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Retrovirus Shuttle Vector for Study of Kinase Activities of pp60$^{c\text{-}src}$ Synthesized In Vitro and Overproduced In Vivo

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We have constructed a recombinant murine retrovirus which efficiently transduces avian pp60$^{c\text{-}src}$ into murine cells and which is easily rescued from infected cells in plasmid form. To characterize the virus, several randomly selected NIH 3T3 lines were isolated after infection with recombinant retroviral stocks. All lines overproduced avian pp60$^{c\text{-}src}$ and appeared morphologically normal. Immunoprecipitates made from these lines with antisera specific for pp60$^{c\text{-}src}$ were tested for their kinase activities in vitro. We find that both autokinase and enolase kinase activities increase proportionately with the level of pp60$^{c\text{-}src}$ in the immunoprecipitates. To further test the authenticity of the pp60$^{c\text{-}src}$ encoded by the retroviral vector, these analyses were repeated in the presence of polyomavirus middle T antigen. Avian pp60$^{c\text{-}src}$ was activated as a protein kinase, indicating that the virally encoded pp60$^{c\text{-}src}$ interacts normally with middle T antigen. Interestingly, by increasing the intracellular levels of pp60$^{c\text{-}src}$ 15-fold over normal endogenous levels, we were unable to obtain a proportionate increase in the amount of middle-T-antigen–pp60$^{c\text{-}src}$ complex. Finally, using the shuttle features designed into the vector, we have isolated the first fully processed cDNA encoding functional avian pp60$^{c\text{-}src}$. pp60$^{c\text{-}src}$ synthesized in vitro with this cDNA had intrinsic protein kinase activity and no detectable phosphatidylinositol kinase activity.

The cellular proto-oncogene *src* encodes pp60$^{c\text{-}src}$, a phosphoprotein of 60,000 molecular weight (60K) (5, 19, 23, 28). Amino acid sequences inferred from DNA sequence analysis indicate that pp60$^{c\text{-}src}$ is structurally very similar to its viral counterpart, pp60$^{v\text{-}src}$. Both pp60$^{c\text{-}src}$ and pp60$^{v\text{-}src}$ possess intrinsic tyrosine kinase activities (6, 14, 21, 30) and are associated with the plasma membrane (8, 20, 27, 40) due to an amino-terminal myristylation (2, 29). Despite these similarities, however, pp60$^{c\text{-}src}$ and pp60$^{v\text{-}src}$ differ in subtle but important ways. First, a number of point mutations have resulted in amino acid substitutions within the amino-terminal domain of pp60$^{v\text{-}src}$ (only eight such substitutions in the case of the Schmidt-Ruppin A strain of Rous sarcoma virus [RSV]). Second, the last 19 amino acids at the carboxy terminus of pp60$^{c\text{-}src}$ have been replaced by a different sequence of 12 amino acids in pp60$^{v\text{-}src}$ (37). These structural differences are important because chicken (16) and rodent (24, 31) cells are not transformed by elevating avian pp60$^{c\text{-}src}$ expression to levels equal to or higher than the amount of pp60$^{v\text{-}src}$ sufficient to elicit cellular transformation. In addition, pp60$^{c\text{-}src}$ overproducer lines do not contain elevated levels of total cell phosphotyrosine as reported in RSV-infected (15) or RSV-transformed (11) cells. Thus, pp60$^{v\text{-}src}$ may have escaped a negative control mechanism that regulates the activity of pp60$^{c\text{-}src}$ in vivo.

Recently, Courtneidge and Smith (9, 10) demonstrated a fraction of the intracellular pp60$^{c\text{-}src}$ to be associated with a subpopulation of the polyomavirus middle tumor antigen (MTAg) in polyomavirus-transformed cells. The tyrosine kinase activity of pp60$^{c\text{-}src}$ in vitro is significantly enhanced in the presence of MTAg (1). The population of pp60$^{c\text{-}src}$ associated with MTAg contains a novel tyrosine phosphorylation within the amino-terminal region of the molecule (41). This modified form of pp60$^{c\text{-}src}$ is detected in polyomavirus-transformed cells labeled in vivo with $^{32}P_i$ in the presence of sodium vanadate and after immunocomplex kinase assays of lysates from polyomavirus-transformed cells in vitro. Novel amino-terminal tyrosine phosphorylations are also associated with "activated" pp60$^{v\text{-}src}$ molecules (7, 26). Thus, through its interactions with pp60$^{c\text{-}src}$, MTAg may cause the cellular proto-oncogene to behave like its viral counterpart.

The systems currently available for introducing c-*src* sequences into cells include RSV variants in which the v-*src* gene has been replaced with the c-*src* gene and plasmid constructions encoding pp60$^{c\text{-}src}$ under the control of various eucaryotic promoters (16, 17, 24, 31). In this study, we have constructed a recombinant retrovirus which efficiently transduces both avian pp60$^{c\text{-}src}$ and a selectable marker into murine cells. We have tested the usefulness of this system for the study of pp60$^{c\text{-}src}$ in several ways. A number of NIH 3T3 cell lines were selected for drug resistance after infection with recombinant retroviral stocks and analyzed for the presence of avian pp60$^{c\text{-}src}$. All drug-resistant lines tested were positive for the presence of avian pp60$^{c\text{-}src}$. This efficiency of transmission, coupled with the ability of the retrovirus to be integrated at a single copy per recipient cell, are clear advantages of this system over plasmid-based systems, which rely on transfection (17, 24, 31). To test the authenticity of the pp60$^{c\text{-}src}$ encoded by the recombinant retrovirus, immunoprecipitates from overproducer lines were analyzed for their kinase activities in vitro. These analyses were carried out in both the presence and absence of polyomavirus MTAg. We find that both autokinase and enolase kinase activities increase proportionately with the level of pp60$^{c\text{-}src}$ in the immunoprecipitates. In addition, polyomavirus MTAg is able to activate the protein kinase activity of avian pp60$^{c\text{-}src}$ in vitro. This ability indicates that the virally encoded pp60$^{c\text{-}src}$ interacts normally with MTAg. However, the majority of MTAg and pp60$^{c\text{-}src}$ remains unassociated even when one of the components (pp60$^{c\text{-}src}$) is

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present in large excess. Finally, using the shuttling properties designed into the retroviral vector, we have isolated the first functional cDNA encoding avian pp60$^{c\text{-}src}$. The shuttling properties and ease of rescue designed into this vector but absent from the RSV vectors (16) make it an excellent choice for structure-function studies.

MATERIALS AND METHODS

Construction of pp60$^{c\text{-}src}$ retrovirus vector. The plasmid pHB5, containing sequences encoding avian pp60$^{c\text{-}src}$ and retaining a single intron of 79 base pairs, was the kind gift of H. Hanafusa (16). This construct was cleaved at the *Nco*I site, which contains the initiation codon of pp60$^{c\text{-}src}$, and was ligated to a 50-fold molar excess of a synthetic oligonucleotide (5′ CATGGTGATCAC 3′), which creates a *Bcl*I site and regenerates the *Nco*I site. Dam$^-$ bacterial cells (which lack the methylase that methylates the N^6 position of the adenine residues in the sequence GATC) were transformed, and ampicillin-resistant colonies were screened for the presence of both *Nco*I and *Bcl*I sites. The *Bcl*I-engineered construct (pHB5-B) was restricted with *Bcl*I and *Bgl*II (the *Bgl*II site is about 6 base pairs downstream of the termination codon of pp60$^{c\text{-}src}$), and the pp60$^{c\text{-}src}$-containing fragment (1.7 kilobases) was isolated. This fragment was ligated into the *Bam*HI site of pDOL retroviral vector (A. J. Korman, J. D. Frantz, J. L. Strominger, and R. C. Mulligan, Proc. Natl. Acad. Sci. USA, in press), and the mix was used to transform *Escherichia coli* HB101 cells. Kanamycin-resistant colonies were screened for the presence of pp60$^{c\text{-}src}$ sequences present in both the sense (pDoPy H-6) and antisense (pDoPy H-10) orientations with respect to the 5′ long terminal repeat (LTR).

