Temperature-Sensitive Transformation by Rous Sarcoma Virus and Temperature-Sensitive Protein Kinase Activity

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The transforming protein of Rous sarcoma virus, $p60^{src}$, has associated with it a protein kinase activity. We examined whether a correlation exists between the cellular concentration of enzymatically active $p60^{src}$ and the degree to which chick cells are transformed by mutants of Rous sarcoma virus which are temperature-sensitive for transformation. Such a correlation does exist, but cells infected with some mutants could be shown to contain, at the nonpermissive temperature, an amount of protein kinase activity equal to 30 to 40% of that in a wild-type transformed cell. We quantified the amount of virus-induced protein kinase activity by precipitation of $p60^{src}$ with an excess of antitumor antiserum. Our initial measurements of activity were serious underestimates, due to the lability of the protein kinase activity associated with $p60^{src}$ of at least four temperature-sensitive mutants. In fact, no activity at all was associated with $p60^{src}$ of tsLA90 when immunoprecipitation was performed by standard means. However, when immunoprecipitation was performed with procedures which minimize inactivation, it became apparent both that cells transformed by tsLA90 contained protein kinase activity and that cells infected with either NY68 or BK5 contained at the nonpermissive temperature, one-third to one-half as much activity as wild-type transformed cells. This level of activity was much more than that arising from $p60^{src}$ in uninfected cells. In uninfected cells we found an amount of protein kinase activity which varied from 3 to 5% as much as that in a virally transformed cell. The lability of the protein kinase activity of each of these mutants is a further demonstration that this activity is essential for the transformation of cells by Rous sarcoma virus. So as to explain the high protein kinase levels in cells infected with NY68 and BK5 at the nonpermissive temperature, the idea that transformation may be a response to a small quantitative change in the total activity of $p60^{src}$ and the possibility that there may be more than one viral function which is essential for transformation are discussed.

The proper functioning of the product of the *src* gene of Rous sarcoma virus (RSV) is essential for the morphological transformation of cells in tissue culture. This gene is expressed as a 60,000-dalton phosphoprotein, $p60^{src}$ (4, 10, 14, 15). A protein kinase activity which is able to phosphorylate the heavy chain of bound antibodies has been shown to be associated with $p60^{src}$ (6, 10, 14, 16). Although it has not been proven that $p60^{src}$ is itself a protein kinase, it is clear that a protein kinase is invariably associated with biologically active immunoprecipitable $p60^{src}$. This has led to the hypothesis that protein phosphorylation is involved in the process by which RSV causes the malignant conversion of cells in tissue culture (6).

Several mutants of RSV have been isolated which are temperature-sensitive in their ability to both initiate and maintain transformation in tissue culture, e.g., tsBK5 (SR-RSV-A) (11), tsNY68 (SR-RSV-A) (9), tsLA24 (PR-RSV-A)

(19), and tsGI251 (PR-RSV-A) (2). By using antitumor sera which are able to precipitate enzymatically active $p60^{src}$, it has been demonstrated that cells infected with NY68 contain at the nonpermissive temperature less immunoprecipitable protein kinase activity than do cells at the permissive temperature (6, 16), and that the protein kinase activity of $p60^{src}$ encoded by GI251 is more thermolabile than that encoded by wild-type virus (14). These observations support the idea that the protein kinase activity of $p60^{src}$ is essential for transformation and suggest that these mutants fail to transform at the nonpermissive temperature because they fail to produce sufficient active protein kinase.

We wished to correlate the concentration of protein kinase activity with the degree to which cells infected with mutants temperature-sensitive for transformation were transformed. We therefore have gone to some lengths to estimate accurately the amount of immunoprecipitable

protein kinase activity in cells infected by a variety of temperature-sensitive mutants. We have found quite high apparent protein kinase activities in some phenotypically normal cells and quite low apparent protein kinase activities in some phenotypically transformed cells.

MATERIALS AND METHODS

Cells and viruses. All experiments were done with secondary or tertiary cultures of chicken embryo fibroblasts derived from embryonated *gs⁻ chf⁻* eggs (flock Illinois 23) from SPAFAS, Roanoke, Ill. SR-RSV-A and the mutant derived from it, tsNY68 (9), originated in the laboratory of H. Hanafusa, The Rockefeller University, New York, N.Y. A second stock of SR-RSV-A and the mutant derived from it, tsBK5 (11), were a gift of G. S. Martin, the University of California, Berkeley. SR-RSV-D and the mutants derived from it, tsLA90 and tsLA91 (P. K. Vogt, unpublished data), were obtained from G. Giotta, The Salk Institute.

