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Reversion from transformed to normal phenotype by inhibition of protein synthesis in rat kidney cells infected with a temperature-sensitive mutant of Rous sarcoma virus

(concanavalin A receptors/nonmuscle cell myosin/microfilaments/membrane mobility)

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ABSTRACT **By the use of a rat kidney cell line infected with a temperature-sensitive Rous sarcoma virus, we have shown that, at permissive temperatures where the cells are transformed, concanavalin A induces a clustering of its cell membrane receptors into patches, and the intracellular smooth muscle myosin-like protein is in a disordered state. By contrast, with infected cells grown at nonpermissive temperatures, the addition of concanavalin A does not alter the uniform distribution of its receptors, and the smooth muscle myosin-like protein is arranged in an ordered filamentous structure. These results are consistent with the hypothesis that the myosin protein is part of an intracellular aggregating-disaggregating complex. In the normal cell it is in its aggregated state and inhibits the lateral mobility of the concanavalin A receptors in the membrane; in the transformed cell the complex is relatively disaggregated and permits the concanavalin A receptors to be mobile.**

The addition of protein synthesis inhibitors to infected cells grown at the permissive temperature causes the cells to change from the transformed phenotype to the normal. Removal of the reversible inhibitors causes the cells to revert to the transformed phenotype. These results show that (*i*) protein synthesis, presumably of an unstable product of the transforming gene of the temperature-sensitive virus, is required to maintain the transformed state in these infected cells at the permissive temperature; and (*ii*) protein synthesis is not required for the intracellular myosin-containing complex to revert from its disordered transformed state to its ordered normal state. This suggests that the product of the transforming gene directly or indirectly causes the disaggregation of the myosin-containing complex in the process of transformation.

A number of different receptor molecules in the cell membranes of cultured fibroblasts show a greater lateral mobility in transformed than in normal cells (1–4). It has been suggested (5, 6) that these differences in receptor mobility are directly connected to the loss of control of normal cell growth which occurs upon transformation. That is, if cell growth requires the mobility of receptors in the cell membrane (to allow membrane expansion and cytokinesis to occur, for example), then normal cell growth can be inhibited by restricting receptor mobility by means of mechanisms that the transformed cell is no longer able to use (5). Several molecular mechanisms have been proposed to account for these mobility differences, no one of which has yet been generally accepted. We have a working hypothesis (5, 7, 8) to explain these mobility differences. (*a*) There is an intracellular smooth muscle actomyosin-like protein complex present in nonmuscle cells (9) that forms at least part of the microfilament assemblies that have been observed inside these cells (10–12). (*b*) These actomyosin-like proteins are, at least in part, peripheral proteins (13) bound to the cytoplasmic surfaces of these cell membranes (8), attached noncovalently to membrane-spanning receptor molecules at sites where these molecules protrude from the inner membrane surface. (*c*) This actomyosin-like complex can undergo a reversible self-aggregation while bound to the membrane, and the extent of this self-aggregation is metabolically controlled. (*d*) In its more highly aggregated state (corresponding to the normal cell), the actomyosin-like complex ties together the receptor molecules that are attached to the complex, thereby inhibiting the lateral mobility of the receptors in the membrane; while in its less aggregated state (corresponding to the transformed cell), the complex does not markedly hinder the lateral mobility of the receptor molecules (see Fig. 2 in ref. 8).