The modifications made to the pZipNeoSV(X)-1 vector to create pDOL include deletion of the 3′ splice acceptor site, placement of the neomycin resistance gene under the control of a simian virus 40 promoter, and replacement of pBR322 sequences with polyomavirus-specific sequences. The first two changes help reduce interference by upstream sequences on the expression of the neomycin resistance gene. We found this modification particularly useful because the pp60$^{c\text{-}src}$ gene utilized throughout this study retains a single intron. This intron adds an additional 5′ splice donor and a 3′ splice acceptor to the construct. Thus, aberrant splicing events which interfere with the expression of the drug resistance gene are possible. Our initial attempts at expressing pp60$^{c\text{-}src}$ with pZipNeoSV(X)-1 were unsuccessful, possibly for this reason. The third modification places a polyomavirus origin on the expression vector as well as sequences encoding polyomavirus large T antigen, thus promoting greater replication of transfected vectors in murine packaging cells (psi-2) (22). These final modifications are outside of the LTRs and therefore are not transmitted by the recombinant retrovirus.

Generating infectious, helper-free retroviruses from shuttle vector plasmids. pDOL, pDoPy H-6, and pDoPy H-10 were transfected into psi-2 cells (22) by the calcium phosphate method (12). At 24 h posttransfection, medium supernatants were removed and filtered through a membrane filter (Millipore Corp.). These 24-h supernatants constitute transient viral stocks. At 48 h posttransfection, 5×10^6 cells were seeded per 100-mm tissue culture dish, and neomycin (G418) selection was imposed the following day (34). G418-resistant colonies of psi-2 cells were trypsinized in cloning cylinders and transferred to microtiter wells, where they were cloned individually. Supernatants from clonal populations of psi-2 cells were collected 12 h after feeding, filtered (Millipore), and stored at −70°C. Titers of medium supernatants from cloned neomycin-resistant psi-2 cell colonies or transient viral supernatants from transfected psi-2 cells were determined by infecting NIH 3T3 cell cultures in the presence of 8 μg of Polybrene (Aldrich Chemical Co., Inc.) per ml and selecting with 40 μg of G418 per ml 1 to 2 days after infection.

Screening G418-resistant murine cells for avian pp60$^{c\text{-}src}$. G418-resistant NIH 3T3 cell colonies were trypsinized in cloning cylinders and transferred to microtiter wells, where they were cloned individually. Cells (10^6) from expanded clonal populations of G418-resistant NIH 3T3 cells were rinsed once in methionine-free medium and then incubated for 4 h in the presence of 1 mCi of [^{35}S]methionine per ml. Equivalent numbers of cells from each line were washed with cold phosphate-buffered saline (PBS), rinsed with 137 mM NaCl–20 mM Tris (pH 8.0)–1 mM $MgCl_2$–1 mM $CaCl_2$ (buffer A), and lysed in buffer A containing 10% glycerol, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 10 μg of Aprotinin (Sigma Chemical Co.) (lysis buffer) per ml at 4°C for 20 min. The cellular lysate was clarified at 10,000 × *g* for 10 min at 4°C. Cell lysates were normalized for protein content by the Bio-Rad Laboratories protein assay and were incubated with monoclonal antibodies specific for either avian pp60$^{c\text{-}src}$ (EC10) or both avian and murine pp60$^{c\text{-}src}$ (GD11) at 4°C for 20 min (25). Sepharose Cl4B-protein A (Sigma Chemical Co.) was added, and incubation continued for an additional 20 min at 4°C. The immunoprecipitates were collected and washed once with phosphate-buffered saline, twice with 0.5 M LiCl–0.1 M Tris (pH 7.4), and finally with H_2O. The immunoprecipitates were then suspended in Laemmli buffer, boiled for 5 min at 100°C, and analyzed directly by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). Alternatively, the immunoprecipitates were suspended in kinase buffer (20 mM Tris [pH 7.4], 5 mM $MnCl_2$) and 5 to 20 μCi [γ-^{32}P]ATP for 20 min at 25°C prior to SDS-PAGE. For enolase reactions, 10 μM ATP and 2 μg of acid-denatured rabbit muscle enolase (the kind gift of Brian Schaffhausen) were utilized. All measurements of protein levels reported in the text were quantitated by excising gel bands after autoradiography and measuring radioactivity in a scintillation counter. Lipid and kinase assays were linear over the time of the assay and were dependent on the amount of immunoprecipitated cell lysate added.

Interactions between polyomavirus MTAg and avian pp60$^{c\text{-}src}$. G418-resistant NIH 3T3 cells were either mock infected or infected with polyomavirus at a multiplicity of infection of 10. At 20 h postinfection, cells were washed once in methionine-free medium and then incubated for 4 to 6 h in methionine-free medium supplemented with 0.1 mCi of [^{35}S]methionine per ml. Cell extracts were prepared as described above and incubated with 2 μl of rabbit anti-T serum (D. Pallas, M. E. Mahoney, D. R. Kaplan, and T. M. Roberts, unpublished data) or with monoclonal antibodies specific to pp60$^{c\text{-}src}$ (EC10 or GD11). Immunoprecipitations were carried out as described above, and the results were analyzed directly for ^{35}S-labeled proteins or analyzed by kinase assays to measure protein kinase or phosphatidylinositol (PI) kinase activity. For protein kinase assays, washed immunoprecipitates were incubated in kinase buffer supplemented with 5 to 50 μCi of [γ-^{32}P]ATP for 20 min at 25°C. For PI kinase assays, immunoprecipitates were washed as described above, with an additional wash performed in 100 mM NaCl–10 mM Tris (pH 7.4)–1 mM EDTA (TNE).

Immunoprecipitates were then assayed for PI phosphorylation activity as described elsewhere (39).

Rescue protocol. Avian pp60$^{c\text{-}src}$-containing DNAs were rescued from G418-resistant NIH 3T3 cell lines 6-4, 6-6, and 6-8 by the rescue-fusion protocol. G418-resistant NIH 3T3 cells were trypsinized and plated at a ratio of 1:1 with COS-1 cells such that confluency was reached within 48 h. Cells were rinsed three times with medium lacking serum and were fused by the addition of a 50% solution of polyethylene glycol 1000 (J. T. Baker Chemical Co.) in medium lacking serum for 1 min at room temperature. Cells were washed five times with medium lacking serum and were incubated for 3 days in medium containing 10% calf serum. Hirt supernatants were prepared as described elsewhere (13). Cells were lysed in 2.0% SDS–10 mM Tris (pH 7.4)–60 mM EDTA and then adjusted to a final NaCl concentration of 1.25 M. The lysates were stored overnight at 4°C and then centrifuged at 30,000 rpm for 46 min in an SW70.1 rotor. The supernatant was phenol-chloroform extracted and precipitated with isopropanol. The pellet was used to transform HB101, and kanamycin-resistant colonies were screened for pp60$^{c\text{-}src}$ sequences.