Primary or secondary cultures of chicken embryo fibroblasts were infected, at a multiplicity of 0.1 to 0.5 focus-forming units per cell, 4 h after being seeded onto a petri dish, in Dulbecco-modified Eagle medium supplemented with 2% tryptose phosphate broth, 1% calf serum, 1% heat-inactivated chick serum, and in the case of subgroup D viruses, polybrene (2 μ g/ml). These cells were grown for 3 to 4 days at 37°C until morphological transformation was frank and were then subcultured. The cells were reseeded on 35-mm plastic petri dishes (Falcon) at a density of 1.5×10^6 cells per dish in Dulbecco-modified Eagle medium supplemented with 2% tryptose phosphate broth and 4% calf serum. These cultures were then grown at either 36°C or 41°C for 72 h with daily changes of medium. This prolonged incubation at either the permissive or the nonpermissive temperature was to ensure that the protein kinase levels measured were not affected by the prior incubation of the cells at a semipermissive temperature (37°C). Uninfected sister cultures were treated identically.

Cell lysis and immunoprecipitation. Immunoprecipitation was essentially as described before (15, 16), being carried out in RIPA buffer (1% Nonidet P-40 [NP-40], 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.2, 1% Trasylol). Some additional precautions were taken, however, to prevent inactivation of the protein kinase activity of the mutant *p60^{src}*s. Lysis of the cells and all the handling of the precipitates was done in the cold room. Clarification of the lysates was usually done by centrifugation in the SM24 rotor in a refrigerated Sorvall centrifuge. Use of a Brinkman microfuge for clarification was unsatisfactory because it partially inactivated the protein kinase activity of NY68. At no time were the lysates or the precipitates frozen or stored in the cold.

Some experiments employed a lysis buffer referred to as NP-40 buffer. This was essentially RIPA buffer lacking both sodium deoxycholate and SDS. It consisted of 1% NP-40, 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.2, 1% Trasylol, and 1 mM EDTA. The EDTA was included to prevent the use of the

ATP in the cell lysate for the phosphorylation of the heavy chain of the immunoglobulin during the formation of the immune complex.

All cells to be compared were handled identically and lysed simultaneously. Before lysis, cell numbers were determined with a Coulter Counter. All cells were dissolved at a concentration of either 5×10^6 or 1×10^7 cells per ml of lysis buffer. After clarification, all precipitates were formed with 5×10^4 cells (5 to 10 μ l of lysate) and 2 μ l of heat-inactivated antitumor serum. The serum was always heat-inactivated (30 min at 56°C) because it contained an endogenous protein kinase activity which caused the phosphorylation of the heavy chain of the immunoglobulin.

The assay of protein kinase was as described before (16), except that [γ^{32} P]ATP (2,000 to 3,000 Ci/mmol; New England Nuclear Corp.) was used at a concentration of 100 μ Ci/ml. To determine the extent of the reaction, the immune complex was dissolved in gel sample buffer and subjected to SDS-polyacrylamide gel electrophoresis. The area of the gel containing the heavy chain of the immunoglobulin was identified either by staining with Coomassie brilliant blue or by autoradiography, excised, and counted utilizing a toluene-based scintillation cocktail in a scintillation counter.

Antisera. Most antisera used here were prepared as described before (15), using the procedure first described by Brugge and Erikson (4). Several experiments employ an antiserum which cross-reacts with the product of the endogenous *sarc* gene, *p60^{src}*. This serum was produced by using the same protocol as before, but using this time a stock of SR-RSV-D which had been recently rescued from a SR-RSV-D-transformed rat cell line. This virus was a generous gift of L. Rohrschneider, the Fred Hutchinson Cancer Center, Seattle, Wash.

2-Deoxyglucose uptake. The rate of uptake of [3 H]2-deoxyglucose was determined as described before (12, 16).

In vitro translation. SR-RSV-A and NY68 virion RNAs were prepared and translated in the mRNA-dependent rabbit reticulocyte lysate as previously described (3, 16).

