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Structural Analysis of the Avian Sarcoma Virus Transforming Protein: Sites of Phosphorylation

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The avian sarcoma virus (ASV) protein responsible for cellular transformation in vitro and sarcomagenesis in animals was studied structurally with special reference to the sites of phosphorylation on the polypeptide. The product of the ASV *src* gene, pp60^{src}, is a phosphoprotein of 60,000 daltons. We found that pp60^{src} contained two major sites of phosphorylation, one involving phosphoserine and the other involving phosphothreonine, and possible additional minor sites of phosphorylation. By using *N*-formyl[^{35}S]methionyl-tRNA$_f$ as a radiolabeled precursor in the cell-free synthesis of the *src* protein in conjunction with partial proteolysis mapping, we determined that the major phosphoserine residue was located on the amino-terminal two-thirds of the molecule and that the phosphothreonine was located on the carboxy-terminal third. We further determined that the phosphorylation of pp60^{src} in cell extracts involved at least two protein kinases, the one that phosphorylated the major serine site being cyclic AMP dependent and the other, acting on the threonine residue, being a cyclic nucleotide-independent phosphotransferase. Finally, analysis of the pp60^{src} isolated from cells infected with a temperature-sensitive *src* gene mutant of ASV revealed that phosphorylation of the major threonine residue was severely reduced when infected cells were grown at the nonpermissive temperature, whereas a phosphorylation pattern characteristic of the wild-type pp60^{src} was observed at the permissive temperature. As pp60^{src} has an associated protein kinase activity, the possible involvement of phosphorylation-dephosphorylation reactions in the functional regulation of ASV transforming protein enzymatic activity is discussed.

Avian sarcoma viruses (ASV) are able to induce sarcomas in a variety of animals and transform both avian and mammalian cells in culture. A single ASV gene, termed *src* for sarcoma gene, is directly responsible for ASV-induced neoplastic transformation (9, 20). We have recently identified the polypeptide product of the *src* gene, a 60,000-dalton phosphoprotein designated pp60^{src}, and have further determined that pp60^{src} acts as a protein kinase (1, 2, 4, 14). The fact that of the four ASV strains tested (Schmidt-Ruppin [SR], Prague, Bratislava, and Bryan) all encode similar *src* gene polypeptides with phosphotransferase activities (J. S. Brugge et al., J. Virol., in press) and that the kinase activity is growth temperature dependent in cells transformed with an ASV temperature-sensitive transformation mutant (4) suggests that ASV-induced oncogenesis may be due to the aberrant phosphorylation of cellular proteins by the transforming gene product pp60^{src}.

As pp60^{src} is itself phosphorylated, and since phosphorylation-dephosphorylation reactions are known to be involved in the control of enzymes and regulatory proteins (8, 19), we have initiated studies concerning the characterization of the sites of phosphorylation on the pp60^{src} molecule in an effort to understand the functional regulation in cells of the ASV transforming protein. In this communication we report that pp60^{src} contains two major phosphorylated residues, one in phosphoserine and the other in phosphothreonine, and possibly additional minor phosphorylated sites. We have localized these residues within the pp60^{src} molecule and have further determined that at least two protein kinase acitvities are involved in the phosphorylation of the ASV *src* gene protein.

MATERIALS AND METHODS

Cells and virus. Chicken embryo fibroblasts were prepared from 11-day-old embryos (Spafas, Inc., Roanoke, Ill.). The SR subgroup D and the Prague subgroup C strains of ASV were originally obtained from J. Wyke, and ASV-NY68 was obtained from H. Hanafusa. European field vole (*Microtus agrestis*) cells transformed with SR-ASV originally by P. Vogt (clone 1-T) were provided by A. Faras.

Antisera and immunoprecipitation. Tumor-bearing rabbit sera were obtained from rabbits injected subcutaneously as newborns with SR-ASV (1). Tu-

mor-bearing marmoset sera were obtained from marmosets injected with SR-ASV. Tumor-bearing marmoset sera are characterized in detail elsewhere (Brugge et al., J. Virol., in press). For the preparation of cell extracts, unlabeled cultures or cultures radiolabeled for 2 h with either [^{35}S]methionine (700 Ci/mmol, New England Nuclear Corp., Boston, Mass.; 25 μCi/ml) or $^{32}P_i$ (carrier-free, ICN Chemical and Radioisotope Div., Irvine, Calif.; 0.5 to 1.0 mCi/ml) were washed, and the cells were scraped from the dish and then either lysed in RIPA buffer as previously described (1, 4) or disrupted by sonic treatment in 0.01 M Tris-hydrochloride (pH 7.4)-1% Trasylol (FBA Pharmaceuticals, Inc., New York, N.Y.). The cell lysates or sonic extracts were then clarified at 100,000 $\times$ *g* for 30 min. Immunoprecipitation procedures using the *Staphylococcus aureus* immunoadsorbent technique (11) have been described previously (1, 2, 4).

Preparation of *N*-formyl[^{35}S]methionyl-tRNA$_f$. A preparation of chick tRNA was acylated with [^{35}S]methionine and formylated in one step with a crude *Escherichia coli* amino acid:tRNA ligase and formyltransferase preparation in the presence of the formyl donor, folinic acid, all as described by Dubnoff and Maitra (5). After phenol extraction and ethanol precipitation, the initiating species of methionyl tRNA, *N*-formyl[^{35}S]methionyl-tRNA$_f$, was further purified by passage through a benzoylated DEAE-cellulose column. To eliminate unformylated methionyl-tRNA$_f$, the RNA was treated with 10 mM $CuSO_4$ at pH 5.0 for 20 min at 37°C (18). After several ethanol precipitations, the RNA was dissolved in 0.01 M potassium acetate, pH 4.5, at a concentration of 1 mg/ml, and 5 μg was added to each 50-μl cell-free translation reaction. *N*-formyl[^{35}S]methionyl-tRNA$_f$ prepared in this manner had a specific activity of 1×10^6 to 3×10^6 cpm/μg. The purity, as checked by digestion with guanyloribonuclease and electrophoresis at pH 3.5, was greater than 95%.

Cell-free protein synthesis. The preparation and purification of nondefective SR-ASV 70S RNA has been previously reported (6, 14, 15). Subgenomic polyadenylic acid-containing viral RNA was selected by oligodeoxythymidylic acid-cellulose chromatography and sucrose gradient fractionation. Viral polyadenylic acid-containing RNA 21S in size was translated in the mRNA-dependent reticulocyte cell-free system (13) as described previously (6, 15), using either [^{35}S]methionine (25 μCi/50-μl reaction) or *N*-formyl[^{35}S]methionyl-tRNA$_f$ (1×10^6 to 3×10^6 cpm/μg; 5 μg/50-μl reaction) as radiolabeled substrate. In the latter case the wheat germ tRNA was omitted from the reaction mixture, and unlabeled amino acids, including methionine, were added. Under the conditions of cell-free translation used, the 60,000-dalton polypeptide encoded by the *src* gene is the principal product (6, 14).

