JOURNAL OF VIROLOGY, Apr. 1979, p. 311-318
0022-538X/79/04-0311/08$02.00/0

Vol. 30, No. 1

Product of In Vitro Translation of the Rous Sarcoma Virus *src* Gene Has Protein Kinase Activity

BARTHOLOMEW M. SEFTON,* TONY HUNTER, AND KAREN BEEMON

Tumor Virology Laboratory, The Salk Institute, San Diego, California 92112

Received for publication 13 November 1978

In vitro translation of Rous sarcoma virus virion RNA resulted in the synthesis of a protein kinase which, when immunoprecipitated with antitumor serum, phosphorylated the immunoglobulin heavy chain. Even though in vitro translation of virion RNA resulted in the synthesis of a number of polypeptides which were recognized by antitumor serum, control experiments demonstrated that an immunoprecipitable protein kinase activity was found only when an immunoprecipitable $p60^{src}$, the polypeptide product of the *src* gene, was synthesized. A protein kinase with similar properties was therefore intimately associated with $p60^{src}$ which was synthesized in vitro in the reticulocyte lysate, just as it is with $p60^{src}$ which is obtained from transformed chick and mammalian cells. It is therefore highly unlikely that this association is artifactual. ts NY68 is a mutant of Rous sarcoma virus which is able to transform cells at 36 but not at 41°C. In vitro translation of ts NY68 virion RNA at 30°C resulted in efficient synthesis of immunoprecipitable $p60^{src}$, but very inefficient synthesis of an immunoprecipitable protein kinase. The $p60^{src}$ obtained by in vitro translation of wild-type virion RNA was more than 20-fold more active as a protein kinase than was that obtained from ts NY68 RNA. The correlation in the case of ts NY68 of a deficiency in protein kinase activity with an inability to transform cells at high temperature suggests that the protein kinase activity associated with $p60^{src}$ is indeed critical to cellular transformation.

Transformation of chick fibroblasts by avian sarcoma viruses requires the function of the *src* gene. In both avian and mammalian cells transformed by avian sarcoma viruses, this gene is expressed as a 60,000-dalton protein, $p60^{src}$ (2, 4, 5, 8, 9a, 12, 13). Rabbits carrying tumors induced by neonatal injection of Schmidt-Ruppin (SR) Rous sarcoma virus (RSV) have antibodies which precipitate $p60^{src}$ from lysates of cells transformed by some but not all avian sarcoma viruses (5, 13). Collett and Erikson (6) have demonstrated that immunoprecipitates prepared with such antisera from cells transformed by SR RSV contain a protein kinase activity which causes the phosphorylation of the heavy chain of the immunoglobulin in the immunoprecipitate. This most significant observation suggests that protein phosphorylation may be the molecular basis of the neoplastic transformation of cells by avian tumor viruses.

It is important to exclude the possibility that the protein kinase activity associated with immunoprecipitated $p60^{src}$ is due to an adventitiously bound cellular enzyme present in transformed cells. We have approached this problem by the preparation of immunoprecipitates which contain $p60^{src}$ synthesized in vitro in a rabbit reticulocyte lysate. Translation of avian sarcoma virus virion RNA in the nuclease-treated reticulocyte lysate results in the synthesis of a 60,000-dalton polypeptide which is biochemically and immunologically almost indistinguishable from $p60^{src}$ found in transformed cells. Using the assay described by Collett and Erikson (6), we have found that the $p60^{src}$ which is synthesized in vitro also has associated with it protein kinase activity.

Several mutants of SR RSV subgroup A (SR RSV-A) have been isolated which are temperature sensitive for both the initiation and the maintenance of transformation (9, 10). Collett and Erikson (6) have demonstrated that the immunoprecipitates prepared from cells transformed by one such mutant, ts NY68 (9), contain much more protein kinase activity than do immunoprecipitates prepared from sister cultures grown at the nonpermissive temperature. This established that the efficiency with which a protein kinase activity can be precipitated from cells transformed by such a mutant is temperature sensitive. However, this observation is not a direct demonstration that the $p60^{src}$ encoded

by ts NY68 is inactive as a protein kinase at the nonpermissive temperature. In preliminary experiments we found that the protein kinase activity present in immunoprecipitates from cells transformed by ts NY68 was no more thermolabile than the activity prepared in a similar manner from cells transformed by the parent virus, SR RSV-A. We have therefore addressed the question of the temperature sensitivity of the protein kinase associated with the $p60^{src}$ of ts NY68 by comparison of the specific activities of the protein kinase produced by in vitro translation of SR RSV-A and ts NY68 virion RNAs as well as the immunoprecipitable protein kinase activities in cells transformed by SR RSV-A and ts NY68.

