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Temperature-sensitive changes in surface modulating assemblies of fibroblasts transformed by mutants of Rous sarcoma virus

(*ts* mutants/microfilaments/microtubules/growth regulation/neoplastic cells)

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ABSTRACT **The hypothesis that surface modulating assemblies containing microfilaments and microtubules are altered after cellular transformation was tested on cells infected with temperature-sensitive mutants of avian sarcoma virus. Untransformed cells (mouse 3T3 and chick fibroblasts), cells transformed by simian virus 40 (SV 3T3), and chick fibroblasts infected with Schmidt-Ruppin strain of Rous sarcoma virus (SR-RSV-A-infected cells) were first compared for differences in microfilament and microtubule patterns after treatment with fluorescein-labeled antibodies to actin and tubulin. Transformed cells showed disappearance of ordered stress microfilaments and thickened or diffuse alterations of microtubular arrays. At restrictive temperatures (41°), chick fibroblasts infected with a temperature-sensitive mutant (*ts* 68) of Rous sarcoma virus showed normal patterns of stress filaments and radial microtubular arrays originating in 1 or 2 centrioles. At permissive temperatures (37°), these patterns were disordered and resembled those of SR-RSV-A-infected cells. After a shift from 41° to 37°, the changes in microtubules were observed in the majority of cells within 1 hr. These changes were reversible and did not result from the inability of tubulin to polymerize.**

In *ts* 68-infected cells at permissive temperatures, concanavalin A induced much less surface modulation (inhibition of receptor mobility) than at restrictive temperatures. These results suggest that cellular transformation alters both the structure and function of surface modulating assemblies and prompt the hypothesis that products of viral transforming genes may affect these assemblies with a consequent loss of growth control.

Several lines of evidence suggest that neoplastic transformation of fibroblasts by avian sarcoma virus results from the action of a viral gene (1). It is not known, however, whether the gene product acts directly on the nuclear material to alter DNA synthesis, or whether it acts indirectly on cytoplasmic structures. In either case, transformation has marked effects on DNA synthesis and on the structure and function of the cell surface.

We have recently suggested that the behavior of cell surface receptors is controlled by a macromolecular assembly consisting of those receptors, submembranous microfilaments, and microtubules (2, 3). Different components of this surface modulating assembly (SMA) appear to be responsible for global inhibition of receptor mobility (surface modulation), for receptor redistribution, and for alterations in cell movements and shape (2). The microtubular components of the SMA can also play a regulatory role in the commitment of normal lymphocytes to mitogenesis (2, 4). Furthermore, surface modulation, which depends upon microtubules, can be induced by concanavalin A (Con A) in transformed lymphoid cell lines and leads to reversible inhibition of cell division (5). The microtubular proteins, and possibly the actin-like microfilaments of the SMA, may therefore be concerned with the regulation of early biochemical signals that induce the cell to mature and divide (2).

Components of the SMA or molecules regulating the synthesis or assembly of these components may be among the main targets of the products of viral transforming genes. In accord with this hypothesis, recent studies (6) indicate that continuously dividing cell lines have an impairment in their ability to assemble cytoplasmic microtubules. The hypothesis would be particularly strengthened by the occurrence of temperature-induced changes both in SMA structure and in surface modulation of cell lines that had been transformed with temperature-sensitive mutants of sarcoma virus. In the present paper, we describe experiments that provide evidence for such changes.

MATERIALS AND METHODS

Mouse fibroblast 3T3 cells and simian virus 40 transformed 3T3 cells (SV 3T3, kindly supplied by Dr. H. Green, M.I.T., Cambridge, Mass.) were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum and 20 μg/ml of Gentamycin. Chick fibroblasts infected with the Schmidt-Ruppin strain of Rous sarcoma virus-subgroup A (SR-RSV-A) and with a temperature-sensitive mutant (7) *ts NY* 68-SRA (called here "*ts* 68") were kindly provided by Dr. H. Hanafusa (Rockefeller University, New York). Normal chick fibroblast cultures were prepared from 10-day-old chick embryos (SPAFAS, Norwich, Conn.) and, when necessary, were transformed by infecting them with the appropriate viruses (7). Coverslip (18 × 18 mm) cultures were used for immunofluorescence studies. The chick cells were grown in DMEM containing 10% tryptose-phosphate broth, 5% fetal bovine serum, and 100 units of penicillin-streptomycin per ml. The culture media of the transformed chick cells were changed every 24 hr by replacement with prewarmed aliquots of fresh medium.

