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Peptide Analysis of the Transformation-Specific Antigen from Avian Sarcoma Virus-Transformed Cells

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Sera from rabbits bearing tumors induced by avian sarcoma virus (ASV) were used to immunoprecipitate virus-specific proteins from extracts of chicken, hamster, and field vole cells transformed by ASV. Two virus-specific proteins having molecular weights of 76,000 and 60,000 were found in all cell lines examined. The 76,000-molecular-weight protein, Pr76, is the precursor to the internal core proteins of ASV. The 60,000-molecular-weight (60K) transformation-specific antigen from each cell line was subjected to peptide analysis, using chymotrypsin and *Staphylococcus aureus* V8 protease. The resulting peptide maps of the 60K protein from the different ASV-infected cell types were similar for each enzyme, strongly suggesting that the 60K protein is virus coded. Two-dimensional analysis of chymotryptic peptides from Pr76 and 60K reveals that 60K is not related to the gs antigen precursor. Radiolabeling of ASV-transformed cells with inorganic phosphate revealed that 60K is phosphorylated in vivo. The 60K proteins isolated from both ASV-transformed chicken and field vole cells were found to contain one tryptic phosphopeptide. The tryptic phosphopeptides of 60K from both cell lines migrated identically upon two-dimensional peptide analyses, and their migration differed from that of the principal phosphopeptide of Pr76.

Sera from rabbits bearing tumors induced by the Schmidt-Ruppin strain of avian sarcoma virus (SR-ASV) were found to immunoprecipitate the structural proteins of avian sarcoma virus (ASV) (3) as well as a transformation-specific antigen of molecular weight (MW) 60,000 (60K) from chicken and hamster cells transformed by SR-ASV (2). The evidence that 60K is transformation specific follows: (i) 60K is not detectable in chicken cells infected with transformation-defective deletion mutants of ASV or with avian leukosis viruses (21a); (ii) 60K is not precipitated with antibody to any structural proteins of ASV (2); and (iii) when sera from tumor-bearing rabbits (TBR sera) are first adsorbed with the virion proteins of ASV such that immunoprecipitation of virion proteins and their precursors is undetectable, the precipitation of 60K is not diminished (2). Furthermore, a polypeptide of identical MW and antigenicity and containing methionine-containing tryptic and chymotryptic peptides identical to those of the 60K protein found in vivo is produced in a messenger-dependent rabbit reticulocyte cell-free lysate programmed with the 3' one-third of the nondefective SR-ASV genome (21a) or with cytoplasmic 20S polyadenylic acid-containing 20S RNA from SR-ASV-infected chicken cells (Erikson et al., unpublished data). These RNAs have been shown to encompass the sequences encoding the *src* gene (11-13, 17, 25). RNA of the same size class extracted from transformation-defective deletion mutants of SR-ASV fails to program the synthesis of this protein in vitro (21a, 22). All of the above evidence suggests that the 60K protein is a product of the ASV *src* gene.

Genetic analyses have defined the *src* gene as that portion of the viral genome which encodes the protein directly responsible for ASV transformation (1, 14, 19, 24). It is, therefore, expected that if the 60K protein is virus coded and transformation specific, then all cell lines transformed by SR-ASV should contain the identical protein. In this report we have analyzed several different cell types transformed by SR-ASV, including chicken, hamster, and field vole, for the presence of the 60K protein. In addition to establishing that 60K is present in all of the above SR-ASV-transformed cell types, we have further demonstrated that 60K is identical in all of the cell types tested, by analyses of its methionine- and phosphate-containing peptides. Finally, two-dimensional peptide analysis of the 60K protein and Pr76, the precursor to the internal structural proteins of ASV, demonstrated that 60K is not related to Pr76.

MATERIALS AND METHODS

Cells and virus. Chicken embryo fibroblasts were prepared from 11-day-old gs$^-$ embryos (Spafas, Inc., Roanoke, Ill.). The strain of SR-ASV (subgroup D)

was obtained from John Wyke. The SR-ASV-transformed chick cells described in these experiments were used when more than 80% of the cells appeared morphologically transformed. RSV-SH/C1A cells (4) were derived from a primary tumor induced by SR-ASV in a Syrian hamster. SR-3/1a cells, obtained from Harold Varmus, are BHK-21 cells transformed in vitro with SR-ASV (18); SR-RSV-D MA cells, obtained from A. J. Faras, are European field vole (*Microtus agrestis*) cells transformed in vitro with SR-ASV (5). Primary Syrian hamster and secondary field vole cells were used as uninfected controls.

Preparation of antiserum. Tumors were induced in newborn New Zealand rabbits by subcutaneous inoculation of approximately 10^8 focus-forming units of SR-ASV. Five weeks later, when solid tumors were approximately 1 to 2 cm in diameter, the animals were bled. These pooled sera are referred to collectively as TBR serum (2).

