Regulation of pp60^{c-src} and Its Interaction with Polyomavirus Middle T Antigen in Insect Cells

HELEN PIWNICA-WORMS,^{1†*} NIDHI G. WILLIAMS,¹ SENG H. CHENG,² AND THOMAS M. ROBERTS¹

Dana-Farber Cancer Institute and Department of Pathology, Harvard Medical School, Boston, Massachusetts 02115,¹ and Laboratory of Cellular Regulation, Integrated Genetics Inc., Framingham, Massachusetts 01701²

Received 21 June 1989/Accepted 15 September 1989

High yields of soluble, biologically active pp60^{c-src} and middle t antigen (MTAg) of polyomavirus were produced in insect cells, using a baculovirus expression system. In mammalian cells, pp60^{c-src} undergoes a regulatory phosphorylation on Tyr-527 in vivo and is autophosphorylated on Tyr-416 in vitro. In insect cells, pp60^{c-src} was phosphorylated primarily on Tyr-416, although Tyr-527 was detectable at a low level. A kinase-negative mutant of pp60^{c-src} was not phosphorylated on either Tyr-527 or Tyr-416 in insect cells and thus is an excellent biochemical reagent to search for the regulatory kinase that usually phosphorylates Tyr-527 in mammalian cells. MTA g synthesized in insect cells was not phosphorylated on tyrosine residues in vivo or in vitro, suggesting that it did not associate with any endogenous tyrosine kinases. However, MTA g isolated from cells coinfecting with viruses encoding both MTA g and pp60^{c-src} was phosphorylated on tyrosine residues both in vivo and in vitro.

pp60^{c-src} is the cellular homolog of the retroviral transforming protein pp60^{v-src}. pp60^{c-src} and pp60^{v-src} are structurally and functionally related. Both are associated with the plasma membrane because of an amino-terminal myristylation (3, 44). Both possess intrinsic tyrosine kinase activities (12, 23, 32), although the kinase activity of pp60^{v-src} has been estimated to be 10- to 50-fold higher than that of pp60^{c-src} when measured in vitro (21, 24). Both proteins are phosphoproteins, although their sites of tyrosine phosphorylation differ in vivo. pp60^{c-src} is phosphorylated on Ser-17 (11, 22) and Tyr-527 (13) in vivo. Tyr-527 is located in the unique carboxyl terminus of pp60^{c-src} and, therefore, is absent in pp60^{v-src}. Data from a number of laboratories support the hypothesis that phosphorylation of Tyr-527 suppresses the kinase activity and transforming potential of pp60^{c-src} in vivo (5, 6, 14, 16, 17, 24, 28, 29, 40). However, the kinase responsible for this negative regulation has not been identified. Tyr-416 is the site of phosphorylation in vivo in (i) pp60^{v-src} (37, 46); (ii) transforming mutants of pp60^{c-src} (5, 9, 24, 28, 40), and (iii) that fraction of pp60^{c-src} associated with the middle t antigen (MTAg) of polyomavirus (6). In each case, phosphorylation of Tyr-416 correlates with enhanced kinase activity. Thus, full activation of pp60^{c-src} may require concomitant changes in phosphorylation at both Tyr-527 and Tyr-416.

MTAg is the transforming protein of polyomavirus (47). It has no known intrinsic catalytic activities; instead, it is thought to function by interacting with and altering the activities of cellular proteins. Three tyrosine kinases known to interact with MTA g are pp60^{c-src} (19), pp62^{c-yes} (30), and pp59^{c-fyn} (7, 31). MTA g has been shown to activate the kinase activity of pp60^{c-src} and pp62^{c-yes} (2, 30). Genetic studies have demonstrated that an interaction with pp60^{c-src} is necessary for MTA g-mediated transformation (8, 20). How MTA g alters the specific kinase activity of pp60^{c-src} is unclear. The shift in phosphorylation from Tyr-527 to Tyr-416 does not account for the entire activation (5). Complexed

pp60^{c-src} has also been shown to be phosphorylated on one or more amino-terminal tyrosine residues (50). These phosphorylations have been detected in vivo only in the presence of sodium orthovanadate or in vitro in immune complex kinase assays. Thus, their biological significance remains unresolved. In addition, only a small proportion of the total amount of MTA g and pp60^{c-src} present within a cell at any given time is found stably associated (1, 20). Levels of the complex can be increased only marginally by overexpressing either pp60^{c-src} or MTA g (39, 41, 43) in mammalian cells. Factors controlling the association of these two molecules are presently not known. Unfortunately, the two proteins fail to interact when they are coproduced in bacteria (H. Piwnica-Worms, D. Pallas, and T. M. Roberts, unpublished data) or in yeast cells (S. Kornbluth and H. Hanafusa, personal communication) or when they are cotranslated in vitro in a rabbit reticulocyte lysate (H. Piwnica-Worms, and T. M. Roberts, unpublished data). A complete biochemical analysis awaits the development of a system in which purified components can be added and their effects can be assessed individually in vitro.

As a first step towards the development of an assay, we have overproduced MTA g and avian pp60^{c-src}, using a baculovirus expression system. We have analyzed the post-translational modifications undergone by pp60^{c-src} in insect cells, including myristylation and phosphorylation. In addition, by coinfecting cells with recombinant viruses encoding both pp60^{c-src} and MTA g, we have obtained pp60^{c-src}-MTAg complexes.

MATERIALS AND METHODS

Cell lines. *Spodoptera frugiperda* (Sf9) cells were obtained from the American Type Culture Collection. All procedures relating to viral propagation, isolation, and plaquing were performed as suggested by Summers and Smith (49).