Antisera directed against tubulin and actin were prepared by injecting rabbits with porcine brain tubulin and with mouse striated muscle actin each denatured by sodium dodecyl sulfate (8). The specificities of these antisera for mouse and chick tubulins and actins are described elsewhere (I. Yahara, J. Hemperly, and G. M. Edelman, in preparation). The methods used for immunofluorescent staining of fixed fibroblasts were essentially the same as those described by Weber *et al.* (9).

Restriction of cell surface receptor mobility (surface modulation) was tested using turkey antibodies against chick retina cells, fluorescein-labeled rabbit antibodies against turkey Ig, and Con A according to the method described previously (10). The turkey antibodies were obtained after adsorption on glutaraldehyde-fixed chick retina cells immobilized on Sephadex G-50 and elution with pH 3.0 buffer (B.-A. Sela and G. M. Edelman, unpublished results).

Abbreviations: DMEM, Dulbecco's Modified Eagle's Medium; *ts* 68, temperature-sensitive mutant (*ts NY* 68 SRA) of Rous sarcoma virus; SR-RSV-A, Schmidt-Ruppin strain of Rous sarcoma virus-subgroup A; SV 3T3, mouse 3T3 cells transformed by simian virus 40; SMA, surface modulating assembly; Con A, concanavalin A.

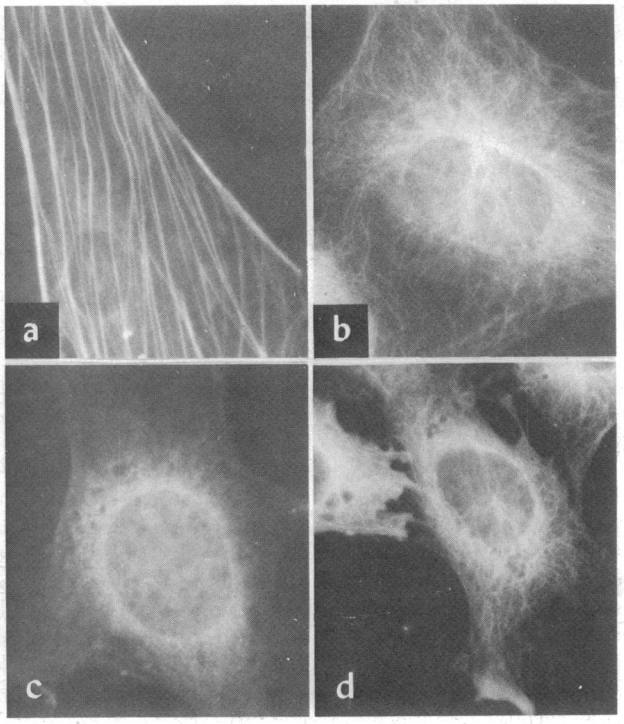

FIG. 1. Patterns of 3T3 and SV 3T3 cells after staining with fluorescein-labeled antibodies to actin and tubulin: (a) 3T3, anti-actin; (b) 3T3, anti-tubulin; (c) SV 3T3, anti-actin; (d) SV 3T3, anti-tubulin (magnification, ×740).

RESULTS

Comparison of normal and transformed mouse fibroblasts

We first compared the staining patterns of anti-actin and anti-tubulin antibodies labeled with fluorescein on mouse 3T3 cells and SV 3T3 cells. With anti-actin antibodies, 3T3 cells showed a pattern of more or less parallel stress fibers (Fig. 1a) similar to the microfilament bundles observed by other workers (8). Staining with anti-tubulin revealed a complex array (Fig. 1b) of bundles radiating from the cell center that was also comparable to the patterns found by others (11). The origin of these bundles was in the centrioles of the cell (ref 11; I. Yahara, J. Hemperly, and G. M. Edelman, in preparation). In contrast to these orderly pictures, and in accord with previous reports (12), the SV 3T3 cells lacked a consistent pattern of stress fibers and instead showed a stained perinuclear aggregate with "fluffy" protrusions (Fig. 1c). The SV 3T3 cells also showed anti-tubulin patterns that were denser, less lacy, and more disorganized (Fig. 1d). In view of the altered morphology of the cells, it was not possible to determine by these means whether the microtubular network was also diminished (6) in amount.