One-dimensional limited proteolysis mapping. To isolate [^{35}S]methionine-labeled, *N*-formyl[^{35}S]methionine-labeled, or ^{32}P-labeled pp60^{src} from preparative sodium dodecyl sulfate-containing polyacrylamide gels for peptide analyses, unstained and unfixed gels were covered with Saran Wrap and exposed to Kodak X-Omat R film at 4°C. The pp60^{src} protein to be analyzed was then excised, and one-dimensional peptide mapping by limited proteolysis during re-electrophoresis was conducted as described by Cleveland et al. (3), using *S. aureus* V8 protease (Miles Laboratories, Inc., Elkhart, Ind.).

Two-dimensional tryptic peptide fingerprinting. Radiolabeled pp60^{src}, localized in gels and excised as described above, was eluted in 0.05 M NH_4HCO_3 (pH 8.5)-0.1% sodium dodecyl sulfate at 41°C. Carrier protein (100 μg of bovine serum albumin) was added, and the proteins were precipitated three times with 20% trichloroacetic acid. After performic acid oxidation and two lyophilizations, the proteins were digested with tolylsulfonyl phenylalanyl chloromethyl ketone-treated trypsin (Worthington Biochemicals Corp., Freehold, N.J.) in 0.05 M NH_4HCO_3, pH 8.5, at an enzyme-protein ratio of 1:20 for 4 to 5 h at 37°C, and then lyophilized. The resultant peptides were then subjected to ascending chromatography on plastic-backed cellulose sheets (Polygram Cel 300, 20 by 40 cm; Macherey-Nagel and Co., Düren, Germany) in *sec*-butanol-*n*-propanol-isoamyl alcohol-pyridine-water (1:1:1:3:3), followed by electrophoresis in the second dimension at either pH 3.5 (pyridine-acetic acid-water, 1:10:189) or pH 6.5 (pyridine-acetic acid-water, 100:3:879).

Phosphoamino acid analysis. ^{32}P-labeled proteins or polypeptide fragments were dried, dissolved in 30 μl of 2 N HCl, and hydrolyzed in sealed glass capillary tubes 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 1,500 V for 75 min. After electrophoresis, the paper was stained with ninhydrin (0.4% ninhydrin-1% acetic acid in acetone), dried, and subjected to autoradiography.

Cell-free phosphorylation reactions. Cell-free extracts of transformed cells were obtained by sonic treatment in 0.01 M Tris-hydrochloride (pH 7.4)-1% Trasylol as described above. Reaction mixtures (75 μl) consisted of 100 to 300 μg of the sonically treated cell-free extract in 10 mM Tris-hydrochloride (pH 7.4), 5 mM $MgCl_2$, 650 μM theophylline, and 1 μM [γ-^{32}P]-ATP (made according to the procedure of Glynn and Chappell [7], 1,000 to 1,400 Ci/mmol). Reactions were carried out at 30°C for 7 min and terminated by addition of EDTA to 30 mM. After dilution, reaction mixtures were immunoprecipitated with either tumor-bearing rabbit serum or tumor-bearing marmoset serum as described (1, 4). Cyclic nucleotides (cGMP or cAMP; Sigma Chemical Co., St. Louis, Mo.) were included in various reactions as detailed in the legends to the figures. Acid- and heat-stable cAMP-dependent protein kinase inhibitor was provided by David Andrews, Department of Pharmacology, University of Colorado Medical Center, Denver, or was purchased from Sigma.

RESULTS

One-dimensional partial proteolysis of pp60^{src}. As a means of analyzing various structural features of pp60^{src}, we used the limited proteolysis procedure of Cleveland et al. (3). By using a variety of proteases, characteristic diges-

tion patterns can be obtained for pp60^{src}. The use of *S. aureus* V8 protease results in an especially simple partial digestion map. [^{35}S]methionine-labeled *src* protein synthesized in the cell-free reticulocyte system, when subjected to V8 protease partial proteolysis, produced two major digestion products (Fig. 1A, tracks 1, 3, and 5). At the highest protease concentration (track 5) smaller peptides were revealed. The two principal digestion products had molecular weights of approximately 34,000 and 26,000, respectively, suggesting that they may represent peptides generated by protease cleavage at one site within the pp60^{src} molecule. Furthermore, the pattern of digestion implied that the 26,000-dalton fragment may have been an end product of the V8 protease digestion, as its intensity did not change upon hydrolysis with increased amounts of enzyme. In contrast, the 34,000-dalton peptide appeared to be lost at the same time that the group of small peptides was generated. (These speculations are confirmed below.) Thus, we performed experiments to determine which of these two major V8 protease fragments represented the NH_2 terminus of the pp60^{src} molecule. This was accomplished by the cell-free synthesis of the *src* protein, using *N*-formyl[^{35}S]methionyl-tRNA$_f$ as the radiolabeled precursor. The initiator amino acid *N*-formyl[^{35}S]methionine is incorporated only at the NH_2-terminal position of the resultant polypeptide (5). The *N*-formyl-[^{35}S]methionine-labeled *src* protein was then subjected to limited proteolysis with V8 protease and run in a gel next to the uniformly [^{35}S]-methionine-labeled protein. Only the 34,000-dalton fragment contained the *N*-formyl[^{35}S]methionine label (Fig. 1A, tracks 2, 4, and 6), indicating that this peptide represented the NH_2-terminal 60% of the *src* protein. This result also allows us to conclude that the 26,000-dalton fragment is derived from the COOH terminus of the protein. Hereafter we will refer to these V8 protease digestion fragments as the V8-NH_2 and the V8-COOH peptides, respectively.

In initial experiments directed at investigating the phosphorylation of pp60^{src}, we performed a similar V8 protease partial digestion analysis on [^{35}S]methionine-labeled and ^{32}P-labeled pp60^{src} isolated by immunoprecipitation of ASV-transformed cell extracts. Figure 1B (tracks 2, 4, and 6) shows the pattern of the [^{35}S]methionine-labeled protein. It can be seen by comparing this pattern to the pattern of the cell-free-synthesized protein (Fig. 1A, tracks 1, 3, and 5) that the in vivo (immunoprecipitated) and the in vitro (cell-free-synthesized) proteins are identical, confirming previous results (14). Figure 1B (tracks 1, 3, and 5) shows the cleavage pattern