Generation of c-src baculoviruses. pSP68-4(RBS) (39) was restricted with *Hind*III, and a *Bam*HI linker was inserted to generate pSP68-4RBS(Bam). pSP68-4RBS(Bam) was restricted with *Nco*I, and a *Bcl*II linker (39) was inserted to generate pSP68-4RBS(Bcl-Bam). pSP68-4RBS(Bcl-Bam) was restricted with *Bcl*II and *Bam*HI, and the fragment

* Corresponding author.

† Present address: Department of Physiology, Tufts Medical School, 136 Harrison Ave., Boston, MA 02111.

encoding pp60^{c-src} was isolated. This c-src fragment was cloned into *Bam*HI-restricted and phosphatased pAC373 (49) to generate pAC373(c-src). A mixture of 2 µg of pAC373(c-src) and 1 µg of purified wild-type viral DNA was transfected into Sf9 cells. Four days later, medium supernatants were removed and centrifuged at 1,000 rpm for 10 min. Clarified supernatants containing wild-type and recombinant viruses were plaque on a monolayer of Sf9 cells. Occlusion negative plaques were picked and seeded onto 2.5×10^6 cells. Three days later, cells and medium supernatants were removed and centrifuged at 1,000 rpm for 10 min. The supernatant containing the virus was stored at 4°C. The pelleted cells were lysed and immunoprecipitated with EC10 serum (a monoclonal antibody specific for avian pp60^{c-src} [36]), and kinase assays were performed in vitro as described previously (39). All lysates tested positive for the presence of kinase-active pp60^{c-src}. One supernatant was selected and taken through three rounds of plaque purification. A c-src mutant encoding methionine rather than lysine at position 295 was constructed by site-directed mutagenesis as described previously (40). The Lys-295 to Met mutant was then cloned into pAC373 to generate pAC373(295) and recombinant viruses were generated as described above.

Generation of MTA_g baculovirus. p890 (26) was restricted with *Bcl*I and *Bam*HI, and the fragment encoding MTA_g was cloned into pAC373 which had been linearized with *Bam*HI and phosphatased. The recombinant plasmid was designated pAC373(MTA_g). A mixture of pAC373(MTA_g) and wild-type viral DNA was transfected into Sf9 cells as described above for pp60^{c-src}. Twenty occlusion-negative plaques were picked and seeded onto cells as described above. After 6 days, cells and medium supernatants were removed and clarified. Viral supernatants were stored at 4°C. Cells were lysed and normalized for protein content with the Bio-Rad Laboratories protein assay kit, and proteins were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electro-

phoresis (SDS-PAGE). Proteins were blotted onto nitrocellulose and probed with PY19 serum (polyclonal antibody specific for polyoma t antigens [34]) as described in the protoblot immunoblotting system from Promega Biotec. One viral stock was selected and taken through three rounds of plaque purification.

Time course of protein production and accumulation. Cells, 3×10^6 , were seeded into each of 13 60-mm tissue culture dishes. One plate was mock infected, six were infected with recombinant baculovirus encoding pp60^{c-src}, and the remaining six were infected with recombinant baculovirus encoding MTA_g. Cells were lysed at 15, 20, 24, 29, 40, and 52 h postinfection (p.i.), and their protein concentration was measured with the Bio-Rad protein assay kit. Total cell protein, 30 µg, was then resolved by SDS-PAGE, transferred electrophoretically to a 0.45-µm nitrocellulose filter. The filter was then blocked in 1% bovine serum albumin in TBST (10 mM Tris hydrochloride [pH 8.0], 150 mM NaCl, 0.05% Tween 20) for 1 h at 37°C. The pp60^{c-src} blot was incubated overnight with EC10 serum (1:2,500 dilution), and the MTA_g blot was incubated for 4 h with PY19 serum (1:2,500 dilution). Both blots were then washed, incubated with anti-mouse (pp60^{c-src} blot) or anti-rabbit (MTA_g blot) alkaline phosphatase-conjugated immunoglobulin G for 45 min, and then developed by using the enzymatic color reaction kit from Promega Biotec.

Myristylation of pp60^{c-src}. Cells, 3×10^6 , were seeded into each of two 60-mm tissue culture dishes. After attachment, the cells were infected with recombinant baculovirus encoding pp60^{c-src} at a multiplicity of infection of 10. At 40 h p.i., one plate was rinsed in methionine-free medium and then labeled in methionine-free medium supplemented with 2 mM glutamine and 0.67 mCi of [³⁵S]methionine per ml. The second plate was labeled with 2 mCi of [³H]myristic acid (Dupont, NEN Research Products) in a final volume of 1.5 ml. The [³H]myristic acid was lyophilized and redissolved in

FIG. 1. MTA_g and pp60^{c-src} production and accumulation. Sf9 cells were either mock infected (lane 7, both panels) or infected with recombinant baculoviruses encoding pp60^{c-src} (left panel) or MTA_g (right panel) at a multiplicity of infection of 10. Cells were harvested at various times after infection; cell lysates were prepared, and proteins were resolved by SDS-PAGE. The proteins were blotted onto nitrocellulose and probed with EC10 (left panel) or PY19 (right panel) serum. Lane 1, 52 h p.i.; lane 2, 40 h p.i.; lane 3, 29 h p.i.; lane 4, 24 h p.i.; lane 5, 20 h p.i.; lane 6, 15 h p.i.; lane 7, mock infected.

15 μ l of dimethyl sulfoxide prior to labeling. After 2 h, cells were lysed and proteins were analyzed directly by SDS-PAGE or immunoprecipitated with EC10 serum prior to SDS-PAGE.

Intrinsic and associated kinase activities. Cells, 9×10^6 , were seeded into each of three 100-mm tissue culture dishes. After attachment, cells were mock infected or infected with recombinant baculovirus encoding MTA_g or both pp60^{c-src} and MTA_g. Cell lysates were prepared at 40 h p.i. and normalized for protein content. A 200- μ g amount of protein from each lysate was immunoprecipitated with either EC10 or PY19 serum, and kinase assays were performed in vitro.

