel electrophoresis. Furthermore, actin is the only major component accounting for more than 85% of the protein migrating with a molecular weight of 45,000. The remaining 15% is accounted for by a variety of minor protein species

of the cell which share with actin the property of an identical polypeptide chain molecular weight. The small amount of contaminating protein in the original 45,000 molecular weight antigen was not expected to interfere with the specificity of the antiserum in fluorescence microscopy because the antibody titer of the serum against actin was good (1:150 in complement fixation) (see Lazarides and Weber 1974), and because actin is a major structural protein of all tissue culture cells accounting for approximately 10% of the cell protein. Furthermore, recent experiments show that antibodies obtained against homogeneous SDS-denatured actin show the same immunofluorescent pattern as previously observed with the original antibody preparation (Lazarides and Lindberg, in prep.).

In agreement with the conservative protein structure of actin (Bray 1972), the antibody does cross-react with actin from cells from a variety of different species, e.g., human, monkey, rat, hamster, mouse and chicken.

Actin Filaments in 3T3 Cells Visualized with Actin Antibody

The 3T3 mouse cell line was derived from Swiss mouse embryo cultures (Todaro and Green 1963). This line is growth controlled but aneuploid.

Experimental conditions for immunofluorescence have been reported previously (Lazarides and

Weber 1974). When observed with darkfield UV optics, the vast majority of 3T3 cells on a coverslip display a beautiful array of bright fluorescent fibers ranging downward in size from heavy cables running the length of the cell to thin threads barely resolved with a 100 × objective.

Although the pattern of fibers varies significantly from one cell to the other in a growing culture, there are certain general features common to most 3T3 cells. The fibers very often run parallel to each other for very long distances through the cell. In some regions they appear to converge in "focal points" or show branching. The fibers seem to span the periphery of the cell and to be concentrated toward the adhesive side of the cell (see, e.g., Fig. 1).

Light microscopic studies were performed on fixed 3T3 cells to determine whether any of these structures could also be resolved by phase contrast optics. These studies were restricted to 3T3 cells, which were well spread (Goldman et al. 1974). Within these cells a distinct array of phase dense fibers may be detected, which is birefringent in polarized light. Differential focusing with Nomarski optics revealed that the phase dense fibers are concentrated toward the adhesive side of the cell. The pattern of fibers in living cells is similar to the pattern of actin fibers observed in fixed and acetone-treated cells after antibody staining. Since the phase dense fibers are similar to "stress fibers" previously described in rat embryo cells (Buckley

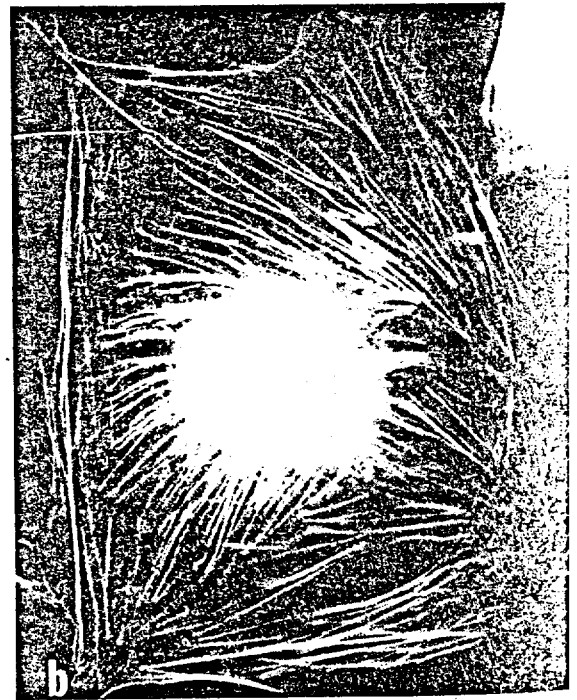


Figure 1. The same well-spread 3T3 cell fixed and stained with actin antibody is viewed with (a) phase contrast and (b) UV darkfield optics. Note relationship between phase dense fibers and fluorescent fibers. (Reprinted, with permission, from Goldman et al. 1974).

and Porter 1967) and in the BHK hamster cell line (Goldman and Knipe 1972), we conclude that these stress fibers contain actin. This point is emphasized in Figure 1. Here the same fixed and stained cell is viewed with phase optics and fluorescence optics in order to demonstrate the identity of phase dense fibers and fluorescent antibody-stained fibers in a well-spread cell. Stress fibers, the thick actin-containing bundles seen in Figure 1, are not necessarily the only form of actin organization in cells (see Discussion).