Transcription and translation of c-*src* cDNA in vitro. The rescued plasmid 6(8)-4 was restricted with *Xba*I and *Sal*I, and the pp60$^{c\text{-}src}$-containing fragment was subcloned between the *Xba*I and *Sal*I sites of pSP65 (SP6 transcription system; Promega Biotec) to generate pSP68-4. pSP68-4 was then restricted with *Xba*I and *Nco*I to remove the LTR-*gag* sequences which originated from the retroviral vector. This fragment was replaced with the oligomer 5′ CTAGAAAG GAGGTACGACTTT 3′ and 5′ CCTCCATGCTGGTAC 3′ to generate the plasmid pSP68-4 (RBS). In addition, pHB5-B, which contains the gene for pp60$^{c\text{-}src}$ and retains the 79-base-pair intron (see above), was restricted with *Bcl*I and *Eco*RI, and the 1.7-kilobase fragment containing c-*src* was cloned between the *Bam*HI and *Eco*RI sites of pSP64 to yield pSP64src. After linearization with *Sal*I (pSP68-45 [RBS]) or *Eco*RI (pSP64src), the vectors were transcribed in vitro with SP6 RNA polymerase. RNA transcripts were purified and translated in a micrococcal nuclease-treated rabbit reticulocyte lysate (New England Nuclear Corp.) in the presence of [^{35}S]methionine. Samples were analyzed directly by SDS-PAGE or immunoprecipitated prior to SDS-PAGE. To test the kinase activities of the pp60$^{c\text{-}src}$ translated in vitro, translation reactions were carried out in the absence of [^{35}S]methionine. After a 1-h incubation period, 1 ml of lysis buffer and 1 μl of a rabbit anti-pp60$^{c\text{-}src}$ polyclonal serum (H. Piwnica-Worms, D. Pallas, and T. M. Roberts, unpublished data) were added to the translation mix. The immunoprecipitations and kinase assays (protein and PI) were performed as described above. As a positive control for the PI kinase assays, 5 × 10^5 RSV-NRK cells (Schmidt-Ruppin avian sarcoma virus-trans-formed rat kidney cells) were lysed and immunoprecipitated with either a rabbit preimmune serum or a rabbit anti-pp60$^{c\text{-}src}$ polyclonal serum. PI kinase assays were then performed on the immunoprecipitates.

***Staphylococcus* V8 protease mapping.** Cells (2 × 10^6) from the G418-resistant NIH 3T3 line 6-8 were labeled in methionine-free medium supplemented with 2.0 mCi of [^{35}S]methionine per ml for 4 h. Immunoprecipitations were carried out as described above with 2 μl of pp60$^{c\text{-}src}$-specific monoclonal antibody (GD11). In addition, the rescued cDNA (pSP68-4 [RBS]) was transcribed, translated, and immunoprecipitated as described above. [^{35}S]methionine-labeled immunoprecipitates were electrophoresed on a 10% polyacrylamide gel. After autoradiography, gel slices containing [^{35}S]methionine-labeled pp60$^{c\text{-}src}$ were excised and homogenized in 200 μl of Cleveland gel fingerprint buffer (10 mM sodium bicarbonate, 0.1% SDS, 0.1% 2-mercaptoethanol, 20% sucrose). The homogenized gel slices were then split into four 50-μl aliquots, and 10 μl of V8 protease dilutions were added (0.0, 0.01, 0.1, and 1.0 mg/ml). Incubations were at 37°C for 30 min. Samples were analyzed on a 15% polyacrylamide gel.

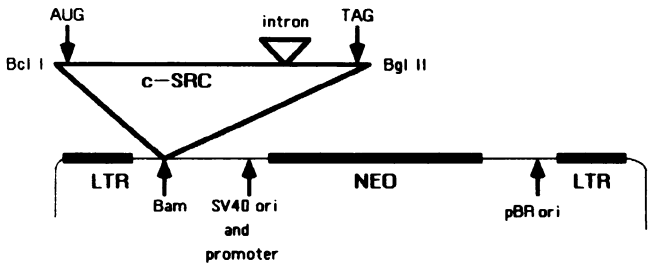

FIG. 1. Construction of the c-*src* shuttle vectors. Sequences encoding pp60$^{c\text{-}src}$ and retaining a single intron of 79 base pairs were obtained from plasmid pHB5 (16). The c-*src*-containing restriction fragment (*Nco*I site to *Bgl*II) was made BamHI compatible by inserting a synthetic oligomer at the *Nco*I site to create a *Bcl*I site. The *Bcl*I-to-*Bgl*II fragment was cloned into the *Bam*HI site of pDOL. This change placed the expression of pp60$^{c\text{-}src}$ under the control of the MoMuLV promoter present within the 5′ LTR. AUG, Initiation coden; TAG, termination codon; NEO, neomycin resistance gene.

RESULTS

Construction of the c-*src* shuttle vectors and generation of infectious recombinant retroviruses. The c-*src* gene utilized throughout this study was obtained from plasmid pHB5 (16). pHB5 contains a partially processed gene of approximately 1.7 kilobases encoding avian pp60$^{c\text{-}src}$. In pHB5, the c-*src* gene is flanked at its 5′ end by an *Nco*I site which encodes the initial methionine and at its 3′ end by a *Bgl*II site approximately 6 base pairs downstream of the termination codon. To facilitate the cloning of this gene into the retroviral vector, we used a synthetic oligonucleotide to insert a *Bcl*I site just upstream from the *Nco*I site, creating a new plasmid, pHB5-B. The *Bcl*I-*Bgl*II fragment containing c-*src* from pHB5-B was ligated into the *Bam*HI site of pDOL (Fig. 1) (Korman et al., in press). This change places the expression of c-*src* under the control of the promoter present within the 5′ LTR of Moloney murine leukemia virus (MoMuLV). Kanamycin-resistant bacterial colonies were screened for recombinant plasmid vectors carrying c-*src* sequences in both the sense (pDoPyH-6) and antisense (pDoPyH-10) orientations with respect to the 5′ LTR.

The c-*src* recombinant shuttle vectors described above encode no retroviral proteins. Thus, for packaging to occur, these functions must be supplied in *trans*. We utilized psi-2 cells, which are NIH 3T3 cells containing stably integrated, packaging-deficient mutants of MoMuLV (22). The c-*src*-containing constructs (pDoPyH-6 and pDoPyH-10) as well as the parental vector lacking c-*src* sequences (pDOL-1) were transfected into psi-2 packaging cells by the calcium phosphate method (12). Transiently expressed retroviruses present in the medium were harvested 24 h later. The titers of these viral stocks ranged from 100 to 500 neomycin-resistant colonies per ml of medium supernatant. In addition, transfected psi-2 cells were incubated for a further 24 h, and