Comparison of *ts* 68-infected chicken fibroblasts at restrictive and permissive temperatures

At the restrictive temperature (41°) in the nonconfluent state, the *ts* 68-infected chicken cells showed a flattened and scalloped morphology (Fig. 2a) which was converted to a predominantly spindle-shaped form (Fig. 2d) at the permissive temperature (37°). At the restrictive temperature, the anti-actin and anti-tubulin patterns were very similar to those observed for normal 3T3 cells: more or less parallel stress filaments with labeled anti-actin (Fig. 2b) and radiating complex arrays with anti-tubulin (Fig. 2c). After growth under permissive conditions, however, the anti-actin patterns were blurred and diffuse with only a few remaining stress filaments and with occasional stained foci of variable size (Fig. 2e). The anti-tubulin patterns were also drastically altered (Fig. 2f) to a diffuse or fluffy appearance with the persistence of a concentration of the stain at a central location in the cell.

To exclude the possibility that these changes in actin bundles and microtubular arrays resulted trivially from a direct effect of elevating the temperature, both normal chicken fibroblasts and cells infected with "wild-type" SR-RSV-A were compared at 37° and 41°. At both temperatures, the patterns of the normal cells resembled those of *ts* 68-infected cells at the restrictive temperature, whereas the cells infected with SR-RSV-A showed patterns comparable to those of *ts* 68-infected cells at the permissive temperature (Fig. 3).

Table 1. Modulating effect of Con A on patch and cap formation in *ts* 68-infected cells* as a function of temperature

Cell pattern		Distribution of fluorescence†		
		Cap	Patch	Diffuse
"Normal" (*ts* 68 at 41°)	−Con A	83 (93)	6 (7)	0 (0)
	+Con A	24 (18)	36 (26)	76 (56)
"Transformed" (*ts* 68 at 37°)	−Con A	41 (72)	16 (28)	0 (0)
	+Con A	68 (52)	44 (34)	19 (14)

* Fluorescein-labeled turkey antibodies (50 μg/ml) directed against cell surface antigens were used to induce cap and patch formation. When present, Con A was at 100 μg/ml.
† Number of stained cells; % stained cells is included in parentheses.

Rate of change of SMA patterns with change of temperature

It was important to determine the rate at which the observed changes in actin and tubulin patterns took place. An estimate was obtained by performing a "shift-down" experiment (Fig. 4) in which a change from restrictive to permissive temperatures was checked by scoring of "normal" and "altered" patterns as a function of time. After the temperature shift, the majority of cells showed altered patterns within a period of 1–2 hr.

Similar observations were made in a shift-up experiment. It was found that the "normal" pattern could also be established well within 60 min. These effects appeared to be reversible both after shift-down or shift-up.

Effect of Con A on surface modulation in *ts* 68-infected cells

We next attempted to correlate the changes in the patterns of the components of the SMA with the capacity of Con A to induce restriction of cell surface mobility [anchorage modulation (2)]. A loss of microtubular structure might be expected (2) to result in reduction or abolition of this form of global modulation of the cell surface.

Binding of Con A to *ts* 68-infected cells induced a restriction of mobility of surface molecules reactive with fluorescein-labeled antibodies to chick retina cells (Table 1). At 37°, the caps formed in the absence of Con A were "central" or perinuclear and were reduced in amount as compared to 41°, at which temperature the caps were plate-shaped and found mainly at