It has just come to our attention, that Gabbiani and coworkers have used a smooth muscle auto-antibody obtained from patients with chronic aggressive hepatitis. This antibody stained smooth muscle cells from a variety of different origins as well as myo-fibroblasts from rat and human skin wounds and cultivated fibroblasts (Gabbiani et al. 1973). The staining pattern obtained in these cells is rather similar to the one found with actin antibody.

Comparison of Transformed and Normal Cells

Table 1 summarizes the properties of a variety of transformed cells, their revertants and the corresponding normal cells. The majority of the lines used in this study are SV40-transformed clones (Todaro et al. 1963; Pollack et al., unpubl.); only one MSV-transformed clone is included (Ozanne and Vogel 1974). Revertants were isolated from some of these lines by negative selection with FdU or BrdU light (Pollack et al. 1968; Vogel et al. 1973; Vogel and Pollack 1973). The table shows the sensitivity of the different lines to restriction by serum, density and anchorage and also gives the production of

the activator of plasminogen in the different lines (Pollack et al., unpubl.).

The different clones were grown on glass coverslips and subjected to immunofluorescence. On each coverslip 50 to 100 individual cells were characterized by at least two persons independently and without knowledge of the identity of the cell line studied. Cells in this study were characterized as "positive" if strong actin cables were visualized throughout the cell, and "negative" if only thin structures or general fluorescence was observed. The number of positive cells as judged by two different investigators was in good agreement and is expressed as "average percent of cells with actin cables" in Tables 2 and 3. Figure 2 shows immunofluorescent patterns of some of the cell lines studied.

The regulation of proliferation of fibroblast cultures is complex. Well-regulated cell lines, such as the mouse line 3T3, are sensitive to inhibition by at least three different environmental signals: contact with other cells, deprivation of serum, and absence of a solid substrate. SV40 virus-transformed 3T3 clones may lose sensitivity to all three signals and thus grow despite cell-cell contact, grow even when serum is reduced by 10-fold, and form spherical colonies when suspended in methyl cellulose gel (see Table 1). This loss of growth control can be reversed. By applying negative selection pressures to transformants, cell lines, called revertants, have been obtained which exhibit growth control comparable to that of normal cells. Some revertants have regained sensitivity to contact regulations and anchorage regulations without regaining a high serum requirement for growth. These have been termed "density revertants" (Table 1). Other re-

Table 1. Expression of Parameters of Growth Control by Cells of Mouse and Rat Origin

Species	Line	Transforming virus	Class	Sensitivity to restriction ^a			Production of activator of plasminogen ^e
				serum ^b	density ^c	anchorage ^d	
Swiss mouse	3T3	none	line, normal	+	+	+	-
Swiss mouse	SV101	SV40	fully transformed	-	-	-	+
Swiss mouse	LS	SV40	serum-dependent revertant	+	+	+	+
Swiss mouse	F1	SV40	density-dependent revertant	-	+	+	-
Balb/c mouse	3T3B	none	line, normal	+	+	+	-
Balb/c mouse	KA31	(KMLV[MSV])	fully transformed	-	-	-	-
Balb/c mouse	M22	MSV	fully revertant	+	+	+	-
Rat	RE cells	none	primary, normal	+	+	+	-
Rat	SVRE3	SV40	transformed	-	-	-	+
Rat	SVRE5	SV40	transformed	-	-	-	+
Rat	SVRE9	SV40	transformed	-	-	-	+
Rat	SVRE12	SV40	transformed	-	-	+	-

For details, see Oey et al. (1974).

^a (+) Restriction, leading to maintenance of cell number with little or no increase; (-) absence of restriction, leading to increase in cell number.

^b To assay sensitivity to serum-dependent restriction, growth was measured after inoculation of cultures sparsely in 1% calf serum.

^c To assay sensitivity to density-dependent restriction, growth was measured in cultures inoculated in 10% calf serum and permitted to grow to confluence with periodic replacement of medium.

^d To assay sensitivity to anchorage-dependent restriction, cultures were inoculated as single cells suspended in methyl cellulose gel and the fraction of cells able to form visible colonies was determined after 21 days.

^e See Risser et al. (this volume); Pollack et al. (1974).

Table 2. Actin-bearing Cables in Growing Cultures

Line	Growing ^a	cAMP conc. ^b (pmole/mg protein)	Av. % of cells with cables ^c	Range
3T3	+	25	94	90-100
SV101	+	12	3	0-8
LS	+	24	4	3-5
F1	+	23	20	10-25
Balb	+	ND	76	71-81
KA	+	ND	0	0
M22	+	ND	24	9-29
RE cells	+	ND	98	96-100
SV3	+	ND	2	1-3
SV5	+	ND	heterogeneous ^d	
SV9	+	ND	5	3-6
SV12	+	ND	14	10-30

All cultures were plated in 10% CS at 2×10^3 cells/cm² and fixed for antibody staining 48 hr after plating.

