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# Protein kinase activity associated with the avian sarcoma virus *src* gene product

(*src* gene product p60$^{src}$/immunoprecipitation/avian sarcoma virus temperature-sensitive mutant/avian sarcoma virus-transformed mammalian cells/[$\gamma$-$^{32}$P]ATP)

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ABSTRACT **Incorporation of phosphorus from [$\gamma$-$^{32}$P]ATP into protein was catalyzed by specific immunoprecipitates from avian sarcoma virus (ASV)-transformed avian and mammalian cells. This incorporation was observed only when antiserum from tumor-bearing rabbits able to specifically precipitate the ASV sarcoma gene product, p60$^{src}$, was used to immunoprecipitate antigens from transformed cell lysates. Immunoprecipitates of extracts from normal cells or cells infected with a transformation-defective ASV mutant showed no activity in this assay, nor did any immune complexes formed with normal rabbit serum and any of the cell extracts tested. The expression of the protein kinase activity (ATP:protein phosphotransferase, EC 2.7.1.37) was growth temperature-dependent in cells infected with an ASV mutant temperature-sensitive for transformation. These results on an enzymatic activity associated with the ASV transforming protein are discussed in terms of protein phosphorylation as a mechanism for viral transformation.**

Four genes have been identified and mapped on the avian sarcoma virus (ASV) genome (1, 2). Three of these genes—*gag*, *pol*, and *env*—code for the virion structural proteins, including the group-specific (gs) viral core proteins, the RNA-directed DNA polymerase, and the envelope glycoproteins, respectively. The fourth gene, designated *src*, has been identified through the isolation of temperature-sensitive (ts) and deletion mutants defective in transformation *in vitro* and sarcoma induction *in vivo* (3–7).

Recent work from this laboratory has resulted in the identification of a protein of molecular weight ($M_r$) 60,000 that appears to be the product of the ASV *src* gene (8–10). Determination that this protein is actually the product of the *src* gene is based on the following data: (*i*) It was detected as a nonstructural, transformation-specific antigen in ASV-transformed chicken cells, ASV-transformed mammalian cells, and ASV-induced mammalian tumor cells, by immunoprecipitation of radiolabeled cell extracts with serum from ASV-tumor-bearing rabbits. (*ii*) *In vitro* cell-free translation of the 3′ third of nondefective ASV viral RNA, the region of the genome that contains the *src* gene, resulted in the synthesis of a polypeptide of $M_r$ 60,000. (*iii*) The polypeptide of $M_r$ 60,000 made *in vitro* by cell-free translation and the transformation-specific antigen isolated by immunoprecipitation of all types of ASV-infected cell extracts tested are identical as determined by peptide analyses (ref. 10; J. S. Brugge, E. Erikson, M. S. Collett, and R. L. Erikson, unpublished results). We feel that it is consistent with these data to conclude that the protein of $M_r$ 60,000 is the product of the ASV *src* gene and consequently give it the following designation: p60$^{src}$.

In order to further elucidate the mechanism of ASV-induced oncogenesis it is necessary to determine the function of the p60$^{src}$ polypeptide. We report here an enzymatic activity ascribable to the ASV *src* gene product: protein phosphorylation. In this preliminary communication we describe the identification and association of protein kinase activity (ATP: protein phosphotransferase, EC 2.7.1.37) with p60$^{src}$.

## MATERIALS AND METHODS

**Cells and Virus.** Chicken embryo fibroblasts were prepared from 11-day-old embryos (Spafas, Inc., Roanoke, IL). The Schmidt–Ruppin (SR) strain of ASV, subgroup D, was originally obtained from J. Wyke, and transformation-defective (td) SR-ASV and ASV-NY68 were obtained from H. Hanafusa.

Baby hamster kidney cells transformed by SR-ASV (BHK-SR3/1a) and a morphological revertant cell line (BHK-SR3/11R) (11) were provided by H. Varmus.

Normal European field vole (*Microtus agrestis*) cells and vole cells transformed with SR-ASV originally by P. Vogt (clone 1-T) (12) were provided by A. Faras.