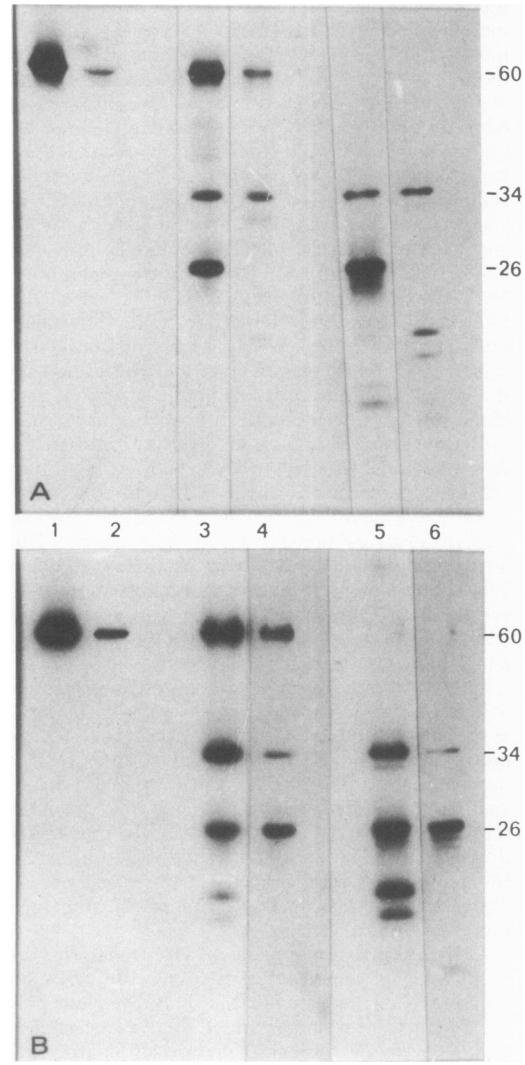


FIG. 1. *One-dimensional limited proteolysis mapping of pp60^{src}. The 60,000-dalton ASV src protein bands localized by autoradiography of preparative polyacrylamide gels were excised and subjected to partial proteolysis by S. aureus V8 protease during re-electrophoresis. Protease concentrations: tracks 1 and 2, no enzyme; tracks 3 and 4, 0.005 μg of enzyme; tracks 5 and 6, 0.05 μg of enzyme. The numbers in the margin represent approximate molecular weights in kilodaltons. (A) The SR-ASV src protein was synthesized in the reticulocyte cell-free system from the viral RNA as described in the text, using either [^{35}S]methionine (tracks 1, 3, and 5) or N-formyl[^{35}S]methionyl-tRNA$_f$ (tracks 2, 4, and 5) as radiolabeled precursor. (B) [^{35}S]methionine-labeled (tracks 2, 4, and 6) and ^{32}P-labeled (tracks 1, 3, and 5) pp60^{src} were prepared by immunoprecipitation of radiolabeled SR-ASV-transformed vole cell extracts with tumor-bearing rabbit serum.*

of ^{32}P-labeled pp60^{src}. Two major fragments, with electrophoretic mobilities identical to those of the [^{35}S]methionine peptides, were produced. Furthermore, the 26,000-dalton fragment appeared to be a digestion end product, whereas the 34,000-dalton fragment was lost with attendant appearance of smaller peptide fragments upon increasing the protease concentration. These results suggest that the two major ^{32}P-labeled V8 protease fragments were identical to the V8-NH_2 and V8-COOH peptides. In addition, these data imply that the pp60^{src} protein contains multiple sites of phosphorylation.

Two-dimensional tryptic peptide mapping of pp60^{src}. To further investigate the possibility that pp60^{src} contained multiple sites of phosphorylation, we performed various two-dimensional fractionation techniques on exhaustively trypsinized ^{32}P-labeled pp60^{src}. The first dimension of the fractionation in all cases involving ascending chromatography and was followed by electrophoresis at either pH 3.5 or pH 6.5. Figure 2 displays the resultant *src* protein phosphopeptide maps when pp60^{src} was obtained from chicken cells infected with SR-ASV (Fig. 2A and C) and from vole cells infected with SR-ASV (Fig. 2B and D). Considerably different maps were obtained when the pH of electrophoresis in the second dimension was changed from 3.5 to 6.5. At pH 3.5, one major phosphopeptide was apparent (Fig. 2A and B), as previously reported (2). However, when, after chromatography, the second dimension of the separation was carried out at pH 6.5, two major phosphopeptides were revealed, as well as several minor phosphorylated spots (Fig. 2C and D). The nature and significance of these minor peptides will not be pursued in this study.

Thus, two-dimensional tryptic fingerprinting of pp60^{src} suggests, as did the one-dimensional partial V8 protease analyses, that the *src* protein contains at least two major sites of phosphorylation. To conclusively demonstrate this, we performed both phosphoamino acid analysis and two-dimensional tryptic fingerprinting on the ^{32}P-labeled V8-NH_2 and V8-COOH peptides. We have previously indicated that pp60^{src} contains both phosphoserine and phosphothreonine (2). The V8-NH_2 peptide contained only phosphoserine (Fig. 3, track 2), whereas the V8-COOH fragment contained exclusively phosphothreonine (Fig. 3, track 3). Furthermore, the smaller peptides generated by V8 protease contained only phosphoserine (Fig. 3, track 4), confirming that these fragments were derived from the V8-NH_2 fragment.

The two-dimensional tryptic phosphopeptide maps of the V8-NH_2, V8-COOH, and small peptide fragments are presented in Fig. 4. It is clearly seen that one of the major tryptic phosphopeptides was present both in the V8-NH_2 fragment and in a smaller peptide derived from this fragment and that the second major tryptic phosphopeptide originated from the V8-COOH fragment. Furthermore, phosphoamino acid analysis of the two major tryptic phosphopeptides revealed that the major tryptic peptide from the V8-NH_2 fragment and its smaller fragments, which migrated toward the cathode, contained only phosphoserine and that the tryptic peptide from the V8-COOH fragment, which migrated toward the anode, contained exclusively phosphothreonine (data not shown).

Cell-free phosphorylation of pp60^{src}. In an effort to understand the phosphorylation of pp60^{src} and, ultimately, the possible functional regulation of pp60^{src} enzymatic activity by phosphorylation-dephosphorylation modifications, we investigated conditions whereby pp60^{src} could be phosphorylated in crude, cell-free extracts. Unlabeled ASV-infected cell extracts were prepared by sonic treatment, and the soluble portion was obtained after ultracentrifugation. These soluble sonic extracts were then adjusted to kinase reaction conditions by the addition of $MgCl_2$ and [γ-^{32}P]ATP and incubated with and without the further addition of cyclic nucleotides. The phosphorylation of pp60^{src} was monitored by immunoprecipitation of the reaction mixtures, followed by sodium dodecyl sulfate-containing polyacrylamide gel electrophoresis. Figure 5 illustrates the results obtained when SR-ASV-transformed vole cell extracts were used in such an experiment. pp60^{src} was phosphorylated under these conditions in the absence of any added cyclic nucleotides (track 1). This result may indicate that not all of the intracellular pp60^{src} molecules were phosphorylated at any one time, since the protein could be further phosphorylated in vitro. Alternatively, our detection of the phosphorylation of pp60^{src} in vitro may reflect dephosphorylation-phosphorylation exchange reactions occurring in our cell-free system. We are currently unable to distinguish between these possibilities.