^a (+) = Doubling time of ≥ 30 hr; (-) = Doubling time of ≤ 70 hr.

^b All lines assayed while growing in sparse culture, at less than 2×10^4 cells per cm², in 10% calf serum (see Oey et al. 1974).

^c Cables were visualized with antibody to SV101 actin. See text for details.

^d Single cells = no cables; clumps have cables.

vertants have regained a high serum requirement for growth and, in addition, sensitivity to either contact alone, or to both contact and anchorage. These are called "serum revertants" (Table 1). (For a discussion of these lines, see Oey et al. 1974.)

In the absence of any restriction on growth (10% serum, sparse culture), normal, untransformed fibroblasts contain "actin cables" (see 3T3, REF and Balb) (Table 2 and Fig. 2). Transformed clones isolated after infection with SV40 or MSV show a drastic loss of strong actin fibers, as documented for 3T3 versus SV101, REF versus REFSV3, REFSV9 and REFSV12, and Balb/c versus KA, the only MSV-transformed clone included in this study (Table 2 and Fig. 2). So far only a few revertants have been studied and the results are also incorporated in Table 2. The two density-sensitive revertants F1SV and M22 show a remarkable recovery of the "cabling pattern" originally present in 3T3 or Balb but lost upon transformation with either SV40 (SV101) or MSV (KA) (see also Fig. 2). The serum-sensitive revertant LS shows a pattern rather similar to SV101 and is therefore quite distinctly different from the other two revertants. Of these three revertants, M22 and F1 show an actual contact inhibition of DNA synthesis (Pollack

and Vogel 1973), whereas LS maintains a low density by continued cell division coupled with shedding (Vogel and Pollack, unpubl.).

Neither serum concentration (Table 3) nor, by implication, the level of cellular cyclic AMP (Tables 2 and 3) seem to have any marked effect on the expression of actin cables. Although cells generally flatten out in low serum (0.1%), and although intracellular cAMP always increases, these effects are not accompanied by any change in the number of cells containing actin cables (Tables 2 and 3). In particular, cell lines unable to grow in low serum (3T3, REF, Balb, LS and M22) show no noticeable increase in the expression of cables. Furthermore, lines which are able to grow in low serum but have a marginal degree of actin cables in 10% serum do not recover a higher degree of expression of cables in low serum, although they definitely show a flatter morphology.

Data on intracellular concentration of cyclic AMP are available in the 3T3 series for 10 and 1% serum and are given in Tables 2 and 3 together with the number of cells showing actin cables in both media. There is no obvious correlation between the two sets of data. In spite of a more than 3-fold increase in internal cyclic AMP level, when the serum-sensitive line LS is shifted from 10 to 1% serum, no noticeable recovery of actin cables is seen. Since cyclic AMP may have a rather complex mechanism of influencing cellular properties, we cannot exclude the possibility of a link between cAMP and expression of cables, but the preliminary experiments reported above do not show such a connection.

DISCUSSION

Normal cells have organized actin into structures large enough to be seen by ordinary microscopy.

Table 3. "Actin Cables" and cAMP in 3T3 System

	Growth in		cAMP (pmole/mg protein) ^a		Av. % cables	
	10% CS	1% CS	10%	1%	10%	1%
3T3	+	-	25	85	>95	>95
SV101	+	+	12	30	3	8
LS (rev)	+	-	25	80	4	2
F1 (rev)	+	+	23	40	20	35

^a See Oey et al. 1974.