**Preparation of Antiserum and Immunoprecipitation.** Antiserum was obtained from New Zealand rabbits in which tumors had been induced by injection of purified SR-ASV as previously described (8, 13). Several preparations of antiserum, referred to as TBR serum, were employed in the studies reported here. All were able to immunoprecipitate virion structural proteins as well as the transformation-specific antigen of $M_r$ 60,000 (8, 13).

For the preparation of cell extracts, unlabeled cell cultures grown on 100-mm dishes were washed several times with STE buffer (0.15 M NaCl/0.05M Tris·HCl, pH 7.2/0.001 M EDTA), scraped from the dishes, and either lysed in 1.2 ml of RIPA buffer [1.0% Triton X-100/1.0% sodium deoxycholate/0.1% sodium dodecyl sulfate ($NaDodSO_4$)/0.15 M NaCl/0.05 M Tris·HCl, pH 7.2] or sonicated in 0.6 ml of 0.01 M Tris·HCl, pH 7.2. All of the above buffers contained 100 kallikrein inactivator units/ml of Trasylol (FBA Pharmaceuticals). The cell lysates were then clarified at 100,000 g for 30 min and the supernatants (adjusted to RIPA buffer) were incubated with 10–30 $\mu$l of serum. After 30 min at 4°, 100–200 $\mu$l of a 10% suspension of the protein A-containing bacterium, *Staphylococcus aureus*, strain Cowan I, were added to adsorb the immune complexes (14). The bacteria were washed four times with RIPA buffer,

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Abbreviations: ASV, avian sarcoma virus; *src*, designation for an ASV gene responsible for transformation of fibroblasts; p60$^{src}$, designation for the protein product of the ASV *src* gene; ts, temperature-sensitive; td, transformation-defective; $M_r$, molecular weight; TBR, serum from rabbits bearing SR-ASV-induced tumors; $NaDodSO_4$, sodium dodecyl sulfate; SR, Schmidt–Ruppin strain of ASV, subgroup D; Pr, Prague strain of ASV, subgroup C; B77, Bratislava strain of ASV, subgroup C; BH-ASV(RAV-50), Bryan strain of ASV, pseudotype Rous-associated virus 50, subgroup D.

once with 0.15 M NaCl/0.05 M Tris·HCl, pH 7.2, and then prepared for the protein kinase activity assay.

**Assay for Protein Kinase Activity.** The detection of protein kinase activity in immunoprecipitates involved the resuspension of the bacteria-bound immune complexes directly in the reaction mixture. The reaction mixtures (25 $\mu$l) contained 20 mM Tris·HCl, pH 7.2, 5 mM $MgCl_2$, and 0.4–1.2 $\mu$M [$\gamma$-$^{32}$P]ATP (310–870 Ci/mmol). [$\gamma$-$^{32}$P]ATP, made according to the procedure of Glynn and Chappell (15), was initially and generously supplied by T. Walker, B. Pace, and N. Pace. Reactions were generally incubated at 30° for 10 min, at which time an equal volume of 2× sample buffer [0.14 M Tris·HCl, pH 6.8/22.4% (vol/vol) glycerol/6% $NaDodSO_4$/0.02% bromophenol blue/10% (vol/vol) 2-mercaptoethanol] was added. After heating at 95° for 1 min, the mixtures were centrifuged and the supernatants were recovered. Samples were then taken for determination of trichloroacetic acid-precipitable radioactivity or polyacrylamide gel analysis.

**Polyacrylamide Gel Electrophoresis and Autoradiography.** Samples were analyzed by electrophoresis through a discontinuous slab gel system with the buffer systems described by Laemmli (16). Gels were stained, destained, and dried onto Whatman 3MM paper (8). Radioactivity was detected by exposure to Kodak X-Omat R film with and without the use of Du Pont Cronex Lightning Plus intensifying screens.