In vivo labeling and phosphopeptide mapping. Sf9 cells were infected with recombinant virus encoding pp60^{c-src}, MTA_g, or both pp60^{c-src} and MTA_g each at a multiplicity of infection of 10. At 36 h after infection, cells were rinsed once in phosphate-free medium and then incubated for 2 h in phosphate-free medium supplemented with 2 mM glutamine, 1.5% dialyzed calf serum, and 2 mCi of ³²P-labeled P_i per ml. Cell lysates were prepared in a phosphate-RIPA buffer (10 mM sodium phosphate [pH 7.2], 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium deoxycholate, 0.1% SDS, 2 mM EDTA) supplemented with 2 mM phenyl-methylsulfonyl fluoride, 0.1% Aprotinin, and 2 mM sodium orthovanadate, and pp60^{c-src} was immunoprecipitated with either EC10 or PY19 serum. Staphylococcus V8 and tryptic protease mapping was performed as described previously (14, 30), and cyanogen bromide mapping was performed as described by Jove et al. (25). Phosphoamino acid analysis was performed as described previously (35).

RESULTS

cDNAs encoding normal pp60^{c-src}, a c-src mutant containing methionine rather than lysine at position 295, and the MTA_g of polyomavirus were cloned into the *Bam*HI site of pAC373 (49) to generate pAC373(c-src), pAC373(295), and pAC373(MTA_g), respectively. Sf9 cells were then transfected with a mixture of wild-type baculoviral DNA and pAC373(c-src), pAC373(295), or pAC373(MTA_g). Five days after transfection, medium supernatants containing both wild-type and recombinant viruses were removed and plaqued on a monolayer of Sf9 cells. Recombinant virus plaques were identified visually by their occlusion-negative phenotype (49). Recombinant virus was purified from contaminating wild-type virus by three rounds of plaque purification.

Protein production and accumulation. The time course of protein production and accumulation in insect cells was examined as follows. Sf9 cells were infected with recombinant viruses encoding either pp60^{c-src} (Fig. 1, left panel) or MTA_g (Fig. 1, right panel) at a multiplicity of infection of 10. At various times after infection, cell lysates were prepared and protein production was examined by Western blotting (immunoblotting). pp60^{c-src} and MTA_g were first detected 20 h p.i. (lane 5), increased linearly until about 40 h (lane 2), and continued to accumulate gradually from 52 to 72 h (lane 1 and data not shown). Breakdown products were detectable by about 30 h p.i. (lane 3), though the bulk of the protein remained stable for at least 3 days (data not shown). By Coomassie blue staining, we estimated that pp60^{c-src} and MTA_g comprise approximately 1% of total cell protein.

pp60^{c-src} is myristylated in insect cells. pp60^{c-src} is myristylated in avian and mammalian cells (3, 44) and in yeast cells (29). To determine whether it was myristylated in insect cells, we infected cells with recombinant baculoviruses

encoding pp60^{c-src} and then labeled the infected cells with either [³H]myristic acid (Fig. 2, right panel) or [³⁵S]methionine (Fig. 2, left panel). Total cell lysates or immunoprecipitates prepared by using antiserum specific for pp60^{c-src} were then resolved by SDS-PAGE. Although pp60^{c-src} was the major protein labeled with [³⁵S]methionine, several additional proteins were also labeled during this time period. Since the labeling period was started at 40 h p.i. when host protein synthesis is shut off, these proteins represent late virus-specific proteins. pp60^{c-src} was the only protein labeled with [³H]myristic acid. We do not know the fraction of pp60^{c-src} that is myristylated.

Intrinsic and associated kinase activities of pp60^{c-src} and MTA_g in insect cells. To determine whether (i) pp60^{c-src} produced in insect cells was enzymatically active, (ii) MTA_g could associate with any endogenous insect tyrosine kinases, and (iii) MTA_g and pp60^{c-src} would associate when coproduced in insect cells, we performed the following experiments: insect cells were mock infected (Fig. 3, lane 1) or infected with recombinant baculoviruses encoding MTA_g (lane 2) or MTA_g and pp60^{c-src} (Fig. 3, lanes 3 and 5). Cell lysates were prepared and immunoprecipitated with either EC10 serum (34) specific for avian pp60^{c-src} (lane 5) or PY19 serum (36) which recognizes MTA_g (lanes 1, 2, 3, and 4). Kinase assays were performed in vitro. MTA_g was phosphorylated in immune complex kinase assays in the presence

FIG. 2. Myristylation of pp60^{c-src}. Sf9 cells were infected with recombinant virus encoding pp60^{c-src} and at 40 h p.i. labeled with either [³⁵S]methionine (left panel) or [³H]myristic acid (right panel). Cell lysates were prepared after 2 h of labeling, and proteins were resolved directly by SDS-PAGE (T) or immunoprecipitated with EC10 serum prior to SDS-PAGE (E). Exposure times were 16 h for ³⁵S and 96 h for ³H. Arrowheads denote pp60^{c-src}.

FIG. 3. Intrinsic and associated kinase activities of $pp60^{c-src}$ and MTA. Sf9 cells were mock infected (lane 1) or infected with recombinant viruses encoding either MTA (lane 2) or MTA and $pp60^{c-src}$ (lanes 3 and 5). Cell lysates were prepared at 40 h p.i. and immunoprecipitated with EC10 (lane 5) or PY19 (lanes 1, 2, 3, and 4) serum, and kinase assays were performed in vitro. Lysates were also prepared from NIH 3T3 cells transformed by MTA (lane 4 [10]) and immunoprecipitated with PY19 serum, and kinase assays were performed in vitro. The protein with the greatest electrophoretic mobility in lane 4 is a MTA breakdown product (39). For lanes 1, 2, 3, and 5, 200 μ g of total cell protein was immunoprecipitated, whereas 1.5 mg of total cell protein was immunoprecipitated in lane 4.