One prediction of this hypothesis is that there should be a correlated change in the mobility of cell surface receptors and the integrity of intracellular myosin-containing structures in going from the normal to the transformed state. Such a correlation can be inferred from various published studies on diverse systems, but cannot be firmly established by them. We therefore sought the least ambiguous experimental system to make such correlations. We have compared the distribution of surface receptors for concanavalin A (Con A) (using fluorescein-labeled Con A) with the distribution of intracellular smooth muscle myosin-like molecules (using the indirect fluorescein-labeled antibody method) in normal and transformed fibroblasts. The system we have chosen is the normal rat kidney (NRK) cell line (14), which is infected with a temperature-sensitive Rous sarcoma virus, *LA*23 (15). At permissive temperatures (34°), 100% of *LA*23-infected cells exhibit the morphological and growth characteristics of the transformed state, whereas at nonpermissive temperature (39°) they exhibit the normal characteristics‡. The appropriate temperature shift converts the infected cells from one state to the other within a few hours, and affords the most direct possible comparison of the two states. At neither temperature do these infected cells produce infectious Rous sarcoma virus. They synthesize nonglycosylated virion proteins which are located in the cytoplasm. At the permissive temperature the surface of these cells probably contains a tumor-specific surface antigen (16).

We first show in this paper that at the nonpermissive temperature, the Con A receptors are uniformly dispersed and relatively immobile in the *LA*23-infected cell surface; in the same cells, the smooth muscle myosin-like molecules are arranged in orderly filamentous arrays. The properties of these cells are indistinguishable from those of uninfected NRK cells. By contrast, in *LA*23-infected cells after a few hours at the

Abbreviations: Con A, concanavalin A; F-Con A, fluorescein-conjugated Con A; NRK, normal rat kidney.

‡ Chen, Y. C., Hayman, M. J. & Vogt, P. K. (1976) "Properties of mammalian cells transformed by temperature-sensitive mutants of avian sarcoma virus," submitted for publication.

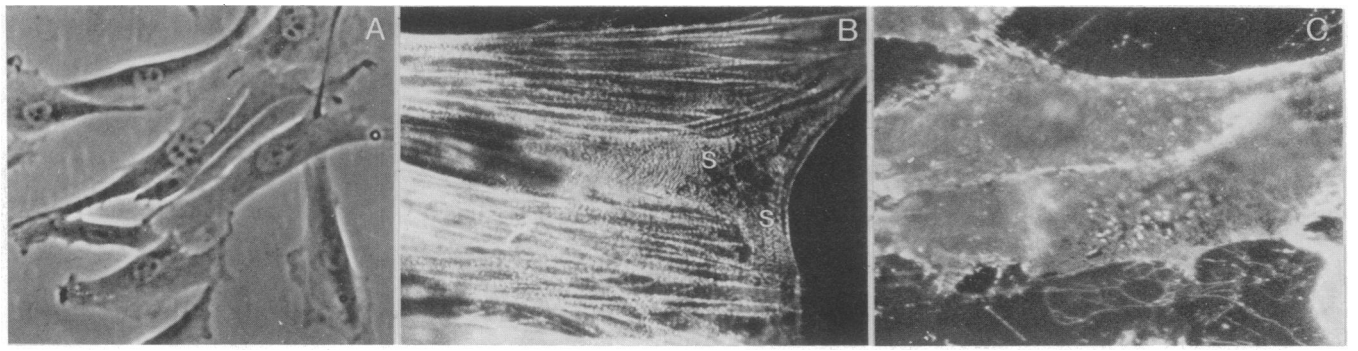

FIG. 1. Normal rat kidney (NRK) cells. (A) Phase contrast micrograph of living NRK cells. (B) Indirect immunofluorescent staining with antibody against myosin showing both filamentous and striped (s) staining patterns in an NRK cell. (C) Surface distribution of F-Con A on several NRK cells. The cells were incubated with F-Con A for 20 min at 34° and then fixed. The surface distribution of F-Con A is uniform. (A) ×280; (B and C) ×725.

permissive temperature, ConA induces clustering of its cell surface receptors, while the intracellular myosin-like molecules are disarrayed and filaments are no longer clearly discerned.