To determine if the addition of cGMP or cAMP was able to stimulate this basal level of cell-free phosphorylation, each cyclic nucleotide was added to reactions at various concentrations (Fig. 5). Tracks 2, 3, and 4 show the effect of cGMP at concentrations of 0.1, 1, and 10 μM, respectively, on the phosphorylation of pp60^{src}. No stimulation was noted at the two lower cGMP concentrations, as determined by direct spectrophotometry of the protein bands from the gel, but a slight (1.5-fold) increase in pp60^{src}

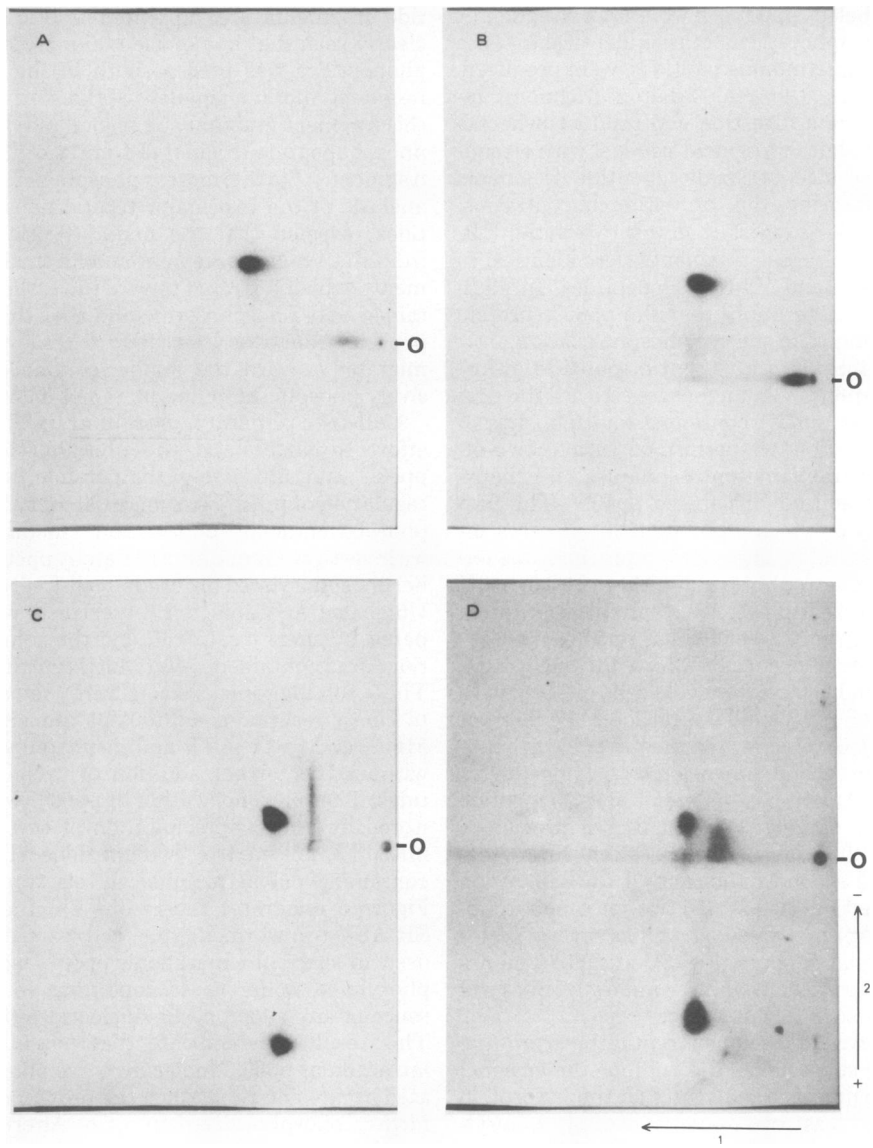


FIG. 2. *Two-dimensional tryptic fingerprints of ^{32}P-labeled pp60^{src}. ^{32}P-labeled pp60^{src} was isolated by immunoprecipitation of either SR-ASV-transformed chick cell extracts (A and C) or SR-ASV-transformed vole cell extracts (B and D), and the proteins were digested with trypsin as described in the text. Fractionation of all phosphopeptide samples involved ascending chromatography in the first dimension and electrophoresis in the second dimension at either pH 3.5 (A and B) or pH 6.5 (C and D). O, Origin.*

phosphorylation was observed at 10 μM cGMP. Due to the extremely high concentration of cGMP and in view of the results to follow, we feel that this stimulation by cGMP was the result of cross-activation of a cAMP-dependent phosphorylating system. When, under the same conditions, cAMP was added to these reactions, considerable (2- to 6-fold) stimulation of the phosphorylation of pp60^{src} was observed at all cAMP concentrations (tracks 5, 6, and 7). Similar results were obtained with SR-ASV- and Prague ASV-transformed chicken cell extracts.

To further demonstrate that this stimulation by cAMP of the phosphorylation of pp60^{src} was due to a cAMP-dependent protein kinase, we added to cAMP-containing reaction mixtures the heat- and acid-stable inhibitory protein (16, 17). This protein is a specific inhibitor of cAMP-dependent protein kinases which interacts with the catalytic subunit of these enzymes (16). Ad-

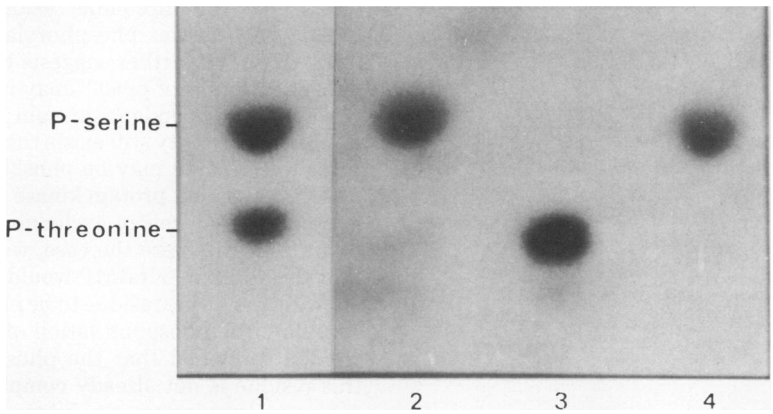


FIG. 3. *Phosphoamino acid analysis of pp60^{src} and of peptide fragments generated by V8 protease digestion. ^{32}P-labeled SR-ASV-transformed vole cell extracts were used for the preparative isolation of pp60^{src} and subsequent preparative V8 protease hydrolysis during re-electrophoresis. The major V8 protease fragments were localized by autoradiography and excised, and the peptides were eluted from the gel pieces as described in the text. Each of the eluted peptides was precipitated with 20% trichloroacetic acid, redissolved, and then subjected to electrophoresis in a second polyacrylamide gel. After autoradiographic localization, excision, and elution, each peptide fragment was subjected to complete tryptic digestion followed by acid hydrolysis and paper electrophoresis, all as described in the text. Tracks: 1, intact pp60^{src}; 2, V8-NH$_2$ peptide; 3, V8-COOH peptide; 4, low-molecular-weight fragment (see Fig. 1B, track 5). Both low-molecular-weight ^{32}P-labeled V8 fragments contained only phosphoserine.*