MATERIALS AND METHODS

Cells and viruses. All viruses were grown in primary or secondary cultures of chicken embryo fibroblasts prepared from embryonated eggs obtained from SPAFAS, Norwich, Conn. The SR RSV-A and the mutant derived from it, ts NY68, were obtained originally from H. Hanafusa, Rockefeller University. B77 virus was obtained from M. Halpern, Wistar Institute, and originated in the laboratory of P. Vogt, University of Southern California. PR RSV-C (stock 1) was obtained from M. Stone, Duke University. PR RSV-C (stock 2) was obtained through the Viral Oncology Program of the National Cancer Institute. We have not determined directly whether these two stocks of virus are indeed of the Prague strain and of subgroup C. Both encode $p60^{src}$ proteins which are essentially identical in size to that of PR RSV-B (3). Stock 1 encodes a $p60^{src}$ which is precipitated by our antisera and which is closely related by two-dimensional peptide mapping to the $p60^{src}$ of SR RSV-A (3). Stock 2 encodes a $p60^{src}$ which is not precipitated by our antisera and which is closely related to that of PR RSV-B by two-dimensional peptide mapping (3). td SR RSV-D was obtained from M. Lai, University of Southern California. ts BK5 and its SR RSV-A parent were obtained from G. S. Martin, University of California, Berkeley.

Purification of viruses, extraction of RNA, and in vitro translation. The procedures for the growth and purification of the viruses, the extraction and isolation of 70S RNA, and the general method of in vitro translation in the mRNA-dependent reticulocyte lysate have all been described previously (2, 13).

Immunoprecipitation and protein kinase assay. For the experiments shown in Fig. 1, a 115-μl in vitro translation reaction containing 3 μg of heat-denatured RSV virion 70S RNA or 0.6 μg of tobacco mosaic virus RNA was set up. A 15-μl portion of each reaction mixture was removed and mixed with 8 μCi of [^{35}S]methionine (Amersham/Searle; specific activity, >500 Ci/mmol). Both parts of each reaction were then incubated for 45 min at 30°C. At this time, 5 μl of the sample containing [^{35}S]methionine was removed for direct analysis of the in vitro translation products. The remaining samples were diluted 10-fold with RIPA buffer (1% Nonidet P-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 0.15 M NaCl, 0.01 M sodium phosphate, pH 7.2, 1% Trasylol) and then divided in half for immunoprecipitation with normal and antitumor sera. A 2-μl amount of serum, which had been heated to 56°C for 30 min to inactivate endogenous kinase activity, was used per 500 μl of diluted reaction. Immunoprecipitation with fixed *Staphylococcus aureus* to precipitate the primary antibody was carried out as described previously (13), up to the last wash in RIPA buffer. At this point, the [^{35}S]methionine-labeled samples were dissolved in 25 μl of solubilizing buffer (14), and 10 μl was analyzed on a 15% acrylamide–0.09% bisacrylamide–sodium dodecyl sulfate gel. The precipitates in the samples being used for measurement of kinase activity were resuspended in 0.5 ml of 0.15 M NaCl in 0.01 M sodium phosphate, pH 7.2, transferred to a microcentrifuge tube, and pelleted in a microcentrifuge at 4°C. The precipitates were washed once with 0.3 ml of 0.15 M NaCl in 0.01 M sodium phosphate, pH 7.2, pelleted again, and suspended in 20 μl of buffer containing 0.1 M sodium phosphate, pH 6.8, 0.005 M $MgCl_2$, 10 μCi of [γ-^{32}P]ATP (specific activity, >2,500 Ci/mmol; New England Nuclear Corp.). The samples were then incubated at 30°C for 10 min before being diluted with 300 μl of cold RIPA buffer. The precipitates were pelleted again, and the supernatant was aspirated. The immunoprecipitates were dissolved in 25 μl of solubilizing buffer and boiled for 1 min, and the bacteria were pelleted. A 10-μl amount of the supernatant was analyzed in each case on a 12.5%–acrylamide–0.1% bisacrylamide–sodium dodecyl sulfate gel.

The experiment in Fig. 2 was carried out in a fashion similar to that described above, except that the starting mixtures were 35 μl in volume and contained 1 μg of either SR RSV-A or ts NY68 heat-denatured 70S virion RNA. A 5-μl amount was removed and mixed with 2 μCi of [^{35}S]methionine. At the end of the reaction, 2 μl of the [^{35}S]methionine-containing incubations was removed for direct analysis. The remaining volumes were subjected to immunoprecipitation with antitumor serum only, as described above. The kinase reaction and gel analysis were also as outlined above.