(lanes 3 and 5) but not in the absence (lane 2) of $pp60^{c-src}$. In immunoprecipitates prepared with EC10 serum (lane 5), both $pp60^{c-src}$ and MTA phosphorylation is evident. EC10 serum immunoprecipitates both complexed and free $pp60^{c-src}$, both of which can be phosphorylated in vitro. This accounts for the significant phosphorylation of $pp60^{c-src}$ in this experiment. In immunoprecipitates prepared with PY19 serum (lane 3), only the $pp60^{c-src}$ complexed to MTA was immunoprecipitated and, since MTA is the preferred substrate, phosphorylation of $pp60^{c-src}$ is less evident. MTA was also phosphorylated in vivo by $pp60^{c-src}$ when both proteins were coproduced in insect cells (Fig. 4). Cells were infected with viruses encoding $pp60^{c-src}$ (Fig. 4, lane 4) or MTA (lane 1) or with both recombinant viruses (lanes 2 and 3) and were labeled with $^{32}P_i$. Cell lysates were prepared and immunoprecipitated with either PY19 (lanes 1 and 2) or EC10 (lanes 3 and 4) serum. $pp60^{c-src}$ coimmunoprecipitated with MTA, using antisera specific for MTA (lane 2), and MTA coimmunoprecipitated with $pp60^{c-src}$, using antisera specific for $pp60^{c-src}$ (lane 3). $pp60^{c-src}$ was phosphorylated primarily on tyrosine and serine residues (Fig. 4, bottom panel). MTA was phosphorylated on serine when synthesized alone in insect cells (lane 1) and was phosphorylated on

FIG. 4. Association between $pp60^{c-src}$ and MTA in vivo. (Top) Cells were infected with viruses encoding MTA (lane 1), $pp60^{c-src}$ (lane 4), or both MTA and $pp60^{c-src}$ (lanes 2 and 3). At 36 h p.i., cells were labeled with $^{32}P_i$. Cell lysates were prepared and immunoprecipitated with either PY19 (lanes 1 and 2) or EC10 (lanes 3 and 4) serum. Immunoprecipitates were resolved by SDS-PAGE. (Bottom) Lanes 1, 2, and 3 are the results of phosphoamino acid analysis performed on MTA isolated from lanes 1, 2, and 3, respectively, of the gel shown in the top panel. Lane 4 is the result of phosphoamino acid analysis performed on $pp60^{c-src}$ isolated from lane 4 of the gel shown in the top panel.

tyrosine as well as serine when coproduced with $pp60^{c-src}$ (lanes 2 and 3). We also found that MTA reacted with an anti-phosphotyrosine antibody in immunoblots from lysates prepared from cells coinfecting with viruses encoding both MTA and $pp60^{c-src}$ but not from lysates prepared from cells infected with virus encoding MTA only (data not shown). Thus, MTA was phosphorylated on tyrosine both in vitro and in vivo only when coproduced with $pp60^{c-src}$ in insect cells.

$pp60^{c-src}$ is phosphorylated primarily on Tyr-416 and Ser-17 in insect cells. To determine where the $pp60^{c-src}$ produced in insect cells was phosphorylated in vitro and in vivo, two-dimensional tryptic phosphopeptide mapping was performed. Tyr-416 was the major site of autophosphorylation (Fig. 5A, B, and C). Tryptic digests of $pp60^{c-src}$ synthesized and labeled in insect cells gave rise to two major tryptic phosphopeptides (Fig. 5D). Species 2 migrated at a position similar to the tryptic peptide containing Ser-17, and species 1 migrated at a position similar to the tryptic peptide containing Tyr-416. To verify this, we mixed tryptic digests of $pp60^{c-src}$ synthesized in insect cells with tryptic digests of a $c-src$ mutant that we have previously shown to be phosphorylated on Ser-17 and Tyr-416 (40) (Fig. 5E). We do not know what fraction of $pp60^{c-src}$ produced in insect cells is phosphorylated on Ser-17, but phosphotyrosine was present in greater quantities than phosphoserine by phosphoamino acid analysis (Fig. 4, bottom panel) and more label was incorporated into the peptide containing Tyr-416 than into that containing Ser-17 (assuming equal recovery of both peptides; Fig. 5D). Thus, it is unlikely that there is a stoichiometric phosphorylation of Ser-17.

Since the phosphopeptide containing Tyr-527 is often difficult to recover during tryptic phosphopeptide mapping, we also performed one-dimensional cyanogen bromide mapping (data not shown). Using this method, we found that

FIG. 5. Tryptic phosphopeptide mapping of pp60^{c-src} labeled in vitro and in vivo. pp60^{c-src} was immunoprecipitated from lysates of (A) infected Sf9 cells or (B) NIH 3T3 cells overproducing pp60^{c-src}, and autokinase assays were performed in vitro. Alternatively, pp60^{c-src} was immunoprecipitated from ³²P-labeled lysates of (D) infected Sf9 cells or (E) NIH 3T3 cells expressing a c-src mutant in which Tyr-527 is replaced by Phe. Immunoprecipitated proteins were digested with trypsin, separated by electrophoresis and chromatography in two dimensions, and autoradiographed. (C) Mix of phosphopeptides from panels A and B; (F) mix of phosphopeptides from panels D and E. Spots 1 and 2 designate the phosphopeptides containing Tyr-416 and Ser-17, respectively. The origins are marked with arrowheads. Electrophoresis at pH 8.9 was performed in the horizontal dimension with the anode at the right.