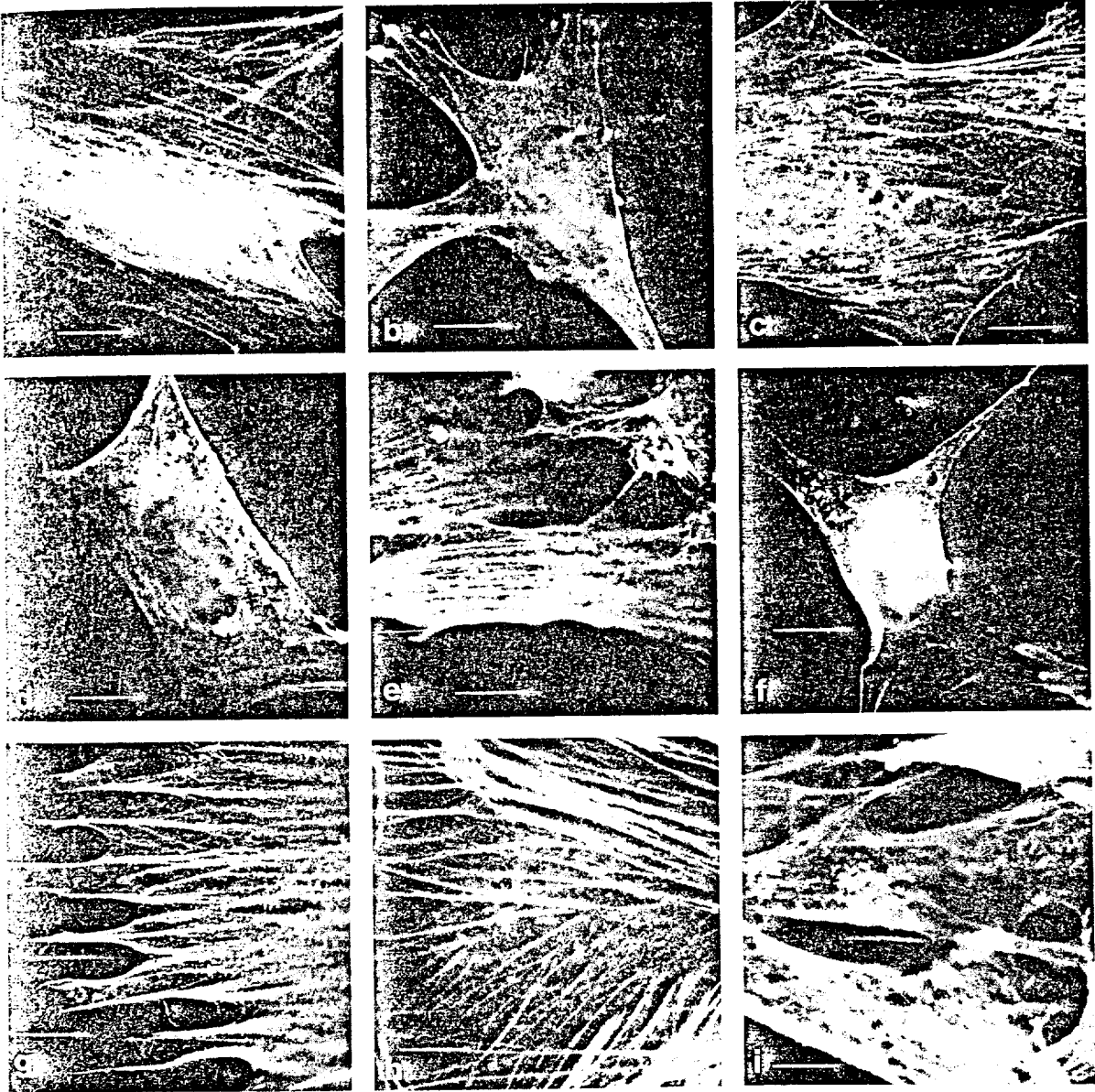


Figure 2. Actin antibody staining of the following cell lines: (a) 3T3; (b) SV101; (c) revertant F1-SV101; (d) revertant LS-SV101; (e) Balb/c 3T3; (f) MSV-Balb/c-3T3-KA31; (g) revertant M22; (h) REF; (i) SVRE9. Bar equals 10 μ .

Precisely because immunofluorescence microscopy is clearly limited in resolution as compared with electron microscopy, it has the advantage that it is a fast and convenient technique allowing the screening of a large number of cells under a variety of experimental conditions. It was this advantage which encouraged us to compare normal, transformed and revertant cell lines in their actin antibody staining properties.

The actin bundles that are clearly resolved in normal cells (3T3, Balb, REF) are not resolved in most of the transformed clones, even when low serum is used to spread out these cells. Although we are still able to observe general fluorescence, the majority of the cells of each transformed line shows very few,

if any, cables, although thinner threads are occasionally visible.

