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Construction of plasmids for expression of Rous sarcoma virus transforming protein, p60^{src}, in *Escherichia coli*

(recombinant DNA/*lac* promoter/*src* gene)

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ABSTRACT We have constructed plasmids that direct the synthesis of the Rous sarcoma virus transforming gene (*src*) product (p60^{src}) in *Escherichia coli*. A 203-base-pair *lac* promoter-operator DNA encoding the first eight amino acids of β-galactosidase was ligated to the 5′ end of the *src* gene from the Prague A strain of Rous sarcoma virus (PrA-RSV) which had been cloned in pBR325. Antiserum, from a tumor-bearing rabbit, directed against pp60^{src} was used to screen bacteria containing the recombinant plasmid for a protein of approximately 60,000 daltons, and several colonies producing a protein immunologically related to pp60^{src} were detected. Partial proteolytic cleavage analysis revealed that the *src*-related protein produced in bacteria is structurally similar to pp60^{src} immunoprecipitated from PrA-RSV-infected chicken cells. Partially purified *src* protein from *E. coli* can be phosphorylated *in vitro* by the catalytic subunit of cAMP-dependent protein kinase. Tryptic phosphopeptide analysis demonstrated that the catalytic subunit phosphorylated a serine-containing tryptic peptide in the bacterial *src* protein that comigrated with the phosphoserine-containing tryptic peptide of pp60^{src} immunoprecipitated from ^{32}P-labeled PrA-RSV-infected chicken cells.

Transformation of cells by Rous sarcoma virus (RSV) is the result of the expression of the viral sarcoma gene, *src* (1, 2). The *src* gene product, pp60^{src}, is a phosphoprotein of 60,000 daltons (3, 4) which contains two major sites of phosphorylation (5). A serine site is present in the NH_2-terminal portion of the molecule and is phosphorylated by a cyclic AMP-dependent kinase. The second site of phosphorylation is located in the COOH-terminal portion at a tyrosine residue and is phosphorylated in a cyclic AMP-independent manner (5–7). Many experiments have shown that pp60^{src} is tightly associated with a protein kinase activity that can transfer the phosphate of ATP to various proteins *in vitro* and *in vivo* (8–13).

To overcome the problem of separating pp60^{src} from possible contaminating cellular protein kinases, we chose to construct a recombinant plasmid that would express the *src* gene product in bacterial cells. With the exception of a reported protein kinase activity in *Salmonella typhimurium* (14), no evidence of protein kinases in bacterial cells has been presented.

In this paper, we describe the construction of plasmids that contain a 203-base-pair (bp) *Escherichia coli lac* promoter-operator sequence joined to the *src* gene which directs the synthesis of the *src* gene product in *E. coli*. We also report the identification and initial characterization of the *src* protein made in bacterial cells.

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MATERIALS AND METHODS

Bacterial Strains and Plasmids. All cloning experiments were performed in the *E. coli* strain HB101 (15) in accordance with National Institutes of Health guidelines. DNA from a previously described λ clone containing the Prague A strain of RSV (PrA-RSV) genome (16) was digested with *Eco*RI under conditions that generated partial restriction products (16). Genomic-length viral DNA was recovered from agarose gels and self-ligated. The ligated viral DNA was cleaved with *Sal* I and inserted at the *Sal* I site of pBR322. This plasmid was the source of the *src* gene fragment used in the cloning experiments described below. The 203-bp *Eco*RI *lac* promoter-operator fragment was constructed by F. Fuller and L. Johnsrud and its use as a "portable" promoter was described by Backman *et al.* (17). We obtained it from David L. Hare and John R. Sadler (University of Colorado Health Sciences Center, Denver, CO).