For the experiment shown in Table 1, primary chicken embryo fibroblasts were infected with SR RSV-A or ts NY68 and incubated for 3 days at 37°C. These cells and uninfected sister cultures were then transferred to 35-mm dishes at a density of 5×10^5 cells per dish and incubated at either 36 or 41°C in Dulbecco modified Eagle medium supplemented with 2% tryptose phosphate broth, 4% calf serum, and 1% heat-inactivated chick serum. The medium was changed at 24 and 48 h to Dulbecco modified Eagle medium supplemented with 2% tryptose phosphate broth and 4% calf serum. At 48 h, both sets of cultures infected with SR RSV-A and the cultures infected with ts NY68 and incubated at 36°C were morphologically fully transformed. The cultures infected with ts NY68 which had been maintained at 41°C were morphologically indistinguishable from the uninfected cells. Appropriate cultures were incubated in complete fresh medium supplemented with [^{35}S]methionine (100 μCi/ml) from 48 to 68 h after transfer. At 68 h, all cells were dissolved in RIPA buffer at a concentration

of 1.35×10^6 cells per 0.1 ml. A 10-μl amount of each [^{35}S]methionine-labeled lysate was assayed directly for acid-precipitable radioactivity. [^{35}S]methionine-labeled extracts derived from 1.1×10^6 cells were immunoprecipitated with 2 μl of either heat-inactivated antitumor serum or normal serum. One-fifth of the immunoprecipitate in each case was analyzed on a 15% acrylamide–0.09% bisacrylamide–sodium dodecyl sulfate gel. For assay of kinase activity, extracts derived from 3.1×10^6 cells were precipitated with 2 μl of either heat-inactivated antitumor serum or normal serum. The kinase reaction was carried out exactly as described above for the in vitro samples, except that one-fifth of the immunoprecipitate of each sample was analyzed.

Uptake of 2-deoxyglucose. Uptake of 2-deoxyglucose was measured as described by Martin et al. (11), with minor modifications. Before labeling, the cells were washed twice with phosphate-buffered saline containing 1% calf serum. Labeling was for 10 min at 37°C in 1 ml of the above solution containing 2 μCi of [^{3}H]-labeled 2-deoxyglucose (Amersham/Searle; 17.5 Ci/mmol). After labeling, the cells were washed four times with serum-free phosphate-buffered saline, and radioactivity and protein content were determined as described (11).

RESULTS

Synthesis of protein kinase activity in vitro. Chick cells transformed by SR RSV-A, SR RSV-D, and one stock of PR RSV-C (stock 1) contain p60^{src} which is precipitable by several antisera which we have obtained from rabbits carrying tumors induced by SR RSV-D (13). In agreement with the observations of Collett and Erikson (6), the immunoprecipitates prepared with these sera from cells transformed by SR RSV-A (data not shown) and SR RSV-D (Fig. 1A) contained a protein kinase activity which could phosphorylate the heavy chain within the immune complex. In vitro translation of 70S RNA from SR RSV-A, SR RSV-D, and PR RSV-C (stock 1) results in the synthesis of a p60^{src} which can be precipitated by our sera and which has been shown by peptide mapping to be related to the p60^{src} present in the respective transformed cells (13). We therefore examined whether in vitro translation resulted in the synthesis of an immunoprecipitable protein kinase activity.

Heat-denatured 70S RNAs from SR RSV-D and PR RSV-C (stock 1) were translated in the nuclease-treated reticulocyte lysate. The in vitro products were immunoprecipitated, and the precipitates were tested for the presence of a protein kinase which would phosphorylate the immunoglobulin heavy chain. The precipitates prepared with antitumor serum both contained such protein kinase activity, whereas the precipitates prepared with normal serum did not (Fig. 1A). Analysis of the immunoprecipitates of the labeled polypeptides resulting from translation of these RNAs in the presence of [^{35}S]methionine in parallel (Fig. 1B) showed that significant amounts of a 60,000-dalton polypeptide were precipitated by the antitumor serum in both cases. We have previously shown by peptide mapping that this polypeptide is essentially identical to p60^{src} present in transformed cells (13). We also found that the translation products of 24S polyadenylated SR RSV-D virion RNA, which, as the immunoprecipitate in Fig. 1B shows, consisted almost entirely of *src*-related polypeptides, contained an immunoprecipitable protein kinase (Fig. 1A).