pp60^{c-src} was also phosphorylated to a small extent on Tyr-527 (~10% of the level seen at Tyr-416). To distinguish between the possibility that Tyr-527 was being phosphorylated by a kinase present in Sf9 cells other than pp60^{c-src} itself, we expressed a kinase-negative mutant of pp60^{c-src} in insect cells and compared its phosphorylation pattern with that of normal pp60^{c-src}, using one-dimensional staphylococcal V8 protease mapping (a protocol in which phosphate on Tyr-527 is stable). The kinase-negative mutant was constructed by replacing lysine residue 295 in pp60^{c-src} with methionine. Lys-295 is in the ATP-binding domain of pp60^{c-src} and is essential for kinase activity (25, 48). The Lys-295 to Met mutant has been shown to be phosphorylated on Tyr-527 in avian cells (25). If an endogenous insect kinase was responsible for phosphorylating Tyr-527, then this residue would be expected to be phosphorylated in the Lys-295 mutant; if, on the other hand, pp60^{c-src} was itself responsible for this phosphorylation, then the Lys-295 mutant would not be predicted to be phosphorylated on Tyr-527. The presence of V8 fragments 1, 3, and 4 (Fig. 6) indicated that the Lys-295 mutant was phosphorylated within its amino terminus, presumably on Ser-17. However, the carboxy-terminal V8 fragment (V2) containing Tyr-416 and Tyr-527 was not present in the map of the Lys-295 mutant (Fig. 6, lane 3), indicating that this mutant was not phosphorylated on either Tyr-527 or Tyr-416.

DISCUSSION

In this report, we describe the application of a baculovirus expression system to (i) produce large quantities of soluble, biologically active pp60^{c-src} and MTA_g; (ii) produce large quantities of a kinase-negative mutant of pp60^{c-src} to be used as substrate to identify and purify kinases that regulate pp60^{c-src} in mammalian cells; and (iii) develop a system for

the study of MTA_g-pp60^{c-src} interactions in vitro. The baculovirus expression system utilizes a helper-independent virus (*Autographa californica* nuclear polyhedrosis virus) which can be grown to high titers in cells adapted to Spinner culture (49). Thus, high levels of recombinant protein can be obtained with relative ease. We have obtained large quantities of soluble, biologically active pp60^{c-src} and MTA_g, putting in the realm of possibility a three-dimensional structural analysis of both proteins. In addition, baculoviruses are noninfectious to vertebrates and their promoters have been shown to be inactive in mammalian cells (4). This is a clear advantage over other eucaryotic viral vector systems when expressing oncogenes or other potentially dangerous proteins. Further, unlike bacterial expression systems, the baculovirus system is a eucaryotic expression system and, therefore, utilizes many of the protein modification, processing, and transport systems which occur in higher eucaryotic cells.

Two of the three posttranslational modifications of pp60^{c-src} which usually occur in higher eucaryotic cells also take place in insect cells. These are myristylation and phosphorylation on Ser-17. Myristylation has also been shown to occur in yeast cells (29). In mammalian cells, Ser-17 is phosphorylated by a cyclic AMP-dependent protein kinase (11, 38). A homologous enzyme with the capacity to recognize avian pp60^{c-src} must therefore exist in Sf9 cells. The only invertebrate homolog of c-src that has been cloned and sequenced is from *Drosophila melanogaster*. The amino terminus of the *Drosophila* gene diverges greatly from its vertebrate homolog, but it does encode a serine at position 26 which appears to be homologous to serine 17 in vertebrate pp60^{c-src} (45). Phosphorylation of Tyr-527, the third modification of pp60^{c-src} that usually occurs in mammalian cells, was also detectable at low levels in Sf9 cells. That the

FIG. 6. Staphylococcal V8 protease mapping. pp60^{c-src} (lanes 1 and 2) and the Lys-295 to Met c-src mutant (lane 3) were immunoprecipitated from ³²P-labeled lysates of infected Sf9 cells and resolved by SDS-PAGE. Gel bands containing the respective proteins were excised and digested with staphylococcal V8 protease, and the proteolytic digestion products were resolved on a 15% polyacrylamide-SDS gel. V8 fragments 1, 3, and 4 are derived from the amino terminus of pp60^{c-src}, and V8 fragment 2 is derived from the carboxyl terminus of pp60^{c-src}.

Lys-295 mutant was not phosphorylated on Tyr-527 suggests that this low level of phosphorylation was due to auto- or transphosphorylation by pp60^{c-src} itself.

Drosophila c-src has been shown to encode a tyrosine residue at a position homologous to Tyr-527 in vertebrate c-src (45). Thus, the invertebrate *src* homolog may be regulated by a mechanism similar to that used in vertebrate cells. The finding that the Lys-295 mutant of pp60^{c-src} was not phosphorylated on Tyr-527 in insect cells can be explained in several ways. (i) A 527 kinase homolog may not be present in insect cells. (ii) The 527 kinase may be present in insect cells but its activity may be saturated by the large quantities of the c-src mutant being produced. However, the Ser-17 kinase was not saturated under these conditions. (iii) The Tyr-527 kinase may be more labile than the Ser-17 kinase. pp60^{c-src} is synthesized late in the viral replication cycle, long after host protein synthesis has been shut off. Thus, the Tyr-527 kinase may have been present prior to viral infection but may have turned over by the time pp60^{c-src} synthesis began. (iv) The determinants that make up the recognition site for the 527 kinase may not be shared between avian pp60^{c-src} and its *Drosophila* homolog. Thus, it is possible that an insect 527 kinase exists but does not recognize avian pp60^{c-src}. (v) Finally, it is still formally possible that pp60^{c-src} is itself the 527 kinase, but that it functions poorly in yeast and insect cells (15, 16, 29).