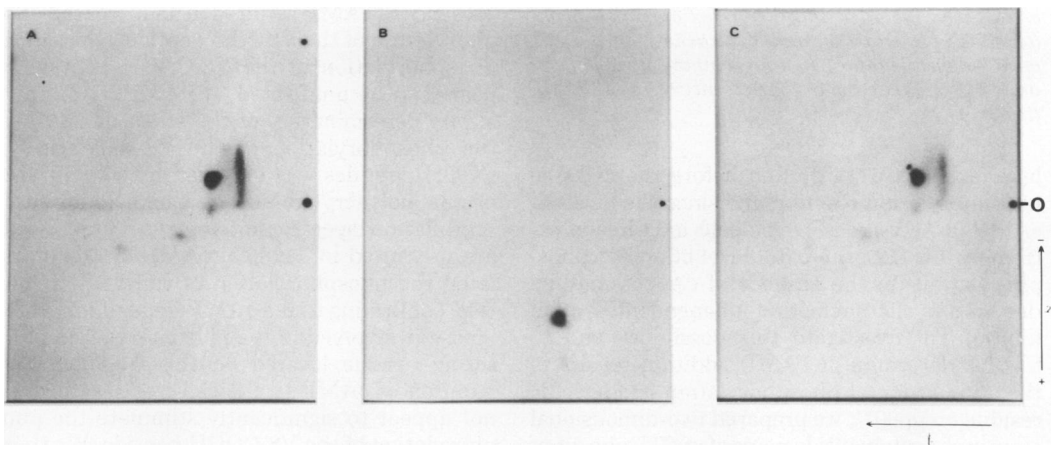


FIG. 4. *Two-dimensional tryptic fingerprints of ^{32}P-labeled pp60^{src} peptide fragments generated by V8 protease digestion. ^{32}P-labeled V8-NH$_2$ and V8-COOH peptide fragments and a low-molecular-weight peptide obtained by V8 protease hydrolysis during re-electrophoresis were prepared as described in the legend to Fig. 3. After complete tryptic digestion, the phosphopeptides were fractionated by chromatography followed by electrophoresis at pH 6.5. O, Origin. (A) V8-NH$_2$ fragment containing phosphoserine; (B) V8-COOH fragment containing phosphothreonine; (C) low-molecular-weight fragment (both low-molecular-weight ^{32}P-labeled V8 protease fragments [Fig. 1B, track 5] produced identical fingerprints).*

dition of this protein kinase inhibitor resulted in a significant reduction in the amount of phosphorylated pp60^{src} (Fig. 5, track 8), compared with an identical reaction run in its absence (Fig. 5, track 7). The extent of inhibition observed in this experiment was 60% and has varied in different experiments from 50 to 85%. This variability and failure to completely inhibit the cAMP-stimulated phosphorylation may reflect either the ability of pp60^{src} to interact directly with the catalytic subunit of the cAMP-dependent protein kinase or an interaction of the inhibitor with pp60^{src} (16).

Above, we have demonstrated that a cAMP-dependent protein kinase is involved in the phosphorylation of pp60^{src}. However, since the cel-

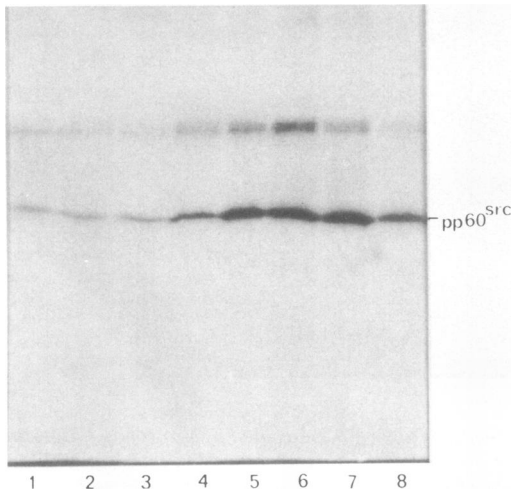


FIG. 5. *Cell-free phosphorylation of pp60^{src}. Sonically treated cell-free extracts from SR-ASV-transformed vole cells were prepared and incubated in the cell-free phosphorylation reaction mixture, followed by immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis, all as described in the text. cGMP was included in reactions at 0.1 (track 2), 1.0 (track 3), and 10 (track 4) μM, and cAMP was included in other reactions, also at 0.1 (track 5), 1.0 (track 6), and 10 (tracks 7 and 8) μM. cAMP-dependent protein kinase inhibitor (15 μg) was added to one reaction in the presence of 10 μM cAMP (track 8).*

lular extracts were diluted before the cell-free phosphorylation reaction and since the levels of cAMP in ASV-transformed cells are already extremely low (12), the basal level of pp60^{src} phosphorylation (in the absence of cAMP) may be due to a cyclic nucleotide-independent protein kinase. To investigate this possibility and to further determine if cAMP addition results in the stimulation of phosphorylation of a specific residue on pp60^{src}, we prepared two-dimensional tryptic phosphopeptide maps of pp60^{src} phosphorylated in the cell-free extracts with and without the addition of cAMP. Figure 6B illustrates the fingerprint of the cell-free-phosphorylated pp60^{src} when cAMP was present in the reaction. It can be seen that the same two major tryptic phosphopeptides observed when pp60^{src} was phosphorus-radiolabeled in vivo were represented, indicating that our conditions of cell-free phosphorylation mimicked those present in the intact cell. However, when cAMP was omitted from the cell-free phosphorylation reaction, only the phosphothreonine residue was phosphorylated (Fig. 6A). These results indicate that phosphorylation of the serine residue, located on the V8-NH_2 peptide, requires cAMP and is therefore carried out by a cAMP-dependent protein kinase. That the threonine residue on the V8-COOH peptide was phosphorylated in the absence of cAMP further suggests that this site of phosphorylation of pp60^{src} may involve a cyclic nucleotide-independent protein kinase. However, the possibility still exists that the phosphothreonine residue may be phosphorylated by a cAMP-dependent protein kinase which requires extremely low levels of cyclic nucleotide for activation. If this were the case, we would expect that the addition of cAMP would, in addition to allowing the serine residue to be phosphorylated, stimulate the phosphorylation of the threonine residue (provided that the phosphorylation of this residue is not already complete in the cell extracts). In an attempt to address this issue, we performed the cell-free phosphorylation of pp60^{src} with and without cAMP in identical-sized reactions with three different cellular extracts (SR-ASV- and Prague ASV-transformed chick and SR-ASV-transformed vole cell extracts) and analyzed the pp60^{src} reaction products by limited V8 protease proteolysis (Fig. 7). In visually comparing the appropriate tracks (digestion with the same enzyme concentration with and without cAMP), it can be seen that the presence of cAMP resulted in an increased phosphorylation of the V8-NH_2 peptide, whereas the phosphorylation of the V8-COOH peptide appeared to be unaffected by cAMP. A more accurate determination of the effect of cAMP on the phosphorylation of the V8-NH_2 and V8-COOH peptides was obtained by solubilization of the polyacrylamide gel bands followed by scintillation spectrophotometry. These results are presented in Table 1. cAMP clearly stimulated the phosphorylation of the V8-NH_2 peptide, confirming that a cAMP-dependent protein kinase is involved in the phosphorylation of the serine residue located on this fragment. The addition of cAMP to the cell-free reactions did not appear to significantly stimulate the phosphorylation of the V8-COOH peptide. We therefore tentatively conclude that the phosphorylation of the threonine residue on this peptide fragment involves a cyclic nucleotide-independent protein kinase activity.

