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Biological Properties of "Partial" Transformation Mutants of Rous Sarcoma Virus and Characterization of Their pp60^{src} Kinase

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We have isolated mutants of Rous sarcoma virus from an unmutagenized stock of the Schmidt-Ruppin strain of Rous sarcoma virus. These mutants induce only a "partial" transformation, and the transformation properties induced show unusual properties or combinations. Cells infected with mutant CU2 have a unique "blebby" morphology, have lost surface fibronectin, form very small colonies in soft agar, and are nearly normal with respect to adhesiveness and hexose transport. Cells infected with mutant tsCU11 have a nearly normal morphology, but grow well in soft agar. Cells infected with mutant CU12 have a fusiform morphology, intermediate levels of hexose transport and fibronectin, and form very large colonies in soft agar. Because the appearance of the different parameters of transformation is dissociated in these mutant-infected cells, these data are interpreted as supporting a model in which the transforming protein pp60^{src} interacts with more than one primary target in generating the transformed phenotype. All of the mutants display levels of pp60^{src} kinase activity less than that of the wild type. In the case of mutant CU12, the lower kinase activity is in part a consequence of a lower steady-state amount of pp60^{src} inside the cell.

Infection of chicken embryo fibroblasts with Rous sarcoma virus leads to a complex array of biological alterations, including changes in cellular morphology, the cytoskeleton, surface fibronectin, plasminogen activator production, adhesiveness, hexose transport, and growth properties (reviewed in reference 12). The establishment and maintenance of these changes is dependent on the continuous expression of the *src* gene (12, 18, 22, 23, 35). Recently, the *src* gene has been shown to code for a 60,000-dalton phosphoprotein (pp60^{src}) which has an unusual protein kinase activity: it transfers phosphate from the γ position of ATP to the hydroxyl of tyrosine (5, 10, 11, 14, 21, 27).

We have considered two models to explain how a single protein kinase could generate such a diverse collection of cellular changes: either pp60^{src} interacts directly with a single primary target, inducing a cascade of secondary changes, or else pp60^{src} phosphorylates several different primary targets, which lead to various of the cellular alterations (Fig. 1). We reasoned that if the second model is correct, it might be possible to isolate mutants in the *src* gene which are hindered in their ability to phosphorylate some targets but not others. The phenotype of such mutants would be "partial transformation defective mutants" (3, 39), i.e., they would induce some parameters of transformation well, but induce others poorly.

We have isolated three mutants of Rous sarcoma virus which we believe to be partial transformation defectives. These mutants all induce only a partially transformed phenotype, and the transformation parameters which are induced show unusual properties or combinations. This report describes the biological properties of these mutants and a partial characterization of the amount and kinase activity of their pp60^{src}.

MATERIALS AND METHODS

Virus strains. All mutants were derived from the Schmidt-Ruppin strain of Rous sarcoma virus subgroup A without prior mutagenesis. The stocks of wild-type virus and of strain tsNY68 were obtained originally from Kawai and Hanafusa (18).

Cells and cell culture. Cells were obtained from 11-day-old gs$^-$ chicken embryos (Spafas; Norwich, Conn.) and cultured as described previously (38). The eggs were from a predominantly chf$^-$ flock, but embryos were not tested for chf. For comparison of transformation parameters with different mutants, secondary cell cultures were infected and then transferred 48 h later and put at 36 or 42°C. Cells were transferred once more at 48 h, plated at 1.4 to 4.0 $\times$ 10^5 cells per

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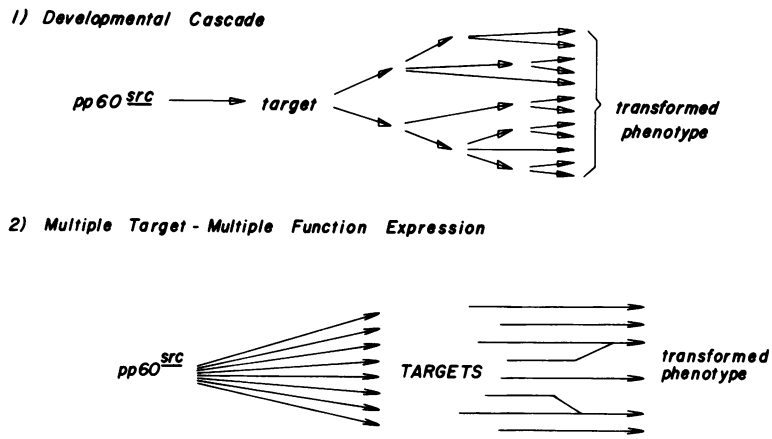

FIG. 1. *Alternative models of pp60^{src} action in inducing the transformed phenotype. Panels: 1, single-target model; 2, multiple-target model.*

35-mm dish, and transformation parameters were measured 48 h later.

Mutant selection. Mutants were obtained in a selection procedure designed to give late function assembly mutants. Secondary cells were plated at 10^6 per 100-mm dish and incubated for 24 h at 36°C. Cells were infected at a multiplicity of infection of 0.1 to 1.0 (based on soft agar colony titer) and incubated at 36°C for 4 h, at which time cultures were shifted to 42°C. At 17 h postinfection 2 μg of cycloheximide per ml was added to stop protein synthesis. At 19 h after infection, cells were washed three times with growth medium plus 2 μg of cycloheximide per ml to remove free virus and maintain inhibition of protein synthesis, with care being taken to maintain cells at 42°C throughout the wash procedure. Cells were then shifted to 36°C; after 1 h, the culture fluid was collected and cloned as soft agar colonies at 36°C. From 10 to 100 soft agar colony-forming particles per ml were generally obtained.

The selection procedure was performed six times with slight variations using mutagenized and unmutagenized virus, and the resulting culture fluids were then cloned. Out of 466 clones examined for replication defectiveness at the nonpermissive temperature (42°C), none was found to be significantly defective in replication. Instead, many of the clones, when picked onto normal cells, produced morphologically abnormal transformation. Of the 35 variant clones that were saved, 33 were spontaneous, having been obtained from nonmutagenized wild-type SR-A virus stock. Three of these spontaneous mutants, CU2, tsCU11, and CU12, were chosen for detailed examination. All of these mutants were cloned in soft agar a minimum of four times, at least once from a sonicated stock.

Measurements of transformation parameters. Most measurements were performed as described by Weber and Friis (39).

For electron microscopy cells were grown on cover slips, fixed in 2% glutaraldehyde, dehydrated through graded alcohol, critical point dried, and sputter coated with gold. They were scanned in a JEOL JSM-U3 scanning electron microscope at 15 kV, tilt 20°.

The casein plaque assay was adapted from the method of Balduzzi and Murphy (2). Focus-forming ability was assayed by the procedure used for the casein assay, but no casein agar layer was added, and 5 ml of soft agar was used. The day before foci were counted 1.0 ml of growth medium was added to each plate. Foci were counted at 36°C on days 9 to 13 and at 42°C on days 7 and 8. Soft agar colony-forming ability was assayed by plating 10^6 cells, exposed to various virus dilutions, in soft agar, above a feeder layer.