Since these results were consistent with our working hypothesis, we entertained the following further possibility. Suppose that the state of aggregation of the intracellular actomyosin-like complex was controlled metabolically by a reversible enzymatic modification (e.g., by phosphorylation–dephosphorylation reactions) of one or more components of the complex. If such were the case, then the function of the product(s) of the viral gene [the *src* gene (17)] responsible for transformation might be to alter, directly or indirectly, the steady-state level of that chemical modification. The temperature sensitivity of the *LA*23 transformation might then reflect the intracellular instability of that *LA*23 *src* gene product. Accordingly, we then treated *LA*23-infected NRK cells *at the permissive temperature* with the protein synthesis inhibitors cycloheximide, puromyocin, or abrin. In each case, the treated cells reverted in 12–16 hr to the normal phenotype with respect to morphology and to Con A receptor and intracellular myosin characteristics. Removal of the reversible inhibitors resulted in the restoration of the transformed phenotype.

MATERIALS AND METHODS

Normal and Virus-Infected Cell Cultures. The sources of NRK, *LA*23-NRK, and B77-NRK cells are elsewhere described.‡ All cells were cultured in a Ham's F12 based medium [100 ml of F12, 10 ml of Tryptose Phosphate Broth (Difco Lab., Detroit, Mich.), 5 ml of fetal calf serum, and antibiotics] under an atmosphere of 10% CO_2 and 90% air. Cells were dissociated with trypsin for passaging or plating onto cover slips (15,000–30,000 cells per 35 mm dish). Cells were cultured for at least 2 days after plating before an experimental treatment. Living cultures were observed with phase contrast optics by means of a Wild inverted microscope, and photographed on Kodak Panatomic X film.

Inhibition and Measurement of Protein Synthesis. Cycloheximide (Sigma Chemical Co., St. Louis, Mo.) was dissolved in ethanol and added to medium at a dilution of 1:1000; this level of ethanol alone had no effect on the cells. Puromycin (Calbiochem, La Jolla, Calif.) was dissolved directly into medium and diluted before use. Abrin (18) (a gift of Dr. Rockford Draper) in Tris-buffered saline was added to medium at a dilution of 1:200. Protein synthesis was measured as incorporation of L-[^{3}H]leucine (New England Nuclear, Boston, Mass.) into trichloroacetic acid-insoluble material. Cultures were first treated with inhibitors for 1 hr and then incubated with the labeled amino acid in medium plus inhibitor for an additional 2.5 hr.

Fluorescence Staining Procedures. The preparation and properties of the rabbit antibodies to smooth muscle myosin from the human uterus has been described (8). Fluorescein-conjugated Con A (F-Con A) was prepared by reaction of Con A bound to Sephadex G-50 with fluorescein isothiocyanate (Calbiochem), and was collected by elution with 0.03 M glycine·HCl, pH 2.0 (19).

By the use of intact cells, the F-Con A stained only cell surface receptors for Con A, whereas the staining for myosin was intracellular and required that the cells be first rendered permeable to the antibody reagents. For the staining of the Con A receptors, F-Con A was dissolved at 50 μg/ml in minimal essential medium and preequilibrated at the temperature and atmosphere of the cell cultures to be treated. The cultures were rinsed with minimal essential medium and incubated with the Con A solution for 20 min, after which they were rinsed and fixed with 2% formaldehyde. F-Con A binding was completely inhibited by the addition of 100 mM α-methylmannopyranoside. For staining of myosin (20), the indirect immunofluorescent procedure was used. The fluorescein-conjugated goat antibodies against rabbit IgG had a ratio of absorbance at 495 nm to that at 280 nm of 0.8. Cells were fixed with 2% formaldehyde in phosphate-buffered saline at room temperature for 20 min, were washed, and then were frozen and thawed once to render them permeable. If the cells were fixed but not frozen and thawed, no immunofluorescent staining occurred. Also, if the rabbit antibodies against myosin were absorbed with human uterine myosin, no staining occurred.

The fluorescent-labeled cells were observed with a Zeiss photomicroscope using a 40 X oil immersion lens and a dark-field condenser. Fluorescence was excited with an Osram HBO 200 watt bulb using a Zeiss FITC interference filter and photographed on Kodak plus X film.