Phosphorylation of pp60^{src} in chicken cells infected with a temperature-sensitive *src* gene mutant of ASV. In an initial attempt to obtain information concerning the possible significance of the phosphorylation of pp60^{src} with respect to *src* function in cellular transformation, we analyzed pp60^{src} from cells infected with a temperature-sensitive *src* gene mutant of SR-ASV. Chicken cells infected with the NY68 temperature-sensitive *src* gene mutant of SR-ASV (10) and grown at the permissive temperature (35°C) are morphologically transformed

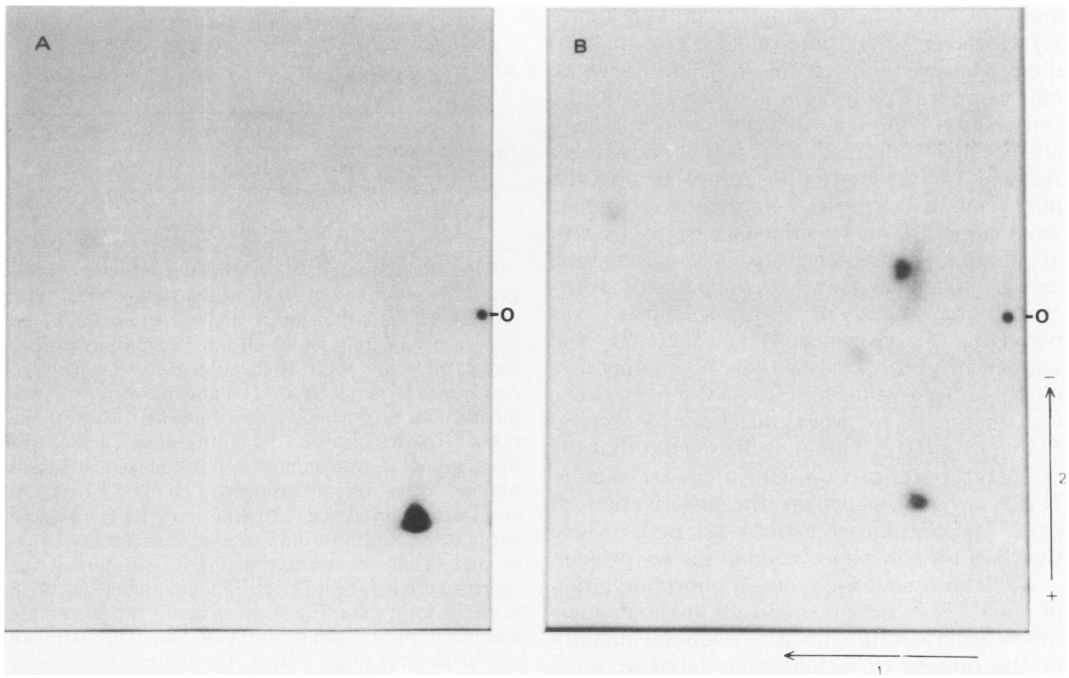


FIG. 6. *Two-dimensional tryptic fingerprints of pp60^{src} phosphorylated in cell-free extracts with and without added cAMP. pp60^{src} was phosphorylated in cell-free extracts of SR-ASV-transformed vole cells in the absence (A) or presence (B) of 10 μM cAMP as described in the legend to Fig. 5 and in the text. The phosphorylated proteins were immunoprecipitated and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and pp60^{src} was eluted and digested with tolylsulfonyl phenylalanyl chloromethyl ketone-treated trypsin. Two-dimensional fractionation involved chromatography followed by electrophoresis at pH 6.5. O, Origin.*

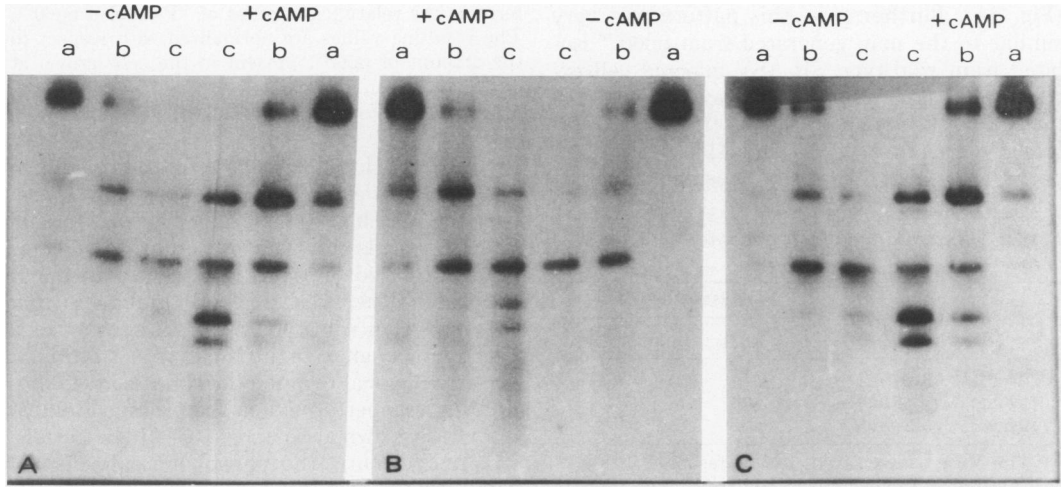


FIG. 7. *One-dimensional limited proteolysis mapping of pp60^{src} phosphorylated in cell-free extracts with and without added cAMP. Cell-free phosphorylation reactions were carried out as described in the text, using extracts derived from SR-ASV-transformed chick cells (A), Prague ASV-transformed chick cells (B) and SR-ASV-transformed vole cells (C) in the presence or absence of 10 μM cAMP as indicated. All reactions were of identical size and were immunoprecipitated (SR-ASV-infected cell extracts with tumor-bearing rabbit serum and Prague ASV-infected cell extracts with tumor-bearing marmoset serum), followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After autoradiographic localization, the pp60^{src} bands were subjected to limited digestion with S. aureus V8 protease during re-electrophoresis. Protease concentrations: track a, 0.001 μg of enzyme; track b, 0.005 μg of enzyme; track c, 0.05 μg of enzyme.*

and contain a functional pp60^{src} protein kinase (4). However, when these cells are grown at the nonpermissive temperature (41°C), although no effect on virus replication is observed (20), the cells exhibit a more normal cellular morphology and the pp60^{src} protein kinase activity is severely reduced (4). At both the permissive and the nonpermissive growth temperatures, similar amounts of [^{35}S]methionine-labeled *src* protein are immunoprecipitable from NY68-infected cell extracts (Table 2). However, a significant reduction in the amount of ^{32}P-labeled pp60^{src} was noted in cells grown at 41°C (Table 2). The amount of phosphorylated pp60^{src} immunoprecipitable from wild-type SR-ASV-infected cells was unaffected by growth at either 35 or 41°C (data not shown). These results suggest that the temperature-sensitive lesion in the *src* gene of NY68, which results in the inactivation of pp60^{src} protein kinase activity (4), may involve the (lack of) phosphorylation of the *src* protein.