For measurement of density inhibition cells were plated in 35-mm dishes and incubated at 36 and 42°C, with cell counts being recorded each 24 h for 96 h.

Indirect immunofluorescence studies of fibronectin and tropomyosin were performed on formaldehyde-fixed cells grown on cover slips as described by Weber et al. (37). In the case of tropomysin, formaldehyde fixation was followed by treatment with acetone to permeabilize the cell to the immune reagents. Cells were viewed with a Zeiss microscope equipped with epifluorescent illumination and were recorded on Kodak Tri-X film rated at ASA 800.

Characterization of pp60^{src}. [^{35}S]methionine or $^{32}PO_4$-labeled mutant or wild-type infected cells were scraped into phosphate-buffered saline and washed once with phosphate-buffered saline. The equivalent of one 35-mm plate was lysed in 250 μl of lysis buffer (32) (0.15 M NaCl, 0.01 M NaH_2PO_4/K_2HPO_4 [pH 7.2], 1% Nonidet P-40, 1 mM EDTA, 1% Trasylol). Protein concentration was determined by the method of Peterson (24), and the specific activity of the lysate was determined by measuring the radioactivity of trichloroacetic acid precipitates. The equivalent of 20 μg of protein of the lysate was preabsorbed with normal rabbit serum and *Staphylococcus aureus* (Calbiochem) and was then incubated for 60 min at 4°C with 5 μl of antiserum from tumor-bearing rabbits (5, 30), 5 μl of lysate of normal cells, 5 μl of lysate of concentrated tdNY101-infected cells, and *S. aureus*. Control experiments demonstrated that antisera and *S. aureus* were in excess. The extract of tdNY101-infected cells blocked the precipitation of virus structural proteins. After centrifugation in an Eppendorf microfuge the pellet was washed four times with lysis buffer and once with 50 mM Tris-hydrochloride (pH 7.4) and

then suspended in Laemmli sample buffer (19) and boiled for 3 min. After centrifugation the supernatant was analyzed on 12% polyacrylamide gels by the method of Laemmli (19), except that *N,N'*-diallyltartardiamide was used as a cross-linker. The labeled proteins were detected by autoradiography.

For quantitating the $pp60^{src}$, cells were labeled for 40 h. with [^{35}S]methionine. After immunoprecipitation and electrophoresis, the [^{35}S]methionine-labeled $pp60^{src}$ was cut out of the gel and the gel slice was eluted overnight at 60°C in 1 ml of 1% sodium dodecyl sulfate–0.1 M NaOH; then Aquasol (New England Nuclear) was added, and the sample was counted in a scintillation counter.

To measure kinase activity, cell lysate containing 20 μg of protein was incubated with 5 μl of antiserum and 25 μl of 20% fixed *S. aureus* solution (Calbiochem) for 60 min at 4°C and precipitated by centrifugation in an Eppendorf microfuge. The precipitate was washed in kinase buffer containing 20 mM Tris-hydrochloride (pH 7.2)–5 mM $MgCl_2$. The pellet was suspended in 20 μl of kinase buffer, 20 μl of kinase buffer containing 0.02 to 0.1 μM [^{32}P]ATP (2,000 Ci/mmol) and 100 μM AMP was added, and the sample was incubated for 10 min at 20°C. The reaction was terminated by adding 2 mM ATP–0.4 M NaCl–1 mM EDTA–10 mM NaH_2PO_4/K_2HPO_4 (pH 7.2)–1% Nonidet P-40–0.25% sodium deoxycholate. After centrifugation the pellet was washed once with the same buffer, suspended in Laemmli sample buffer, and boiled for 3 min. The bacteria were repelleted, and the supernatant was subjected to electrophoresis. Note that the lysis buffer did not contain sodium dodecyl sulfate or deoxycholate, which have been reported to inhibit kinase activity in some cases (32).

Peptide mapping by limited proteolysis with *S. aureus* V-8 protease was carried out essentially as described by Cleveland et al. (8). Extracts labeled with ^{32}P were immunoprecipitated with anti-pp60 serum and run on 12% polyacrylamide-sodium dodecyl sulfate slab gels. The $pp60^{src}$ band was located on the unstained, undried gels by autoradiography and transferred to a second 14% polyacrylamide gel. Digestion was carried out directly in the stacking gel by using 50 ng of protease per sample.

Antisera from tumor-bearing rabbits were prepared as described previously (5, 29). The concentrated virus used in some preparations was a generous gift of L.R. Rohrschneider.

RESULTS

Phenotypic characterization of the mutants: morphology. The most obvious alteration in the transformed phenotype generated by these mutants is in the morphology of the infected cells. Morphological studies at various magnifications are shown in Fig. 2, 3, and 4. Cells infected with CU2 had a flat, nonrefractile appearance in the phase microscope and displayed numerous surface "blebs" which were quite striking at higher magnifications. These blebs were more prevalent at 36°C than at 42°C; when the cells came into contact at either temperature the blebs disappeared, and cell edges became extremely difficult to distinguish (Fig. 3, 42°C). Because CU2-infected cells had a faster growth rate and obtained a higher final cell density than did wild-type virus-infected cells, it is highly unlikely that the blebby appearance resulted from some virus-caused toxicity.

The morphology of tsCU11-infected cells was close to that of normal, uninfected cells.

CU12-infected cells had a "fusiform" morphology of the type previously described by Temin (34) and by Martin (23). This phenotype has not to our knowledge been previously characterized in detail.

Cytoskeleton. The microfilament network was visualized by staining fixed cells with guinea pig anti-tropomyosin antibody and fluorescein-labeled rabbit anti-guinea pig immunoglobulin G (Fig. 5). Normal cells and cells infected with tsNY68 and held at 42°C had a well-developed network of "stress fibers," which was lost upon transformation (e.g., tsNY68-infected cells at 36°C) (1, 25, 36). Cells infected with CU2 or tsCU11 partially lost their microfilament network when held at 36°C, but maintained it at 42°C. Cells infected with CU2 at 36°C and tsNY68-infected cells at 42°C displayed substantial punctate staining with tropomyosin as well as the stress fibers. The significance of the punctate distribution is unclear, but it is worth noting that Burridge and Feramisco observed a similar distribution staining with anti-actin antibody, but only in cells which had been subjected to a microinjection procedure (6). Cells infected with CU12 did not have a well-developed microfilament system, but displayed only a diffuse fluorescence.