RESULTS

We have compared the amounts of enzymatically active *p60^{src}* in detergent lysates of chick cells infected with several RSVs by immunoprecipitation with a significant excess of antitumor serum. The amount of immunoprecipitated protein kinase activity was assayed by determination of the final extent of phosphorylation of the heavy chain of immunoglobulin during the incubation of the immunoprecipitates with [γ^{32} P]ATP. Incorporation here was quantified by electrophoresis of the dissolved immunoprecipitate in an SDS-polyacrylamide gel, excision of the region of the gel containing the heavy chain, and measurement of the incorporation of 32 P by scintillation counting. Phosphorylation in the immunoprecipitate is extremely rapid and limited in extent, probably because only those an-

tibodies which have bound to p60^{src} can serve as substrates (10). Our data therefore do not represent rates of reaction, but instead reflect the total extent of the reaction. We presume that the incorporated radioactivity is proportional to the number of enzymatically active molecules in the precipitate. All incubations with [γ -³²P]ATP were performed for 10 min at 30°C. We report below that the protein kinase activity characteristic of several temperature-sensitive mutants is less than that of the parental wild-type viruses. These relative differences in activity were not affected in magnitude by either the temperature or the length of the incubation with [γ -³²P]ATP. Most of the experiments were performed with an antiserum which does not recognize the product of the endogenous *sarc* gene which we will refer to here as p60^{src}, and therefore our values reflect only the concentration of virally encoded p60^{src}.

Protein kinase activity in cells infected with mutants of SR-RSV-D. Having previously measured protein kinase levels in cells infected with NY68 (16), we wished to extend our studies to mutants derived from strains of RSV other than SR-RSV-A, the parent of NY68. We therefore employed several new mutants derived from SR-RSV-D. We first measured the amount of immunoprecipitable protein kinase in cells infected with, and phenotypically fully transformed by, tsLA90 at 36°C (Fig. 1). This experiment was performed by using RIPA buffer both to lyse the cells and wash the precipitates, a procedure we and others have employed previously (6, 16). We were unable to detect any p60^{src}-associated protein kinase activity in these cells. This was surprising. We knew that our antiserum could precipitate p60^{src} of LA90 efficiently, that the concentration of p60^{src} in cells transformed by LA90 was at least 40% of that in cells transformed by wild-type SR-RSV-D (Fig. 1), and that this antiserum was a good substrate for the protein kinase associated with wild-type p60^{src} (Fig. 1). In addition, it was clear that LA90 caused unambiguous transformation of chick cells at 36°C. Cells infected with LA90 were, at 36°C, indistinguishable morphologically from cells transformed by SR-RSV-D and transported 2-deoxyglucose at the three- to fourfold accelerated rate characteristic of RSV-transformed chick cells. We performed identical experiments with a related temperature-sensitive mutant, tsLA91, and obtained essentially the same unexpected results (data not shown).

Apparently the procedure we used to extract and to precipitate p60^{src} from these cells did not result in the recovery of any molecules which had associated with them an active protein ki-

FIG. 1. The protein kinase activity induced by LA90. Protein kinase was immunoprecipitated from uninfected cells grown at 41°C, cells infected with LA90 and grown at 36°C or 41°C, and cells infected with SR-RSV-D grown at 36°C or 41°C. These cells were lysed in RIPA buffer and immunoprecipitation was performed with an antiserum which does not cross-react with p60^{src}. Immunoprecipitation, assay for protein kinase and electrophoresis were as described in the text. Cells infected with LA90 or SR-RSV-D were labeled for 20 h at either 36 or 41°C with [³⁵S]methionine (50 μ Ci/ml) in Dulbecco-modified Eagle medium containing half the usual concentration of methionine and 4% calf serum. Lysis was in RIPA buffer. Immunoprecipitation was performed as described in the text with an antiserum which does not recognize p60^{src}. Gel electrophoresis was performed as described before (15). All the precipitates displayed here were formed in antigen excess. (A) Protein kinase activity, uninfected cells grown at 41°C; (B) protein kinase activity, LA90-infected cells grown at 36°C; (C) protein kinase activity, LA90-infected cells grown at 41°C; (D) protein kinase activity, SR-RSV-D-infected cells grown at 36°C; (E) protein kinase activity, SR-RSV-D-infected cells grown at 41°C; (F) LA90-infected cells, grown at 36°C, labeled with [³⁵S]methionine; (G) LA90-infected cells, grown at 41°C, labeled with [³⁵S]methionine; (H) SR-RSV-D-infected cells, grown at 36°C, labeled with [³⁵S]methionine; (I) SR-RSV-D-infected cells, grown at 41°C, labeled with [³⁵S]methionine.