Preparation of cell extracts and immunoprecipitation. Cells were seeded in 60-mm dishes at a density of 3×10^6 cells per plate. Twenty-four hours later, the cells were labeled for 3 h, with 25 μCi of [^{35}S]methionine (1,100 Ci/mmol, Amersham) per ml in Eagle medium lacking methionine and containing 5% calf serum, scraped from the dish, washed in a solution of 0.15 M NaCl-0.05 M Tris-hydrochloride, pH 7.2-1 mM EDTA containing 1% Trasylol (FBA Pharmaceuticals, N.Y.), lysed in 0.8 ml of RIPA (10) (1.0% Triton X-100-1.0% sodium deoxycholate-0.1% sodium dodecyl sulfate [SDS]-0.15 M NaCl-0.05 M Tris-hydrochloride, pH 7.2), and clarified at 100,000 × *g*, and the supernatant was incubated with 10 μl of serum. After 30 min at 4°C, 200 μl of a 10% suspension of the protein A-containing bacterium *Staphylococcus aureus* strain Cowan I was added to adsorb immune complexes as described by Kessler (15). The bacteria were washed, and the immunoprecipitated polypeptides were eluted and analyzed on 5 to 15% SDS-polyacrylamide gels as described previously (2).

To radiolabel cells with ^{32}P, cell monolayers were washed several times with phosphate-free growth medium and were then labeled for 2 h with phosphate-free medium containing 2% dialyzed calf serum and either 0.1 mCi or 1 mCi of carrier-free $^{32}P_i$ (ICN) per ml.

One-dimensional partial proteolysis. To isolate [^{35}S]methionine-labeled polypeptides from preparative gels for peptide analysis, a small strip was cut from the edge of the gel after it had been stained with Coomassie brilliant blue and destained. The strip was dried and subjected to autoradiography, while the remainder of the gel was stored in water at 4°C. The gel region containing the protein to be analyzed was excised, and peptide mapping by limited proteolysis was carried out as described by Cleveland et al. (6), using chymotrypsin (Worthington) or *S. aureus* V8 protease (Miles).

Two-dimensional peptide fingerprinting. ^{32}P-labeled and [^{35}S]methionine-labeled immunoprecipitated proteins were subjected to electrophoresis in SDS-polyacrylamide slab gels, located by autoradiography, excised, and eluted in 0.05 M NH_4HCO_3 (pH 8.5)-0.1% SDS at 37°C overnight. Carrier protein (100 μg of bovine serum albumin) was added, and the proteins were precipitated twice with 20% trichloroacetic acid. After performic acid oxidation and two lyophilizations, the proteins were digested with tolylsulfonyl phenylalanyl chloromethyl ketone-treated trypsin (Worthington) or with alpha-chymotrypsin (Worthington), and the resultant peptides were subjected to ascending chromatography followed by electrophoresis at pH 3.5, all as previously described (9).

Phosphoamino acid analysis. ^{32}P-labeled tryptic peptides prepared as described above were dried, dissolved in 30 μl of 2 N HCl, and hydrolyzed in sealed glass capillaries at 100°C for 12 h. The hydrolysates were dried, dissolved in 4 μl of a solution containing 5 μg each of unlabeled phosphoserine and phosphothreonine, and spotted onto Whatman 3MM paper. Electrophoresis was performed in formic acid-acetic acid-water (25:87:888; pH 1.9) at 1,000 V for 2 h. After electrophoresis, the paper was stained with ninhydrin (0.4% ninhydrin, 1% acetic acid in acetone), dried, and subjected to autoradiography.

RESULTS

Identification of 60K protein in various transformed cell lines. The four cell lines examined included SR-ASV-transformed chicken embryo fibroblasts, baby hamster kidney (BHK-21) cells transformed in vitro with SR-ASV (SR-3/1a) (7, 18), RSV-SH/C1A cells (2-4) derived from a primary tumor induced in a newborn Syrian hamster with SR-ASV, and European field vole (*M. agrestis*) cells transformed in vitro with SR-ASV (SR-RSV-D MA) (5, 16). To assay for the presence of the 60K protein, a [^{35}S]methionine-labeled extract from each cell line was immunoprecipitated with TBR serum, and the immunoprecipitated polypeptides were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 1). Normal chick, hamster, and field vole cell extracts immunoprecipitated with either normal rabbit or TBR serum did not yield any proteins in the 60,000-MW region of the gel. TBR serum precipitated two specific proteins migrating with MWs of 76,000 and 60,000 from all four lines of ASV-transformed cells. The 76,000-MW protein (Pr76) has previously been demonstrated to be the cellular precursor to the mature gs antigens (8). The intermediate and mature cleavage products of Pr76 (Pr66, Pr53, Pr33, p27, p19, and p12-15), were detectable in SR-ASV-transformed chicken cells (Fig. 1, track 4), but no cleavage of Pr76 was detectable in any of the mammalian cells examined. The 180,000-MW protein present in ASV-transformed chicken and field vole cells (tracks 4 and 12, Fig. 1) is believed to be the initial translation product of the genes coding for the gs antigens, *gag*, and the virion polymerase, *pol* (20-22). The 60K protein from all three transformed mammalian cell lines appears to migrate identically to the 60K protein