RESULTS

NRK cells

Uninfected NRK cells grow in patches with free cells between patches (Fig. 1A) and stop growing when they reach confluence. Individual cells are roughly rectangular in outline. They show typical fibroblast-like ruffled membrane activity.

When stained with the antibody against smooth muscle myosin and observed with indirect immunofluorescence, these cells show two patterns. One is highly filamentous (Fig. 1B) and has been previously described for myosin staining (12, 20). Another, which is more common in cells 1 day after plating, is

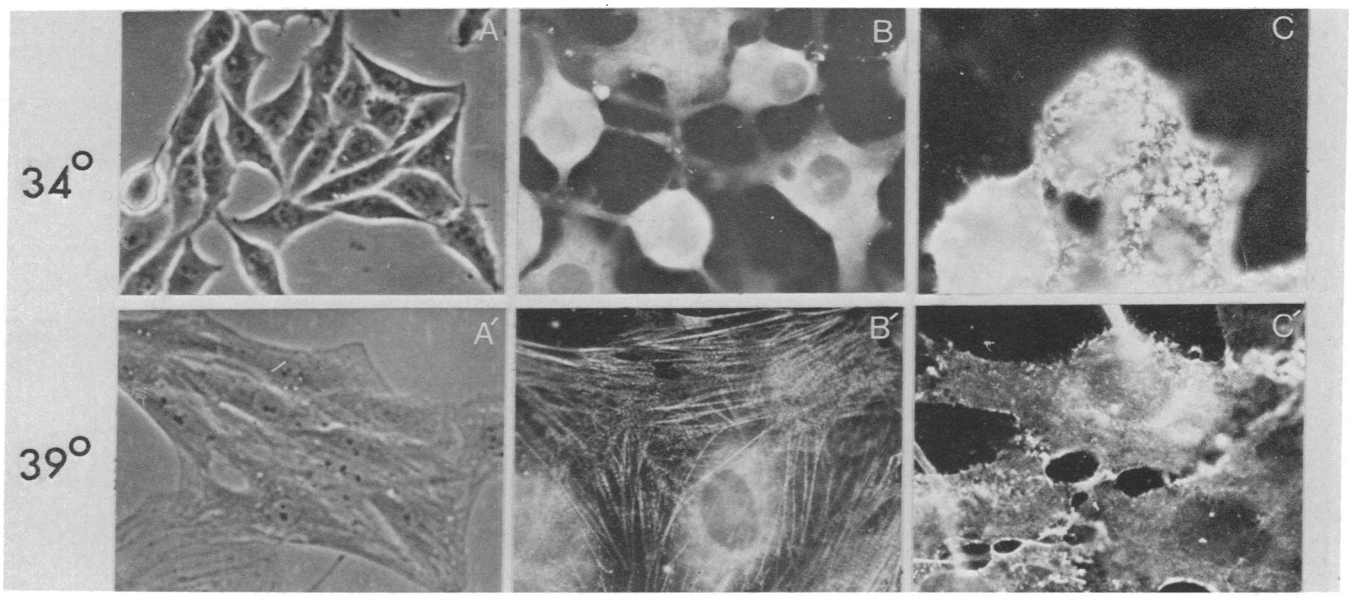

FIG. 2. *LA*23-infected NRK cells: effect of temperature. Top row shows cells grown at 34° (permissive temperature); bottom row shows cells grown at 39° (nonpermissive temperature). Phase contrast micrographs of cells at 34° (A) and 39° (A′) show the effect of temperature on the morphology of these cells. With indirect immunofluorescent staining with antibody against myosin the cells grown at 34° (B) show a lack of ordered staining, as compared with the filamentous staining seen on cells grown at 39° (B′). C and C′ show the surface distribution of F-Con A after 20 min of incubation. On cells grown at 34° (C) the F-Con A is extensively patched, while on cells grown at 39° (C′) the distribution is uniform. (A and A′) ×280; (B–C′) ×725.

a pattern of stripes also seen in the cell in Fig. 1B. The width of the fluorescent stripes in the latter pattern is about 0.5 μm, which would be consistent with laterally ordered arrays of short bipolar myosin filaments (Ash, Wang, and Singer, in preparation). Nonmuscle myosin *in vitro* typically forms short bipolar filaments about 0.3–0.5 μm in length (21, 22).