DNA Constructions. Restriction enzymes were purchased from Bethesda Research Laboratories, and digestions were performed as recommended by the supplier. DNA polymerase I (Boehringer Mannheim) reactions were performed for 1 hr at 15°C in 50 mM Tris·HCl, pH 7.8/5 mM $MgCl_2$/1 mM 2-mercaptoethanol containing 50 μg of bovine serum albumin per ml and dATP, dCTP, dGTP, and TTP at 200 μM each. T4 DNA ligase (New England BioLabs) reactions were performed for 16 hr at 10°C in 50 mM Tris·HCl, pH 7.8/10 mM $MgCl_2$/20 mM dithiothreitol containing 50 μg of bovine serum albumin and 200 μM ATP. For ligation of blunt ends, 600 μM ATP was used, and the reaction mixtures were incubated at room temperature for 5 hr. Exonuclease III (New England BioLabs) and S1 nuclease (a gift of W. Hahn) reactions were performed as described by Roberts and Lauer (18) except sodium fluoride was added to the S1 nuclease reaction at a concentration of 50 mM to inhibit phosphatase activity. By digesting a known sequence for various lengths of time with *Exo* III, we determined that 12 min was optimal for removing about 140 bp. *Eco*RI linkers were purchased from Collaborative Research (Waltham, MA). DNA sequence analysis was performed by the method of Maxam and Gilbert (19).

Radiolabeling of Proteins. Bacterial cells were grown overnight under the conditions of Roberts and Roberts (20) in M9 medium (21) containing 200 μM $MgSO_4$. An aliquot was diluted

Abbreviations: RSV, Rous sarcoma virus; PrA-RSV, Prague strain of RSV subgroup A; *src*, designation for the RSV gene responsible for transformation; pp60^{src}, designation for the protein product of the RSV *src* gene; p60^{src}, designation for the *src* protein made in *E. coli*; TBR serum, serum from rabbit bearing SRD-RSV-induced tumors; bp, base pair(s).

1:20 in M9 medium containing 40 μM $MgSO_4$ and 1 mM isopropyl thiogalactoside and grown at 37°C for 20 min. Carrier-free $H_2{}^{35}SO_4$ (New England Nuclear) was added to a concentration of 400 μCi/ml (1 Ci = 3.7×10^{10} becquerels) and the bacteria were labeled for 2 hr. The bacteria were pelleted by centrifugation and resuspended in 50 mM Tris·HCl, pH 8.0/25% sucrose. Lysozyme was added to a final concentration of 2 mg/ml. After 5 min at 0°C, $MgCl_2$ was added to 5 mM final concentration, followed by DNase I to 60 μg/ml. The bacteria were lysed by the addition of 1% Nonidet P-40/0.5% sodium deoxycholate/0.1 M NaCl/0.01 M Tris·HCl, pH 7.2/1 mM EDTA and centrifuged at 16,000 × *g* for 30 min at 4°C. Labeling of PrA-RSV-infected chicken embryo fibroblasts was performed as described (3).

Bacterial cultures were radiolabeled with [^{32}P]orthophosphate (400 μCi/ml; carrier-free; ICN) for 3 hr in Tris-buffered medium (22). Extracts were prepared as above and either immunoprecipitated with tumor-bearing rabbit serum (TBR serum) before polyacrylamide gel electrophoresis or analyzed directly by polyacrylamide gel electrophoresis.

Immunoprecipitation and Protein Analyses. Immunoprecipitation and gel electrophoresis have been described (9, 23). The TBR serum (3) used for the immunoprecipitations was selected for its ability to immunoprecipitate pp60^{src} encoded by PrA-RSV. One-dimensional peptide mapping by limited proteolysis was carried out as described by Cleveland *et al.* (24). Two-dimensional separation of tryptic peptides was performed as described (5). Immunoaffinity chromatography was conducted as described (25). Assays for phosphorylation by catalytic subunit were performed in 5 mM $MgCl_2$ containing 1 μM [γ-^{32}P]ATP and 10 ng of catalytic subunit purified as described (26) (kindly supplied by James L. Maller) for 30 min at room temperature. Assays for β-galactosidase were carried out by David L. Hare according to the method of Betz and Sadler (27).