Fibronectin. Fibronectin distribution was visualized by staining with rabbit antibody against human cold-insoluble globulin and fluorescein-labeled goat anti-rabbit immunoglobulin G. The cell surface fibronectin was quantitated by lactoperoxidase-catalyzed iodination followed by gel electrophoresis (15). The data are shown in Fig. 6 and 7. In normal cells, fibronectin displays a fibrillar distribution which has been shown to be partially coincident with actin cables (1, 13, 16, 33, 41). Upon transformation, the amount of fibronectin on the surface decreased, and the fibrillar distribution was lost. Cells infected with CU2 possessed low levels of fibronectin at 36 and at 42°C, and the fibronectin which was present was not fibrillar. To the contrary, fibronectin in CU2-infected cells appeared to be predominantly punctate. The peculiar distribution of fibronectin could be related to the fact that cells infected with CU2 were extremely adhesive, even though their fibronectin levels were very low. Perhaps all the fibronectin in these cells accumulates at the adhesion plaques

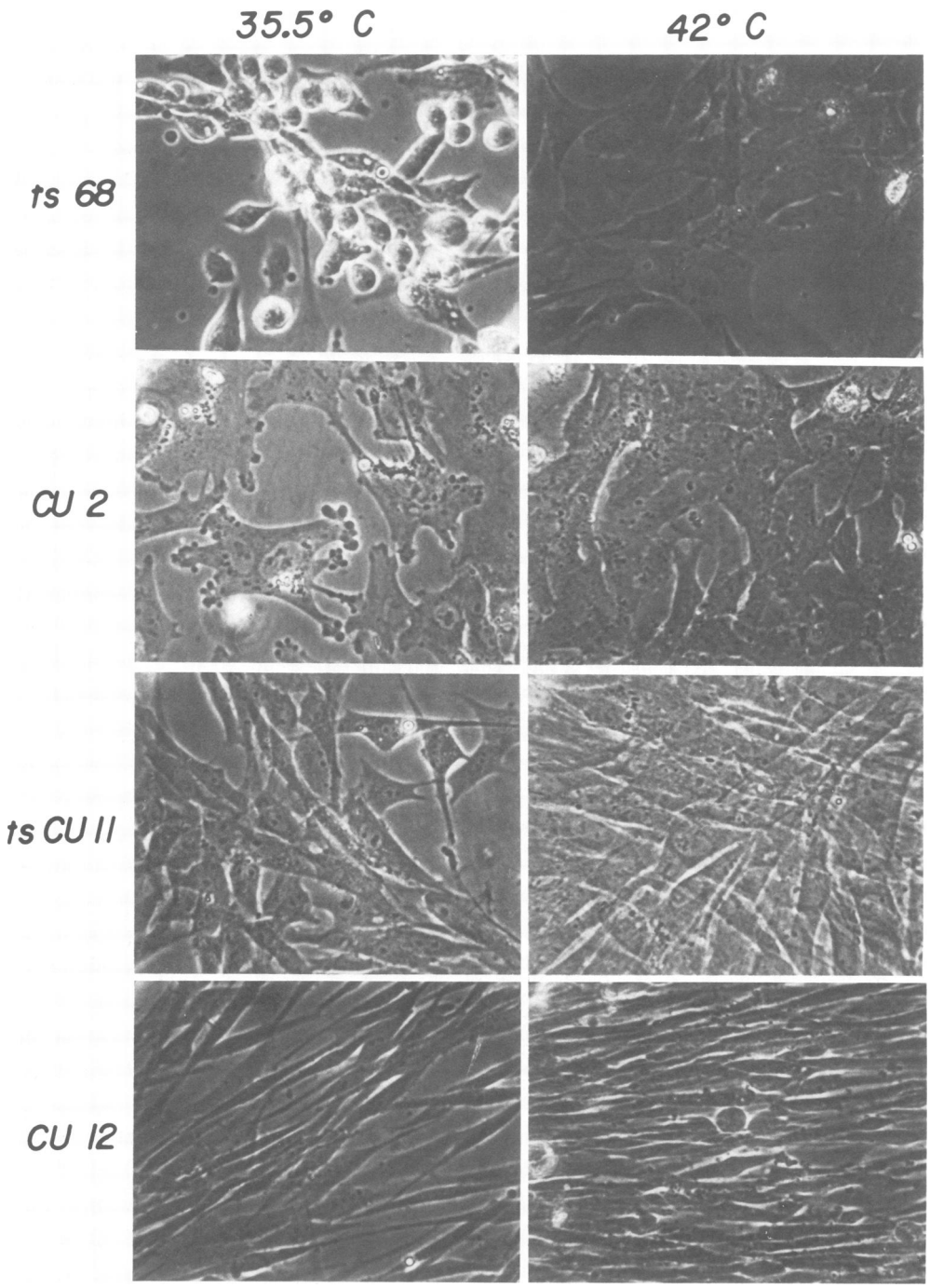

FIG. 2. *Phase micrographs of tsNY68, CU2, tsCU11, and CU12-infected chicken embryo cells grown at 36 or 42°C. Magnification, ×335.*

(1, 30, 33, 40, 41). Cells infected with tsCU11 had very low amounts of fibronectin at 36°C, and cells infected with CU12 had intermediate amounts of fibronectin.

Soft agar colony growth. CU2-, tsCU11-, and CU12-infected cells all formed soft agar colonies at 36°C, and all but tsCU11-infected cells also formed soft agar colonies at 42°C (Fig.