Recent studies strongly suggest that Tyr-527 is a major regulatory site in pp60^{c-src}. Phosphorylation of this residue has been shown to suppress the kinase activity and transforming potential of pp60^{c-src}. Other members of the *src* family of tyrosine kinases possess a Tyr-527 homolog. Thus, suppression by phosphorylation at this residue may be a general mechanism for regulating the biochemical and bio-

logical activities of the entire *src* family of tyrosine kinases. The finding that the Lys-295 mutant is not phosphorylated on Tyr-527 in insect cells makes the baculovirus system ideal for preparing large quantities of this mutant to be used for identifying and isolating the 527 kinase from mammalian cells. In addition, other regulatory kinases, besides that which phosphorylates Tyr-527, may also be identified by using the approach.

We have previously reported a region in the carboxyl terminus of pp60^{c-src}, bordered by Asp-516 and Pro-525, that is required for MTA_g association in mammalian cells (9). Since this region is not conserved in the *Drosophila* pp60^{c-src} homolog (45), we did not predict an interaction to occur between MTA_g and the insect homolog of pp60^{c-src}. Indeed, MTA_g synthesized in Sf9 cells was not detectably associated with any endogenous insect kinases when assayed *in vivo* or in immune complex kinase reactions assayed *in vitro*. However, MTA_g did form a complex with avian pp60^{c-src} in cells coinfecting with viruses encoding both proteins. These results make the baculovirus expression system preferable to other expression systems for studying complex formation because: (i) preparations of MTA_g from higher eucaryotic cells would be expected to be contaminated with endogenous pp60^{c-src} and (ii) pp60^{c-src} and MTA_g do not interact when coproduced in yeast cells (Kornbluth and Hanafusa, personal communication) or bacteria (Piwnica-Worms et al., unpublished data) or when cotranslated *in vitro*, using a rabbit reticulocyte lysate (Piwnica-Worms and Roberts, unpublished data). In addition, overproduction of either component in mammalian cells does not significantly increase levels of complex formation (39, 41, 43). These results suggest that there may be an interplay between both positive and negative factors which act in conjunction to regulate the interaction of pp60^{c-src} and MTA_g in mammalian cells. For example, the posttranslational modifications undergone by MTA_g (i.e., phosphorylations) may be a positive factor regulating complex formation in mammalian cells (42). We can easily test this hypothesis by comparing the posttranslational modifications undergone by MTA_g in insect cells and those which occur in mammalian and yeast systems. Factors that inhibit complex formation in mammalian cells may include regulatory proteins, such as the 527 kinase, that compete with MTA_g for pp60^{c-src} binding. The baculoviral expression system may be the system of choice for identifying and purifying these various factors from mammalian cells.

Complex formation between MTA_g and pp60^{c-src} was assayed both *in vivo* and *in vitro*. We found that MTA_g coimmunoprecipitated with pp60^{c-src}, using antisera specific for pp60^{c-src}, and that pp60^{c-src} coimmunoprecipitated with MTA_g, using MTA_g-specific sera. In addition, MTA_g was phosphorylated on tyrosine *in vivo* only when coproduced with pp60^{c-src} and *in vitro* only when pp60^{c-src} was present in the immunoprecipitates. Because the majority of pp60^{c-src} produced in insect cells is not phosphorylated on Tyr-527, its kinase activity is constitutively high. It has, therefore, been difficult to determine whether the pp60^{c-src} associated with MTA_g is activated compared with its unassociated counterpart. To date, we have been unable to measure any substantial enhancement in kinase activity when pp60^{c-src} is complexed to MTA_g in insect cells. We are currently mapping the complexed pp60^{c-src} and MTA_g to identify any novel posttranslational modifications that occur as a result of complex formation.

There remains uncertainty as to whether the complex formed between MTA_g and pp60^{c-src} in insect cells is func-

tional. Because of the large quantities of protein being produced, the complex formation we observed could be due to a nonspecific aggregation of the two proteins. Using antisera specific for MTA_g, we have observed coimmunoprecipitation of pp60^{v-src} and the *v-fms* gene product when either is coproduced with MTA_g in insect cells (although not to the same extent as with pp60^{c-src}). Neither of these proteins has been demonstrated to associate with MTA_g in mammalian cells. However, we do not observe MTA_g-pp60^{c-src} complexes sedimenting to the bottom of sucrose gradients, as would be expected for large nonspecific aggregates. We also do not observe a major shift in the sedimentation of either MTA_g of pp60^{c-src} from coinfecting cells when compared with their sedimentation from singly infected cells. When isolated from mammalian cells, the complex sediments in the region of the gradient expected for a 220-kilodalton complex (19, 20). These results might suggest that we have no complex formation inside insect cells, but rather the two proteins associate after the cells are lysed and incubated for extended periods, as is the case during immunoprecipitations. Alternatively, the complex isolated from mammalian cells may contain proteins in addition to pp60^{c-src} and MTA_g which contribute to its greater sedimentation velocity, and the complex formed in insect cells may be deficient in these other proteins. Several proteins have been shown to associate with MTA_g in mammalian cells (33), and a potential phosphatidylinositol kinase of approximately 85 kilodaltons has been shown to be associated with MTA_g-pp60^{c-src} complexes isolated from mammalian cells (18, 27). Further studies will be required to determine whether MTA_g and pp60^{c-src} form a functional complex in insect cells.

Regardless of whether a functional complex is formed between pp60^{c-src} and MTA_g within insect cells at the time of their synthesis, this system provides a powerful tool for the study of pp60^{c-src} and MTA_g interactions in vitro. We are currently purifying large quantities of MTA_g, free of contaminating tyrosine kinases, and large quantities of pp60^{c-src} as a first step towards the development of a system in which purified components can be added and their ability to drive complex formation can be assessed individually.

ACKNOWLEDGMENTS

We thank R. Jove for advice on the cyanogen bromide mapping, Debbie Morrison for the baculovirus encoding the *v-fms* gene product, and A. Desai for secretarial assistance.