RESULTS

Construction of Plasmids. Our objective was to construct plasmids that would express the protein product of *src* in *E. coli*. We ligated a 203-bp *Eco*RI restriction fragment constructed by F. Fuller and L. Johnsrud (17) containing the *E. coli* UV-5 *lac* promoter, the *lac* operator, ribosome-binding site, and the ATG and first seven amino acids of β-galactosidase onto the *src* gene of PrA-RSV by the cloning strategy outlined in Fig. 1. The 3.1-kb *Eco*RI restriction fragment containing the *src* gene was isolated from a plasmid containing the entire PrA-RSV genome (see *Materials and Methods*) and inserted into the *Eco*RI site of pBR325. Partial *Eco*RI digestion was used to linearize the *src* plasmid, and DNA polymerase I was used to fill in the ends. After blunt-end ligation, a plasmid was recovered that retained only the 5′ *Eco*RI site. This DNA was digested with *Xho* I, which recognizes a unique sequence approximately 140 nucleotides 5′ to the ATG of the *src* gene (16). Exonuclease III and S1 nuclease were used to remove DNA sequences between the restriction enzyme site and the *src* gene and to obtain plasmids with ends near the ATG of *src*. *Eco*RI linkers were joined to the ends of the plasmid by blunt-end ligation and subsequently digested to remove excess linkers. The *Eco*RI *lac* promoter fragment was inserted and the recombinant plasmids were used to transform HB101. Because the *lac* operator binds the *lac* repressor present in the bacterial cell, causing increased transcription of the cellular β-galactosidase gene, plasmids containing the *lac* promoter-operator fragment were selected on plates containing the indicator 5-chloro-4-bromo-3-indolyl-β-