nase. One could conclude from this result that LA90 was able to transform chick cells without a protein kinase activity comparable to that found with other avian sarcoma viruses. We were concerned, however, that cells transformed by LA90 might indeed contain an immunoprecipitable protein kinase activity but we had in-

activated or dissociated it during the extraction. The experiment presented in Fig. 1 was done by using RIPA buffer for lysis of the cells and for washing the precipitates. Whereas this extraction and washing procedure readily reveals protein kinase activity in cells infected with NY68, we examined whether it was too harsh for cells infected with LA90.

When we used a buffer containing 1% NP-40 but lacking both 1% deoxycholate and 0.1% SDS, both to lyse the cells and to wash the precipitate and took care to keep the lysate and the precipitate ice cold, p60^{src}-specific protein kinase activity was apparent in LA90-infected cells, even against a somewhat higher background than was obtained with other procedures (Fig. 2). The protein kinase activity precipitable from cells transformed by LA90 was less than that precipitable from cells transformed by wild-type virus. Whether this reflects the true situation within the infected cells or is due to lability of the protein kinase activity of p60^{src} of LA90 is not known.

NP-40 buffer appears to allow the extraction of immunoprecipitable protein kinase activity from LA90-infected cells because it is less denaturing than RIPA buffer and not because it is more efficient at the extraction of the p60^{src} polypeptide. Equal amounts of p60^{src} were extracted and precipitated from LA90-transformed cells and from SR-RSV-D-transformed cells with either RIPA buffer or NP-40 buffer (data not shown). Cells transformed by these two viruses also contain small and variable quantities of a *src*-related 52K polypeptide (15). The amounts of the 52K polypeptide were much lower than those of p60^{src} and were the same with each buffer used (data not shown). The precipitates prepared in NP-40 buffer from cells infected with LA90 therefore presumably contain p60^{src} which has a significantly higher specific activity than that isolated in RIPA buffer. More polypeptides are precipitated nonspecifically in NP-40 buffer than in RIPA buffer (data not shown). The increased protein kinase activity we detected is however almost certainly not due to extraneous protein kinases present in the precipitate because the p60^{src}-specific activity is very much greater than that measured with either uninfected cells or with normal serum (Fig. 2).

It should be noted that not all mutants derived from SR-RSV-D encode an easily denatured p60^{src}. Cells infected with the related mutant, tsLA92, contained significant amounts of protein kinase activity which was stable to extraction with RIPA buffer. This mutant is, however, much leakier than LA90 or LA91.

FIG. 2. Extraction of protein kinase activity induced by LA90 with NP-40 buffer. Uninfected cells grown at 41°C, cells infected with LA90 and grown at 36 or 41°C, and cells infected with SR-RSV-D and grown at 36°C or 41°C were lysed in NP-40 buffer. Protein kinase activity was immunoprecipitated with an antitumor serum which did not cross-react with p60^{src}. The procedures for lysis, immunoprecipitation, assay for protein kinase and gel electrophoresis are described in the text. These precipitations were performed in antibody excess. (A) Uninfected cells grown at 41°C and immunoprecipitated with antitumor serum; (B) LA90-infected cells grown at 36°C and immunoprecipitated with antitumor serum; (C) SR-RSV-D-infected cells grown at 36°C and immunoprecipitated with antitumor serum; (D) LA90-infected cells grown at 41°C and immunoprecipitated with antitumor serum; (E) SR-RSV-D-infected cells grown at 41°C and immunoprecipitated with antitumor serum; (F) SR-RSV-D-infected cells grown at 41°C and immunoprecipitated with normal rabbit serum.

Protein kinase activity in cells infected with NY68. We and others have previously reported that cells infected with NY68 contain very much more immunoprecipitable protein kinase at the permissive temperature than at the nonpermissive temperature (6, 15). Knowing, however, that RIPA buffer could completely inactivate the protein kinase activity associated with the p60^{src} of LA90, without affecting the

precipitability of the polypeptide itself, we decided to reexamine the question of how much protein kinase activity was actually present in cells infected with NY68.