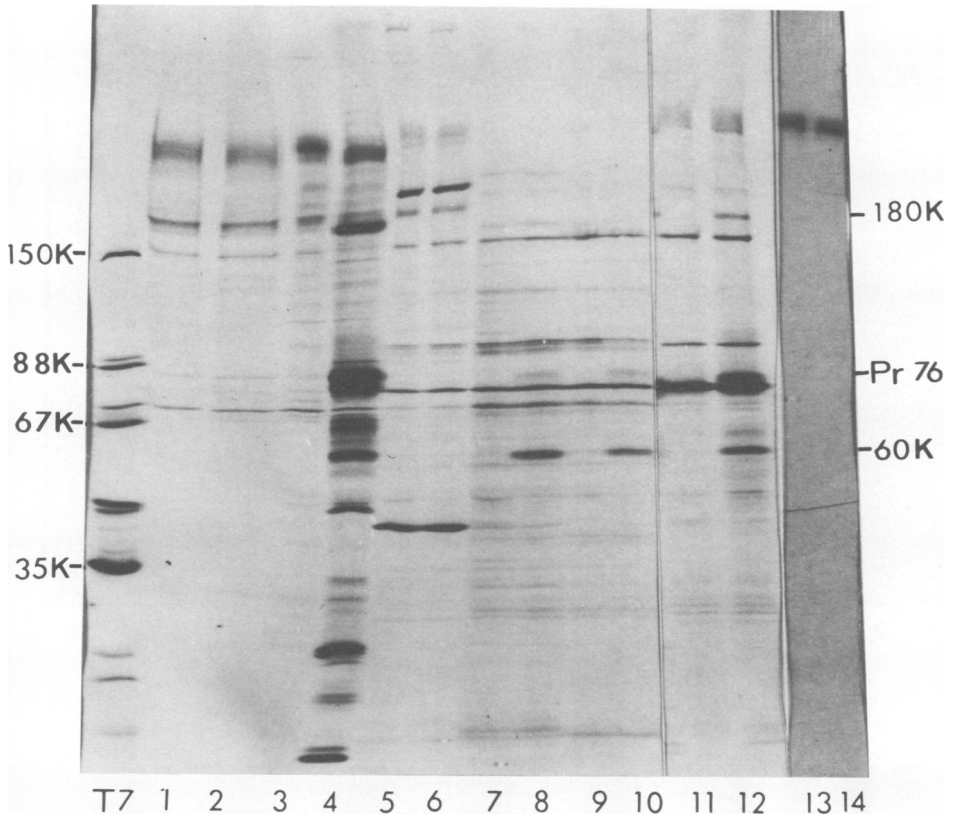

FIG. 1. *Autoradiogram of SDS-polyacrylamide gel analysis of proteins immunoprecipitated from [^{35}S]-methionine-labeled uninfected and ASV-transformed cells. Cells were labeled with [^{35}S]methionine and lysed in RIPA (10), and the cell extracts were clarified and immunoprecipitated as described in the text. The odd-numbered tracks contain proteins immunoprecipitated with normal rabbit serum and the even-numbered tracks, TBR serum. (1, 2) Uninfected chick, (3, 4) SR-ASV-transformed chick, (5, 6) uninfected primary Syrian hamster cells, (7, 8) SR-3/1a, (9, 10) RSV-SH/C1A, (11, 12) SR-ASV-D MA, (13, 14) uninfected field vole cells. T7 proteins were calibrated against Escherichia coli RNA polymerase, ovalbumin, bovine serum albumin, and trypsin.*

from SR-ASV-transformed chicken cells. This protein has previously been shown to be transformation specific (2).

One-dimensional peptide analysis of 60K protein. To determine whether the 60K proteins identified in Fig. 1 are identical, gel slices containing [^{35}S]methionine-labeled 60K from each cell line were treated with various concentrations of *S. aureus* V8 protease or chymotrypsin during re-electrophoresis, according to the procedure of Cleveland and co-workers (6). The peptides generated by this partial proteolysis are shown in Fig. 2. In each case, the first well shows the undigested protein, whereas the subsequent three wells contain the protein digested with increasing concentrations of enzyme. In this way, a unique pattern of peptides was produced for each protein analyzed. Although this technique employs only one-dimensional electrophoresis where peptide migration is based primarily on size, the use of two different proteases allows one to reduce, if not eliminate, the possibility of fortuitous migration of nonidentical peptides. Despite quantitative differences among the peptide patterns, it was clearly seen that the cleavage products of 60K from all four cell lines examined were similar for each enzyme tested, indicating that these proteins are identical. Because this technique of partial proteolysis yields relatively large peptide fragments, differences due to minor host cell modifications of this protein could not be resolved by this technique.

Two-dimensional peptide analysis of Pr76 and 60K protein. To evaluate the relationship between Pr76 and 60K, these polypeptides were subjected to two-dimensional peptide analysis after digestion by trypsin or chymotrypsin. Four tryptic peptides of Pr76 and 60K ap-