This work was supported by Public Health Service grants CA30002 and CA43803 to T.M.R. and R29 CA50767 to H.P.-W. from the National Cancer Institute.

LITERATURE CITED

- Bolen, J. B., V. DeSeau, J. O'Shaughnessy, and A. Shokreh. 1987. Analysis of middle tumor antigen and pp60^{c-src} interactions in polyomavirus-transformed rat cells. *J. Virol.* 61:3299-3305.
- 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.
- 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.
- Carbonell, L. F., M. J. Klowden, and J. K. Miller. 1985. Baculovirus-mediated expression of bacterial genes in dipteran and mammalian cells. *J. Virol.* 56:153-160.
- Cartwright, C. A., W. Eckhart, S. Simon, and P. L. Kaplan. 1987. Cell transformation by pp60^{c-src} mutated in the carboxy-terminal regulatory domain. *Cell* 49:83-91.
- Cartwright, C. A., P. L. Kaplan, J. A. Cooper, T. Hunter, and W. Eckhart. 1986. Altered sites of tyrosine phosphorylation in pp60^{c-src} associated with polyomavirus middle tumor antigen. *Mol. Cell. Biol.* 6:1562-1570.
- Cheng, S. H., R. Harvey, P. C. Espino, K. Semba, T. Yamamoto, K. Toyoshima, and A. E. Smith. 1988. Peptide antibodies to the human c-fyn gene product demonstrate pp59^{fyn} is capable of complex formation with the middle t antigen of polyoma virus. *EMBO J.* 7:3845-3856.
- Cheng, S. H., W. Markland, A. F. Markham, and A. E. Smith. 1986. Mutations around the NG59 lesion indicate an active association of polyoma virus middle-T antigen with pp60^{c-src} is required for cell transformation. *EMBO J.* 5:325-334.
- Cheng, S. H., H. Piwnica-Worms, R. W. Harvey, T. M. Roberts, and A. E. Smith. 1988. The carboxy terminus of pp60^{c-src} is involved in complex formation with the middle-T antigen of polyoma virus. *Mol. Cell. Biol.* 8:1736-1747.
- Cherington, V., B. Morgan, B. M. Spiegelman, and T. M. Roberts. 1986. Recombinant retroviruses that transduce individual polyoma tumor antigens: effects on growth and differentiation. *Proc. Natl. Acad. Sci. USA* 83:4307-4311.
- Collett, M. S., E. Erickson, and R. L. Erikson. 1979. Structural analysis of the avian sarcoma virus transforming protein: sites of phosphorylation. *J. Virol.* 29:770-781.
- Collett, M. S., A. F. Purchio, and R. L. Erikson. 1980. Avian sarcoma virus transforming protein p60^{src} shows protein kinase activity specific for tyrosine. *Nature (London)* 285:167-169.
- Cooper, J. A., K. L. Gould, C. A. Cartwright, and T. Hunter. 1986. Tyr⁵²⁷ is phosphorylated in pp60^{c-src}: implications for regulation. *Science* 231:1431-1434.
- Cooper, J. A., and C. S. King. 1986. Dephosphorylation or antibody binding to the carboxy terminus stimulates pp60^{c-src}. *Mol. Cell. Biol.* 6:4467-4477.
- Cooper, J. A., and A. MacAuley. 1988. Potential positive and negative autoregulation of pp60^{c-src} by intermolecular autophosphorylation. *Proc. Natl. Acad. Sci. USA* 85:4232-4236.
- Cooper, J. A., and K. Runge. 1987. Avian pp60^{c-src} is more active when expressed in yeast than in vertebrate fibroblasts. *Oncogene* 1:297-310.
- Courtneidge, S. A. 1985. Activation of the pp60^{c-src} kinase by middle T antigen binding or by dephosphorylation. *EMBO J.* 4:1471-1477.
- Courtneidge, S. A., and A. Heber. 1987. An 81kd protein complexed with middle t antigen and pp60^{c-src}: a possible phosphatidylinositol kinase. *Cell* 50:1031-1037.
- 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.
- Courtneidge, S. A., and A. E. Smith. 1984. The complex of polyoma virus middle-T antigen and pp60^{c-src}. *EMBO J.* 3:585-591.
- 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-src} relative to pp60^{v-src}. *Mol. Cell. Biol.* 5:2753-2763.
- Cross, F. R., and H. Hanafusa. 1983. Local mutagenesis of Rous sarcoma virus: the major sites of tyrosine phosphorylation of pp60^{c-src} are dispensable for transformation. *Cell* 34:597-607.
- Hunter, T., and B. W. Sefton. 1980. Transforming gene product of Rous sarcoma virus phosphorylated tyrosine. *Proc. Natl. Acad. Sci. USA* 77:1311-1315.
- Iba, H., F. R. Cross, E. A. Garber, and H. Hanafusa. 1985. Low level of cellular protein phosphorylation by nontransforming overproduced p60^{c-src}. *Mol. Cell. Biol.* 5:1058-1066.
- Jove, R., S. Kornbluth, and H. Hanafusa. 1987. Enzymatically inactive pp60^{c-src} with altered ATP-binding site is fully phosphorylated on its carboxy-terminal regulatory region. *Cell* 50:937-943.
- Kaplan, D. R., B. Bockus, T. M. Roberts, J. Bolen, M. Israel, and B. S. Schaffhausen. 1985. Large-scale production of polyoma middle T antigen by using genetically engineered tumors. *Mol. Cell. Biol.* 5:1795-1799.
- Kaplan, D. R., M. Whitman, B. Schaffhausen, D. C. Pallas, M.