When F-Con A is bound to these normal cells for 20 min at temperatures between 40° and 34°, the common resulting pattern is a uniform surface staining, although many cells show some clearing of fluorescence over the nucleus (Fig. 1C). This uniform distribution is essentially the same as is found when the cells are prefixed, or when they are treated with Con A at 0° and then fixed, except that in the latter two cases no clearing over the nucleus occurs.

*LA*23-infected cells

Effect of Temperature. When these cells are grown at the permissive temperature (34°), they have a smaller and more irregular outline and appear to be denser than the parental NRK cells (Fig. 2A). When the cultures become crowded, these cells round up while staying in contact with the surface. These cultures can achieve high cell densities. At nonpermissive temperatures (39–40°) these cells flatten out and show contact inhibition of growth (Fig. 2A′). The typical outline of these cells at nonpermissive temperatures is less rectangular but similar to that of the uninfected NRK cells.

The *LA*23-infected cells grown at permissive temperature and stained with antibody against myosin reveal a uniform unstructured fluorescence. There are neither filaments nor stripes visible (Fig. 2B). In cultures grown at nonpermissive temperatures, however, a more structured filamentous staining pattern is seen (Fig. 2B′). In general, these filaments are not quite as long or as ordered as those in the uninfected NRK cells, but the staining is quite distinct from that seen in these infected cells at the permissive temperature.

When the *LA*23-infected cells grown at permissive temperature are treated with fluorescent Con A for 20 min, the Con A is found to be distributed in patches over the surface of the cells, as in Fig. 2C. The *initial* distribution of Con A binding sites on these cells is uniform, as seen by prefixing the cells or treating them at 0°. Therefore, the patches reflect a redistribution of Con A receptors induced by Con A binding. In contrast, if the *LA*23-infected cells are grown at nonpermissive temperature and treated with Con A at this temperature, the distribution of fluorescence is uniform and similar to that of uninfected NRK cells (Fig. 2C′).

Effect of Protein Synthesis Inhibitors. When cycloheximide (10 μg/ml), puromyocin (10^{-4} M), or abrin (8 ng/ml) is added to cultures of *LA*23-infected cells at permissive temperature, the cells flatten out and at 12–16 hr resemble the parental NRK cells morphologically (Fig. 3A and B). If the cycloheximide or puromycin is washed out at this point the cells round up and resume growing as transformed cells (Fig. 3C). These treatments produce little cell death. Abrin treatment is not reversible (18), and the cells stay flattened for days before dying.

When *LA*23-infected cells grown with inhibitor for 16 hr are fixed and stained with antibodies against myosin, the most common pattern observed is the striped pattern (Fig. 3D), seen also in 1-day cultures of uninfected NRK cells (Fig. 1B). These cells also exhibit myosin-containing filaments (Fig. 3E), although they are not as numerous as in cultures of uninfected NRK cells or in *LA*23-infected cells grown at 39–40° for 2 days.

A 16-hr treatment with inhibitors at 34° prevents the Con A patching on *LA*23-infected cells (Fig. 3F). The staining is uniform and similar to that of uninfected NRK or *LA*23-infected cells grown at nonpermissive temperatures.

Abrin (8 ng/ml), cycloheximide (10 μg/ml), and puromycin (10^{-4} M) inhibit protein synthesis by 99, 96, and 88%, respectively.