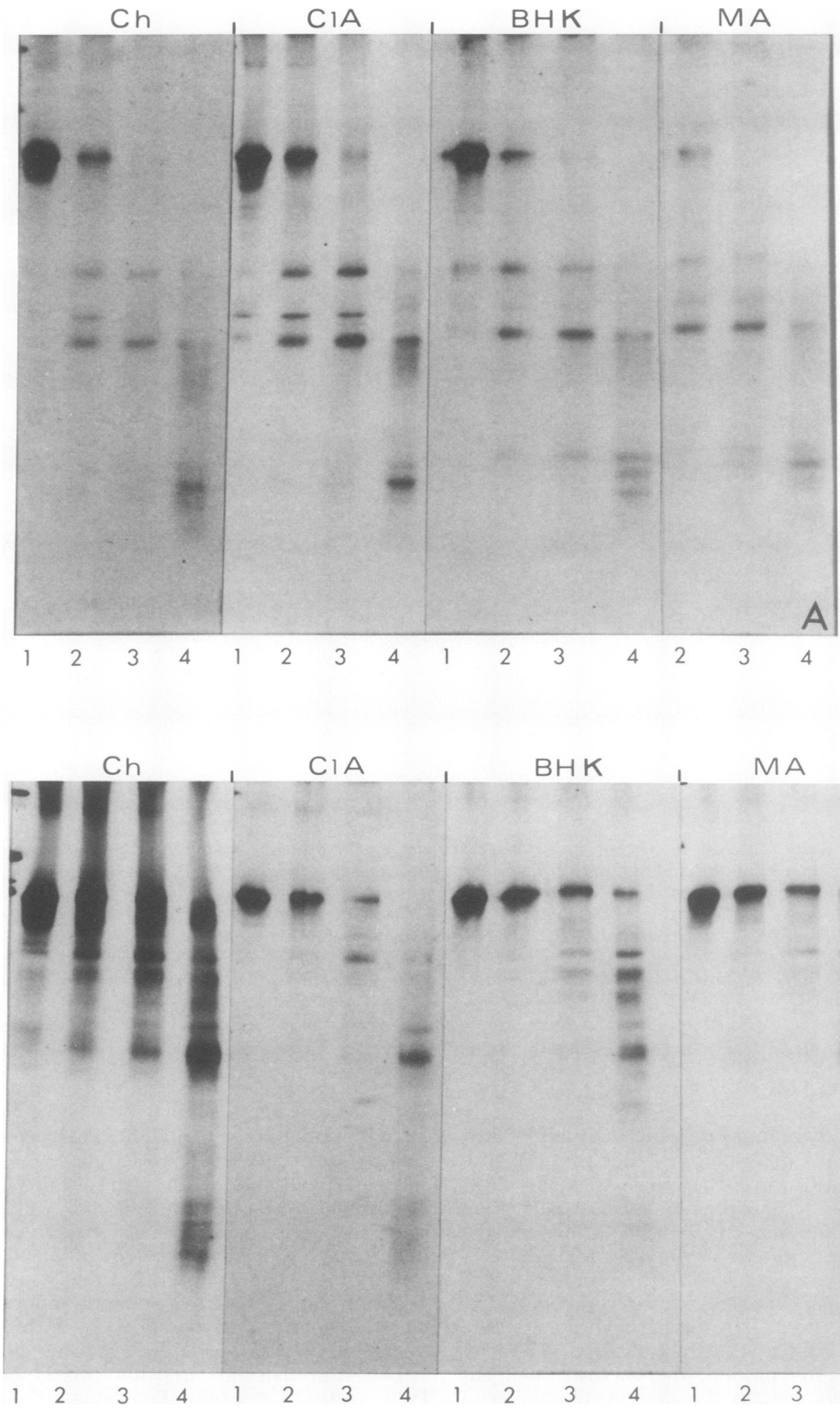

FIG. 2. *Autoradiogram of the cleavage products of 60K digested with S. aureus V8 protease (A) or with chymotrypsin (B) during re-electrophoresis. 60K was immunoprecipitated and isolated from SR-ASV-transformed chick cells (Ch), RSV-SH/C1A (C1A), SR-3/1a (BHK), and SR-RSV-D MA (MA), and digested with no enzyme, 0.005 μg, 0.025 μg, and 0.5 μg of protease (A) or with no enzyme, 0.1 μg, 0.5 μg, and 2 μg of chymotrypsin (B) (tracks 1 to 4, respectively). A description of these cells is given in the text.*

pear to migrate similarly upon two-dimensional fractionation (Fig. 3). In addition, the 60K protein contains at least three other methionine-containing peptides, and Pr76 contains several unique peptides that are not present in molar quantities yet are reproducibly detected in this molecule. Since the comigration of several tryptic peptides from Pr76 and 60K suggests that these proteins might be related, each protein was digested with a different proteolytic enzyme, chymotrypsin, to further clarify this issue. Chymotryptic proteolysis has proven to be a reproducible and accurate means of peptide analysis (23). The chymotryptic peptide maps of Pr76 and 60K each contain a unique pattern of peptides, 9 for 60K and 10 for Pr76 (Fig. 4). Analysis of a mixture of the chymotryptic peptides from Pr76 and 60K revealed that all of the peptides were unique for each molecule with the possible exception of one. The lack of similarity of the chymotryptic peptides from these two proteins demonstrates that the 60K protein is not related to Pr76 and indicates that the comigration of the four tryptic peptides of Pr76 and 60K was fortuitous.

The possibility that the 60K preparation used for the tryptic peptide map was contaminated with Pr76 or one of its cleavage products can be ruled out since identical maps are generated from 60K synthesized in a cell-free protein-synthesizing system programmed with RNA from the 3′ one-third of the ASV genome, a region which does not contain the *gag* gene (12, 13, 17, 25) and which does not program synthesis of Pr76 (21a). In addition, the results below which demonstrate that the tryptic phosphopeptide of Pr76 is different from that of 60K indicate that our preparations of 60K are not contaminated with a *gag* gene product intermediate.