To determine if the reduced phosphorylation of pp60^{src} in NY68-infected cells at the nonpermissive temperature involved a general inability of the protein to be phosphorylated or some more specific deficiency, we analyzed both [^{35}S]methionine-labeled and ^{32}P-labeled pp60^{src} from these cells grown at both 35 and 41°C by V8 protease limited proteolysis mapping (Fig. 8). The [^{35}S]methionine-labeled peptide patterns of pp60^{src} were identical for the proteins obtained from cells grown at either 35 or 41°C (Fig. 8A). Furthermore, this pattern was very similar to the map generated from pp60^{src} isolated from wild-type SR-ASV-infected cell extracts (Fig. 8A, track 1). However, when ^{32}P-labeled peptide maps of pp60^{src} from NY68-infected cells grown at 35 and 41°C were compared, it appeared that the phosphorylation of the V8-COOH peptide of the protein obtained from the cells grown at the nonpermissive temperature was highly deficient. The protein isolated from cells grown at the permissive temperature exhibited phosphorylation of both major V8 protease peptides (Fig. 8B), similar to that observed with wild-type pp60^{src} (Fig. 8B, track 1). To further analyze the NY68 pp60^{src} proteins, two-dimensional tryptic phosphopeptide fingerprinting was performed on the proteins isolated from cells grown at 35 and 41°C. At the permissive temperature, the phosphopeptide map of NY68 pp60^{src} was identical to that of the wild-type protein (Fig. 2C and 9A). However, the *src* protein isolated from cells grown at the nonpermissive temperature showed a nearly complete lack of phosphorylation of the phosphothreonine-containing peptide (Fig. 9B). These results confirm the V8 protease analysis above and indicate that, upon shift to the nonpermissive growth temperature, the threonine residue on

TABLE 1. *Stimulation of phosphorylation of pp60^{src} by cAMP*[a]

Virus/cell	Stimulation by cAMP	
	V8-NH_2	V8-COOH
SR-ASV/chick	3.15	1.25
Prague ASV/chick	2.50	1.15
SR-ASV/vole	3.85	0.95

[a] The V8 protease fragments represented in Fig. 7 were cut out of the gel and solubilized in 30% H_2O_2 at 60°C for 16 h, and their radioactivities were determined. The ratio of the radioactivity of a given peptide from pp60^{src} phosphorylated in the presence and absence of cAMP and digested with the same amount of protease was taken to represent the stimulation of phosphorylation by cAMP. The results above represent the averages of the values obtained from the peptides generated by 0.005 and 0.05 μg of protease. The individual values varied by less than 15%.

TABLE 2. *Growth temperature-dependent phosphorylation of pp60^{src} in chick cells infected with a temperature-sensitive transformation mutant of ASV*[a]

Growth temp (°C)	Relative amt of pp60^{src}	
	^{35}S-labeled	^{32}P-labeled
35	1.00	1.00
41	0.91	0.26

[a] Parallel cultures of NY68-infected chick cells, grown at either 35 or 41°C, were radiolabeled with either [^{35}S]methionine or ^{32}P and immunoprecipitated with tumor-bearing rabbit serum. The immunoprecipitated materials were then subjected to polyacrylamide gel electrophoresis. The amount of pp60^{src} was quantitated as previously described (3a). Briefly, the pp60^{src} bands, localized by autoradiography of the fixed, dried gel, were cut out and solubilized, and their radioactivities were determined. The pp60^{src} region of gel tracks containing extracts immunoprecipitated with normal rabbit serum was used to determine background levels of radioactivity. By determining the specific activity of the [^{35}S]methionine-labeled protein from the total cell extracts, an estimate of the absolute amount of pp60^{src} can be obtained (3a). This estimate will be in error to the extent that the specific activity of the total cell protein and that of pp60^{src} may differ. However, this error is minimized by calculating the ratio of the amount of pp60^{src} (normalized to nanograms of pp60^{src} per milligram of cell protein) present at 35 to that present at 41°C. Similarly, the ratio of the amount of ^{32}P-labeled pp60^{src} (normalized to counts per minute of pp60^{src} per microgram of pp60^{src}) present at the two temperatures may be used to determine the relative quantities of ^{32}P-labeled pp60^{src}. The resulting values are normalized with respect to the amount of pp60^{src} present in the cells grown at 35°C.

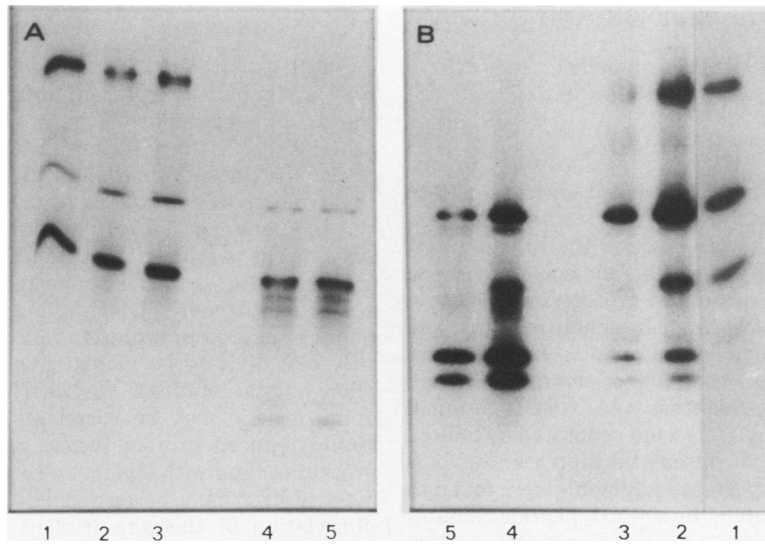


FIG. 8. *One-dimensional limited proteolysis mapping of pp60^{src} from chick cells infected with a temperature-sensitive src gene mutant of ASV. Chick cell cultures infected with the temperature-sensitive src gene mutant of SR-ASV, NY68, grown at either 35 or 41°C, were labeled with either [^{35}S]methionine or $^{32}P_i$ for 2 h, and the pp60^{src} proteins were isolated and subjected to partial proteolysis as described in the text. S. aureus V8 protease concentrations: tracks 1, 2, and 3, 0.005 μg of enzyme; tracks 4 and 5, 0.05 μg of enzyme. (A) [^{35}S]methionine-labeled pp60^{src} from wild-type SR-ASV-transformed vole cells (track 1) and from NY68-infected chick cells grown at 35°C (tracks 2 and 4) or 41°C (tracks 3 and 5). (B) ^{32}P-labeled pp60^{src} from wild-type SR-ASV-transformed vole cells (track 1) and from NY68-infected chick cells grown at 35°C (tracks 2 and 4) or 41°C (tracks 3 and 5).*