Several control experiments were performed to determine whether the kinase activity was due to the presence of p60^{src} or some other protein in the precipitate. First, we prepared precipitates with antitumor serum from translation reactions containing no added RNA or tobacco mosaic virus genomic RNA. These did not have protein kinase activity (Fig. 1A). Second, we translated heat-denatured 70S RNA from td SR RSV-D clone 21, a transformation-defective virus with a large deletion in the *src* gene, from B77 virus, and from another stock of PR RSV-C (stock 2). Translation of RNA from td SR RSV-D does not yield a p60^{src} (2, 12), whereas translation of RNA from B77 virus and PR RSV-C (stock 2) results in the synthesis of a p60^{src} which is clearly related to that of SR RSV-D by peptide mapping, but which is not immunoprecipitable with the antisera used in these experiments (3). Precipitates prepared from these incubations contained no protein kinase activity (Fig. 1A). Analysis of the labeled polypeptides resulting from translation of these RNAs in the presence of [^{35}S]methionine in parallel showed the expected presence of the 76,000-dalton precursor to the internal structural proteins of the virus, a major translation product of genomic RNA, which is brought down by the antitumor serum. No protein corresponding to p60^{src} was precipitated from the translation products of these three viral RNAs (Fig. 1B). In the case of B77 virus and PR RSV-C (stock 2), this negative result was not due to a failure to synthesize p60^{src}. The p60^{src} was obvious among the [^{35}S]methionine-labeled products before precipitation (data not shown). The absence of precipitable protein kinase activity in these controls makes it very unlikely that the immunoprecipitable protein kinase we have observed with SR RSV-A (see below), SR RSV-D, and PR RSV-C (stock 1) is due either to the polypeptides encoded by the *gag*, *pol*, or *env* genes or to an endogenous protein kinase of the reticulocyte

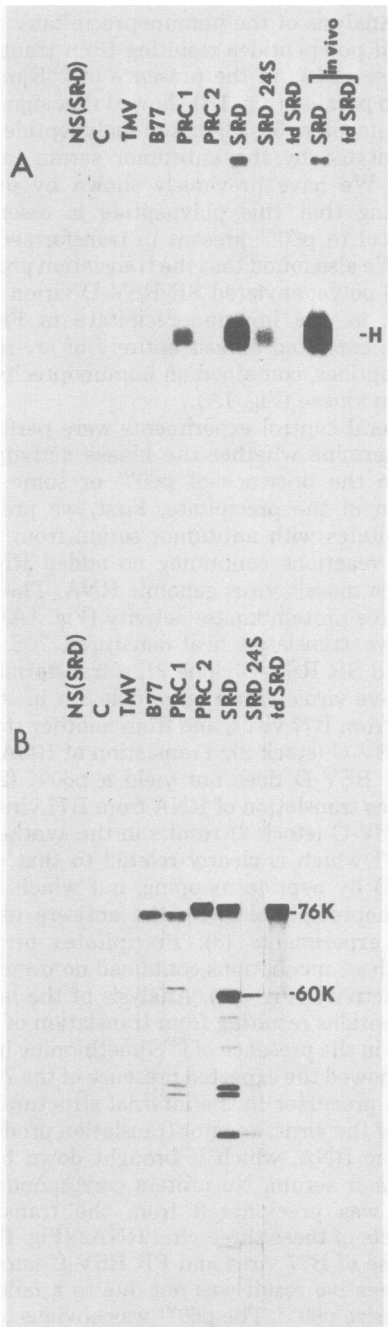

FIG. 1. *Assay for protein kinase activity in in vitro translation products of RSV virion RNAs. The translation reactions, immunoprecipitations, and protein kinase assays were carried out as described in the text. The abbreviations are as follows: C, control, no added RNA; TMV, tobacco mosaic virus genomic RNA; PR-C 1, PR RSV-C (stock 1) virion RNA; PR-C 2, PR RSV-C (stock 2) virion RNA; SR-D, SR RSV-D virion RNA; SR-D 24S, polyadenylated 24S RNA isolated from SR RSV-D virion RNA; td SR-D, td SR RSV-D virion RNA. SR-D and td SR-D in vivo were protein kinase assays performed on cells infected with SR RSV-D and td SR RSV-D, respectively, as described in the text for cells infected with SR RSV-A. All of the samples were precipitated with antitumor serum except the most lefthand tracks [NS(SR-D)]. These tracks show the results of precipitation of the in vitro translation products of SR RSV-D virion RNA with normal serum. Because all of the precipitations with normal serum looked identical, only a single example is shown here. The analyses of the immunoprecipitates of [^{35}S]methionine-labeled SR RSV-D- and td SR RSV-D-infected cells are not shown here. In (A), H refers to the position of the immunoglobulin heavy chain. In (B), the positions of pr76^{gag} (76K) and p60^{src} (60K) are indicated. Each part of the figure is a montage of different tracks from a single gel. The gel in (A) was exposed for 24 h with a fluorescent screen, whereas the fluorogram in (B) was exposed for 100 h. (A) Protein kinase assay; (B) analysis of [^{35}S]methionine-labeled immunoprecipitates.*

lysate. This is also borne out by the result with 24S polyadenylated genomic RNA of SR RSV-D, where we observed synthesis of *src*-related polypeptides but little synthesis of other viral polypeptides.