Therefore, a word of caution must be made with respect to comparative peptide analysis. The comigration of peptides produced from two

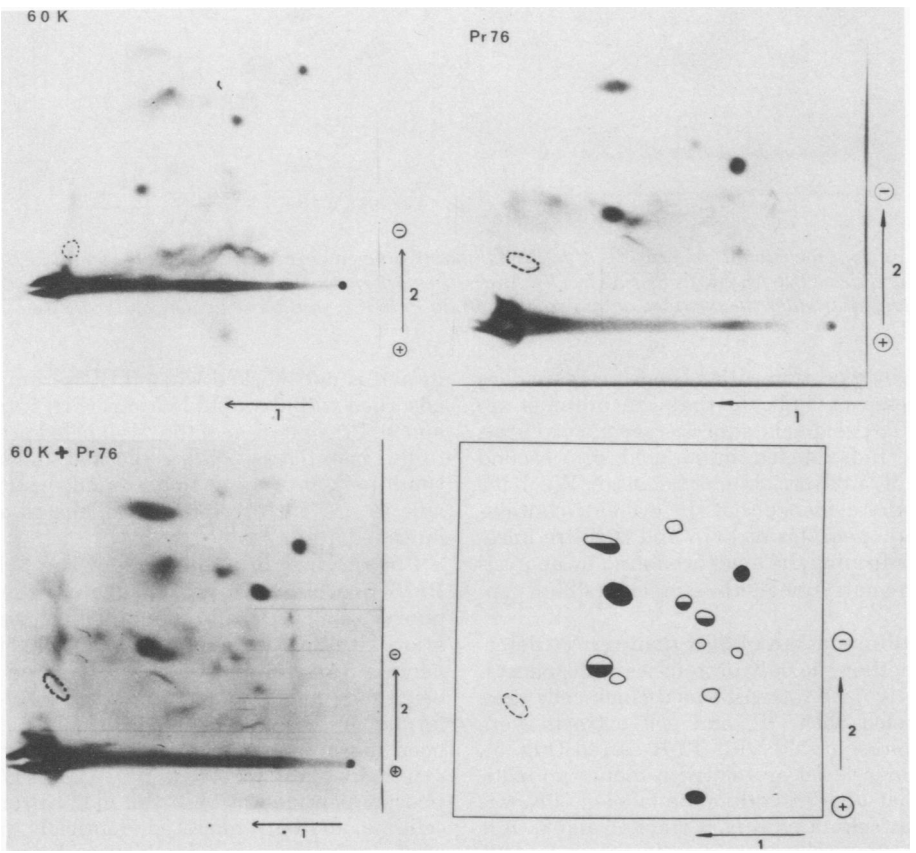

FIG. 3. *Two-dimensional fingerprints of [^{35}S]methionine-labeled tryptic peptides found in Pr76 and 60K, prepared from SR-ASV-infected chick cells, and of a mixture of equal counts from each protein. Analysis was performed as described in the text. The diagram (lower right) illustrates the peptides unique to 60K* (●) *and to Pr76* (○) *and those which comigrate* (◒).