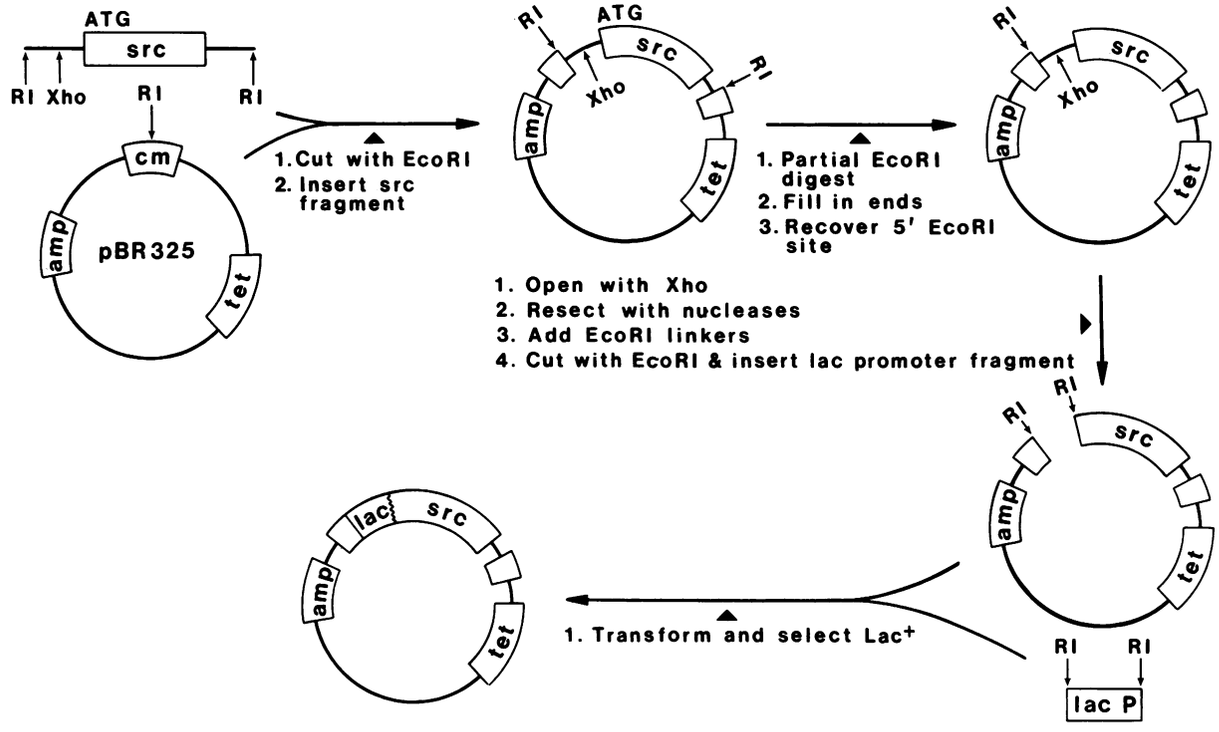

FIG. 1. Plasmid construction. The *Eco*RI fragment containing the *src* gene was isolated from a plasmid containing the entire PrA-RSV genome and inserted into the *Eco*RI site of pBR325. The recombinant plasmid was subjected to partial *Eco*RI digestion, treatment with DNA polymerase I to fill in the *Eco*RI ends, and ligation with T4 DNA ligase to recircularize the molecule. After transformation of HB101, a plasmid that retained only the 5′ *Eco*RI site was recovered. This plasmid was digested with *Xho* I and resected with *Exo* III and S1 nuclease. *Eco*RI linkers were added and the mixture was digested with *Eco*RI. The *Eco*RI fragment containing the UV5 *lac* promoter and the first seven amino acids of β-galactosidase was inserted by ligation. HB101 was transformed with the ligation mixture and plated on 5-chloro-4-bromo-3-indolyl β-D-galactoside plates. Colonies containing the *lac* promoter fragment were identified by their blue color.

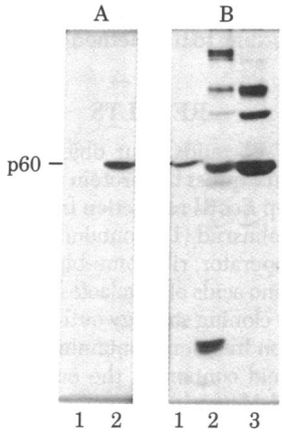

FIG. 2. Immunoprecipitation of the *src* protein from *E. coli* containing a *lac-src* plasmid and from RSV-infected cells. Bacterial cells were labeled with $H_2{}^{35}SO_4$ and animal cells were labeled with [^{35}S]methionine. After immunoprecipitation of extracts with TBR serum, the samples were electrophoresed in NaDodSO$_4$/polyacrylamide gels. (*A*) Lanes: 1, HB101 containing pBR325 with the 203-bp *lac* promoter-operator fragment; 2, HB101 containing a *lac-src* recombinant plasmid. (*B*) Lanes: 1, HB101 containing a *lac-src* recombinant plasmid; 2, PrA-RSV-infected chicken embryo fibroblasts; 3, vole cells transformed by the Schmidt–Ruppin strain of RSV. p60, Position of 60,000-dalton protein.

D-galactoside (28). Recombinants with constitutive synthesis of β-galactosidase metabolized the indicator, producing a blue precipitate around the colony. Blue colonies were then assayed for the production of p60^{src} by immunoprecipitation of bacterial cell extracts.