B77-infected cells

These cells are similar at all temperatures to the *LA*23-infected cells growing at 34°. The B77-infected cells are denser and

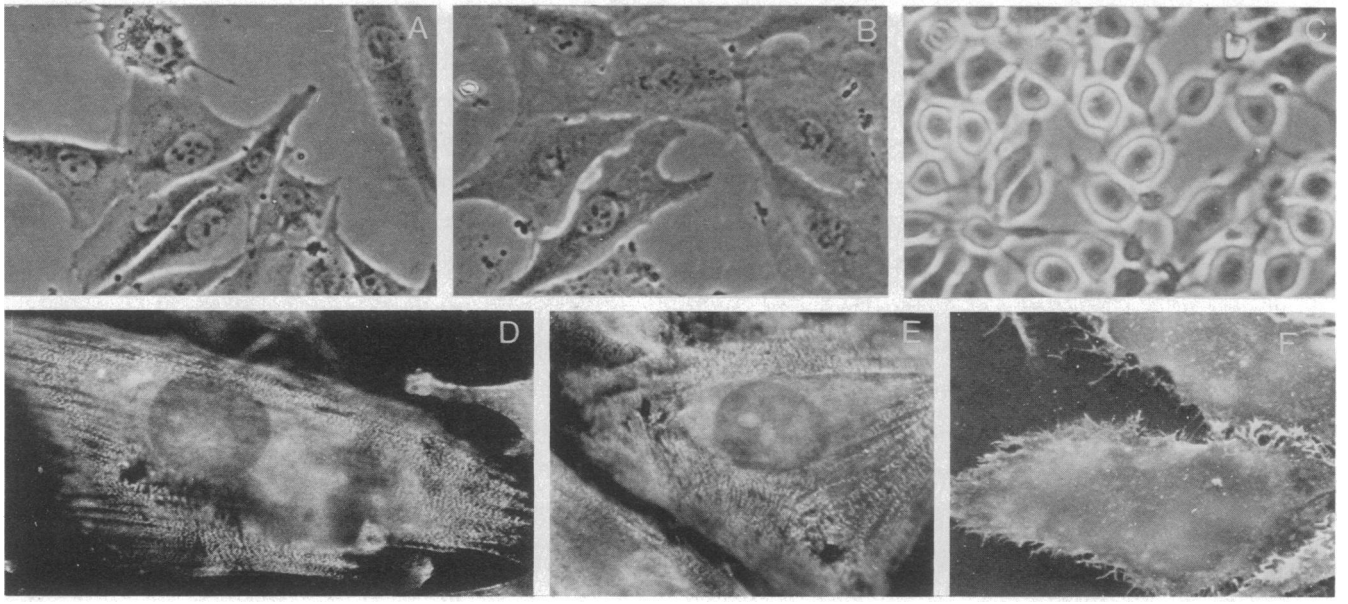

FIG. 3. *LA*23-infected NRK cells: effect of protein synthesis inhibitors. All cells were grown at 34° (permissive temperature). When *LA*23-infected cells are grown in the presence of cycloheximide (A) or puromycin (B) for 14 hr, the cells are spread and resemble the parental NRK cells (Fig. 1A), as seen with phase contrast microscopy. Abrin treatment produces identical results. (C) Cells 48 hr after removal of cycloheximide. The cells have resumed growth and are extremely rounded at this higher cell density. Removal of puromycin yields identical recovery, but abrin treatment is not reversible. (D and E) Staining patterns by indirect immunofluorescent antibody against myosin on cells treated with cycloheximide (D) and abrin (E) for 16 hr. The staining shows a high degree of ordering in the striped pattern (see Fig. 1B), with some filaments also visible. Puromycin treatment yields identical results. In (F), *LA*23-infected cells have been treated with abrin for 16 hr and then incubated with F-Con A for 20 min. The distribution of F-Con A is uniform. Again, cycloheximide and puromycin treatment yields identical results. (A, B, and C) ×280; (D, E, and F) ×725.

more rounded at low cell number than the *LA*23-infected cells. The myosin staining pattern is uniform and unstructured, while Con A forms patches on the surface both at 34° and 39°. These patterns are the same as seen for the *LA*23-infected cells at the permissive temperature.