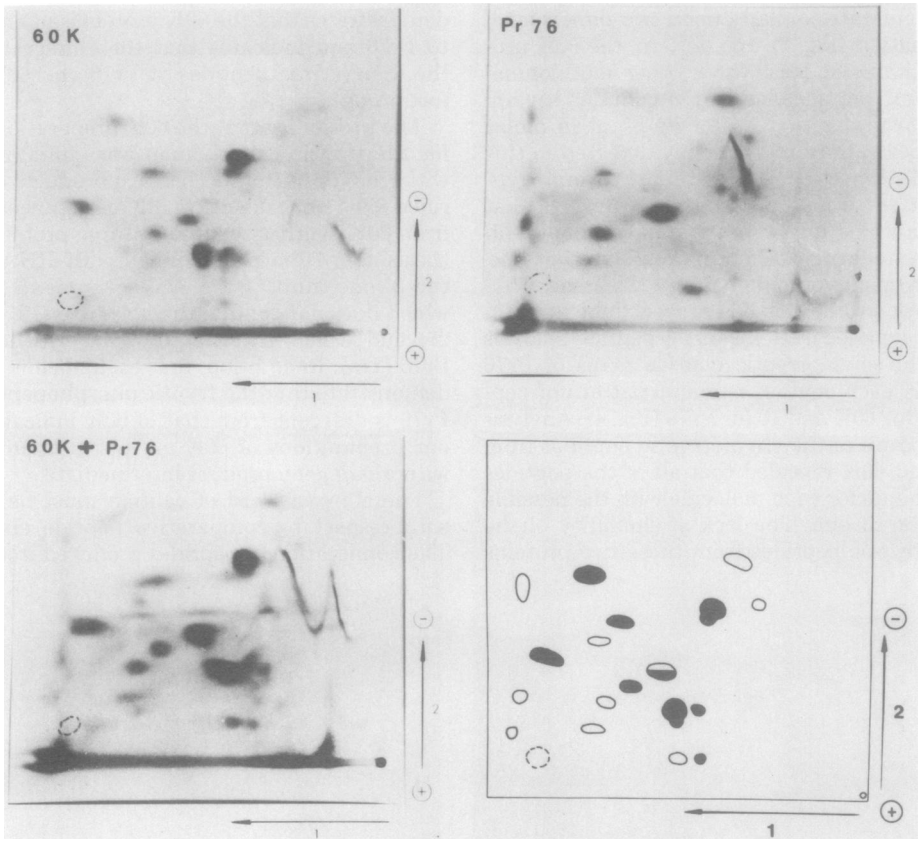

FIG. 4. *Two-dimensional fingerprints of [^{35}S]methionine-labeled chymotryptic peptides found in Pr76 and 60K prepared from SR-ASV-infected chick cells. Analysis was performed as described in the text. The diagram (lower right) illustrates the peptides unique to 60K* (●), *to Pr76* (○), *and those which comigrate* (◒).

proteins by digestion with a single protease does not necessarily indicate that the proteins are related. To prove relatedness, a second protease, a second radiolabeled amino acid, or a second system of analysis should be used. We have preliminary evidence that the cysteine-containing tryptic peptides of Pr76 and 60K are unrelated, confirming the evidence found by analysis of the chymotryptic methionine-containing peptides.

Phosphorylation of 60K protein. To determine whether the 60K protein was phosphorylated in vivo, ASV-transformed chick cells were radiolabeled with ^{32}P, and cell extracts were immunoprecipitated with TBR serum (Fig. 5). A phosphorylated protein with mobility identical to that of [^{35}S]methionine-labeled 60K was immunoprecipitated (Fig. 5, tracks 5 and 8). It is believed that this protein is identical to 60K since it is not precipitated with normal serum (Fig. 5, tracks 4 and 7), it is not found in uninfected cells (Fig. 5, tracks 1 to 3), and its precipitation is not blocked when TBR serum is first adsorbed with disrupted virions (Fig. 5, tracks 6 and 9). Precipitation of the ^{32}P-labeled precursor to the gs antigens, Pr76, is blocked under these conditions, indicating that the adsorption was able to prevent precipitation of the gs antigen intermediates.

Phosphopeptide analysis of the 60K and Pr76 proteins. To further analyze the phosphorylation of 60K and compare its phosphorylation with that of Pr76, two-dimensional tryptic peptide fingerprinting was performed on ^{32}P-labeled 60K and Pr76 isolated from ASV-transformed chick and field vole cells (Fig. 6). It had been shown previously (9) that the same tryptic peptide is phosphorylated in p19 and Pr76. One phosphopeptide was observed in 60K from each cell line, and both migrated identically, indicating that the same peptide is phosphorylated in 60K in divergent cell lines. The phosphopeptide of Pr76 clearly migrates differently than that of the 60K protein.