Immunoprecipitation of the Recombinant Proteins. Bacterial cells were labeled with $^{35}SO_4^{2-}$ and the cellular extracts were immunoprecipitated with TBR serum crossreactive with pp60^{src} from PrA-RSV. The proteins were subjected to NaDodSO$_4$/polyacrylamide gel electrophoresis (Fig. 2). Control immunoprecipitation from HB101 containing the 203-bp *lac* promoter fragment in pBR325 showed no proteins migrating in the 60,000-dalton region. Immunoprecipitation of bacterial extracts revealed that nine recombinant clones yielded a protein of approximately 60,000 daltons and one example is shown (Fig. 2*A*, lane 2; *B*, lane 1). The bacterial product had an apparent molecular size near that of pp60^{src} immunoprecipitated from eukaryotic cells infected with RSV. The frequency of recombinant blue colonies that produced a 60,000-dalton protein was 25%, all producing about the same amount of soluble product. Each producer contained two *lac* promoter fragments as determined by restriction endonuclease digestion and by β-galactosidase assay (27). When the 60,000-dalton band was excised from the gel after immunoprecipitation in antibody excess and its radioactivity was compared to the radioactivity of the total cell extract, we calculated that the 60,000-dalton *src* gene product represented 0.3% of the total labeled soluble protein from *E. coli*.

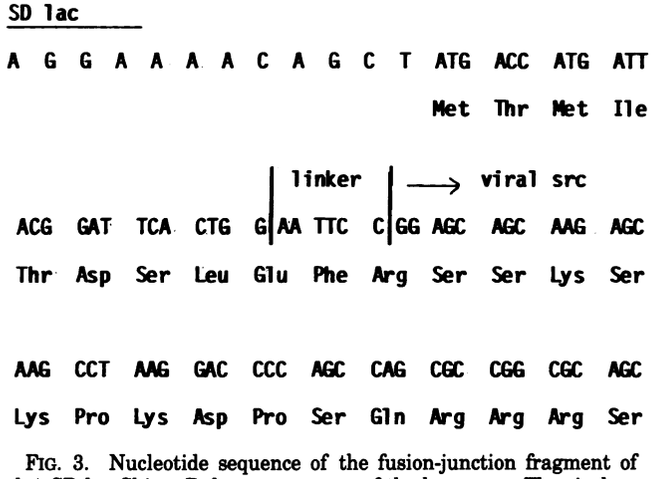

FIG. 3. Nucleotide sequence of the fusion-junction fragment of pls4. SD *lac*, Shine–Dalgarno sequence of the *lac* operon. The viral *src* sequences are indicated and the assignment is based on the published sequence of the *src* gene (29). The *Eco*RI linker is inserted in the second amino acid of the *src* product. The direction of transcription is left to right.

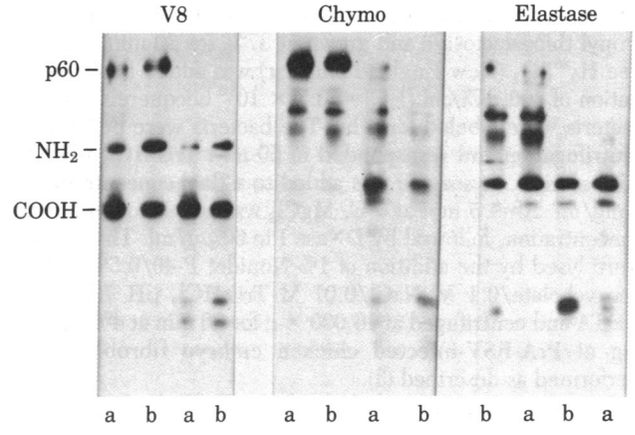

FIG. 4. One-dimensional limited proteolysis mapping of the *src* protein from *E. coli* and from chicken embryo fibroblasts. The [^{35}S]methionine-labeled *src* protein from PrA-RSV-infected chicken embryo fibroblasts (lanes a) and the $^{35}SO_4$-labeled *src* protein from *E. coli* (lanes b) were immunoprecipitated and excised from preparative NaDodSO$_4$/polyacrylamide gels. The protein was subjected to limited proteolysis during reelectrophoresis as described (24) with *Staphylococcus aureus* V8 protease (5 and 50 ng), chymotrypsin (40 and 600 ng), and elastase (10 and 100 ng).