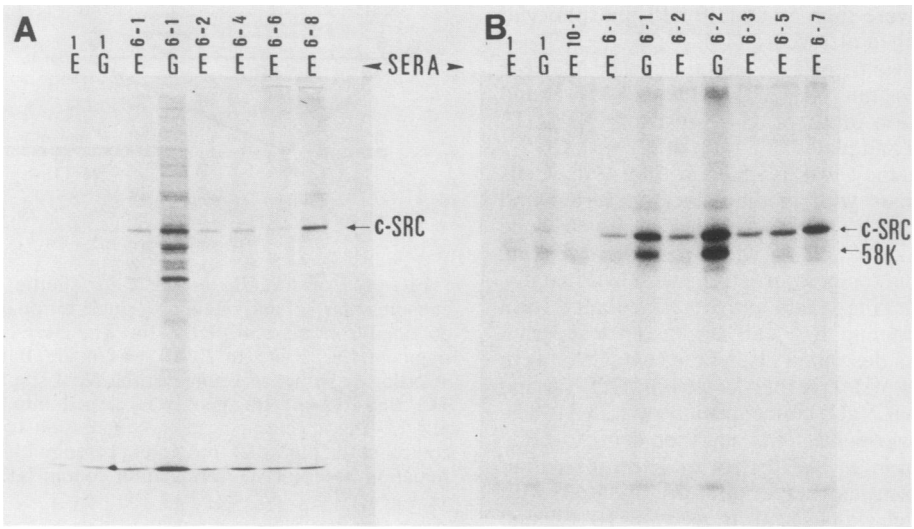

FIG. 2. Screening for the expression of avian pp60$^{c\text{-}src}$ in NIH 3T3 cells. [^{35}S]methionine-labeled extracts were prepared from G418-resistant NIH 3T3 cells generated by infection with viral stocks from psi-2 cells transfected with the original shuttle vector (lanes 1), the shuttle vector containing c-*src* in the antisense orientation (lane 10-1), or the shuttle vector containing c-*src* in the sense orientation (lanes 6-1, 6-2, 6-3, 6-4, 6-5, 6-6, 6-7, and 6-8) with respect to the 5′ LTR. The extracts were immunoprecipitated with monoclonal antibodies specific for either avian (E) or avian and murine (G) pp60$^{c\text{-}src}$ and were analyzed directly by SDS-PAGE (A). Alternatively, kinase assays were performed on unlabeled immunoprecipitates (B).

then neomycin (G418) selection was imposed (34). Neomycin-resistant colonies were clonally expanded to generate stable psi-2 producer lines. Medium supernatants from these lines were collected 12 h after feeding and were filtered (Millipore). The yields of infectious virus we obtained varied from clone to clone, ranging from 5×10^3 to 1×10^5 neomycin-resistant colonies per ml of medium supernatant. Titers were highest from lines producing retroviruses lacking c-*src* sequences, followed by lines producing retroviruses carrying c-*src* in the antisense orientation with respect to the promoter present within the 5′ LTR. The lowest titers were obtained from packaging lines producing recombinant retroviruses carrying c-*src* in the sense orientation with respect to the 5′ LTR.

Analysis of the virally encoded avian pp60$^{c\text{-}src}$ in infected NIH 3T3 cells. To determine if the medium supernatants contained recombinant retroviruses encoding avian pp60$^{c\text{-}src}$, NIH 3T3 cells were infected with filtered supernatants, G418 selection was imposed, and drug-resistant colonies were clonally expanded. G418-resistant NIH 3T3 cells were screened for the presence of avian pp60$^{c\text{-}src}$ with monoclonal antibodies specific for pp60$^{c\text{-}src}$ (25). Immunoprecipitations were carried out on [^{35}S]methionine-labeled cell extracts (Fig. 2A) or alternatively on unlabeled cell extracts when kinase assays were performed (Fig. 2B). To distinguish avian pp60$^{c\text{-}src}$ from endogenous murine pp60$^{c\text{-}src}$, a monoclonal antibody (EC10) that recognizes only avian pp60$^{c\text{-}src}$ (lanes E) was utilized. Alternatively, a monoclonal antibody (GD11) recognizing both avian and murine pp60$^{c\text{-}src}$ (lanes G) was used to determine total levels of pp60$^{c\text{-}src}$ in G418-resistant lines. Control cell lines generated by infection of NIH 3T3 cells with retroviruses lacking c-*src* (Fig. 2, lanes 1) or containing c-*src* in the antisense orientation with respect to the 5′ LTR (lane 10-1) lack avian pp60$^{c\text{-}src}$. These lines do, however, contain low levels of endogenous murine pp60$^{c\text{-}src}$, which is kinase active. Eight randomly selected G418-resistant cell lines made by infection with retroviruses encoding c-*src* in the sense orientation with respect to the 5′ LTR (lanes 6-1 through 6-8) were tested for the expression of avian pp60$^{c\text{-}src}$. All eight murine lines were positive for the presence of kinase-active avian pp60$^{c\text{-}src}$, although the levels varied between individual cell lines (the amount of kinase activity correlated with the level of pp60$^{c\text{-}src}$ as measured by [^{35}S]methionine incorporation). This variability in levels of pp60$^{c\text{-}src}$ expression may be due to differences in the chromosomal sites of retroviral integration. Cell lines 6-1 and 6-8 were the highest pp60$^{c\text{-}src}$ producer lines, with levels of pp60$^{c\text{-}src}$ 15-fold higher than in control lines. Despite the high level of pp60$^{c\text{-}src}$ expression, all lines appeared morphologically normal and failed to form foci. This result is in agreement with earlier reports (16, 24, 31). The second protein (58K), present in GD11 immunoprecipitates, is a proteolytic cleavage product of pp60$^{c\text{-}src}$. This cleavage product is generated by a protease present in some batches of GD11 serum. It should be noted that the avian pp60$^{c\text{-}src}$-specific monoclonal antibody EC10 precipitated pp60$^{c\text{-}src}$ with low efficiency. This fact is in contrast with its reported high reactivity with pp60$^{v\text{-}src}$ (25). GD11 serum, which recognizes both murine and avian pp60$^{c\text{-}src}$, was more efficient at immunoprecipitating pp60$^{c\text{-}src}$ and thus is more indicative of total levels of pp60$^{c\text{-}src}$ in the overproducer lines. pp60$^{c\text{-}src}$ immunoprecipitates from the overproducer lines were also tested for their ability to phosphorylate an exogenous protein substrate (rabbit muscle enolase). Enolase phosphorylation increased proportionately with the increase in pp60$^{c\text{-}src}$ in each of the overproducer lines (data not shown).

Polyomavirus MTAg has been shown to interact with pp60$^{c\text{-}src}$ in cells from a variety of species (1, 18). However, only a small fraction of the total MTAg present in polyomavirus-infected cells participates in this interaction. To test whether we could drive the association further by increasing the concentration of one component (pp60$^{c\text{-}src}$), we infected our highest overproducer line (6-8) with polyomavirus and measured the degree of association by protein kinase assays (Fig. 3). G418-resistant NIH 3T3 control lines (10-1) and pp60$^{c\text{-}src}$ overproducer lines (6-8)

were mock infected or infected with polyomavirus. Cell lysates were prepared and immunoprecipitated with antisera specific for polyomavirus early-region proteins (PY19) or with monoclonal antibodies specific for pp60$^{c\text{-}src}$ (EC10 or GD11). The immunoprecipitates were analyzed by kinase assays. Lanes 3 and 4 of Fig. 3 represent the total kinase-active fraction of MTAg present in the control (10-1) and pp60$^{c\text{-}src}$ overproducer (6-8) lines after infection. By [^{35}S]methionine labeling, both lines were shown to contain equivalent amounts of MTAg; however, there was 20 times more pp60$^{c\text{-}src}$ in line 6-8 compared with line 10-1 (data not shown). The larger quantity of phosphorylated MTAg (approximately twofold more) in line 6-8 [Fig. 3, lane 4]) indicates that a larger percentage of MTAg is associated with pp60$^{c\text{-}src}$ in the overproducer line. The pp60$^{c\text{-}src}$-MTAg associations did not increase proportionately with the levels of pp60$^{c\text{-}src}$, however. Thus, the majority of MTAg and pp60$^{c\text{-}src}$ remains unassociated even in the presence of a large excess of pp60$^{c\text{-}src}$. Other factors involved in regulating the association between pp60$^{c\text{-}src}$ and MTAg remain to be elucidated. Also seen in Fig. 3, lanes 3 and 4, are large T antigen and a 45K proteolytic cleavage product of MTAg (3). The proteolytic cleavage fragment is associated with pp60$^{c\text{-}src}$ immunoprecipitates, whereas the large T antigen is not (Fig. 3, lanes 8, 10, and 12).