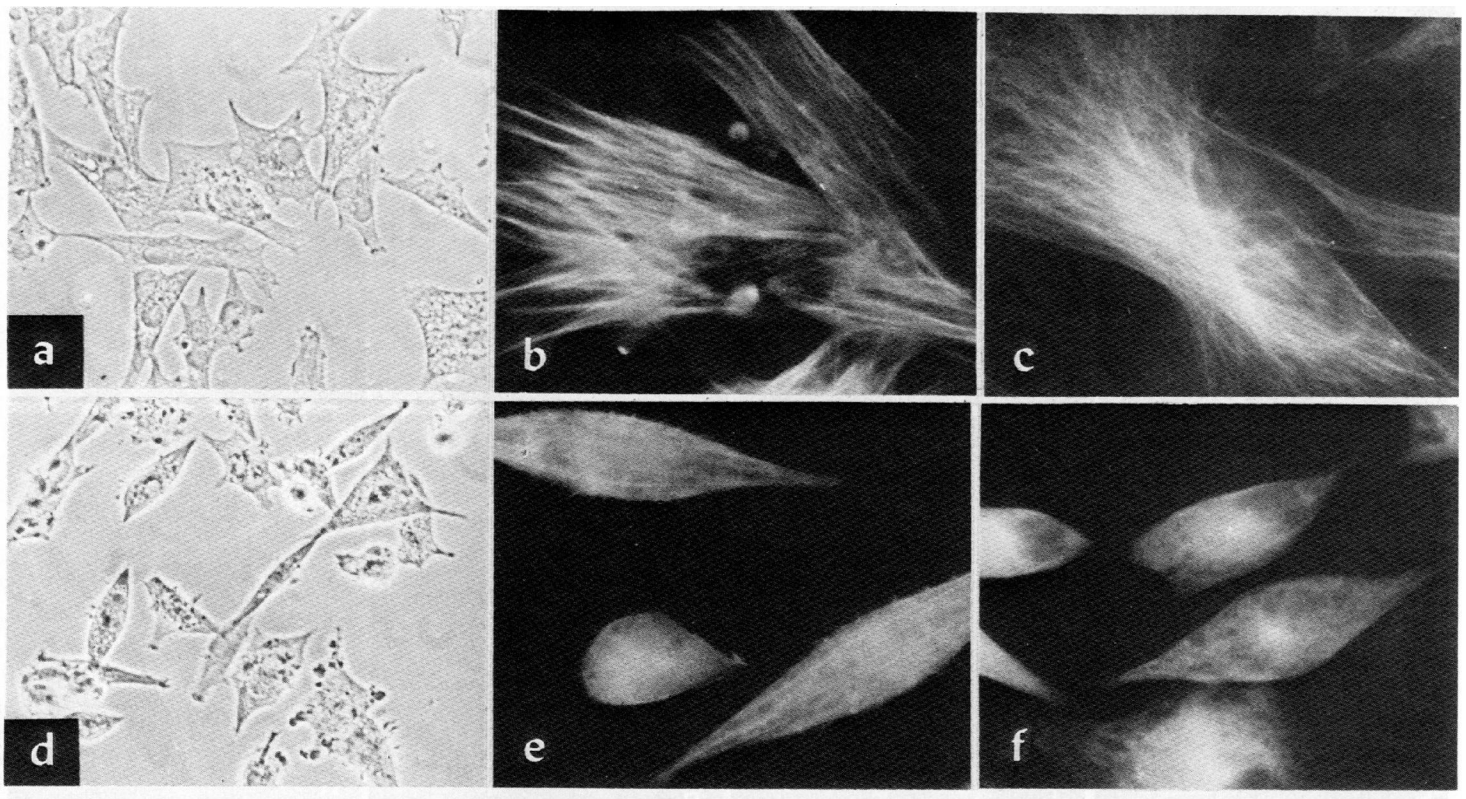

FIG. 2. Comparison of *ts* 68-infected cells at restrictive (41°) and permissive (37°) temperatures after binding of fluorescein-labeled antibodies to actin and tubulin: (a) 41°, phase contrast; (b) 41°, anti-actin; (c) 41°, anti-tubulin; (d) 37°, phase contrast; (e) 37°, anti-actin; (f) 37°, anti-tubulin (magnification a and d, ×360; b, c, e, and f, ×900).

the cell boundary. At the permissive temperature, modulation by Con A (indicated by the percentage of cells with diffuse staining) occurred in only 14% of stained cells as contrasted with 56% at the restrictive temperature. Anchorage modulation (2) was therefore more marked in cells at the restrictive temperature, at which the microtubular assemblies were more nearly normal (Fig. 2). The percentage of central or perinuclear caps was increased by treatment with 10^{-6} M colchicine. At both temperatures, surface modulation was reversed by treatment with this concentration of colchicine, which also caused a complete loss of microtubular structure and the appearance of a diffuse staining pattern with fluorescein-labeled antibodies to tubulin.