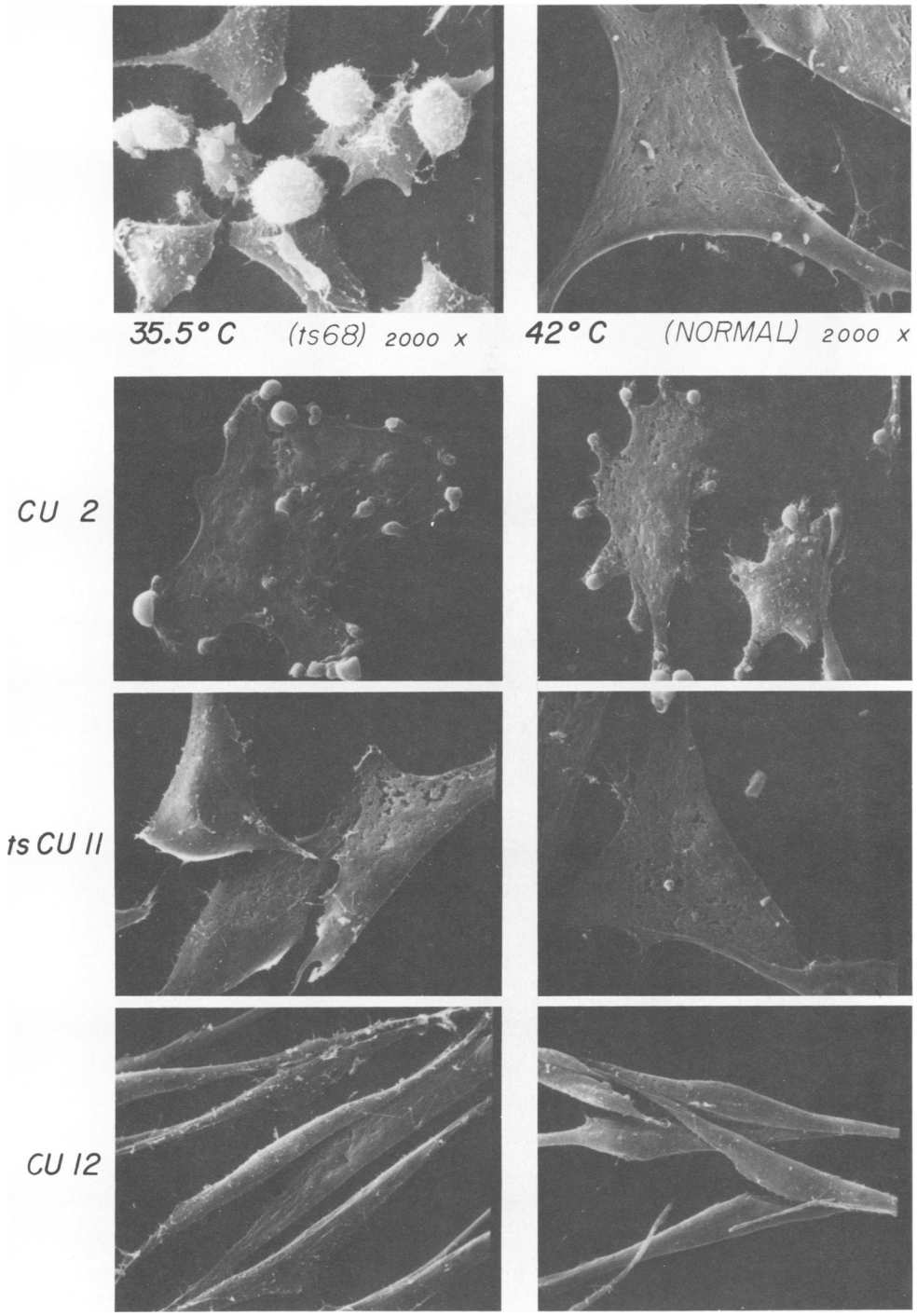

FIG. 3. *Scanning electron micrographs of tsNY68-infected cells grown at 36°C, normal cells grown at 42°C, and CU2-, and tsCU11-, and CU12-infected cells grown at either 36 or 42°C.*

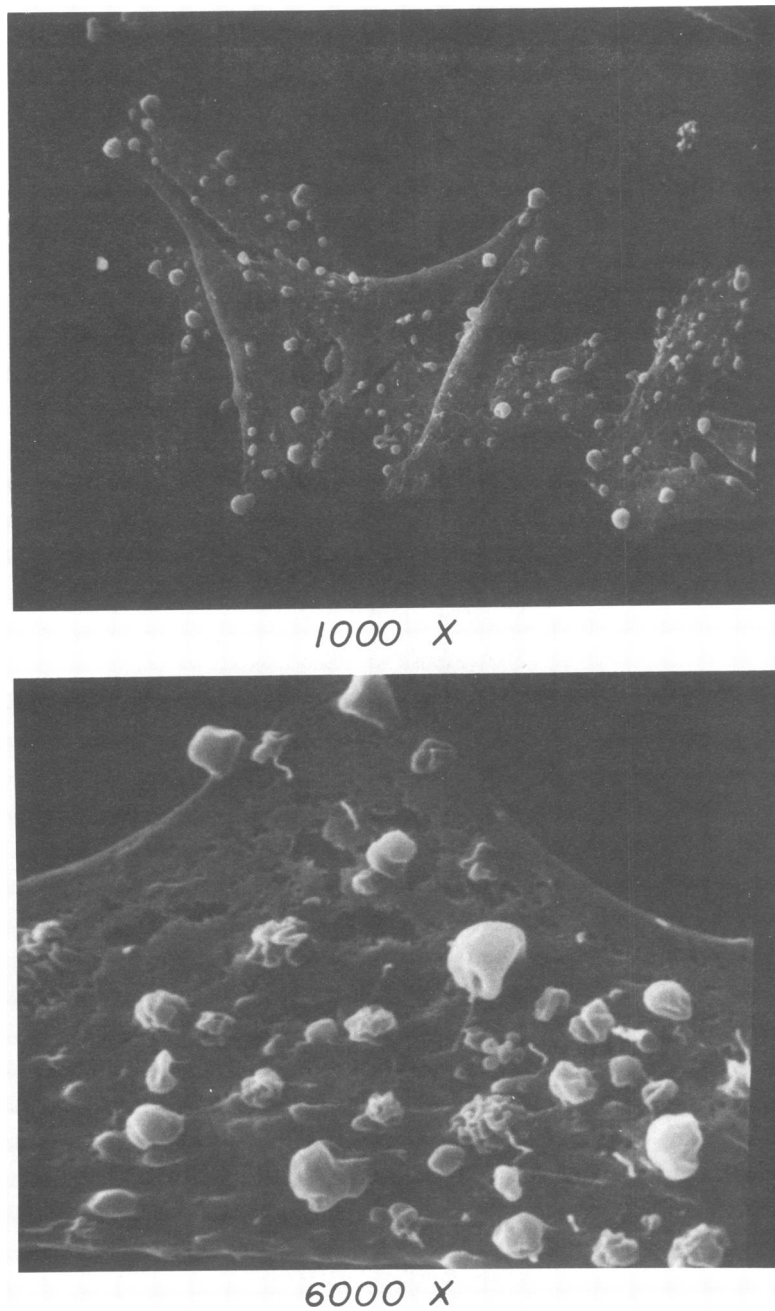

FIG. 4. *Scanning electron micrographs of CU2-infected cells grown at 36°C.*

7). There were significant differences in the appearance of CU2- and CU12-infected colonies compared with those made by wild-type virus-infected cells. At both temperatures, CU2-infected cells formed "minute" colonies which were extremely difficult to see by eye and to quantitate; refeeding or growth for longer periods of time (or both) did not increase their size. CU12-infected cells, on the other hand, formed giant colonies approximately two to three times the size of those formed by wild-type virus-infected cells.