## RESULTS

**Detection of a Protein Kinase Activity in Immunoprecipitates from ASV-Transformed Avian and Mammalian Cells.** Antigens immunoprecipitable from ASV-infected cell extracts by TBR serum include precursors to the mature internal structural proteins, their intermediate and mature cleavage products, the viral DNA polymerase, small amounts of the virion membrane glycoprotein, and the viral nonstructural, transformation-specific antigen of $M_r$ 60,000 (8–10, 13). The protein of $M_r$ 60,000 is believed to be the product of the ASV *src* gene (10) and will hereafter be designated p60$^{src}$.

To determine if immunoprecipitated p60$^{src}$ contained protein phosphorylating activity, bacteria-bound immune complexes were subjected to standard protein kinase assay conditions. It is not unreasonable to expect enzymatic activity in such complexes because of the observation that antisera from both tumor-bearing rabbits and tumor-bearing hamsters were able to precipitate the viral DNA polymerase but failed to inhibit its enzymatic activity (13). Thus, extracts from normal chick cells, avian leukosis virus (RAV-2)-infected chick cells, ASV-transformed chick cells, and chick cells infected with a td mutant of ASV were immunoprecipitated with either normal rabbit serum or TBR serum. The bacteria-bound immune complexes were incubated with [$\gamma$-$^{32}$P]ATP in the absence of any exogenous protein substrates. The reaction was terminated, the bacteria were separated from the IgG and precipitated antigens with electrophoresis sample buffer, and the latter were subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis (Fig. 1). It can be clearly seen that under these assay conditions, protein phosphorylation can be detected only in extracts of ASV-transformed cells immunoprecipitated with TBR serum (Fig. 1, track 3b). A single protein band of approximate $M_r$ 53,000 was highly phosphorylated. Because this radiolabeled material comigrates with the Coomassie blue-stained IgG heavy chain band, and also comigrates with the stained high molecular weight undissociated IgG when analyzed on gels in the absence of 2-mercaptoethanol (data not shown), we believe the phosphorylated protein to be an immunoglobulin. Further indications that phosphorylation is occurring in the antigen–IgG complex are the observations that the kinetics of the reaction are extremely rapid (complete within 1 min at 23°) and that among the few tested, such as histone and casein, exogenously added substrates are not phosphorylated. That the radioactive band is indeed a phosphorylated protein was demonstrated by tryptic peptide and phosphoamino acid analyses. The phosphorylated protein was found to contain a single tryptic phosphopeptide containing exclusively phosphothreonine (data not shown).