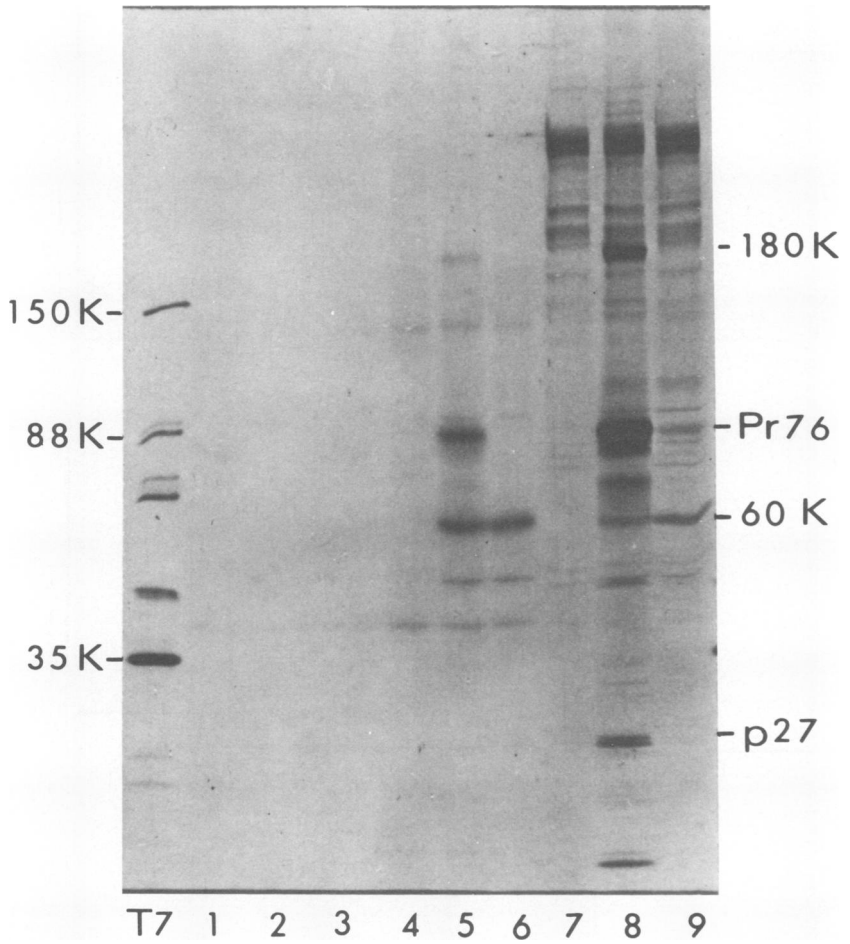

FIG. 5. *Autoradiogram of SDS-polyacrylamide gel analysis of proteins immunoprecipitated from ASV-transformed chick cells labeled with [^{35}S]methionine or $^{32}P_i$. The cells and sera are the following: tracks 1 to 3, ^{32}P-labeled uninfected chick cells, normal serum, TBR serum, TBR serum blocked; tracks 4 to 6, ^{32}P-labeled SR-ASV-transformed chick cells, normal serum, TBR serum, TBR serum blocked; tracks 7 to 9, [^{35}S]-methionine-labeled SR-ASV-transformed chick cells, normal serum, TBR serum, TBR serum blocked. For the blocking reactions, the serum was incubated for 30 min with 125 μg of disrupted SR-ASV before addition of the cell extract.*

Identification of the phosphoamino acid in Pr76 and 60K. To ensure that the ^{32}P label found in the immunoprecipitated Pr76 and 60K proteins was covalently linked to the polypeptides, and not due to an adventitious association of other phosphate-labeled material, analysis of the acid hydrolysis products of both phosphorylated proteins was carried out. Figure 7 demonstrates the existence of primarily phosphoserine in both Pr76 and 60K. Whereas, no phosphothreonine was detected in Pr76, a small amount was reproducibly found in hydrolysates of the 60K protein. In view of the two-dimensional tryptic fingerprint of 60K (Fig. 6, one phosphopeptide), the significance of the phosphothreonine spot is at present unknown. It may represent the phosphorylated amino acid of a minor contaminating protein, or, alternatively, a minor phosphorylated variant of the 60K protein.

DISCUSSION

This report provides independent evidence that the 60,000-MW protein immunoprecipitated from ASV-transformed cells with sera from rabbits bearing ASV-induced tumors is coded for by the ASV genome. The 60K protein was identified in four different ASV-transformed cells: chick, field vole, and hamster cells transformed in vitro, and ASV-induced hamster tu-

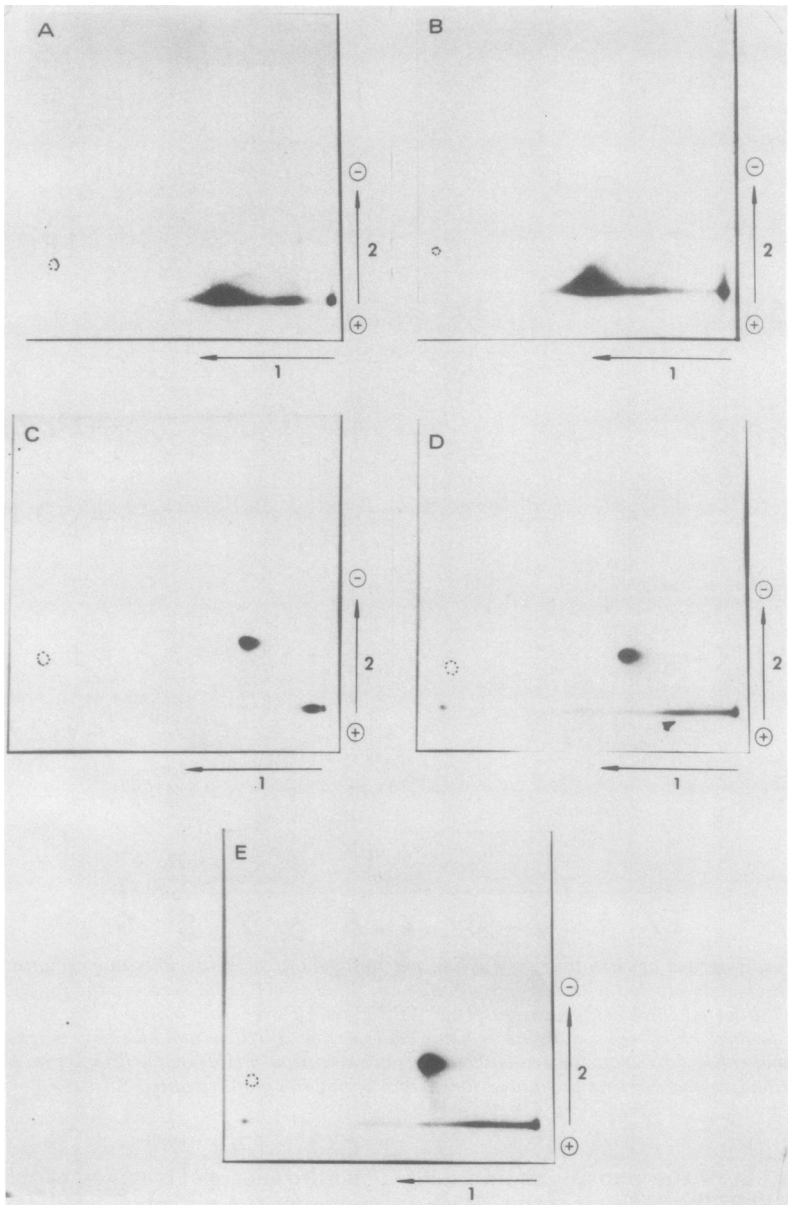

FIG. 6. *Two-dimensional fingerprints of tryptic peptides from ^{32}P-labeled Pr76 and 60K isolated from SR-ASV-transformed chicken embryo fibroblasts and field vole cells. Analysis was performed as described in the text. (A) Pr76, chick; (B) Pr76, vole; (C) 60K, chick; (D) 60K, vole; (E) 60K mix, chick and vole.*