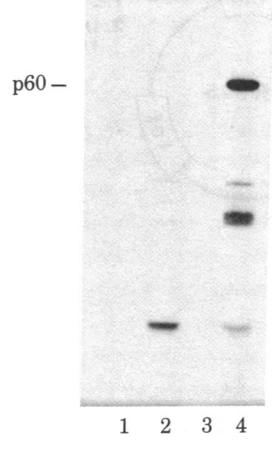

FIG. 5. Phosphorylation of *src* protein from *E. coli* by catalytic subunit of cAMP-dependent protein kinase. Protein preparations from bacterial extracts were purified by immunoaffinity column chromatography as described (25) and incubated at 22°C for 30 min with purified catalytic subunit of cAMP-dependent protein kinase in 10 mM Tris, pH 7.2/5 mM $MgCl_2$/1 μM [γ-^{32}P]ATP and electrophoresed on a NaDodSO$_4$/polyacrylamide gel. Protein preparations from HB101 containing pBR325 with 203-bp *lac* promoter-operator fragment were incubated without catalytic subunit (lane 1) or with catalytic subunit (lane 2). Protein preparations from HB101 containing a *lac-src* plasmid were incubated without catalytic subunit (lane 3) or with catalytic subunit (lane 4).

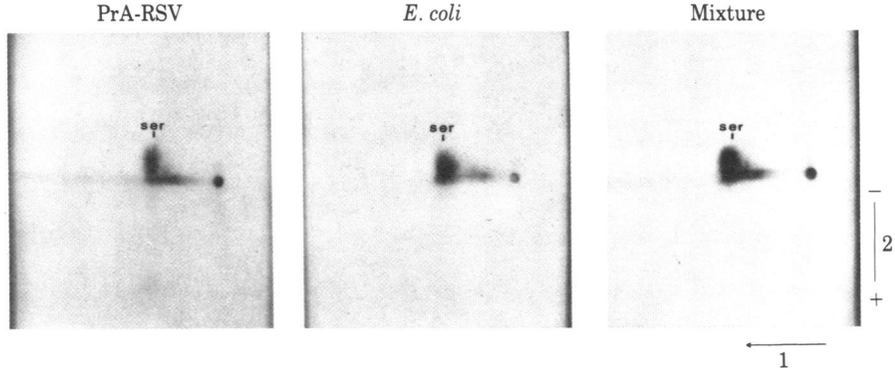

FIG. 6. Two-dimensional analysis of tryptic peptides of ^{32}P-labeled *src* protein isolated from PrA-RSV-infected chicken embryo fibroblasts (*Left*) or from *E. coli* containing *lac-src* plasmid and phosphorylated *in vitro* by catalytic subunit (*Center*) or of a mixture of the two labeled proteins (*Right*). Tryptic digests were prepared as described (5); fractionation was by chromatography in the first dimension and electrophoresis at pH 6.5 in the second dimension.

Sequence Determination. The DNA sequence of one of the plasmids expressing the 60,000-dalton product, designated pls4, was determined by the method of Maxam and Gilbert (19). These analyses revealed that the *src* gene sequences were in the correct reading frame with the ATG of β-galactosidase and would allow translation of the *src* protein beginning with the *lac* control sequences (Fig. 3). Because the *Eco*RI linker was inserted in the *src* gene at a position corresponding to the second amino acid, the fusion protein produced in *E. coli* should be lacking the NH_2-terminal methionine and contain 11 additional amino acids from β-galactosidase and the linker. However, the NH_2-terminal methionine of β-galactosidase has been reported to be cleaved from the polypeptide chain in the bacteria (30).