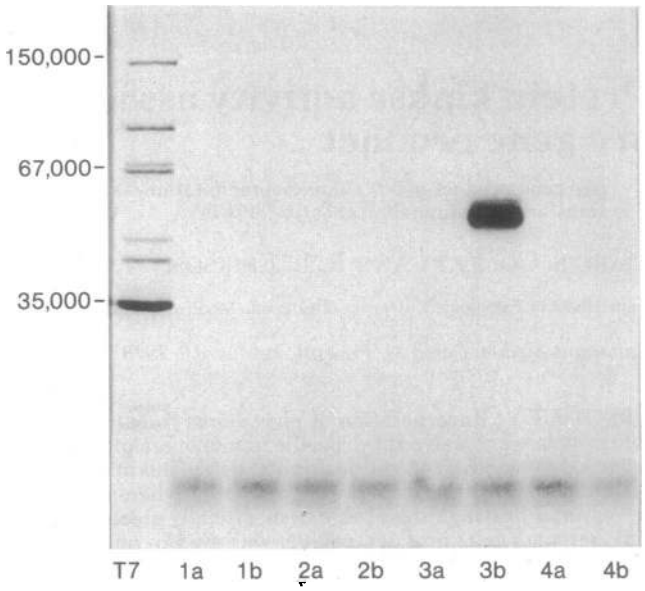


FIG. 1. Detection of a protein kinase activity in immunoprecipitates of chick cell lysates. Cell extracts were prepared from chick embryo fibroblast cultures that were either uninfected (tracks 1), infected with the avian leukosis virus RAV-2 (tracks 2), infected with SR-ASV (tracks 3), or infected with a transformation-defective deletion mutant of SR-ASV (tracks 4). Each extract (1800–3600 $\mu$g of protein) was immunoprecipitated with either normal rabbit serum (a tracks) or TBR serum (b tracks), and a portion of the bacteria-bound immune complexes was incubated in the protein kinase reaction mixture. After termination of the reaction by heating to 95° for 1 min in sample buffer and pelleting of the bacteria, the supernatant was subjected to electrophoresis in a discontinuous $NaDodSO_4$/polyacrylamide slab gel. The stacking gel contained 4.2% acrylamide and 0.1% bisacrylamide, and the separation gel was 10% polyacrylamide (38:1 acrylamide/bisacrylamide), 11 cm long. The figure represents an autoradiogram of the dried gel. Phage T7 virion proteins are included as molecular weight markers.

To further demonstrate that the phosphorylating activity detected in ASV-infected chick cell extracts immunoprecipitated with TBR serum was due to the presence of the viral sarcoma gene product, several mammalian cell lines were subjected to a similar analysis. Baby hamster kidney cells transformed by SR-ASV (BHK-SR3/1a) also demonstrated the IgG phosphorylating activity when cell extracts were immunoprecipitated with TBR serum (Fig. 2, track 1b). However, when a morphological revertant subclone of the BHK-SR3/1a cells (17) was analyzed, no phosphorylating activity was detected in the immunoprecipitates (Fig. 2, track 2b). Analysis of radiolabeled antigens immunoprecipitated with TBR serum has revealed that the expression of p60$^{src}$ in these revertant cells is undetectable (data not shown).

A second mammalian cell type investigated for the presence of the sarcoma-specific protein phosphorylating activity was the European field vole cell. Both normal and SR-ASV-transformed vole cells were analyzed and, as is seen in Fig. 2, (track

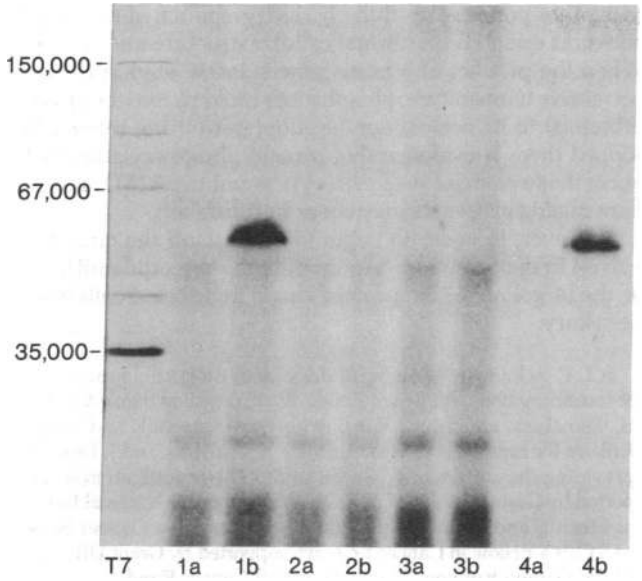


FIG. 2. Presence of a protein kinase activity in immunoprecipitates of SR-ASV-transformed mammalian cells. Cell extracts were prepared from cultures of SR-ASV-transformed baby hamster kidney cells (BHK-SR3/1a) (tracks 1), a normal phenotypic revertant clone of these cells (BHK-SR3/11R) (tracks 2), normal field vole cells (tracks 3), and SR-ASV-transformed field vole cells (tracks 4). Immunoprecipitation with either normal (a tracks) or TBR serum (b tracks) and assay of phosphorylating activity were as described in the legend to Fig. 1.

4b) only the transformed cells immunoprecipitated with TBR serum contained the protein kinase activity.