Release from density inhibition. Both CU2- and CU12-infected cells showed release

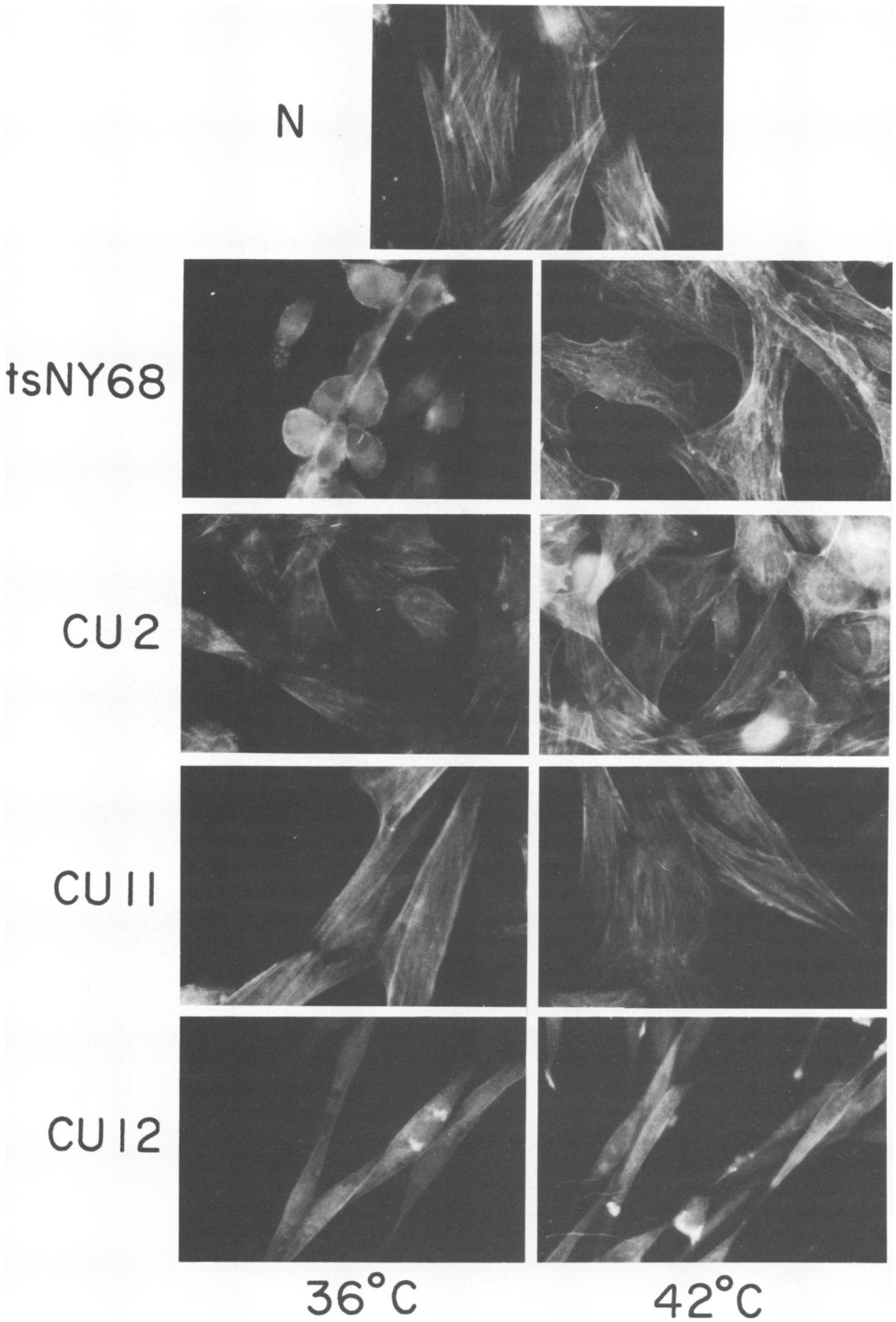

FIG. 5. *Microfilament bundles of normal cells (N) and mutant-infected cells, visualized by staining fixed cells with antitropomyosin antibody.*

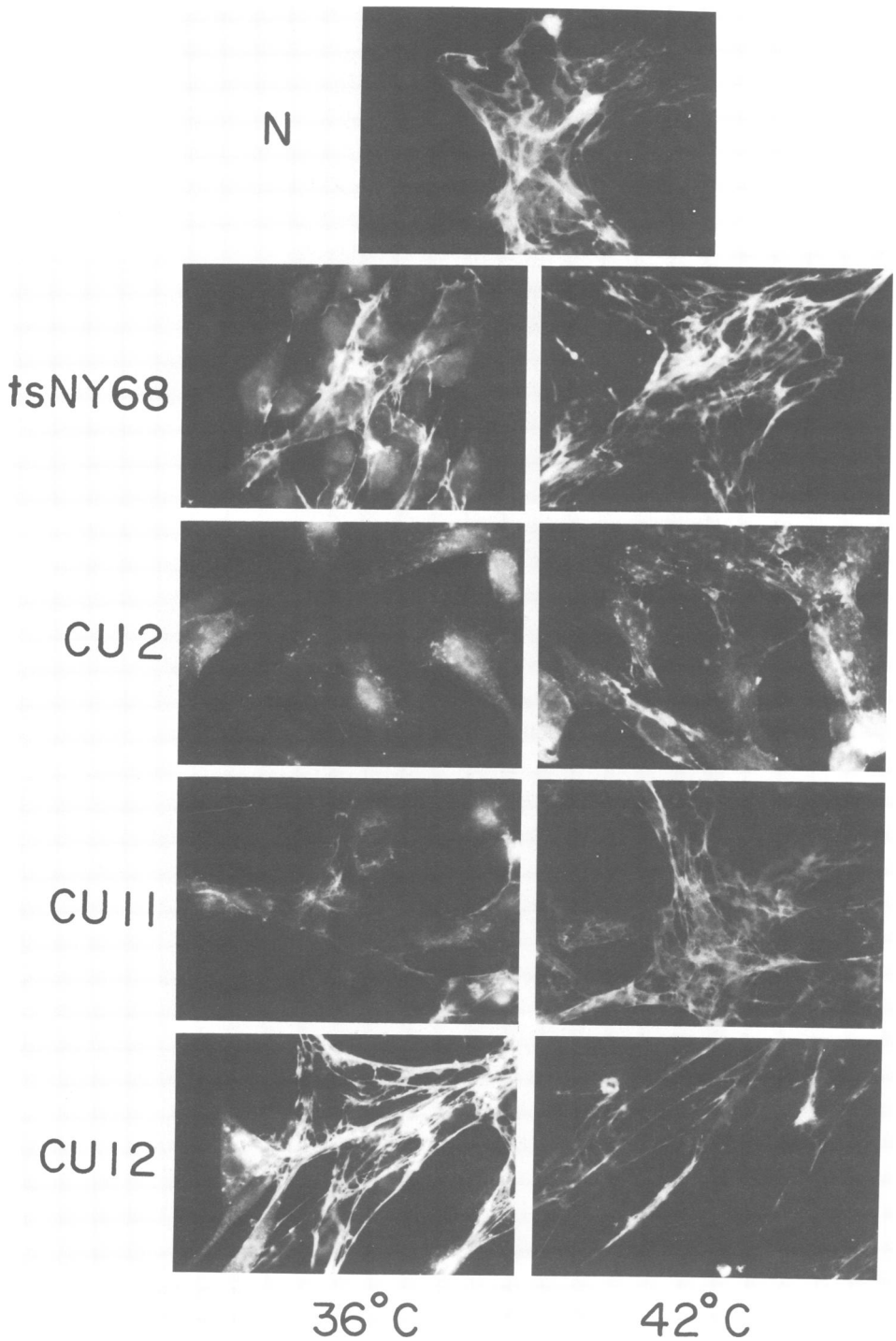

FIG. 6. *Fibronectin distribution on normal cells (N) and mutant-infected cells, visualized by staining with antibody against human cold-insoluble globulin.*