To ensure that we were assaying the amount of antigen in the cells and not the amount of antibody we used for immunoprecipitation, we first measured the ability of a given amount of antiserum (1 μ l) to precipitate p60^{src}-specific protein kinase activity from cells transformed by SR-RSV-A (Fig. 3). The protein kinase activity detected was proportional to the number of cells in the lysate up to 3.2×10^5 cells per μ l of antiserum and thereafter reached a saturation plateau. Other batches of serum gave approximately similar results. We therefore performed all the precipitations described below with 1 μ l of antiserum for each 2.5×10^4 cells. Extracts prepared by lysis of cells infected with NY68 in NP-40 buffer contained amounts of immunoprecipitable protein kinase nearly comparable to extracts from cells transformed by the wild-type virus. At the permissive temperature, the concentration of immunoprecipitable protein kinase in cells infected with NY68 was indistinguishable from that in wild-type transformed cells (Table 1). At the nonpermissive temperature the concentration of immunoprecipitable protein kinase was approximately 40% of wild type (Table 1). This was much more protein kinase activity than we and others had previously found in phenotypically normal cells infected with NY68. This high level of activity was not peculiar to the batch of serum we have used here. Sera from two other rabbits gave the same result. It was also not due to leakiness of our stock of virus.

FIG. 3. Assay for protein kinase activity. The amount of protein kinase activity in cells transformed by SR-RSV-A was determined by lysing the cells in NP-40 buffer and performing immunoprecipitation with 1 μ l of antitumor serum and an amount of lysate containing the indicated number of cells, in 0.1 ml of NP-40 buffer. The other details of the procedures for immunoprecipitation and incubation with [γ -³²P]ATP are described in the text.

TABLE 1. Protein kinase activity in cells infected with tsNY68 and SR-RSV-A: the effect of lysis buffer^a

Infecting virus	Growth temp (°C)	Kinase activity (cpm/10 ⁵ cells) in lysis buffer:		Rate of 2-deoxyglucose uptake (cpm/ μ g of protein per 10 min)
		NP-40	RIPA	
tsNY68	36	13,123	3,582	71
SR-RSV-A	36	12,672	20,334	116
None	41	228	132	13
tsNY68	41	5,610	534	16
SR-RSV-A	41	14,580	10,950	90

^a All experimental details are described in the text.

The cells infected with NY68 and grown at 41°C were indistinguishable morphologically from uninfected cells. Because estimates of morphological transformation are inherently subjective, we also measured the rate of 2-deoxyglucose uptake by these cultures. Accelerated 2-deoxyglucose uptake has been shown to correlate well with transformation (9, 12). Although obviously not a measure of all aspects of transformation, rates of 2-deoxyglucose uptake are extremely useful in that they are wholly quantitative measurements. Cells infected with NY68 and grown at the nonpermissive temperature had a rate of uptake of 2-deoxyglucose which was very similar to that of uninfected sister cultures and much less than that of transformed cells (Table 1).

To test whether these high levels of protein kinase activity were due to the lysis buffer used here, we measured the protein kinase activity in precipitates from sister cultures prepared by exactly the same procedure, except that RIPA buffer was used (Table 1). Extraction in RIPA buffer reduced the precipitable kinase activity of NY68 markedly. At the permissive temperature, NY68-infected cells had only 18% as much activity as did wild-type transformed cells. At the nonpermissive temperature, the mutant-infected cells had less than 5% as much activity as wild-type transformed cells. Although less dramatic than in the case of LA90, the immunoprecipitable protein kinase activity induced by NY68 appeared to be labile during extraction in RIPA buffer. Furthermore, the protein kinase activity induced by the mutant at the nonpermissive temperature appeared to be noticeably more labile than that induced at the permissive temperature. The large quantitative differences between the values we have obtained here and those we reported previously (16) are probably due, therefore, to the inactivation of the protein kinase of the mutant during extraction. Our earlier experiments differed in one other respect; the precipitations were done in antigen excess.

They measured the specific activity rather than the total activity of $p60^{src}$. This, in fact, has only a minimal effect on the relative values obtained because the concentrations of $p60^{src}$ in cells infected with the wild-type virus and the mutant are in fact very similar.