When polyomavirus-infected lines were immunoprecipitated with GD11 serum, which recognizes both avian and endogenous murine pp60$^{c\text{-}src}$, the majority of the kinase-active fraction of MTAg coimmunoprecipitated with pp60$^{c\text{-}src}$ (compare lanes 4 and 12 in Fig. 3). It is important to note that the 58K proteolytic cleavage product of pp60$^{c\text{-}src}$, present in GD11 immunoprecipitates, migrated with an electrophoretic mobility indistinguishable from that of intact MTAg (compare lanes 4 and 11 in Fig. 3). Thus, the 58K band seen in lanes 10 and 12 is actually a mixture of both intact MTAg and the pp60$^{c\text{-}src}$ proteolytic cleavage fragment. Therefore, that fraction of MTAg which associates with pp60$^{c\text{-}src}$ is best represented by the 45K proteolytic cleavage product of MTAg (compare lanes 4 and 12 in Fig. 3).

When polyomavirus-infected lysates from line 6-8 were immunoprecipitated with EC10 serum, which recognizes avian pp60$^{c\text{-}src}$ only, MTAg coimmunoprecipitated with pp60$^{c\text{-}src}$ (compare lanes 7 and 8, Fig. 3). This result demonstrates that MTAg can indeed interact with the avian pp60$^{c\text{-}src}$ encoded by the recombinant retrovirus. Line 10-1 contains only endogenous murine pp60$^{c\text{-}src}$ and thus was negative in the EC10 immunoprecipitation (Fig. 3, lanes 5 and 6). We also find that MTAg can enhance the abilities of both murine and avian pp60$^{c\text{-}src}$ species to phosphorylate themselves. We consistently find a two- to fourfold increase in the autophosphorylating ability of pp60$^{c\text{-}src}$ in the presence of polyomavirus MTAg (compare pp60$^{c\text{-}src}$ in lanes 7 and 8, lanes 9 and 10, and lanes 11 and 12). The ability of pp60$^{c\text{-}src}$ to phosphorylate exogenous protein substrates in the presence of MTAg was also enhanced (data not shown).

Whitman et al. (39) recently reported a PI kinase activity which coimmunoprecipitates with pp60$^{c\text{-}src}$-MTAg complexes in vitro with MTAg-specific serum or monoclonal antibodies specific for pp60$^{c\text{-}src}$. We analyzed immunoprecipitates from a number of pp60$^{c\text{-}src}$ overproducer lines to determine if the level of PI kinase increased proportionately with pp60$^{c\text{-}src}$. In the absence of polyomavirus, the overproducer lines consistently showed a zero- to threefold increase in associated PI kinase activity when compared with control lines (data not shown). This is despite a 3- to 15-fold overproduction of pp60$^{c\text{-}src}$. The low levels of PI kinase observed are consistent with background variation in the assay and did not correlate with levels of pp60$^{c\text{-}src}$ protein and enolase kinase present in the extracts.

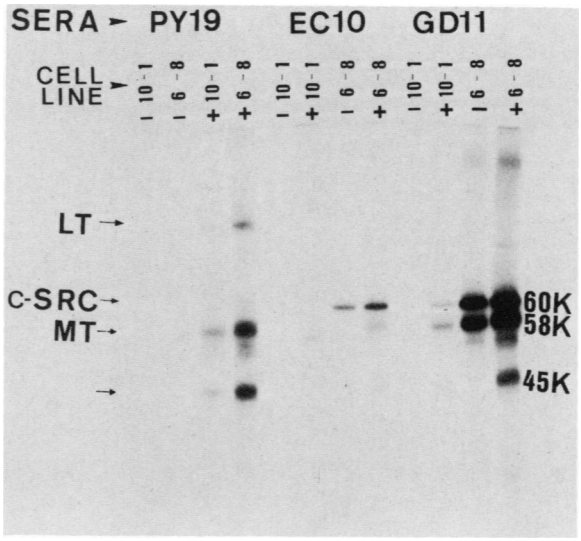

FIG. 3. Interaction between avian pp60$^{c\text{-}src}$ and polyomavirus MTAg. NIH 3T3 cells overproducing avian pp60$^{c\text{-}src}$ (lanes 6-8) and control lines (lanes 10-1) were infected with polyomavirus (+) or were mock infected (−). At 25 h postinfection, cell extracts were prepared and immunoprecipitated with polyomavirus early-region-specific serum (PY19) or with monoclonal antibodies specific to avian (EC10) or avian and murine (GD11) pp60$^{c\text{-}src}$. Kinase assays were performed, and the products were analyzed by SDS-PAGE. LT, Large T antigen; MT, MTAg.

Isolation of a c-*src* cDNA. Cepko et al. (4) demonstrated the usefulness of the pZipNeoSV(X)-1 shuttle vector to rapidly isolate cDNA copies of cloned genomic DNA sequences. Removal of intron sequences from a gene carried in a retrovirus occurs by splicing of the retrovirus prior to encapsidation (32, 33). To isolate a fully processed cDNA of c-*src*, avian pp60$^{c\text{-}src}$-positive NIH 3T3 lines (6-1, 6-4, and 6-8) were fused with COS-1 cells, which caused the integrated provirus form of the vector to be released as a covalently closed circle. Hirt supernatants were used to transform *E. coli* HB101 cells, and kanamycin-resistant colonies were selected. Restriction mapping of the isolated plasmids indicated that clones rescued from the 6-8 line, but not the 6-1 and 6-4 lines, were cDNA copies. To confirm this, the c-*src*-containing fragment from representative plasmids was subcloned into pSP65 and transcribed in vitro with SP6-specific RNA polymerase. The RNA was purified and translated in vitro with rabbit reticulocyte lysates in the presence of [^{35}S]methionine. A protein migrating at approximately 60K was synthesized with RNA transcribed from the rescued c-*src* fragment from line 6-8 (Fig. 4A, lane S2). When RNAs from pSP65 constructions carrying either the starting pp60$^{c\text{-}src}$ gene that retains the 79-base-pair intron or the c-*src* genes rescued from lines 6-1 and 6-4 were transcribed and translated in vitro, a protein of approximately 47K was obtained (Fig. 4A, lane S1). This shortened protein presumably results from termination within the 79-base-pair intron. Both the 47K and 60K translation products were specifically immunoprecipitated with pp60$^{c\text{-}src}$-specific serum (Fig. 4B).