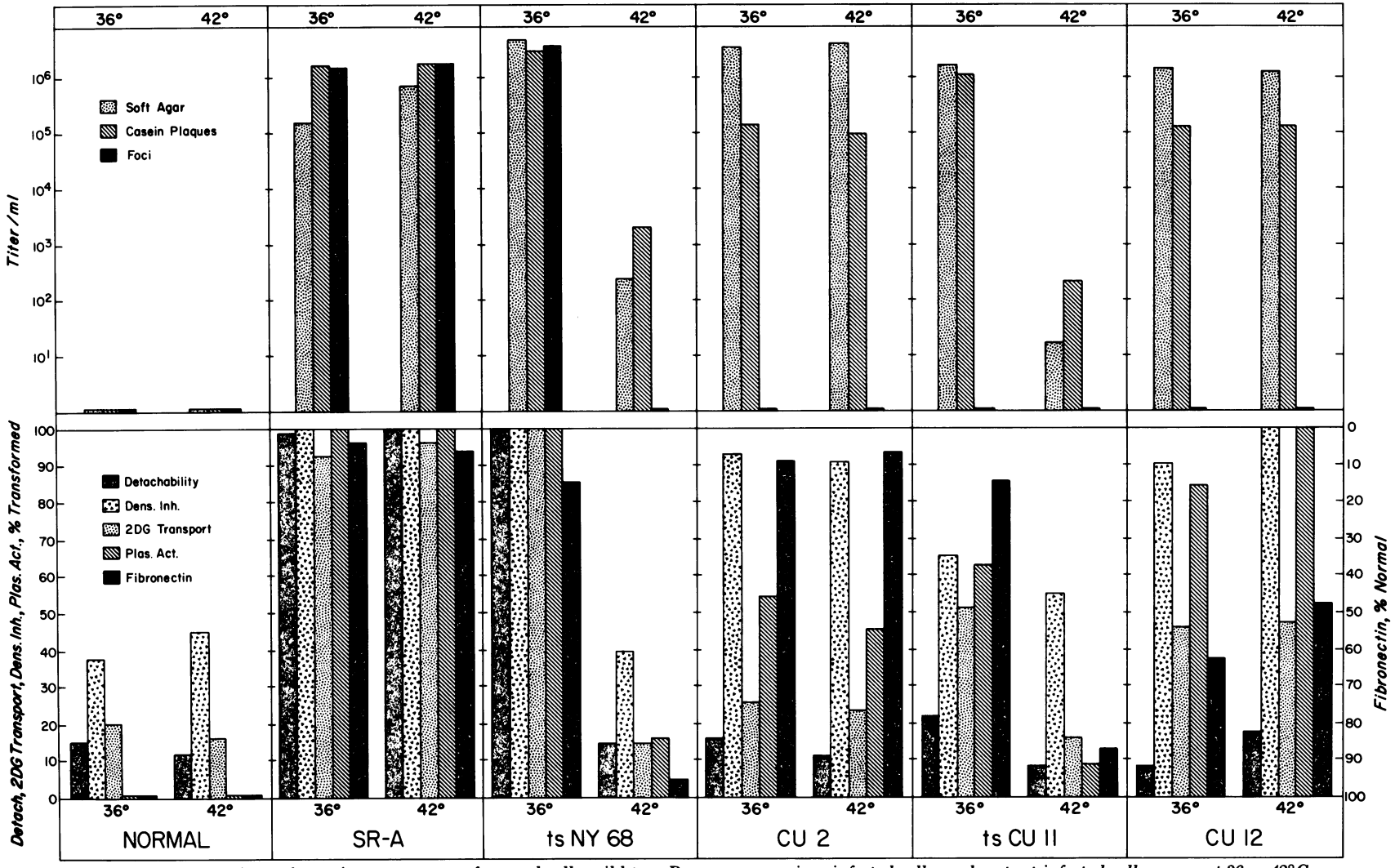

FIG. 7. *Summary of transformation parameters of normal cells, wild-type Rous sarcoma virus-infected cells, and mutant-infected cells grown at 36 or 42°C. The data are plotted so that a fully transformed value for any transformation parameter will be near the top of the panel and a fully normal value will be near the bottom of the panel.*

from density inhibition at both 36 and 42°C (Fig. 7). tsCU11-infected cells partially lost density inhibition at both temperatures, and the cells formed multilayers.

Focus formation. Neither CU2-, tsCU11-, nor CU12-infected cells were able to form typical wild-type foci at either 36 or 42°C (Fig. 7). At the cell density at which wild-type virus-infected cells began to form foci, mutant-infected cells formed multiple layers on the dish. If the culture fluid was frequently changed, CU2- and CU12-infected cells, and, to a lesser extent, tsCU11-infected cells, could be forced to aggregate into piles of cells, but these were not well defined or readily quantitated.

Casein plaque formation. Cells infected by all three mutants formed casein plaques at 36°C, and CU2- and CU12-infected cells formed plaques at 42°C (Fig. 7). As with soft agar colonies CU2 and CU12 casein plaques had a unique morphology. CU2 plaques were minute and difficult to see by eye or to count. CU12 formed very large plaques which were diffuse and ill-defined compared with the clear, sharply defined plaques characteristic of wild-type virus-infected cells.

Increased detachability. All three of the mutant-infected cell cultures were more adhesive than were wild-type-transformed cells, as determined by our assay which measures the percentage of cells in a culture detachable by a stream of medium (40). CU2-infected cells were far more adhesive even than normal uninfected cells and sometimes required prolonged treatment with trypsin and EDTA to remove cells from the dish. Normal chicken embryo cells are generally detached by a few minutes of trypsinization, even without EDTA.

Although the data in Fig. 7 indicate that CU12-infected cells were nearly normal in detachability, we have found that increasing the force of the stream of medium which is used to detach the cells can detach CU12-infected cells without detaching uninfected cells. Thus, CU12-infected cells are clearly less detachable than wild-type-infected cells and more detachable than normal cells, but their position along that continuum depends on the assay conditions.

Hexose transport. tsCU11-infected cells showed temperature-conditional changes in hexose transport rate. At 42°C 2-deoxyglucose uptake was at a low normal level, but at 36°C the rate was raised to an intermediate level. CU2-infected cells showed slightly raised levels, and CU12-infected cells showed an intermediate level of 2-deoxyglucose uptake (Fig. 7).

Characterization of pp60^{src}. The size, amount, and kinase activity of the pp60^{src} coded for by these mutants was determined by established procedures (5, 9–11, 21, 27, 32) (Fig. 8 and 9 and Table 1). All of the mutants coded for a pp60^{src} indistinguishable from that of the wild type in molecular weight and in the sizes of the peptides generated by limited proteolytic digestion with V8 protease from *S. aureus*, as would be expected for point mutants or small deletions. However, CU12-infected cells, in many experiments, displayed a 62,000-dalton phosphoprotein as well as pp60^{src} in the immunoprecipitates. The relationship of this protein to pp60^{src} and the reasons for its variable appearance are under investigation.