The protein kinase activity of $p60^{src}$ of NY68 synthesized in vitro. We earlier found that in vitro translation of genomic RNA from NY68 virions resulted in the synthesis of a $p60^{src}$ which was at least 20-fold less active as a protein kinase than that produced by translation of wild-type virion RNA (16). In this experiment we had isolated the in vitro products by immunoprecipitation in RIPA buffer. To examine whether this striking difference in activity which we had measured after immunoprecipitation was a result of the buffer we had used, we repeated the experiment, using instead NP-40 buffer to wash the precipitates. The $p60^{src}$ of NY68 produced by in vitro translation had significant activity when isolated in NP-40 buffer (23% of wild type) and again negligible activity when isolated in RIPA buffer (Fig. 4). These differences in activity reflect differences in the specific activities of the $p60^{src}$'s because the amount of protein immunoprecipitated was the same in each sample. It appears that even the $p60^{src}$ produced by in vitro translation of genomic RNA of wild-type virus is rendered less active by RIPA buffer. This strengthens our belief that the protein kinase levels we measure with NP-40 buffer are not artifactually high, but rather that the values measured with RIPA buffer are artifactually low. $p60^{src}$ synthesized in vitro appears to be especially sensitive to inactivation with RIPA buffer. $p60^{src}$ of wild-type RSV from transformed chick cells is just as active when extracted in RIPA buffer as it is when extracted with NP-40 buffer (data not shown).

Protein kinase activity in cells infected with BK5. Induction of a protein kinase activity which is quite significant at the nonpermissive temperature, 41°C, and very labile during lysis in RIPA buffer is not a property unique to NY68. Using BK5 (11), a temperature-sensitive mutant similar in many properties to NY68, we performed an experiment identical to that presented in Table 1 (Table 2). We obtained quite similar results. Cells infected with BK5 contained 31% as much protein kinase activity at the nonpermissive temperature as did cells transformed by the wild-type virus, when extraction was done with NP-40 buffer, but only 4% as much activity when the extraction was done in RIPA buffer. Again, the high level of activity at 41°C was not due to leakiness; the cells infected with BK5 were phenotypically quite normal at 41°C, as gauged by cell mor-

FIG. 4. Protein kinase activity of $p60^{src}$'s of SR-RSV-A and NY68 synthesized in vitro. Ten-microliter translation reactions containing either SR-RSV-A or NY68 virion RNA at a final concentration of 40 μ g/ml were incubated for 60 min at 30°C before immunoprecipitation with antitumor serum, either in RIPA buffer or NP-40 buffer. The protein kinase activity in the immunoprecipitates was assayed as described in the text. Parallel incubations carried out with [35 S]methionine were analyzed by immunoprecipitation in either RIPA buffer or NP-40 buffer followed by gel electrophoresis. This showed that there were essentially equal amounts of $p60^{src}$ present in all four immunoprecipitations. The figure shows an autoradiogram of the relevant region of the gel of the samples assayed for protein kinase activity. The autoradiogram was exposed for 10 h with a fluorescent screen. (A) SR-RSV-A in vitro products immunoprecipitated in RIPA buffer; (B) SR-RSV-A in vitro products immunoprecipitated in NP-40 buffer; (C) NY68 in vitro products immunoprecipitated in RIPA buffer; (D) NY68 in vitro products immunoprecipitated in NP-40 buffer.

phology and measurements of the rates of 2-deoxyglucose uptake (Table 2).

It is worth pointing out that there are two factors which have possibly affected the levels of activity we have detected in cells infected with mutants. One is the possibility of renaturation of mutant $p60^{src}$ during lysis of the cells at 4°C. We do not know whether this occurs. The

other is renaturation of the mutant $p60^{src}$ caused by the binding of the antibody. We have done these experiments with several different sera which we know recognize different determinants on the molecule and have obtained similar results. Nonetheless, we cannot absolutely exclude this possibility.

Protein kinase activity in uninfected cells. Uninfected chick cells contain a polypeptide, $p60^{src}$, which is a homolog of viral $p60^{src}$ (5, 13). This polypeptide is a 60,000-molecular-weight phosphoprotein which possesses protein kinase activity (13). It is almost certainly encoded by the gene present in normal chick cells which has nucleic acid homology with the viral *src* gene. The concentration of this polypeptide in uninfected chick cells is reported to be 40- to 100-fold less than that of $p60^{src}$ in virally transformed cells (5, 8, 13). Using an antiserum which recognizes the products of both the cellular and the viral *src* genes, we compared the amount of protein kinase activity in rapidly growing uninfected cells, rapidly growing but phenotypically normal cells infected with NY68 at 41°C, and fully transformed cells (Fig. 5). When these cells were gently lysed, the amount of protein kinase activity in uninfected cells was 5% of that in fully transformed cells and 12% of that in cells infected with, but not transformed by, NY68. This is a typical result. We have found that the amount of $p60^{src}$ -associated protein kinase activity varied from 3 to 5% of that in a virally transformed cell. This level was not markedly affected by the growth rate or the density of the cells, as was suggested by the measurements of Collett et al. of the amount of the $p60^{src}$ polypeptide in cells growing at different rates (5).