To determine whether pp60$^{c\text{-}src}$ translated in vitro was

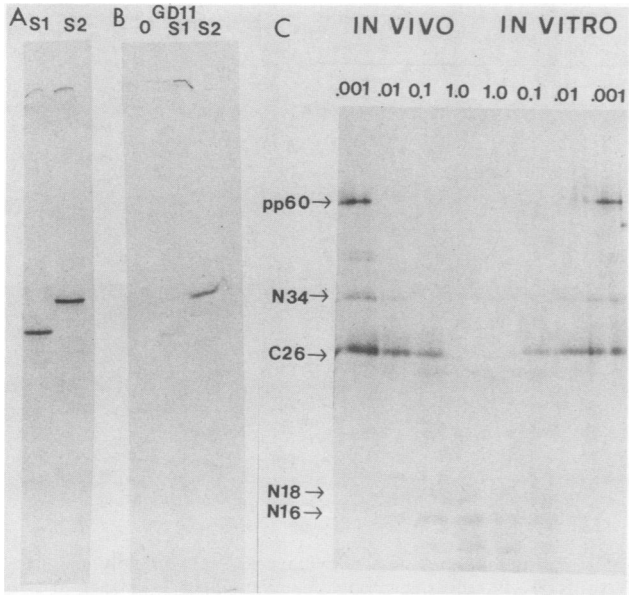

FIG. 4. Transcription, translation, and mapping of pp60$^{c\text{-}src}$ synthesized in vitro. Rescued DNAs from lines 6-4, 6-6, and 6-8 were restricted with *Xba*I and *Sal*I, and the c-*src*-containing fragment was subcloned into pSP65 (SP6 transcription system; Promega Biotec). After linearization with *Sal*I, transcription was carried out in vitro with SP6-specific RNA polymerase. RNA transcripts were purified and translated in a rabbit reticulocyte lysate in the presence of [^{35}S]methionine. Samples were analyzed directly by SDS-PAGE (A) or immunoprecipitated with monoclonal antibody specific for pp60$^{c\text{-}src}$ (GD11) (B). Panel B shows results with c-*src* RNA transcribed from the gene retaining the intron (lane S1), c-*src* RNA transcribed from the gene lacking the intron (lane S2), and no added RNA (lane 0). pp60$^{c\text{-}src}$ immunoprecipitated from cell line 6-8 or from translation reactions carried out in vitro was analyzed by *Staphylococcus* V8 protease mapping (C). Gel slices containing pp60$^{c\text{-}src}$ were homogenized in Cleveland digestion buffer. A 10-μl volume of each of various V8 protease dilutions (1.0, 0.1, 0.01, 0.001 mg/ml) was added to 50 μl of homogenized pp60$^{c\text{-}src}$. Samples were incubated at 37°C for 30 min and analyzed on a 15% polyacrylamide gel.

identical to that synthesized in vivo, partial proteolytic digestions were carried out with *Staphylococcus aureus* V8 protease (Fig. 4C). V8 protease cleaves pp60$^{c\text{-}src}$ into two fragments, i.e., V1, a 34K peptide containing the amino-terminal portion of pp60$^{c\text{-}src}$ and V2, a 26K peptide containing the carboxyl-terminal portion of pp60$^{c\text{-}src}$. Higher concentrations of enzyme result in further cleavage of the V1 peptide at two sites to yield two phosphorylated peptides, V3 (18K) and V4 (16K), which contain the amino-terminal portion of pp60$^{c\text{-}src}$. The peptide map in Fig. 4 confirms that the proteins synthesized in vitro and in vivo were indistinguishable.

To further characterize the proteins synthesized in vitro, kinase assays were performed (Fig. 5). Translation reactions were carried out in the presence of pp60$^{c\text{-}src}$-specific RNA from a gene retaining the 79-base-pair intron (lanes S1) or pp60$^{c\text{-}src}$-specific RNA from a gene lacking the 79-base-pair intron (lanes S2) or with no added RNA (lanes 0). Immunoprecipitations were carried out in the presence of monoclonal antibody specific for avian pp60$^{c\text{-}src}$ (EC10). Kinase assays were then performed in the absence (Fig. 5A) or presence (Fig. 5B) of enolase. pp60$^{c\text{-}src}$ was able to phosphorylate itself (Fig. 5A, lane S2) and was able to phosphorylate enolase (Fig. 5B, lane S2). The truncated pp60$^{c\text{-}src}$ (S1) was not active in the kinase assay. In addition, both translation products (intact pp60$^{c\text{-}src}$ and truncated pp60$^{c\text{-}src}$) were negative when tested for PI kinase activity (Fig. 5C).

DISCUSSION

We have constructed a recombinant murine retrovirus which efficiently transduces avian pp60$^{c\text{-}src}$ into murine cells. The expression of pp60$^{c\text{-}src}$ is regulated by the promoter present within the 5′ LTR of MoMuLV. We chose a murine retroviral LTR to maximize the expression of pp60$^{c\text{-}src}$ in rodent cells. We have infected numerous mouse and rat cell lines, in addition to the NIH 3T3 lines reported here, with the recombinant retroviruses and have isolated many clones, all of which stably express avian pp60$^{c\text{-}src}$. These results indicate that the viral vector is highly stable. Levels of pp60$^{c\text{-}src}$ in the overproducer cell lines ranged from 3- to 15-fold above the endogenous murine levels in control lines. All overproducer lines remained morphologically normal, and none formed foci despite this high level of pp60$^{c\text{-}src}$ production. These findings are in agreement with previous studies which have overexpressed pp60$^{c\text{-}src}$ with either an avian retroviral LTR (16) or simian virus 40 promoter (24, 31). That expression of pp60$^{c\text{-}src}$ does not lead to oncogenic transformation, even at protein levels comparable to or greater than those observed for pp60$^{v\text{-}src}$ in RSV-transformed cells, emphasizes the importance of the structural differences in conferring transforming capabilities to pp60$^{v\text{-}src}$ but not its cellular homolog.

Johnson et al. (17) have also expressed avian pp60$^{c\text{-}src}$ from a MoMuLV promoter in murine cells. When these constructions were transfected into NIH 3T3 cells, foci were

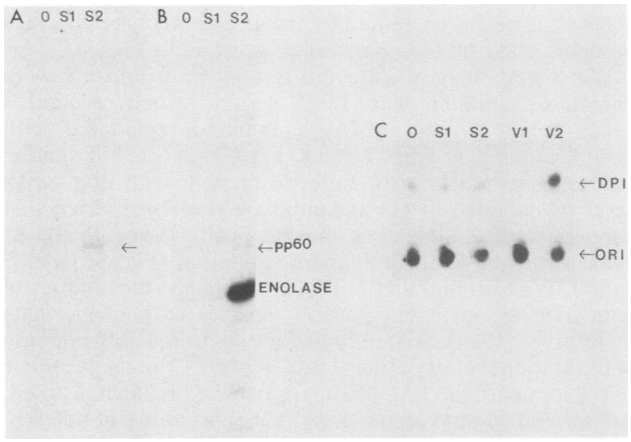

FIG. 5. Kinase activities of pp60$^{c\text{-}src}$ synthesized in vitro. Translation reactions were carried out in the presence of no added RNA (lanes 0), RNA transcribed from a c-*src* gene retaining the intron (lanes S1), or RNA transcribed from a c-*src* cDNA (lanes S2). Immunoprecipitations were carried out with a rabbit anti-pp60$^{c\text{-}src}$ polyclonal serum. Kinase assays were performed in the absence (A) or presence (B) of enolase. Alternatively, PI kinase assays were performed on the immunoprecipitates (C). As a positive control for the PI kinase assays, RSV-NRK cells were lysed and immunoprecipitated with either a rabbit preimmune sera (lane V1) or an anti-pp60$^{c\text{-}src}$ polyclonal sera (lane V2), and PI kinase assays were performed on the immunoprecipitates. DPI, phosphatidylinositol 4-phosphate; ORI, origin.