DISCUSSION

Several coordinated changes in growth control, cell shape, and cell metabolism occur upon transformation of chick fibroblasts by avian sarcoma virus (7). Inasmuch as a single viral gene (*src*) may be responsible for transformation (1), the site of action of this gene or its product becomes of cardinal significance. The proposal that the SMA may be a central regulator of cell growth, shape, and interaction (2, 3) raises the possibility that one or more of the components of the SMA may be targets of the *src* gene product. In accord with this hypothesis, the present studies provide evidence for a correlation between the temperature sensitivity of *ts* 68-infected cells and greatly altered patterns of their actin-like microfilaments and microtubules. The alterations at the permissive temperature were also accompanied by a diminished capacity of the cells to undergo surface modulation induced by Con A and thus they appear to reflect an alteration in SMA function. The structural and functional changes in the SMA may be related to previous observations (13) in surface ruffling patterns of *ts* 68 mutants.

In contrast to the orderly arrangements in untransformed cells (8, 9), the transformed cells of mouse and chick showed highly disordered patterns. There was a loss of the regular actin bundles making up stress filaments with the appearance of a more diffuse pattern of staining. In addition, the normal tubulin pattern, consisting of a complex array of microtubules radiating from one or two central positions, was replaced by a fluffy pattern with multiple spots (SV 3T3 cells) or by a more diffuse pattern with a central concentration of stain (transformed chick fibroblasts).

The striking new observation is that the *ts* 68-infected cells showed the normal pattern at the restrictive temperature and the disordered pattern at the permissive temperature. This was a reversible shift, and in a shift-down experiment (see Fig. 4), the alteration took about 1 hr to appear in the majority of cells. The results obtained in comparisons at both temperatures of SR-RSV-infected and normal chick fibroblasts (see Fig. 3) indicate that this effect is not due to alteration of tubulin by the change in temperature itself.

It is particularly significant that the capacity of Con A to induce cell surface modulation was markedly diminished in cells at the permissive temperature. Inasmuch as microtubules and associated structures are essential for anchorage modulation (2), it is reasonable to attribute this effect to the altered structure of the microtubules at the permissive temperature. At present, however, definitive proof must await a detailed analysis of the effect of different microtubule assembly states on surface modulation.

Two key questions emerge from these findings: (*i*) is the alteration in the structure and function of the SMA a result of defects in the tubulin or actin or in their assembly? (*ii*) Does a defect in the assembly or structure of one component of the SMA (either tubulin or actin) induce the alteration observed in the other? In preliminary experiments, supernatants of ho-