Temperature sensitivity of the p60^{src}-associated protein kinase. If a virally coded protein kinase is essential for the transformation of cells, it would be reasonable to expect a virus temperature sensitive in its ability to transform cells to encode a temperature-sensitive protein kinase. We examined this first by comparing the thermolability of the protein kinase precipitated by antitumor serum from cells transformed by ts NY68 and ts BK5 (another SR RSV-A mutant temperature sensitive for transformation [10]) with that from cells transformed by the respective parental viruses of these mutants. Precipitates prepared from cells grown at the permissive temperature were incubated at 45°C for various intervals, and the surviving ability to phosphorylate the immunoglobulin in the precipitate was then measured. The rates of inactivation of the protein kinase activities prepared from cells transformed by the two mutant viruses were essentially indistinguishable from those of the respective parental wild-type viruses (data not shown).

It seemed possible that any difference in the thermolability of the protein kinase activities encoded by the wild-type and mutant viruses might be obscured when the proteins were immobilized in an immune complex. We therefore attempted to compare the thermolability, before immunoprecipitation, of the protein kinase activities arising from translation of RNA from SR

RSV-A and ts NY68. Heat-denatured 70S RNAs from SR RSV-A and ts NY68 were translated in parallel at 30°C, and both the amount of protein kinase activity and the amount of p60^{src} labeled with [^{35}S]methionine, which could be immunoprecipitated, were quantified by densitometry of the relevant autoradiograms (Fig. 2). RNA from both ts NY68 and SR RSV-A directed the efficient synthesis of p60^{src} (data not shown). The efficiency of precipitation of the p60^{src} from the translation products was essentially the same for SR RSV-A and ts NY68 (about 15% in both cases). In the experiment shown in Fig. 2 slightly more p60^{src} was made from ts NY68 RNA than from SR RSV-A RNA, as judged by immunoprecipitation. In contrast, however, protein kinase activity was barely detectable in the immunoprecipitate of the translation products of ts NY68, although it was readily detectable in the precipitate from the lysate incubated with SR RSV-A (Fig. 2A). The difference in the specific activities of the two preparations of protein kinase was more than 20-fold, where specific activity is defined as the ratio of immunoprecipitable protein kinase activity to the amount of immunoprecipitable p60^{src}. This result was somewhat surprising because in vitro synthesis was performed at 30°C, a temperature much lower than the permissive temperature for cellular transformation. We have also tried translation at temperatures lower than 30°C and for times shorter than 45 min, but neither approach yielded significant protein kinase activity in the case of ts NY68. It is interesting to note that ts NY68 p60^{src} synthesized in vitro attains normal antigenic reactivity without corresponding enzymatic activity.

Knowing that the p60^{src} of ts NY68 synthesized in vitro at 30°C was much less active as a protein kinase than that of SR RSV-A, we then carefully compared the specific activity of p60^{src} present in cells transformed by these two viruses at both the permissive and nonpermissive temperatures. At the same time, we assessed the degree of transformation by measuring the rate of 2-deoxyglucose uptake. The amount of immunoprecipitable p60^{src} per cell, as measured by the incorporation of [^{35}S]methionine for 20 h, was rather similar in cells transformed by ts NY68 and SR RSV-A at both the permissive temperature of 36°C and the nonpermissive temperature of 41°C (Table 1). In contrast, we found that the amount of immunoprecipitable protein kinase was 2- to 3-fold lower in cells transformed by ts NY68 at 36°C and approximately 10-fold lower in cells transformed by ts NY68 at 41°C (Table 1). Thus, even in fully transformed cells,