Inhibition of protein synthesis as described above killed greater than 50% of the cells at 12–16 hr of treatment at 34°. The more rounded cells detach first and lyse. If the inhibitor is washed out at 16 hr and is replaced with fresh medium, cells continue to die, although clusters of dividing cells can be found in dishes several days after the medium is changed. The lethal effect of the inhibitors on these cells is in striking contrast to that seen with these drugs on the *LA*23-infected cells.

DISCUSSION

The use of the NRK cell line infected with a temperature-sensitive Rous sarcoma virus allows a direct comparison to be made of normal and transformed phenotypes with the same infected cells. No ambiguities arise from problems of cell selection upon transformation or the insertion of viral capsid protein in the membranes of the transformed cells. We have shown that in the transformed state (as defined by morphological and growth characteristics), the distribution of smooth muscle myosin-like molecules inside the cell shows no order at the light microscopic level of resolution, and in this state the Con A surface receptor molecules become clustered into patches upon treatment with Con A. This is true of NRK cells infected with *LA*23 virus and grown at the permissive temperature as well as cells infected with wild-type (B77) virus at all temperatures. By contrast, after 1 day of plating of *LA*23-infected cells at the nonpermissive temperature, as well as of normal uninfected NRK cells, the myosin-like molecules show a significant degree of order (Fig. 2B′), while the Con A receptor molecules are uniformly distributed and cannot be clustered upon the addition of Con A. The evidence therefore confirms, in a more direct way than has heretofore been demonstrated, that an increase in mobility of Con A receptors in the plane of the fibroblast surface membrane occurs upon transformation and is correlated with a disordering of myosin-containing structures inside the cell. The results are consistent with the hypothesis (see introduction) that the mobility of Con A receptors is controlled by the state of the myosin-containing structures. Elsewhere, we provide clear evidence for such direct control (Ash and Singer, in preparation).

Pollack *et al.* (23), working with mouse and rat cells transformed with temperature-sensitive mutants of simian virus 40, have previously observed similar effects of transformation on the integrity of intracellular actin- and myosin-containing structures, but no studies of surface receptor distribution were carried out at the same time.

The further and most intriguing findings of this study are that inhibition of protein synthesis in these *LA*23-infected cells, maintained at the permissive temperature, causes them to revert to the normal phenotype; and subsequent reinitiation of protein synthesis causes the reverted cells to become transformed again. The fact that three mechanistically different inhibitors produce this effect strongly suggests that the reversion is a result of the inhibition of protein synthesis and not due to possible other activities of the three compounds. These results show that: (*a*) protein synthesis is required to maintain the transformed state in *LA*23-infected cells at the permissive temperature; and (*b*) protein synthesis is *not* required for the myosin-containing structures to revert from the transformed to normal state (i.e., the components of those structures are not irreversibly altered upon transformation). NRK cells infected with wild-type Rous sarcoma virus (B77) are killed by the protein synthesis inhibitors rather than show any change of phenotype. To what extent these effects of protein synthesis inhibitors apply to other temperature-sensitive transforming viruses and other host cells remains to be determined.

These findings are therefore consistent with the hypothesis (see introduction) that the product of the viral *src* gene is directly or indirectly involved in a reversible chemical modification of components of the myosin-containing complex inside the cells, and this modification controls the state of aggregation of that complex. We are fully aware that there are many alternative molecular explanations of these results, which we will not attempt to detail here. The possibility that the myosin-containing complex is chemically modified in a temperature-sensitive manner in *LA*23-infected NRK cells can be investigated experimentally.

As this paper was being prepared for publication, we learned of the results of Edelman and Yahara (24), with another temperature-sensitive Rous sarcoma virus, ts 68, infecting chick embryo fibroblasts. They showed that Con A-mediated surface modulation, actin distribution, and microtubule integrity were correlated with the interconversion from the normal to the transformed state. No studies with protein synthesis inhibitors were reported, however.

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