observed at 1% the level seen with a similar construction encoding pp60$^{v\text{-}src}$. These focus-selected c-*src*-overexpressing cells had altered morphology and limited growth in soft agar. In contrast with these results, we have never observed focus formation even with our highest pp60$^{c\text{-}src}$ overproducer lines. This discrepancy may result, in part, from the way the constructions were introduced into the murine cells (transfection versus infection). Many more copies of c-*src* would be expected on a per cell basis after transfection than after infection, in which a single copy per genome is expected. The lack of focus formation, in our hands, makes the retroviral shuttle vector system an excellent one for isolating mutants which activate pp60$^{c\text{-}src}$ as a transforming protein. We have succeeded in placing the rescued c-*src* cDNA back into the retroviral vector. Every cell line examined after viral infection and drug selection expressed avian pp60$^{c\text{-}src}$, and, as expected, all lines were morphologically normal. We are presently mutagenizing the rescued c-*src* cDNA and rebuilding the retroviral vectors with the mutagenized pool. Activated mutants are easily assayed (focus formation) and rescued for sequencing. The shuttling properties and ease of rescue unique to this system makes it particularly useful over other systems (16) for structure-function studies of pp60$^{c\text{-}src}$.

The MTAg of polyomavirus plays an indispensible role in viral-mediated transformation, as it alone is capable of transforming established cell lines (38). A subpopulation of MTAg is found complexed to the cellular proto-oncogene pp60$^{c\text{-}src}$ (9, 10). MTAg is known to stimulate pp60$^{c\text{-}src}$ as a protein kinase in vitro (1). In addition, there is an associated PI kinase activity in immunoprecipitates containing MTAg-pp60$^{c\text{-}src}$ complexes which is specifically enhanced in the presence of transformation-competent MTAg (39). We consistently observed enhancement in the tyrosine kinase activity of pp60$^{c\text{-}src}$ in the polyomavirus-infected pp60$^{c\text{-}src}$ overproducer lines compared with infected control lines. In addition, there was a 20- to 30-fold enhancement in the associated PI kinase activity as measured in vitro in the presence of transformation-competent MTAg. This finding indicates that the recombinant retrovirus encodes functional avian pp60$^{c\text{-}src}$. Interestingly, by increasing the intracellular levels of pp60$^{c\text{-}src}$ 15-fold over normal endogenous levels, we were unable to obtain a proportionate increase in the amount of MTAg-pp60$^{c\text{-}src}$ complex. The majority of pp60$^{c\text{-}src}$ and MTAg remained unassociated. This result is similar to the findings of Schaffhausen et al. (B. Schaffhausen, B. Bockus, D. R. Kaplan, K. Berkner, and T. M. Roberts, Proc. Natl. Acad. Sci. USA, in press), in which a 100-fold overproduction of MTAg expressed from an adenovirus vector resulted in only a 3-fold increase in the amount of MTAg-pp60$^{c\text{-}src}$ complex. What factors control the association of these two molecules is presently not understood.

Recently, Whitman et al. (39) demonstrated the presence of a PI kinase activity in immunoprecipitates from cells infected with transformation-competent polyomavirus. The identity of the PI kinase was not determined, leaving open the possibility that pp60$^{c\text{-}src}$ was itself a PI kinase. We have examined our pp60$^{c\text{-}src}$ overproducer lines for enhanced PI kinase activity in vitro. Immunoprecipitates from uninfected overproducer lines were enhanced in their ability to phosphorylate exogenous protein substrates, but not PI substrates, compared with the uninfected nonoverproducer lines. The enhanced protein kinase activity correlated with the levels of pp60$^{c\text{-}src}$ in immunoprecipitates (15-fold), whereas the associated PI kinase activity did not. This result suggests that pp60$^{c\text{-}src}$ by itself has little or no PI kinase activity. This possibility is consistent with the results of Sugano and Hanafusa (35), who reported that overproduction of pp60$^{c\text{-}src}$ in chicken embryo fibroblasts has no effect on the total level of PI kinase in whole-cell extracts and that immunoprecipitations from these extracts with pp60$^{c\text{-}src}$-specific antiserum has no effect on the level of PI kinase activity remaining in the extracts. By examining the temperature sensitivity of PI and tyrosine kinase activities in membranes extracted from cells infected with a temperature-sensitive mutant of RSV, Sugimoto and Erikson (36) have come to a similar conclusion for pp60$^{v\text{-}src}$. More directly, we have measured the kinase activities associated with pp60$^{c\text{-}src}$ translated in vitro and associated with pp60$^{c\text{-}src}$ immunoprecipitates from pp60$^{c\text{-}src}$ overproducer cell lines. The lack of any detectable PI kinase signal in the presence of a strong intrinsic protein kinase signal strongly suggests that pp60$^{c\text{-}src}$ by itself has little or no PI kinase activity.

One of the more useful features of the c-*src* retroviral vector reported here is its ease of rescue from infected cells. Using the rescue fusion protocol described by Cepko et al. (4), we have isolated the first functional cDNA encoding pp60$^{c\text{-}src}$. When this cDNA was transcribed and translated in vitro, full-length pp60$^{c\text{-}src}$ was produced. Mapping studies indicated that the product synthesized in vitro was identical to that made in vivo. The availability of a functional c-*src* cDNA has allowed us to engineer the expression of this protein in bacterial, yeast, and insect virus vectors. This capacity should allow, for the first time, large-scale production and purification of pp60$^{c\text{-}src}$, thus facilitating detailed biochemical and genetic studies.

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LITERATURE CITED

1. **Bolen, J. B., C. J. Thiele, M. A. Israel, W. Yonemoto, L. A. Lipsich, and J. S. Brugge.** 1984. Enhancement of cellular src gene product associated tyrosyl kinase activity following polyoma virus infection and transformation. Cell **38**:767–777.
2. **Buss, J. E., and B. M. Sefton.** 1985. Myristic acid, a rare fatty acid, is the lipid attached to the transforming protein of Rous sarcoma virus and its cellular homolog. J. Virol. **53**:7–12.
3. **Carmichael, G. G., B. S. Schaffhausen, D. I. Dorsky, D. B. Oliver, and T. L. Benjamin.** 1982. Carboxy terminus of polyoma middle-sized tumor antigen is required for attachment to membranes, associated protein kinase activities and cell transformation. Proc. Natl. Acad. Sci. USA **79**:3579–3583.
4. **Cepko, C. L., B. E. Roberts, and R. C. Mulligan.** 1984. Construction and application of a highly transmissible murine retrovirus shuttle vector. Cell **37**:1053–1062.
5. **Collett, M. S., J. S. Brugge, and R. L. Erikson.** 1978. Characterization of a normal avian cell protein related to the avian sarcoma virus transforming gene product. Cell **15**:1363–1369.
6. **Collett, M. S., A. F. Purchio, and R. L. Erikson.** 1980. Avian sarcoma virus transforming protein p60src shows protein kinase activity specific for tyrosine. Nature (London) **285**:167–169.
7. **Collett, M. S., S. K. Wells, and A. F. Purchio.** 1983. Physical modification of purified Rous sarcoma virus pp60$^{v\text{-}src}$ protein after incubation with ATP/Mg^{2+}. Virology **128**:285–297.
8. **Courtneidge, S. A., A. D. Levinson, and J. M. Bishop.** 1980. The protein encoded by the transforming gene of avian sarcoma virus (pp60src) and a homologous protein in normal cells (pp60proto-src) are associated with the plasma membrane.