**Correlation of the Protein Kinase Activity and the Immunoprecipitation of p60$^{src}$.** The immunoprecipitation of p60$^{src}$ by the preparations of TBR serum used in these experiments is strain specific. Antibody produced in rabbits bearing tumors induced by SR-ASV does not immunoprecipitate a transformation-specific protein from cells transformed by other strains of ASV (J. S. Brugge and R. L. Erikson, unpublished results). Therefore, to determine if a protein kinase activity was present in immunoprecipitates of chick cells transformed by the Prague strain (Pr-ASV), the Bratislava strain (B77-ASV), and the Bryan strain [BH-ASV(RAV-50)] of ASV, cell lysates were prepared, immunoprecipitated with TBR serum, and subjected to the protein kinase assay. The results, presented in Table 1 along with a summary of the data presented in Figs. 1 and 2, indicate that even though the virus-infected cells are transformed, the protein kinase activity is not found in cell extracts from which no p60$^{src}$ can be immunoprecipitated. It should be pointed out here that because the *src* genes of the various ASV strains are very similar, as judged by molecular hybridization (18) and cell-free translation of subgenomic viral RNA (9, 10), similar gene products are probably present in cells transformed by other strains of ASV, but they are not detected because of a lack of crossreaction with the currently available antisera.

**Temperature Sensitivity of the Protein Kinase Activity in Chick Cells Infected with a Temperature-Sensitive Transformation Mutant of ASV.** To demonstrate more directly that the protein kinase activity is the result of expression of the ASV *src* gene, studies were undertaken to determine if the observed protein phosphorylation was thermosensitive in chick cells infected with a ts mutant in the *src* gene. Previous experiments have suggested that the *src* gene product encoded by such mutants is irreversibly denatured at 41°, the nonpermissive

Table 1. Correlation of the protein kinase activity and the immunoprecipitation of p60$^{src}$

| Virus | Cell | Transformation | p60$^{src}$ | Protein kinase activity |
|---|---|---|---|---|
| — | Chick | − | − | − |
| RAV-2 | Chick | − | − | − |
| td-SR-ASV | Chick | − | − | − |
| SR-ASV | Chick | + | + | + |
| SR-ASV | Hamster | + | + | + |
| SR-ASV | Hamster revertant | − | − | − |
| — | Vole | − | − | − |
| SR-ASV | Vole | + | + | + |
| Pr-ASV | Chick | + | − | − |
| B77-ASV | Chick | + | − | − |
| BH-ASV (RAV-50) | Chick | + | − | − |

Uninfected cell cultures or cultures transformed with the various strains of ASV were either radiolabeled with [$^{35}$S]methionine (for the detection of p60$^{src}$) or left unlabeled (for the detection of protein kinase activity). Cell extracts were prepared and immunoprecipitated with TBR serum as described in *Materials and Methods*. Na-DodSO$_4$/polyacrylamide gel electrophoresis was used to determine the presence (+) or absence (−) of [$^{35}$S]methionine-labeled p60$^{src}$ (8). Protein kinase activity was determined as described in the legend to Fig. 1.

temperature (6). Parallel cultures of chick cells infected with nondefective SR-ASV and the SR-ASV ts mutant NY68 (6) were maintained at both 35° and 41°. Cell extracts were prepared from each of the four cultures, immunoprecipitated with TBR serum, and then analyzed for protein kinase activity in the bacteria–immune complex assay. Cells infected with nondefective ASV showed a slight (2-fold) increase in phosphorylating activity in *src* protein immunoprecipitates when grown at 41° compared to those grown at 35° (Table 2). In contrast to these results, the NY68-infected cells, when grown at the nonpermissive temperature (41°), showed a dramatic decrease in phosphorylating activity (Table 2). These data indicate that the expression of the protein kinase activity detected in *src* protein immunoprecipitates is dependent on the temperature at which

Table 2. Growth temperature-dependent expression of *src* protein-immunoprecipitated phosphorylating activity in chick cells infected with a ts transformation mutant of ASV

| Virus | Growth temperature, °C | Phosphorylating activity: $^{32}$P incorporated, fmol/mg protein | Phosphorylating activity: Normalized values |
|---|---|---|---|
| SR-ASV (nd) | 35 | 16.6 | 1.00 |
| SR-ASV (nd) | 41 | 35.8 | 2.16 |
| SR-NY68 | 35 | 19.8 | 1.00 |
| SR-NY68 | 41 | 1.7 | 0.09 |