- White, L. Cantley, and T. M. Roberts. 1987. Common elements in growth factor stimulation and oncogenic transformation: 85kd phosphoprotein and phosphatidylinositol kinase activity. *Cell* 50:1021-1029.
28. Kmiecik, T. E., and D. Shalloway. 1987. Activation and suppression of pp60^{c-src} transforming ability by mutation of its primary sites of tyrosine phosphorylation. *Cell* 49:65-73.
 29. Kornbluth, S., R. Jove, and H. Hanafusa. 1987. Characterization of avian and viral pp60^{src} proteins expressed in yeast. *Proc. Natl. Acad. Sci. USA* 84:4455-4459.
 30. Kornbluth, S., M. Sudol, and H. Hanafusa. 1987. Association of polyomavirus middle-T antigen with c-yes protein. *Nature (London)* 325:171-173.
 31. Kypta, R. M., A. Hemming, and S. A. Courtneidge. 1988. Identification and characterization of pp59^{fyn} (a src like protein tyrosine kinase) in normal and polyoma virus transformed cells. *EMBO J.* 7:3837-3844.
 32. 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.
 33. Pallas, D. C., V. Cherington, W. Morgan, J. DeAnda, D. Kaplan, B. Schaffhausen, and T. M. Roberts. 1988. Cellular proteins that associate with the middle and small t antigens of polyomavirus. *J. Virol.* 62:3934-3940.
 34. Pallas, D. C., C. Scley, M. Mahoney, E. Harlow, B. S. Schaffhausen, and T. M. Roberts. 1986. Polyomavirus small t antigen: overproduction in bacteria and utilization for monoclonal and polyclonal antibody production. *J. Virol.* 60:1075-1084.
 35. Pallas, D. C., and F. Soloman. 1982. Cytoplasmic microtubule associated proteins: phosphorylation at novel sites is correlated with their incorporation into assembled microtubules. *Cell* 30:407-414.
 36. Parsons, S. J., D. J. McCarley, C. M. Ely, D. C. Benjamin, and T. Parsons. 1984. Monoclonal antibodies to Rous sarcoma virus pp60^{c-src} react with enzymatically active cellular pp60^{src} of avian and mammalian origin. *J. Virol.* 51:272-282.
 37. Patschinsky, T., T. Hunter, F. S. Esch, J. A. Cooper, and B. M. Sefton. 1982. Analysis of the sequence of amino acids surrounding sites of tyrosine phosphorylation. *Proc. Natl. Acad. Sci. USA* 79:973-977.
 38. Patschinsky, T., T. Hunter, and B. M. Sefton. 1986. Phosphorylation of the transforming protein of Rous sarcoma virus: direct demonstration of the phosphorylation of serine 17 and identification of an additional site of tyrosine phosphorylation in pp60^{v-src} of Prague Rous sarcoma virus. *J. Virol.* 59:73-81.
 39. Piwnica-Worms, H., D. R. Kaplan, M. Whitman, and T. M. Roberts. 1986. Retrovirus shuttle vector for study of kinase activities of pp60^{c-src} synthesized in vitro and overproduced in vivo. *Mol. Cell. Biol.* 6:2033-2040.
 40. Piwnica-Worms, H., K. B. Saunders, T. M. Roberts, A. E. Smith, and S. H. Cheng. 1987. Tyrosine phosphorylation regulates the biochemical and biological properties of pp60^{c-src}. *Cell* 49:75-82.
 41. Raptis, L., H. Lamfrom, and T. L. Benjamin. 1985. Regulation of cellular phenotype and expression of polyomavirus middle t antigen in rat fibroblasts. *Mol. Cell. Biol.* 5:2476-2485.
 42. Schaffhausen, B. S., and J. Benjamin. 1981. Comparison of phosphorylation of two polyomavirus middle T antigens in vivo and in vitro. *J. Virol.* 40:184-196.
 43. Schaffhausen, B. S., B. J. Bockus, K. L. Berkner, D. Kaplan, and T. M. Roberts. 1987. Characterization of middle T antigen expressed by using an adenovirus expression system. *J. Virol.* 61:1221-1225.
 44. Schultz, A. M., L. E. Henderson, S. Oroszlan, E. A. Garber, and H. Hanafusa. 1985. Amino terminal myristylation of the protein kinase p60^{src}, a retroviral transforming protein. *Science* 227:427-429.
 45. Simon, A. M., B. Dress, T. Kornberg, and J. M. Bishop. 1985. The nucleotide sequence and the tissue specific expression of Drosophila c-src. *Cell* 42:831-840.
 46. Smart, J. E., H. Oppermann, A. P. Czervlofsky, A. F. Purchio, R. L. Erikson, and G. M. Bishop. 1981. Characterization of sites for tyrosine phosphorylation in the transformed protein of Rous sarcoma virus (pp60^{v-src}) and its normal cellular homologue (pp60^{c-src}). *Proc. Natl. Acad. Sci. USA* 78:6013-6017.
 47. Smith, A. E., and B. K. Ely. 1983. The biochemical basis of transformation by polyoma virus. *Adv. Viral Oncol.* 3:3-30.
 48. Snyder, M. A., J. M. Bishop, J. P. McGrath, and A. D. Levinson. 1985. A mutation at the ATP binding site of pp60^{v-src} abolishes kinase activity, transformation, and tumorigenicity. *Mol. Cell. Biol.* 5:1772-1779.
 49. Summers, M. D., and G. E. Smith. 1987. A manual of methods for baculovirus vectors and cloned insect cell culture procedures. Texas Agricultural Experiment Station, College Station.
 50. Yonemoto, W., M. Javis-Morar, J. S. Brugge, J. B. Bolen, and M. A. Israel. 1985. Tyrosine phosphorylation within the amino-terminal domain of pp60^{c-src} molecules associated with polyoma virus middle-sized tumor antigen. *Proc. Natl. Acad. Sci. USA* 82:4568-4572.