Proc. Natl. Acad. Sci. USA 77:3783–3787.
9. **Courtneidge, S. A., and A. E. Smith.** 1983. Polyoma virus transforming protein associates with the product of the c-src cellular gene. Nature (London) **303**:435–439.
10. **Courtneidge, S. A., and A. E. Smith.** 1984. The complex of polyoma virus middle-T antigen and pp60c-src. EMBO J. **3**:585–591.
11. **Coussens, P. M., J. A. Cooper, T. Hunter, and D. Shalloway.** 1985. Restriction of the in vitro and in vivo tyrosine protein kinase activities of $pp60^{c\text{-}src}$ relative to $pp60^{v\text{-}src}$. Mol. Cell. Biol. **5**:2753–2763.
12. **Graham, F. L., and A. J. Van Der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456–467.
13. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. **26**:365–369.
14. **Hunter, T., and B. W. Sefton.** 1980. Transforming gene product of Rous sarcoma virus phosphorylates tyrosine. Proc. Natl. Acad. Sci. USA **77**:1311–1315.
15. **Iba, H., F. R. Cross, E. A. Garber, and H. Hanafusa.** 1985. Low level of cellular protein phosphorylation by nontransforming overproduced $pp60^{c\text{-}src}$. Mol. Cell. Biol. **5**:1058–1066.
16. **Iba, H., T. Takeya, F. R. Cross, T. Hanafusa, and H. Hanafusa.** 1984. Rous sarcoma virus variants that carry the cellular src gene instead of the viral src gene cannot transform chicken embryo fibroblasts. Proc. Natl. Acad. Sci. USA **81**:4424–4428.
17. **Johnson, P. J., P. M. Coussens, A. V. Danko, and D. Shalloway.** 1985. Overexpressed $pp60^{c\text{-}src}$ can induce focus formation without complete transformation of NIH 3T3 cells. Mol. Cell. Biol. **5**:1073–1083.
18. **Kaplan, P. L., S. Simon, and W. Eckhart.** 1985. Polyomavirus middle T protein encoded by a retrovirus transforms nonestablished chicken embryo cells. J. Virol. **56**:1023–1026.
19. **Karess, R. E., W. S. Hayward, and H. Hanafusa.** 1979. Cellular information in the genome of retroviral avian sarcoma virus directs the synthesis of transforming protein. Proc. Natl. Acad. Sci. USA **76**:3154–3158.
20. **Krueger, J. G., E. Wang, and A. R. Goldberg.** 1980. Evidence that the src gene product of Rous sarcoma virus is membrane associated. Virology **101**:25–40.
21. **Levinson, A. D., H. Oppermann, H. E. Varmus, and J. M. Bishop.** 1980. The purified product of the transforming gene of avian sarcoma virus phosphorylates tyrosine. J. Biol. Chem. **255**:11973–11980.
22. **Mann, R., R. C. Mulligan, and D. Baltimore.** 1983. Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. Cell **33**:153–159.
23. **Oppermann, H., A. D. Levinson, H. E. Varmus, L. Levintow, and J. M. Bishop.** 1979. Uninfected vertebrate cells contain a protein that is closely related to the production of the avian sarcoma virus transforming gene (src). Proc. Natl. Acad. Sci. USA **76**:1804–1808.
24. **Parker, R. C., H. E. Varmus, and J. M. Bishop.** 1984. Expression of v-src and chicken c-src in rat cells demonstrates qualitative differences between $pp60^{v\text{-}src}$ and $pp60^{c\text{-}src}$. Cell **37**:131–139.
25. **Parsons, S. J., D. J. McCarley, C. M. Ely, D. C. Benjamin, and J. T. Parsons.** 1984. Monoclonal antibodies to Rous sarcoma virus $pp60^{src}$ react with enzymatically active cellular $pp60^{src}$ of avian and mammalian origin. J. Virol. **51**:272–282.
26. **Purchio, A. F., S. K. Wells, and M. S. Collett.** 1983. Increase in the phosphotransferase specific activity of purified Rous sarcoma virus $pp60^{v\text{-}src}$ protein after incubation with ATP plus Mg^{2+}. Mol. Cell. Biol. **3**:1589–1597.
27. **Rohrschneider, L. R.** 1979. Immunofluorescence on avian sarcoma virus-transformed cells: localization of the src gene product. Cell **16**:11–24.
28. **Rohrschneider, L. R., R. N. Eisenman, and C. R. Leitch.** 1979. Identification of a Rous sarcoma virus transformation related protein in normal avian and mammalian cells. Proc. Natl. Acad. Sci. USA **76**:4479–4483.
29. **Schultz, A. M., L. E. Henderson, S. Oroszlan, E. A. Garber, and H. Hanafusa.** 1985. Amino terminal myristylation of the protein kinase p60src, a retroviral transforming protein. Science **227**:427–429.
30. **Sefton, B. M., T. Hunter, K. Beemon, and W. Eckhart.** 1980. Evidence that the phosphorylation of tyrosine is essential for cellular transformation by Rous sarcoma virus. Cell **20**:807–816.
31. **Shalloway, D., P. M. Coussens, and P. Yuciuk.** 1984. Overexpression of the c-src protein does not induce transformation of NIH-3T3 cells. Proc. Natl. Acad. Sci. USA **81**:7071–7075.
32. **Shimotohno, K., and H. M. Temin.** 1982. Loss of intervening sequences in genomic mouse β-globin DNA inserted in an infectious retrovirus vector. Nature (London) **299**:265–268.
33. **Sorge, J., and S. H. Hughes.** 1982. Splicing of intervening sequences introduced into an infectious retroviral vector. J. Mol. Appl. Genet. **1**:547–559.
34. **Southern, P. J., and P. Berg.** 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. J. Mol. Appl. Genet. **1**:327–341.
35. **Sugano, S., and H. Hanafusa.** 1985. Phosphatidylinositol kinase activity in virus-transformed and nontransformed cells. Mol. Cell. Biol. **5**:2399–2404.
36. **Sugimoto, Y., and R. L. Erikson.** 1985. Phosphatidylinositol kinase activities in normal and Rous sarcoma virus-transformed cells. Mol. Cell. Biol. **5**:3194–3198.
37. **Takeya, T., and H. Hanafusa.** 1983. Structure and sequence of the cellular gene homologue to the RSV src gene and the mechanism for generating the transforming virus. Cell **32**:881–890.
38. **Treisman, R., U. Novak, J. Favaloro, and R. Kamen.** 1981. Transformation of rat cells by an altered polyoma virus genome expressing only the middle-T protein. Nature (London) **292**:595–600.
39. **Whitman, M., D. R. Kaplan, B. Schaffhausen, L. Cantley, and T. M. Roberts.** 1985. Association of phosphatidylinositol kinase activity with polyoma middle-T competent for transformation. Nature (London) **315**:239–242.
40. **Willingham, M. C., G. Jay, and I. Pastan.** 1979. Localization of the ASV src gene product to the plasma membrane of transformed cells by electron microscopy immunocytochemistry. Cell **18**:125–134.
41. **Yonemoto, W., M. Jarvis-Morar, J. S. Brugge, J. B. Bolen, and M. A. Israel.** 1985. Tyrosine phosphorylation within the amino-terminal domain of pp60c-src molecules associated with polyoma virus middle-sized tumor antigen. Proc. Natl. Acad. Sci. USA **82**:4568–4572.