DISCUSSION

Protein kinase activity is invariably associated with functional $p60^{src}$. We have examined here whether this activity of $p60^{src}$ is essential for transformation by RSV of cells in tissue culture.

TABLE 2. Protein kinase activity in cells infected with tsBK5 and SR-RSV-A: the effect of lysis buffer^a

Infecting virus	Growth temp (°C)	Kinase activity cpm/10 ⁵ cells in lysis buffer:		Rate of 2-deoxyglucose uptake (cpm/μg of protein per 10 min)
		NP-40	RIPA	
tsBK5	36	17,286	3,810	48.0
SR-RSV-A	36	24,126	18,054	50.5
None	41	396	144	9.6
tsBK5	41	5,340	354	10.4
SR-RSV-A	41	16,326	14,928	62.0

^a All experimental details are described in the text.

It is clear from our experiments that the protein kinase activity of $p60^{src}$ encoded by at least four mutants temperature sensitive for transformation is very sensitive to inactivation by a combination of NP-40, SDS, and deoxycholate. A firm correlation therefore exists between a labile protein kinase activity and a thermosensitivity in the ability of these mutants to transform. These results, together with previous data, suggest very strongly that the protein kinase activity of $p60^{src}$ is essential for transformation. These experiments do not, however, test whether this activity is sufficient for transformation.

We have compared the total amount of protein kinase activity associated with $p60^{src}$ in cells infected with mutants temperature-sensitive for transformation at the permissive and the nonpermissive temperatures. We made two quite unexpected observations. First, cells transformed by either LA90 or LA91 contain apparent amounts of protein kinase activity much lower than that of cells transformed by wild-type virus. Secondly, cells infected with NY68 and BK5 contain at the nonpermissive temperature very significant amounts of protein kinase activity, at times as great as 40% of that of wild-type transformed cells. The actual protein kinase levels in mutant-infected cells therefore do not correlate nearly as well with the temperature-sensitive property of these mutants as does the lability of this activity after cell lysis.

Obviously the simple idea that transformed cells contain a high level and normal cells a low level of protein kinase activity, as measured by phosphorylation of immunoglobulin, is incorrect. Either our thinking is too unsophisticated or the assay for protein kinase used here, phosphorylation in the immunoprecipitate, is inappropriate. We will consider the former possibility first. If one accepts, for the purposes of this discussion, that the high protein kinase levels we have detected in cells infected with NY68 or BK5 at the nonpermissive temperature are accurate, there are two explanations which can reconcile these data with the idea that this protein kinase activity is indeed essential for transformation.

First, it may be that the transformation caused by $p60^{src}$ is an exquisitely quantitative process. A level of protein kinase activity 40% of that in a virally transformed cell may be below some critical threshold below which no measurable transformation occurs. There is reason to believe that such a threshold may exist. It seems likely that the rASV's isolated by Hanafusa and colleagues (7, 8, 18) have regained the ability to transform cells by acquisition of at least part of the gene which encodes endogenous $p60^{src}$. This

FIG. 5. Protein kinase activity in uninfected chick cells. Uninfected chick cells, cells infected with NY68, and cells infected with SR-RSV-A were grown at 41°C for 72 h. The cells were then lysed in NP-40 buffer and $p60^{src}$ and $p60^{src}$ were precipitated by using an excess of an antitumor antiserum which reacts with both polypeptides. The procedures for the immunoprecipitation, protein kinase assay, and gel electrophoresis are described in the text. All precipitations were done in duplicate. The four tracks labeled UN were prepared from a lysate of uninfected cells, the four labeled NY68 were from a lysate of phenotypically normal NY68-infected cells, and the four labeled SR-A were from a lysate of SR-RSV-A-transformed cells. In each case, the two tracks labeled N were prepared with normal rabbit serum and the two labeled T were prepared with antitumor antiserum.