All of the mutants except CU12 produced amounts of [^{35}S]methionine-labeled pp60^{src} similar to those obtained with tsNY68-infected cells (all of the data in Table 1 are normalized to tsNY68-infected cells at 36°C). Cells infected with CU12 at 36°C consistently displayed one-half to two-thirds the amount of methionine-

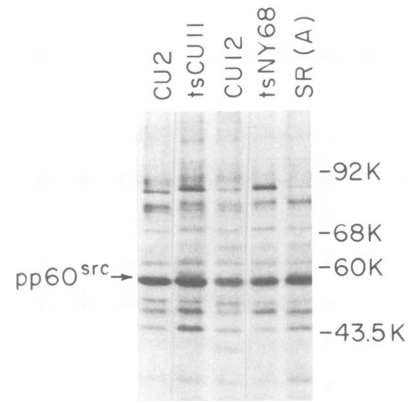

FIG. 8. *Electropherogram of an immunoprecipitate of a [^{35}S]methionine-labeled extract of cells infected with mutant or wild-type Rous sarcoma virus. The band labeled pp60^{src} was not precipitated by normal rabbit serum, was present only in infected cells, and could be labeled with $^{32}PO_4$, consistent with its identification as pp60^{src}.*

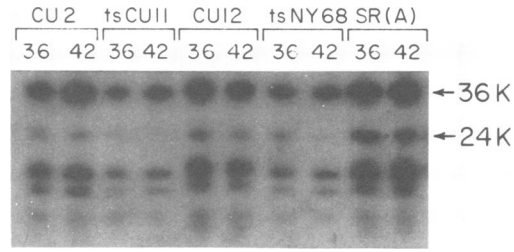

FIG. 9. *Limited proteolysis peptide maps of pp60^{src} coded for by wild-type and mutant virus. The region of a 12% polyacrylamide gel containing $^{32}PO_4$-labeled pp60^{src} was excised and subjected to proteolysis by S. aureus V-8 protease as described previously (8).*

TABLE 1. *Relative amounts and kinase activity of pp60^{src} in mutant and wild-type Rous sarcoma virus-infected cells*[a]

Strain	Temp	Amt of pp60^{src}[b]	Kinase activity[c]	Specific kinase activity[d]
CU2	36°C	180 ± 79 (6)	118 ± 49 (9)	84 ± 37 (4)
tsCu11		124 ± 64 (6)	108 ± 33 (8)	68 ± 25 (4)
CU12		68 ± 16 (6)	118 ± 28 (8)	167 ± 50 (4)
tsNY68		100	100	100
Wild type		151 ± 32 (6)	266 ± 89 (9)	191 ± 85 (4)
CU2	42°C	95 ± 30 (4)	79 ± 55 (9)	48 ± 9 (3)
tsCU11		82 ± 32 (4)	50 ± 14 (8)	73 ± 14 (4)
CU12		70 ± 10 (4)	143 ± 37 (9)	236 ± 45 (4)
tsNY68		83 ± 5 (4)	56 ± 17 (9)	49 ± 17 (3)
Wild type		122 ± 32 (4)	202 ± 93 (9)	158 ± 91 (4)

[a] All data are normalized to tsNY68 at 36°C.

[b] Determined by long-term labeling with [^{35}S]methionine followed by immunoprecipitation. Data are given as percent tsNY68 values, ± standard deviations. Numbers within parentheses are the numbers of independent determinations. Different amounts of unlabeled methionine were utilized in different experiments, between 1 and 25% of the amount found in complete Dulbecco medium. This did not affect the results obtained. Radioactivity in the tsNY68 pp60^{src} at 36°C varied between 427 and 1,178 cpm in different experiments. Specific radioactivity of [^{35}S]methionine in trichloroacetic acid-precipitable protein generally differed by less than 20% between the mutants and never differed by more than a factor of two.

[c] Measured in vitro by transfer of ^{32}P from ATP to immunoglobulin G. Radioactivity incorporated in the tsNY68, 36°C sample varied between 693 and 2,993 cpm in different experiments.

[d] Relative kinase activity divided by relative amount of pp60^{src} for those experiments in which both measurements were performed in parallel.

labeled pp60^{src} shown by cells infected with the other mutants. This is unlikely to be a consequence of differences in cell physiology since all cultures used in a comparison were passaged and labeled identically and in parallel and were subconfluent during the labeling period. In addition, all data are corrected for differences in the specific activity of [^{35}S]methionine incorporation into trichloroacetic acid-precipitable protein. It is possible that the CU12 pp60^{src} is less stable than the wild-type and turns over at a faster rate.

The kinase activity of pp60^{src} was measured by its ability to phosphorylate immunoglobulin G in an immune complex (10, 21, 32). At 36°C, all of the mutants showed a kinase activity comparable to that of tsNY68 and significantly lower than that of the wild type. At 42°C, the kinase activity of tsCU11 and tsNY68 decreased by a factor of approximately two. The CU2 pp60^{src} kinase activity may decrease slightly also, but because of the unavoidable scatter in the data it is not possible to make a firm conclusion. The pp60^{src} kinase activity of CU12-infected cells was not decreased by growth at 42°C, in agreement with our finding that the transformed phenotype generated by this mutant was not temperature conditional.

The pattern of phosphopeptides generated by V8 proteolysis was consistent with the kinase data; the 24,000-dalton peptide, which contains the phosphotyrosine (9, 14) was phosphorylated in all mutant-infected cells at 36°C, but was phosphorylated only slightly at 42°C in tsCU11- and tsNY68-infected cells.

DISCUSSION

It is clear from the data we have presented that CU2, tsCU11, and CU12 induce a partial transformation in infected cells (Fig. 7 and Table 2). Since the various transformation parameters were dissociated from each other in the mutant-infected cells, we believe that these results support a model for transformation in which pp60^{src} directly interacts with more than one primary target, as schematized in Fig. 1. The work presented here, taken together with our previous findings in collaboration with Friis (39) and the work of Calothy and Pessac (7), Becker et al. (3), Royer-Pokora et al. (31), and Beug et al. (4) provide strong, although still indirect, evidence that pp60^{src} acts on more than one primary target.