mor cells. That the 60K proteins isolated from four diverse cells are so similar with respect to their methionine- and phosphate-containing peptides strongly supports the notion that it is virus encoded. Additional confirmation of this finding is provided by experiments which demonstrated that the 3′ one-third of the ASV genome is able to program the synthesis of a 60K protein which is identical to the protein immunoprecipitated from transformed cells with respect to MW, antigenicity, partial protease digestion products, and methionine-containing tryptic and chymotryptic peptides (21a; unpublished data).

Because processing of Pr76 into the mature gs antigens involves production of several intermediate cleavage products, it is necessary to exclude the possibility that the 60K protein is

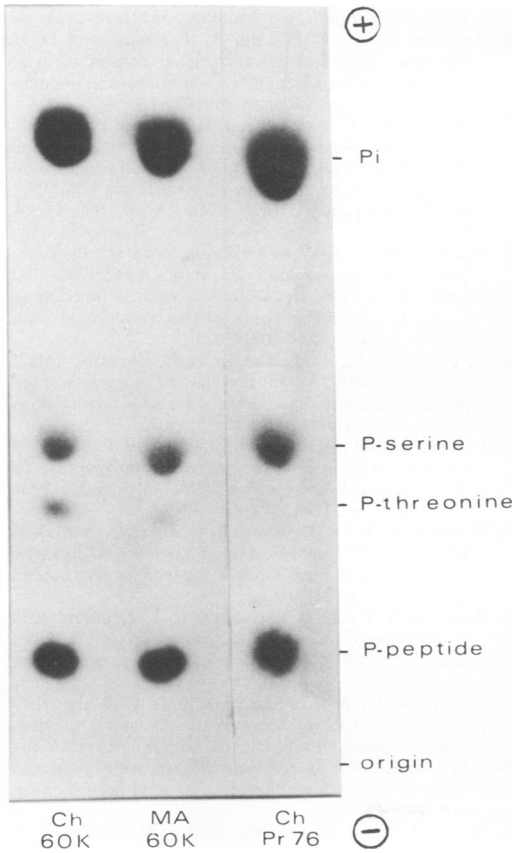

FIG. 7. *Phosphoamino acids of Pr76 and 60K.* ^{32}P*-labeled Pr76 and 60K were immunoprecipitated from lysates of SR-RSV-infected chick cells and of SR-RSV-D MA cells, isolated from SDS-polyacrylamide gels, and digested with trypsin. A portion of the tryptic peptides of each protein was subjected to acid hydrolysis, followed by paper electrophoresis, all as described in the text.*

one of these intermediates. The following evidence from previous reports strongly suggests that 60K is not related to Pr76. (i) When TBR serum is first adsorbed with disrupted virions, the precipitation of the mature gs antigens, as well as all the known intermediates, is blocked, whereas the precipitation of 60K is not reduced (2, 21a). (ii) The 60K protein is not precipitated by antibody to p27 (2). According to the processing scheme of Pr76 (8), p27 should be present in a 60,000-MW intermediate. (iii) The 60K protein is not found in transformation-defective virus-infected cells which contain Pr76 and the cleavage products of this protein (21a). (iv) The 60K protein is synthesized in cell-free protein-synthesizing systems programmed with RNA from the 3′ one-third of a nondefective sarcoma virus genome; this RNA contains the *src* gene and does not contain *gag* gene sequences (11–13, 17, 25), nor does it program the synthesis of Pr76 (21a, 22). (v) There is no evidence that Pr76 is processed into mature antigens in any of the mammalian cells examined in this report, yet 60K is present in every SR-ASV-transformed mammalian cell studied to date.

As presented in this report, the two-dimensional chymotryptic peptide maps of 60K and Pr76 (Fig. 4) clearly demonstrate that these proteins are different. In addition, the tryptic phosphopeptide found in Pr76 is identical to that in p19 (9), but different from the tryptic phosphopeptide found in 60K (Fig. 6). Again it is unlikely that such a result would be obtained if 60K were a cleavage product of Pr76.

In addition, we have evidence that the precipitation of 60K by TBR serum 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. Brugge and R. L. Erikson, unpublished data). Since the precipitation of the gs antigens and all of their precursors is group specific, it is unlikely that 60K is derived from Pr76. Moreover, the *N*-formyl methionine tryptic peptides of Pr76 and 60K synthesized in cell-free extracts migrate differently upon one-dimensional electrophoresis, indicating that Pr76 and 60K do not share translational initiation sites. (A. Siddiqui, A. F. Purchio, and R. L. Erikson, manuscript in preparation).

The evidence presented in this report and previous reports (2, 21a, 22) on the 60K protein proves that this protein is transformation specific, virus coded, and unrelated to the structural proteins of ASV. In view of these findings, we propose that this protein be designated $p60^{src}$ by the convention currently being used to identify ASV gene products.

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