suggests that endogenous $p60^{src}$ may cause transformation when expressed at the elevated levels characteristic of a virally transformed cell. Completely normal uninfected chick cells contain the $p60^{src}$ polypeptide at a concentration of at least 1 to 2.5% of that of $p60^{src}$ in a virally transformed cell (5, 13), and an amount of protein kinase activity which is 3 to 5% of that in a virally transformed cell. Although it could be argued that endogenous $p60^{src}$ does not transform a normal cell because of a fundamental and as yet undetected difference between it and viral $p60^{src}$, it can equally well be argued that uninfected cells, like cells infected with temperature-sensitive mutants at the nonpermissive temperature, simply do not contain so much of this specific protein kinase that they undergo transformation. Second, there may be an activity or a function of $p60^{src}$, in addition to the protein kinase activity, which is also essential for transformation. Inability of the mutants to transform at the nonpermissive temperature could then be due to loss of this second hypothetical function, which we are not measuring, rather than to the reduction in protein kinase activity.

It could also be argued that our results do not reflect the activity of $p60^{src}$ in a transformed cell

because the assay in the immunoprecipitate is artificial. It is possible that the $p60^{src}$'s of NY68 and BK5 are functional in the transfer of phosphate from ATP to protein at the nonpermissive temperature but have lost the ability to bind to their normal substrates. A lesion such as this would not be detected by measuring protein kinase activity in an immunoprecipitate because, there, even a mutant $p60^{src}$ is bound essentially irreversibly to its polypeptide substrate.

We feel, however, that the assay in the immunoprecipitate does detect a biologically important activity. Ambros et al. (1) observed that at least one manifestation of transformation, surface membrane ruffling, was apparent within 30 min of shifting NY68-infected cells from the nonpermissive temperature to the permissive temperature in the presence of an inhibitor of protein synthesis. This observation suggests that renaturation of some activity of $p60^{src}$ which is involved in transformation occurs under these conditions. By using the assay in the immunoprecipitate, we (manuscript in preparation) and others (R. Friis, personal communication) have found that an immediate increase in protein kinase activity accompanies the shift of these cells to the permissive temperature in the ab-

sence of protein synthesis. This result suggests that the protein kinase assay in the immunoprecipitate actually measures an essential activity of p60^{src} which is affected by the temperature-sensitive lesion. This experiment in particular gives us confidence that the values we have reported here are biologically significant.

Our results with LA90 exemplify the real usefulness of the assay in the immunoprecipitate. The protein kinase activity of LA90 is so labile that it is difficult to detect the activity even in fully transformed cells. Isolation of p60^{src} by more traditional means would almost certainly completely inactivate the protein kinase activity of the mutant. The low levels we have detected are probably minimum estimates and it is likely that the mutant proteins have lost some of their activity even during rapid isolation. There may be no better way to estimate the amount of protein kinase activity present in intact cells infected with temperature-sensitive mutants than by immunoprecipitation and assay of protein kinase in the immunoprecipitate.

Our value for the amount of protein kinase activity in uninfected cells, 3 to 5% of that in virally transformed cells, is comparable to the values obtained by others for the relative concentrations of the endogenous p60^{src} and viral p60^{src} polypeptides (5, 8, 13). Together these results suggest that the specific activity of p60^{src} as a protein kinase, as measured by phosphorylation in the immunoprecipitate, is similar to that of viral p60^{src}. Karess et al. (8) have reported, however, that the specific activity of endogenous p60^{src} is approximately 100-fold less than that of viral p60^{src}. There are several possible explanations for this discrepancy. One is that Karess et al. (8) may have used an antiserum which is a poor substrate for p60^{src}. We have obtained one such serum. It is able to precipitate efficiently both p60^{src} and p60^{src} but will serve as a good substrate for the protein kinase activity of only p60^{src}. Another is that the NP-40 buffer we have used may allow the extraction of p60^{src} which has a greater specific activity than does that extracted with modified RIPA buffer (8).

We interpret the lability of the protein kinase activities of five mutants which are temperature sensitive for transformation as demonstration that this activity is essential for transformation. For the reasons just discussed, we feel that our measurements of high protein kinase levels in cells infected with some temperature-sensitive mutants at the nonpermissive temperature may well be accurate. Taken together, these results suggest that the possibility that there is a viral function in addition to the protein kinase activity which is essential for transformation and the

possibility that the transformed phenotype of a chick cell is dependent critically on the actual concentration of *src*-encoded protein kinase activity must both be considered seriously.

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