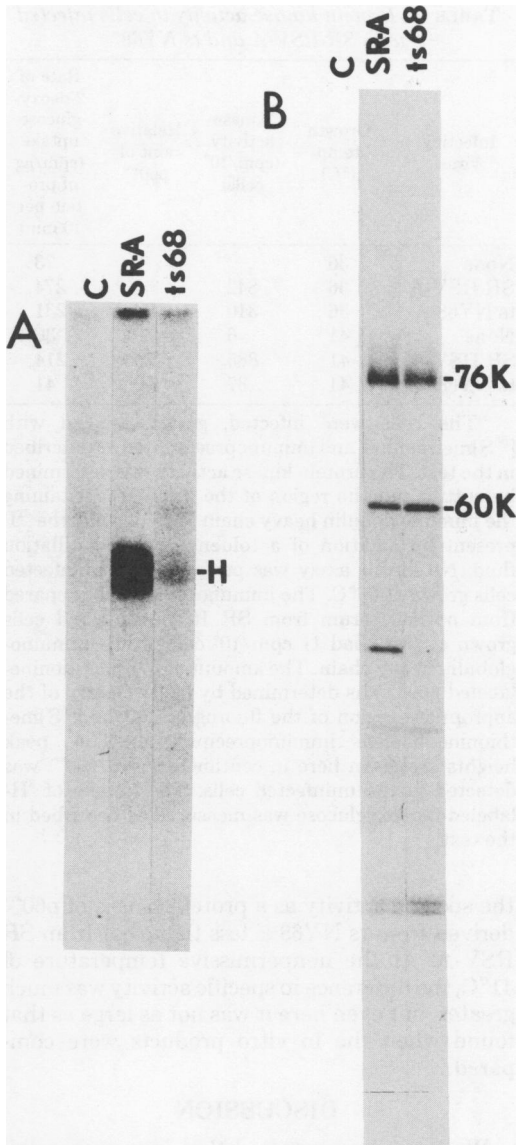

FIG. 2. *Assay for protein kinase activity in in vitro translation products of SR RSV-A and ts NY68 RNAs. The translation reactions, immunoprecipitations, and protein kinase assays were carried out as described in the text. The abbreviations are as follows: C, control, no added RNA; SR-A, SR RSV-A virion RNA; ts68, ts NY68 virion RNA. In (A), H refers to the position of the immunoglobulin heavy chain. In (B), the positions of pr76^{gag} (76K) and p60^{src} (60K) are indicated. Each part of the figure is a montage of different tracks from a single gel. The gel in (A) was exposed for 48 h, whereas the fluorogram in (B) was exposed for 90 h. (A) Protein kinase assay; (B) analysis of [^{35}S]methionine-labeled immunoprecipitates.*

TABLE 1. *Protein kinase activity in cells infected with SR RSV-A and ts NY68*[a]

Infecting virus	Growth temp (°C)	Kinase activity (cpm/10^6 cells)	Relative amt of $p60^{src}$	Rate of 2-deoxyglucose uptake (cpm/μg of protein per 10 min)
None	36		0	73
SR RSV-A	36	842	3.3	274
ts NY68	36	310	4.2	231
None	41	6	0	36
SR RSV-A	41	885	3.9	214
ts NY68	41	87	3.0	41

[a] The cells were infected, grown, labeled with [^{35}S]methionine, and immunoprecipitated as described in the text. The protein kinase activity was determined by cutting out the region of the dried gel containing the immunoglobulin heavy chain and counting the ^{32}P present by addition of a toluene-based scintillation fluid. No kinase assay was performed on uninfected cells grown at 36°C. The immunoprecipitate prepared from normal serum from SR RSV-A-infected cells grown at 41°C had 11 cpm/10^6 cells in the immunoglobulin heavy chain. The amount of [^{35}S]methionine-labeled $p60^{src}$ was determined by densitometry of the appropriate region of the fluorogram of the [^{35}S]methionine-labeled immunoprecipitates. The peak heights are given here in centimeters. No $p60^{src}$ was detected in the uninfected cells. The uptake of ^{3}H-labeled 2-deoxyglucose was measured as described in the text.

the specific activity as a protein kinase of $p60^{src}$ derived from ts NY68 is less than that from SR RSV-A. At the nonpermissive temperature of 41°C, the difference in specific activity was much greater, but even here it was not as large as that found when the in vitro products were compared.