In making this conclusion it is important to be cognizant of the fact that the assays for the different transformation parameters differ widely in their sensitivity, and that it might be possible to obtain what appear to be partially transformed cells even with a single target if one used a weakly oncogenic virus (e.g., a leaky mutant) which left certain transformation-related alterations below the threshold of detection. This point, that a phenotypically leaky mutant could appear to dissociate various transformation parameters, has been discussed in detail previously (39). If simple leakiness were responsible for the results reported here, we would expect to be able to rank the mutants into a hierarchy of effectiveness with respect to their ability to induce transformation. However, we do not believe that our results can be explained by leakiness, since there was no consistent hierarchy into which the mutants could be placed. For example, in CU2- and tsCU11-infected cultures, fibronectin was almost completely lost, in agreement with our previous findings that loss of fibronectin is a parameter of transformation which is extremely sensitive to even small amounts of pp60^{src} activity (39). However, CU12-infected cultures retained substantial amounts of fibronectin even though these cells were more transformed than CU2- and tsCU11-infected cultures by other criteria, such as morphology and plasminogen activator. Similarly, CU2-infected cultures formed only very small soft agar

TABLE 2. *Dissociation of transformation parameters*

	CU2	tsCU11 (36°C)	CU12
Transformed	Fibronectin Density inhibition	Fibronectin Anchorage dependence Casein plaques Plasminogen activator	Anchorage dependence Density inhibition Casein plaques Plasminogen activator
Intermediate or abnormal	Anchorage dependence Casein plaques Blebby morphology Plasminogen activator	Density inhibition Hexose transport	Hexose transport Fibronectin Fusiform morphology Adhesiveness[a]
Untransformed	Hexose transport Adhesiveness[b] Focus formation	Morphology Adhesiveness Focus formation	Adhesiveness[a] Focus formation

[a] Value varied with assay conditions.
[b] Adhesiveness even greater than normal cell control.

colonies compared with those formed at 36°C by tsCU11 or wild-type virus. In addition, CU2-infected cells were less transformed than tsCU11- or CU12-infected cells with respect to glucose transport and adhesiveness. However, CU2 had more dramatic effects on cellular morphology than did tsCU11. Moreover, we did not induce the phenotypes of CU2, CU11, or CU12 when cells infected with tsNY68 were held at temperatures intermediate between the permissive and restrictive temperature to generate intermediate levels of active pp60^{src} (39). Thus, we believe that it is extremely difficult to explain these data if pp60^{src} has only one primary target, and the results are most consistent with a multiple-target model.

In the course of this work, a report appeared demonstrating that pp60^{src} catalyzes the phosphorylation of tyrosine (14). This unusual catalytic activity provides a way to identify potential primary substrates of pp60^{src}. At the time of this writing, four proteins had been shown to contain phosphotyrosine in Rous sarcoma virus-transformed cells: a 36,000-dalton protein which is rapidly phosphorylated during transformation (28); a 50,000-dalton protein which coprecipitates with pp60^{src} during immunoprecipitation (14); pp60^{src} itself (14); and vinculin, a 130,000-dalton cytoskeletal protein (personal communications from G. S. Martin, B. M. Sefton, and L. R. Rohrschneider). Thus, our prediction based on genetic evidence has been strengthened by direct biochemical investigation. Of course, the existence of phosphotyrosine on a cellular protein does not prove it to be a pp60^{src} substrate or demonstrate its function in transformation. The partial transformation mutants reported here should prove useful in determining the functional significance of these various phosphorylations, and such studies are in progress.

In addition to their potential utility in analyzing the primary events in Rous sarcoma virus-induced transformation, these mutants are also useful in understanding the possible cause-effect relationships between various manifestations of the transformed phenotype. For example, we have previously suggested that increased hexose transport is not required for anchorage-independent growth, since cells infected with tsGI251 were able to grow in soft agar at 42°C even though their hexose transport rate was low at this temperature (39). This suggestion was recently confirmed by Pouysségur et al. (26), utilizing cell mutants of transformed cells. Based on the properties of tsCU11, which induced little morphological alteration but stimulated anchorage independent growth, we now suggest that gross morphological alterations are also unnecessary for loss of anchorage dependence. This conclusion has also been reached by Lau et al., based on their results with revertants of vole cells transformed by Rous sarcoma virus (20). Based on the properties of CU2, we can conclude that decreased adhesiveness is not required for the loss of growth control. The partial transformation mutants should also be useful in determining which aspects of the transformed phenotype are required for tumor formation, and these studies are underway.

Since these mutants were all isolated in a procedure designed to select for late function assembly mutants, we have been particularly concerned that the properties of these mutants might be a consequence not of an *src* mutation but of a replication defect. For example, it might be possible for transformation to appear partial because a culture is only partially infected. For the following reasons, we do not believe that replication defectiveness could be responsible for the biological properties of these mutants.

(i) Attempts to detect a significant defect in replication at either 36 or 42°C have proven

negative whether production is measured by soft agar colonies, immunoprecipitation of virus structural proteins, or reverse transcriptase, although some measurements indicate the possibility of a slight replication defect in tsCU11.

(ii) Repeated passaging of the infected cultures as many as 10 times did not alter the phenotype of the infected cells.

(iii) Isolation of single soft agar clones or coinfection with a subgroup C or D *src* deletion yielded cells which were not more transformed, as determined by morphological criteria.

(iv) Recombinants formed between CU2 or CU12 and Carl Zilber associated virus (a subgroup D *src* deletion mutant) induced a cellular morphology and a soft agar colony morphology indistinguishable from the parental partial mutants. We are in the process of constructing a tsCU11 recombinant.

(v) The partial mutants all displayed defects in pp60^{src} kinase comparable in magnitude to that seen with tsNY68.

(vi) All of the mutants were isolated from unmutagenized stocks and thus are likely to be single mutants.

We do not know whether or how our selection procedure led to the isolation of these transformation mutants. Conceivably, careful screening of any virus stock would yield such mutants even without selection.

It is worth pointing out that all of the partial transformation mutants of Rous sarcoma virus reported to date—both our mutants and those isolated by Becker and collaborators (3)—were obtained without mutagenesis. It seems likely to us that mutagenesis would produce many multiply mutated viral genomes, thus diminishing the likelihood of obtaining a mutant which would be hindered only in its affinity for one of several targets. Indeed, tsNY68, which we have used as a model for a complete temperature-conditional transformation mutant (i.e., a mutant which is coordinately temperature conditional for all parameters of transformation) was isolated from a mutagenized stock (18) and probably carries more than one mutation in the *src* gene (17).

Cells infected by all of the partial mutants displayed levels of pp60^{src} kinase activity significantly lower than those of wild type-infected cells. TsCU11, which is clearly temperature conditional for its ability to alter the cellular phenotype, also is temperature conditional for the level of pp60^{src} kinase. CU2 is marginally temperature conditional with respect to both its biological effects and its kinase activity, and CU12 is not temperature conditional. CU2- and tsCU11-infected cells contained amounts of pp60^{src} which were comparable to that obtained with cells infected with tsNY68; thus, the decreased pp60^{src} kinase in these cells is due in part to a decrease in the specific kinase activity of the protein relative to the wild type. On the other hand, CU12-infected cells contained reproducibly less [^{35}S]methionine-labeled pp60^{src} at steady state than did tsNY68-infected cells, and the specific kinase activity of this protein was actually higher than that obtained with tsNY68 at 36°C. Thus, for this mutant, the decreased kinase activity appears to be due at least in part to a decreased amount of the protein.

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