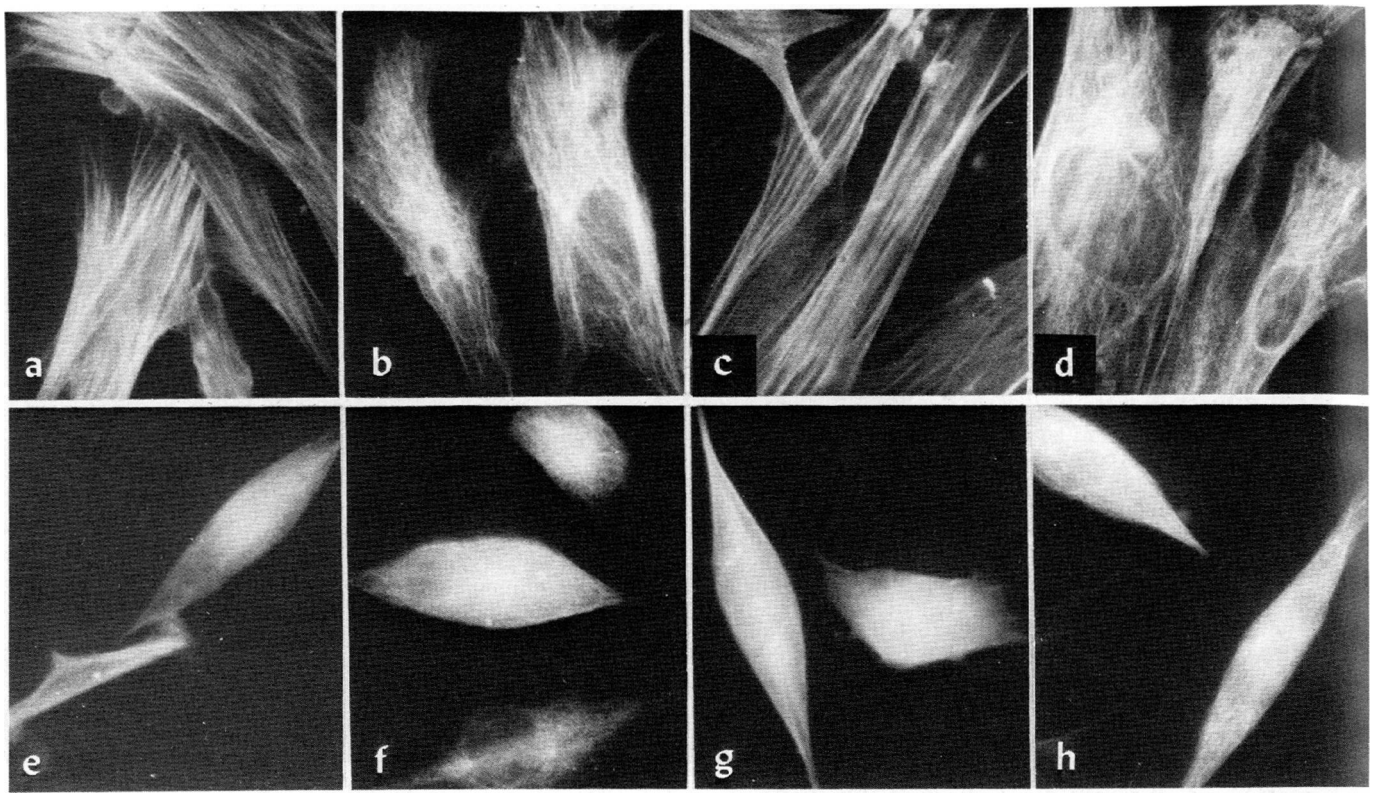

FIG. 3. Comparison of patterns with anti-actin and anti-tubulin in chick fibroblasts and SR-RSV-A-infected fibroblasts at 41° and 37°: (a–d) chick fibroblasts: (a) 41°, anti-actin; (b) 41°, anti-tubulin; (c) 37°, anti-actin; (d) 37°, anti-tubulin; (e–h) SR-RSV-A-infected cells: (e) 41°, anti-actin (f) 41°, anti-tubulin; (g) 37°, anti-actin; (h) 37°, anti-tubulin (magnification ×800).

mogenates of both normal and SR-RSV-A-transformed cells both showed similar amounts of microtubule assembly in media favoring assembly. Furthermore, the colchicine-binding activities and amounts of tubulin precipitated by vinblastine were similar in both types of supernates (J. Hemperly and I. Yahara, unpublished observations). Thus, although definitive comparisons on purified tubulin remain to be made, the *in vitro* assembly into microtubules in SR-RSV-A-infected chick cells appears to be unaffected. This suggests that the temperature-induced changes do not result from a structural defect in tubulin but rather from alteration of some stage of *in vivo* microtubule assembly; additionally, there may be a defect in the rate of synthesis of tubulin. This provisional answer to the first question is supported by the data on normal and SR-RSV-A-infected cells

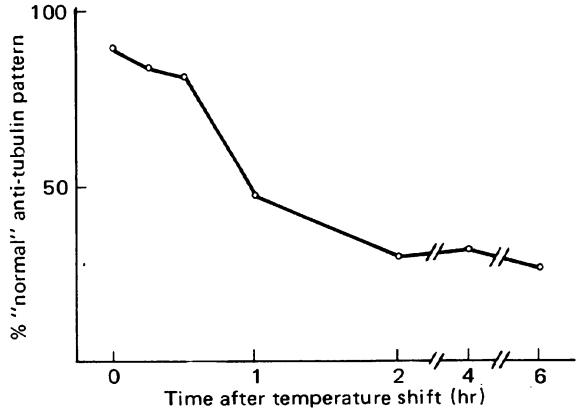

FIG. 4. Kinetics of appearance of cells with altered anti-tubulin patterns after shift-down from restrictive to permissive temperatures. (Two hundred cells were counted at each time point; ordinate refers to the % of these cells having the "normal" pattern.)