DISCUSSION

We have demonstrated that immunoprecipitates which contain $p60^{src}$ synthesized by in vitro translation of RSV virion RNA contain a protein kinase which specifically phosphorylates the immunoglobulin heavy chain. Collett and Erikson (6) have shown previously that immunoprecipitates which contain $p60^{src}$ from cells transformed by RSV contain a similar protein kinase. Even though in vitro translation of RSV virion RNA results in the synthesis of a number of polypeptides which are precipitated by antitumor serum (13), it is clear for three reasons that only those precipitates which contain $p60^{src}$ have protein kinase activity. First, translation products of td SR RSV-D virion RNA are similar to those produced from SR RSV-D virion RNA, except that those coded by the *src* gene are absent (2). Precipitates of the translation products of td SR RSV-D virion RNA with antitumor serum do not contain protein kinase activity. Second, PR RSV-C (stock 2) and B77 virus encode a $p60^{src}$ which is synthesized efficiently in vitro, but which cannot be precipitated by our antitumor sera (3). No protein kinase activity is precipitated from the translation products of RNA from these viruses. And third, the major product of translation of 24S polyadenylated RNA from SR RSV-D virions is $p60^{src}$. The synthesis of $pr76^{gag}$ and other viral polypeptides can barely be detected. Translation products of this RNA do contain a precipitable protein kinase activity. Because the immunoprecipitation of $p60^{src}$ synthesized either in the rabbit reticulocyte lysate or in transformed chick cells results in the precipitation of protein kinases having apparently identical properties, it is quite unlikely that this activity is due merely to adventitious binding of a nonspecific kinase. This conclusion is supported by the finding that a similar protein kinase can be immunoprecipitated from hamster, field vole, and rat cells (6, 9a) transformed by SR RSV. It seems clear that $p60^{src}$ is either a protein kinase itself or functions as a subunit of one protein kinase present in transformed fibroblasts and of another present in rabbit reticulocytes.

There is, however, no compelling reason to believe that $p60^{src}$ is a noncatalytic subunit of a protein kinase rather than a protein kinase itself. Only one polypeptide besides $p60^{src}$ is specifically precipitated from transformed chick cells by antitumor sera. This protein is 80,000 daltons in size, is unrelated to $p60^{src}$, as determined by peptide mapping, and is precipitated presumably because it is associated with $p60^{src}$ (13). Three observations suggest, however, that this polypeptide is not responsible for the protein kinase activity seen in the immunoprecipitates. First, the precipitation of $p60^{src}$ from phenotypically normal cells infected with ts NY68 at 41°C results in the efficient co-precipitation of the 80,000-dalton polypeptide (data not shown) but very inefficient precipitation of a protein kinase activity (Table 1). Second, mammalian cells transformed by RSV do not contain a precipitable polypeptide obviously analogous to the 80,000-dalton polypeptide (Sefton, unpublished data), even though they do contain significant amounts of immunoprecipitable protein kinase activity (6; Sefton, unpublished data). And third, no newly synthesized polypeptide analogous to the 80,000-dalton protein is present in immunoprecipitates of $p60^{src}$ synthesized in vitro (13). It is nevertheless still formally possible that $p60^{src}$

is neither a protein kinase nor a subunit of one, but merely appears to be a protein kinase because it has bound to it small amounts of a very active protein kinase.

We have observed that in vitro translation of ts NY68 RNA at 30°C produces a p60^{src} which is immunoprecipitable but, relative to that of the wild-type SR RSV-A, very deficient in protein kinase activity. This may seem surprising, because it is clear that the ts NY68 p60^{src} has enough activity to transform chick cells at 36°C. It may be that wild-type p60^{src} assumes an enzymatically active conformation somewhat inefficiently when synthesized in vitro in the reticulocyte lysate and that a mutant p60^{src} is at an even greater disadvantage. We found that the p60^{src} encoded by ts NY68 was two- to threefold less active as a protein kinase than was p60^{src} encoded by SR RSV-A in fully transformed cells grown at 36°C. Apparently this difference in activity is magnified in products synthesized in vitro. The observation that the protein kinase obtained by immunoprecipitation from cells transformed by ts NY68 at 36°C is no more thermolabile in the immunoprecipitate than that obtained from cells transformed by SR RSV-A is not necessarily in conflict with our results with p60^{src} synthesized in vitro. It may well be that the protein kinase is stabilized by the antibody to which it is bound or that it is as stable as that of wild type if it is allowed to fold correctly during synthesis at the permissive temperature in a transformed cell.

The temperature dependence of the protein kinase activity of ts NY68 p60^{src} synthesized both in vivo and in vitro supports the idea that the kinase activity of this polypeptide is critical for the transformation of cells by RSV. Thus, it is perhaps surprising that p60^{src} isolated from phenotypically normal ts NY68-infected chick cells actively growing at 41°C has considerable residual kinase activity. This activity could be due to renaturation of inactive p60^{src} molecules during the immunoprecipitation, although our results with the translation products of ts NY68 RNA suggest that this is unlikely. Alternatively, the 10- to 20-fold difference in the total p60^{src}-associated kinase activity at the two growth temperatures may be sufficient to determine the different phenotypes, if, for instance, there is a critical threshold of phosphorylation for one or more proteins involved in transformation. A further possibility is that the deficiency in p60^{src} kinase activity at the nonpermissive temperature is accentuated by a decreased ability of the kinase to interact with its intracellular substrates.