Our inability to detect the display of thick actin bundles in transformed cells is most likely not due to a problem of resolution of the fluorescence microscope, but rather a distinct property of the transformed cell line. In the case of KA31, which is a rather small cell, we cannot rule out the possibility that we are unable to resolve the hypothetically still present actin bundles because they are too closely spaced to be distinguished by light microscopy. In the case of SV101, LS and SVREF, however, it is obvious (Fig. 2) that we should not be limited by resolution, and the fluorescent pattern of "thinner threads" rather than cables is indicative of the

diminished ability of the transformed cells to express the thick cables typical for the untransformed parent cells. We know that the amount of actin in the different cell lines is rather constant (Lazarides, unpubl.), a finding which is in agreement with an independent study of actin in Rous-transformed and normal chick fibroblast. This result, together with the general fluorescence seen in the transformed cells, forces us to consider that actin may be present in different types of organization in all cells and that the ratio of these forms of organization may be changed in transformed cells. From our present results one may readily assume at least four different levels of organization:

(1) The "*thick actin bundles*," typical for the majority of 3T3, Balb and REF cells and also noticeable in a variety of other cultured fibroblasts. This type of organization may be rather similar to the so-called "*stress fibers*" observed by phase microscopy in a variety of flat and well-spread cells, including REF (Buckley and Porter 1967) and 3T3 (Goldman et al. 1974).

(2) The "*thinner bundles or threads*" may be typical for a majority of SV101 and REFSV cells, but also present to a lesser extent in some cells of a 3T3, Balb or REF population.

(3) *Shorter and thinner actin aggregates*, which may exist as "chain- and network-like" structures and which may be present in all cells. Local condensation of such structures may give rise to strong fluorescent staining on the light microscopic level without any individual resolvable structure. Preliminary experiments show that certain organelles—especially ruffles—might have such an actin organization.

(4) *G actin or soluble actin*, which we most likely would not recognize in indirect immunofluorescence, but which is present in the cells studied and has been found in at least some transformed cells (Lazarides, unpubl.), and which may be present in a higher amount in transformed than in the corresponding nontransformed cells.

Very little is known about the relative stability of the different forms of actin organization and their turnover during the cell cycle and various growth conditions. We also do not know yet which other cellular proteins are present together with actin in these structures. If indeed the relative amount of cellular actin is constant, then the preferential loss of one form of actin structure, the thick cables, upon transformation of fibroblast indicates a mechanism that interferes with the expression of actin in the form of thick cables. One or more of the following possibilities could account for this result: a covalent chemical change of the actin molecule itself; a change in concentration or activity of some other protein(s) present in these structures; or a change in concentration of intra- or extracellular metabolite(s) affecting the expression of these fibers. Alternatively one could assume the presence of a negatively acting

protein which adversely affects the expression of thick cables, or a change in a positively acting protein which promotes the expression of this structure in untransformed cells. Future biochemical and ultrastructural experiments may shed some light on this problem.

McNutt and coworkers (McNutt et al. 1973) obtained some evidence that SV40-transformed Balb/c 3T3 cells show a less pronounced microfilament system when compared to the parent Balb/c 3T3 cells. In agreement with this finding, Dermer and coworkers (Dermer et al. 1974) reported that two mouse sarcoma virus-transformed "normal" rat kidney (NRK) cells are deficient in cytoplasmic filaments in comparison to NRK cells.

We recognize the inherent difficulty in any attempt to predict from our results obtained by immunofluorescence microscopy any ultrastructural changes in the transformed cells. Although microfilament bundles are known to contain actin, they are by no means the only organizational form of actin. Electron microscope studies performed on platelets (Behnke et al. 1971) and cultured cells have shown that there is a variety of different forms of actin-containing fibers, since sheath microfilaments as well as microfilamentous networks can both occur (Ludueno and Wessels 1973), and in some round cells the expression of both structures can be lost (Goldman et al., this volume), although actin is still present in high concentration in these cells as judged by biochemical analysis.

Comparison of the degree of expression of actin-containing cables clearly demonstrates that infection of 3T3, REF or Balb/c by either SV40 or MSV gives rise to transformants which have few, if any, cables. The recovery of these cables in the three revertants studied is incomplete, as is the recovery of growth control. Although F1 and M22 recover at least to some degree the expression of these cables, the LS revertant does not. The properties of the different lines as summarized in Tables 1, 2 and 3 do not allow any simple relationship between intracellular cyclic AMP concentration and the expression of thick cables. However, the regulation of the expression of the actin fiber system may be a rather complex process and any simple comparison as attempted in these tables may be impossible. On the other hand, there seems to be a fair comparison of the expression of cables with the production of plasminogen activator (Risser et al., this volume; Pollack et al. 1974). Cell lines showing a high level of this activator are also those characterized by a low degree of expression of cables. At the present time it is not possible to draw such a direct conclusion, and further experiments will be necessary in order to correlate the ability of all clones to produce plasminogen activator (a property very well characterized as typical for transformed cells) with a lower degree of fiber expression in transformed cells and the recovery from this phenomenon in revertant lines.

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