at both 37° and 41°. The second question remains unanswered, but has an important bearing upon the postulated interactions (2) between microfilaments and microtubules.

Perhaps the most challenging problem posed by the present findings is whether the observed alterations of the SMA are only one of many consequences of transformation and are therefore unlinked to alterations of growth control, or whether a protein product resulting from the action of the *src* gene alters the SMA and *therefore* alters growth control. Recent evidence suggesting that there is a single *src* gene (1) bears on this problem of the main mechanism of transformation in altering cell growth. If the site is nuclear, and the gene is present in a single copy, then it is difficult to understand how so many coordinated changes (growth, shape, and metabolism) could occur in transformation unless regulatory genes are affected (Fig. 5I.). On the other hand, a *src* gene product, or a structural gene product induced by the presence of the *src* gene, could bind to one or more sites in the cytoplasm and alter a variety of regulatory and metabolic events (Fig. 5II. and III.).

Conceivably, a single protein could alter a site or a single growing point of the complex assemblies of the SMA (2–4), thereby altering its structure and its proposed regulatory role in growth (Fig. 5III.). A test of this model requires the demonstration that there is a cytoplasmic activator interactive with components of the SMA, and that the *src* gene product acts at or near this point in the growth control pathway. It is pertinent that Con A induces reversible inhibition of growth (5) in a transformed lymphoid cell line (P388), and that this is correlated with surface modulation of these cells (I. Yahara, unpublished observations). Moreover, use of a recently developed cell-free assay for initiation of DNA replication (14) indicates that at least one high-molecular-weight cytoplasmic protein of this cell line is necessary for induction of DNA replication;

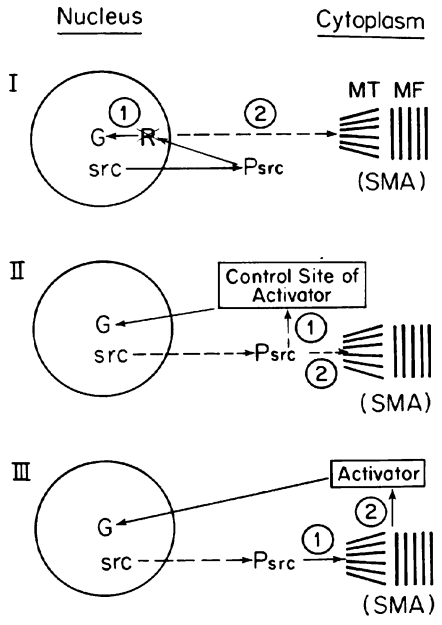

FIG. 5. Three alternative models for action of the *src* gene on both the surface modulating assembly (SMA) and on growth and replication control (G). I. P_{src}, the *src* gene product, (*1*) affects a regulator gene (R) with independent effects on G and SMA (*2*). II. P_{src} alters G by affecting (*1*) a control site for a cytoplasmic activator and (*2*) independently altering shape and surface properties by alteration of the SMA. III. P_{src} alters SMA (*1*) releasing an activator (*2*) (or removing a control protein) which in turn alters G to favor continuous growth.

this factor is not found in normal nonproliferating lymphocytes. Similar activity has also been found in the cytoplasm of *ts* 68-infected cells at permissive temperatures, but not at restrictive temperatures.

We are presently extending these approaches, and are searching for the locus of alteration in the SMA, for evidence of concerted alteration in tubulin and actin assembly, and for possible interactions of a *src* gene product with other cellular components. Obviously, such a search may uncover more complex regulatory pathways than those depicted here. In any case, the alterations in the SMA found after cellular transformation are likely to be an important basis for the changes in shape, mobility, and cell interactions seen in neoplastic cells.

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