The demonstration that p60^{src} synthesized in vitro from genomic RNA of SR RSV has an associated protein kinase activity has also recently been made by Erikson et al. (7). Although direct proof that p60^{src} catalyzes phosphate transfer from ATP is still lacking, it seems evident that a protein kinase is critical in the transformation of cells by RSV. The identification of the substrates of this protein kinase is of paramount importance to our understanding of the molecular mechanism of transformation. The disruption of the cytoskeleton seen in the transformed state (1, 14), taken together with the cytoplasmic location (12a) of p60^{src}, suggests that at least one substrate of the protein kinase is a protein involved in the organization of the cytoskeleton.

ACKNOWLEDGMENTS

We thank Claudie Berdot for help with the experiments and Ray Erikson for communicating his results before publication.

This work was supported by Public Health Service grants CA-17289, CA-17096, and CA-23896 from the National Cancer Institute.

LITERATURE CITED

1. **Ash, J. F., P. K. Vogt, and S. J. Singer.** 1976. 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. Proc. Natl. Acad. Sci. U.S.A. **73**:3603-3607.
2. **Beemon, K., and T. Hunter.** 1978. Characterization of Rous sarcoma virus *src* gene products synthesized in vitro. J. Virol. **28**:551-566.
3. **Beemon, K., T. Hunter, and B. M. Sefton.** 1979. Polymorphism of avian sarcoma virus *src* proteins. J. Virol. **30**:190-200.
4. **Brugge, J., E. Erikson, M. S. Collett, and R. L. Erikson.** 1978. Peptide analysis of the transformation-specific antigen from avian sarcoma virus-transformed cells. J.Virol. **26**:773-782.
5. **Brugge, J. S., and R. L. Erikson.** 1977. Identification of a transformation-specific antigen from avian sarcoma virus transformed cells. Nature (London) **269**:346-348.
6. **Collett, M. S., and R. L. Erikson.** 1978. Protein kinase activity associated with the avian sarcoma virus *src* gene product. Proc. Natl. Acad. Sci. U.S.A. **75**:2021-2024.
7. **Erikson, E., M. S. Collett, and R. L. Erikson.** 1978. *In vitro* synthesis of a functional avian sarcoma virus transforming gene product. Nature (London) **274**:919-921.
8. **Kamine, J., J. G. Burr, and J. M. Buchanan.** 1978. Multiple forms of *sarc* gene proteins from Rous sarcoma virus RNA. Proc. Natl. Acad. Sci. U.S.A. **75**:366-370.
9. **Kawai, S., and H. Hanafusa.** 1971. The effects of reciprocal changes in temperature on the transformed state of cells infected with a Rous sarcoma virus mutant. Virology **46**:470-479.

9a. **Levinson, A. D., H. Oppermann, L. Levintow, H. E. Varmus, and J. M. Bishop.** 1978. Evidence that the transforming gene of avian sarcoma virus encodes a protein kinase associated with a phosphoprotein. Cell **15**:561-572.

10. **Martin, G. S.** 1970. Rous sarcoma virus: a function required for the maintenance of transformation. Nature (London) **227**:1021-1023.

11. **Martin, G. S., S. Venuta, M. Weber, and H. Rubin.** 1971. Temperature-dependent alterations in sugar transport in cells infected by a temperature-sensitive mutant of Rous sarcoma virus. Proc. Natl. Acad. Sci. U.S.A. **68**:2739–2741.
12. **Purchio, A. F., E. Erikson, J. S. Brugge, and R. L. Erikson.** 1978. Identification of a polypeptide encoded by the avian sarcoma virus *src* gene. Proc. Natl. Acad. Sci. U.S.A. **75**:1567–1571.
12a. **Rohrschneider, L. R.** 1979. Immunofluorescence on avian sarcoma virus-transformed cells: localization of the *src* gene product. Cell **16**:11–24.
13. **Sefton, B. M., K. Beemon, and T. Hunter.** 1978. Comparison of the expression of the *src* gene of Rous sarcoma virus in vitro and in vivo. J. Virol. **28**:957–971.
14. **Weber, K., E. Lazarides, R. D. Goldman, A. Vogel, and R. Pollack.** 1974. Localization and distribution of actin fibers in normal, transformed and revertant cells. Cold Spring Harbor Symp. Quant. Biol. **39**:363–369.