Parallel cultures of chick cells infected with nondefective (nd) SR-ASV and cells infected with the ts transformation mutant of SR-ASV, NY68, were maintained at either the permissive (35°) or nonpermissive (41°) temperature. Cell extracts were prepared from the four cultures, samples were taken for determination of protein content, and the remainder was immunoprecipitated with TBR serum as described in *Materials and Methods*. Phosphorylating activity by the bacteria-bound immunoprecipitated complexes was also determined. The resulting activity values, determined by quantitation of the phosphorylated IgG bands from a polyacrylamide gel, are normalized with respect to the amount of cell extract protein used for immunoprecipitation and the activity present in the respective cells grown at 35°.

chick cells infected with an ASV ts mutant in the *src* gene are grown.

## DISCUSSION

The results presented in this communication demonstrate that a protein kinase is the product of the ASV *src* gene, or is closely associated with it. Biosynthetic radiolabeling permits the detection by immunoprecipitation of only one polypeptide in nd virus-infected cells which is not in td virus-infected cells, and that is the polypeptide with a $M_r$ of 60,000 previously identified as the product of the ASV *src* gene (10). Using TBR serum to immunoprecipitate $p60^{src}$, we have shown here that a protein kinase activity is associated only with cells, both avian and mammalian, infected with transforming virus, that expression of this activity is growth temperature-dependent in cells infected with a *src* gene ts mutant virus, and that the protein kinase activity is dependent on the immunoprecipitation of $p60^{src}$. Additional experiments in this laboratory further support the association of the protein kinase activity with $p60^{src}$; the enzymatic activity and $p60^{src}$ cosediment during glycerol gradient centrifugation and coelute from ion exchange columns run singly or sequentially. However, the possibility remains that a highly active kinase that cannot be detected by biosynthetic radiolabeling specifically associates with $p60^{src}$ and, therefore, appears to be specifically immunoprecipitable. Even if this proves to be the case, such a result may permit a better understanding of ASV-induced oncogenesis. The purification of the relevant enzymes from cells infected by nondefective and ts virus will serve to better resolve these issues.

The role of protein phosphorylation in the functional regulation of a variety of cellular processes is well documented (see ref. 19 for a review and further references). Phosphotransferase reactions may be catalyzed by enzymes that are dependent on the presence of cyclic nucleotides for maximal activity (cyclic nucleotide-dependent protein kinases) or their activity may be unaffected by cyclic nucleotides (phosphoprotein kinases) (19). Both classes of protein kinases are generally capable of phosphorylating a number of substrates *in vitro* that cannot be considered the normal *in vivo* targets. Consequently, the phosphorylation of IgG observed here probably provides no clues as to the biologically significant phosphorylation in ASV-induced transformation.

In this regard, it has been previously suggested that the product of the ASV *src* gene may be directly or indirectly involved in a reversible chemical modification of components involved in the maintenance of cell structure (20). This idea is based on indirect experiments employing ts mutants of ASV and inhibitors of protein synthesis. These experiments indicated that all the components necessary for normal cellular architecture were present, although disaggregated, in transformed cells, and were reassembled upon inactivation of the ts *src* gene product, without the necessity of protein synthesis. Thus, one might speculate, for example, that a component(s) of the cytoskeleton complex when phosphorylated may fail to interact properly with other proteins, resulting in disaggregation of the protein networks essential for normal cellular structure and function. When the product of a ts *src* gene is inactivated at the nonpermissive temperature, phosphatases could restore the affected protein(s) to its normal composition, permitting reassembly. Indeed there is evidence that protein phosphorylation influences shape changes in erythrocyte membranes (21), and perhaps analogous events may occur in fibroblasts.

However, in order to begin to understand the circuits involved in oncogenic transformation, the direct identification of the target of the *src* protein kinase in infected cells will be necessary.

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