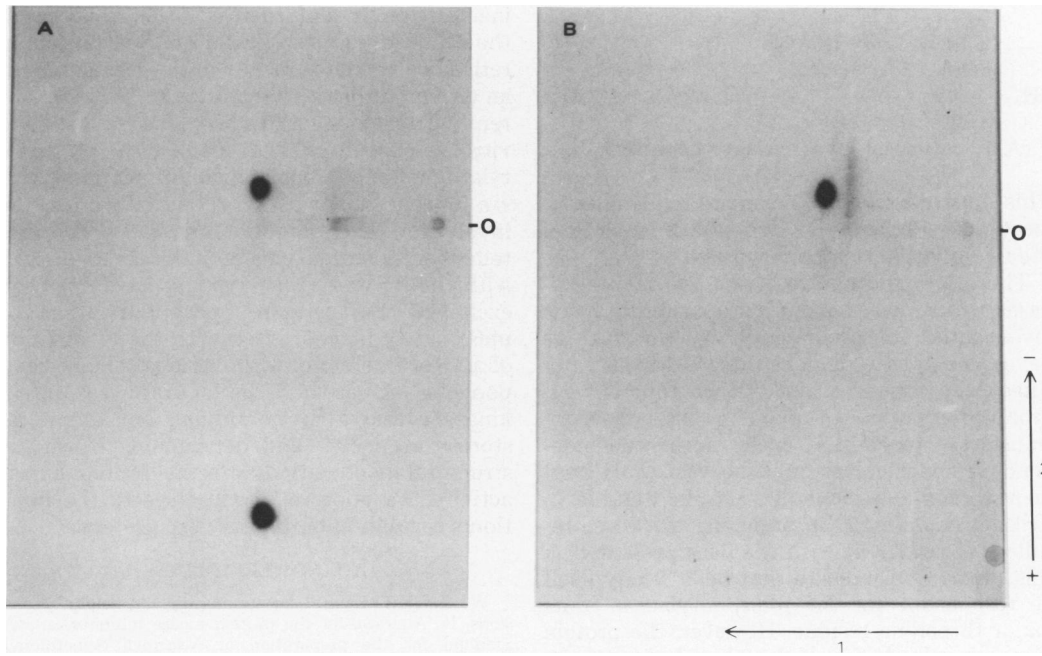
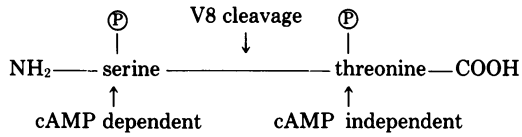


FIG. 9. *Two-dimensional tryptic fingerprints of ^{32}P-labeled pp60^{src} from chick cells infected with a temperature-sensitive src gene mutant of ASV. NY68-infected chick cells grown at either 35 or 41°C were labeled with $^{32}P_i$. The pp60^{src} proteins were isolated by immunoprecipitation and gel electrophoresis and hydrolyzed with trypsin as detailed in the text. The resultant phosphopeptides were fractionated by chromatography followed by electrophoresis at pH 6.5. O, Origin. (A) pp60^{src} from cells grown at 35°C, showing both major phosphopeptides. (B) pp60^{src} from cells grown at 41°C, showing that the major phosphothreonine-containing peptide, which migrates toward the anode, is missing.*

the V8-COOH peptide of pp60^{src} from NY68-infected cells is no longer phosphorylated.

DISCUSSION

In this report we present our initial structural characterizations of the ASV transforming protein with special reference to the sites of phosphorylation in the pp60^{src} molecule. By using the technique of limited proteolysis during re-electrophoresis, we have been able to obtain two polypeptide fragments of pp60^{src}, one representing the NH_2-terminal 60% of the molecule and the other representing the COOH-terminal 40%. These fragments were generated by limited digestion with *S. aureus* V8 protease. Furthermore, both V8 protease fragments were found to be phosphorylated, the V8-NH_2 peptide containing phosphoserine and the V8-COOH peptide containing phosphothreonine. From cell-free phosphorylation studies, we have demonstrated that a cAMP-dependent protein kinase is responsible for the phosphorylation of the V8-NH_2 peptide serine residue and that most likely a cyclic nucleotide-independent protein kinase is involved in the phosphorylation of the V8-COOH peptide threonine residue. These results are summarized by the following diagram:



This illustration is not meant to imply specific locations within the V8 protease fragments of the two phosphorylated residues.

The origin and nature of the two protein kinases which act on pp60^{src} are currently being investigated. In preliminary experiments, we have been able to demonstrate, in partially purified preparations of pp60^{src}, the apparent autophosphorylation of pp60^{src}. This phosphotransferase reaction is cyclic nucleotide independent and involves only the V8-COOH fragment threonine residue (M. S. Collett and R. L. Erikson, manuscript in preparation). These results are consistent with the data presented in this report and indicate that pp60^{src} may itself be responsible for the phosphorylation of its major threonine residue. However, the protein kinase involved in the phosphorylation of the pp60^{src} NH_2-terminal serine residue remains to be identified. It is likely that this cAMP-dependent protein kinase will be of cellular origin, and experiments designed to resolve this issue are in progress.

As phosphorylation-dephosphorylation reactions are known to represent on-off switches for the activity of many enzymes and regulatory proteins (8, 19), the question exists as to whether or not the phosphorylation of pp60^{src} is related to its role in neoplastic transformation. By using the temperature-sensitive *src* gene mutant of ASV, NY68, we have found that on transfer of infected cultures to the nonpermissive temperature, the normally phosphorylated pp60^{src} protein was altered to a protein phosphorylated at only one of the two major sites. The lack of phosphorylation of the V8-COOH peptide threonine residue may be correlated with the severely reduced protein kinase activity of this protein (4) and with the fact that pp60^{src} kinase activity itself may be responsible for the phosphorylation of this site (Collett and Erikson, manuscript in preparation). However, these data do not strictly demonstrate that the major phosphothreonine residue on pp60^{src} is required for *src* enzymatic activity. The mutation in the NY68 *src* gene presumably involves a change in the primary structure of the *src* protein. Our detection of a change in a secondary protein modification (phosphorylation) at the nonpermissive growth temperature may be completely unrelated to the alteration that results in pp60^{src} inactivation. It is of interest in this respect that the cell-free synthesis of the ASV *src* protein in reticulocyte lysates results in the production of an enzymatically active protein (6). We are currently determining if this protein, synthesized in vitro, is phosphorylated. Finally, the phosphorylation of pp60^{src} may have nothing to do with *src* protein kinase activity, but rather may be involved in other potential functions of this protein. Additional enzymatic activities associated with the ASV *src* gene product have not been excluded. Furthermore, phosphorylation of pp60^{src} may be connected with the recognition of and/or interaction with various cellular components, such as the target(s) of the *src* protein kinase activity. By continuing our structural studies of pp60^{src} and determining if various structural modifications affect its protein kinase activity, we hope to obtain clues to the functional regulation of the ASV *src* protein.

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