Characterization of the *E. coli*-Produced Protein. To determine if the bacterial clones were producing authentic *src* protein, we compared the structural relationship of pp60^{src} produced in RSV-transformed chicken cells with *src* made in *E. coli* by partial proteolytic cleavage analysis (24). Fig. 4 shows the protease cleavage maps of [^{35}S]methionine-labeled pp60^{src} from PrA-RSV-infected chicken cells and $^{35}SO_4$-labeled *src* protein from *E. coli*. The two protein maps appear to be similar for three different proteases. Quantitative differences probably reflect differences in the radiolabel used.

Comparison of the Phosphorylation of the *E. coli src* Protein and the PrA-RSV-Infected Chicken Cell pp60^{src}. Previous reports (5, 7, 9) demonstrated that the viral pp60^{src} contains two major sites of phosphorylation. The NH_2-terminal portion of the molecule contains a serine site that is phosphorylated by the catalytic subunit of cAMP-dependent protein kinase, whereas the COOH terminus is phosphorylated on a tyrosine residue by a cyclic AMP-independent protein kinase activity (5). When extracts of recombinant clones that had been radiolabeled with [^{32}P]orthophosphate were either immunoprecipitated or electrophoresed directly on $NaDodSO_4$/polyacrylamide gels, we failed to detect any phosphorylation of the *src* protein. We then determined whether the *E. coli src* protein could be phosphorylated *in vitro;* the results are shown in Fig. 5. The protein from bacterial extracts was purified by immunoaffinity chromatography (25) and shown to be a substrate for the catalytic subunit of cAMP-dependent protein kinase (Fig. 5, lane 4).

The site of phosphorylation on the bacterial protein was analyzed by two-dimensional tryptic phosphopeptide analysis (Fig. 6). The tryptic map of partially purified 60,000-dalton *src* gene product from *E. coli* that had been phosphorylated *in vitro* with catalytic subunit shows one major tryptic phosphopeptide. Amino acid analyses showed this peptide to contain phosphoserine (data not shown). The mixing experiment indicated that the serine-containing phosphopeptide of pp60^{src} isolated from ^{32}P-labeled PrA-RSV-infected chicken cells comigrated with the tryptic phosphopeptide of *in vitro* phosphorylated bacterial enzyme.

DISCUSSION

In this communication, we describe the construction of plasmids containing the *src* gene of PrA-RSV fused to the *E. coli lac* promoter-operator segment. Bacteria containing these plasmids were screened with antibody directed against pp60^{src} encoded by PrA–RSV, and the production of a protein of approximately 60,000 daltons was detected from bacteria containing *lac-src* plasmids but not from *E. coli* containing a plasmid with only the *lac* promoter region. All *lac-src* plasmids contained two copies of the *lac* promoter, and restriction endonuclease mapping of the two plasmids indicated that the promoter region was oriented to transcribe toward the *src* gene. As predicted from this arrangement and confirmed by nucleotide sequence determination, synthesis of the 60,000-dalton protein begins at the ATG of β-galactosidase and proceeds through the *Eco*RI linker into the *src* gene sequences.

Peptide analyses of the *src* protein produced in PrA-RSV-infected chicken embryo cells and *E. coli* containing *lac-src* plasmids show that the two proteins are structurally similar. To date, we have been unable to detect any phosphorylation of the *src* protein made in *E. coli* under the ^{32}P labeling conditions used, and therefore we have designated it "p60^{src}." However, we have shown that p60^{src} can be phosphorylated *in vitro* by the purified catalytic subunit of cAMP-dependent protein kinase. Two-dimensional tryptic phosphopeptide analyses revealed that the major peptide, on the bacterial protein, phosphorylated by catalytic subunit comigrates with the phosphoserine-containing peptide of pp60^{src} immunoprecipitated from ^{32}P-labeled PrA-RSV-infected chicken cells.

We have identified the protein p60^{src} produced in *E. coli* which is structurally and antigenically related to pp60^{src} isolated from RSV-infected cells. Additional characterization of p60^{src} produced in bacteria shows that it functions as a protein kinase; these results are the subject of another communication (31).

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