Linker Insertion-Deletion Mutagenesis of the *v-src* Gene: Isolation of Host- and Temperature-Dependent Mutants

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The host cell regulators and substrates of the Rous sarcoma virus transforming protein pp60^{v-src} remain largely unknown. Viral mutants which induce a host-dependent phenotype may result from mutations which affect the interaction of pp60^{v-src} with host cell components. To isolate such mutants and to examine the role of different regions of *src* in regulating pp60^{v-src} function, we generated 46 linker insertion and 5 deletion mutations within *src*. The mutant *src* genes were expressed in chicken embryo fibroblasts and in rat-2 cells by using retrovirus expression vectors. Most linker insertions within the kinase domain (residues 260 to 512) inactivated kinase activity and transforming capacity, while most insertions in the N-terminal domain and at the extreme C terminus were tolerated. A number of mutations generated a host-dependent phenotype. Insertions after residues 225 and 227, within the N-terminal regulatory domain (SH2), produced a fusiform transformation in chicken embryo fibroblasts and abolished transformation in rat-2 cells; a similar phenotype also resulted from two deletions affecting SH2 (residues 149 to 174 and residues 77 to 225). Insertions immediately C terminal to Lys-295, which is involved in ATP binding, also produced a conditional phenotype. Insertions after residues 299 and 300 produced a temperature-sensitive phenotype, while insertions after residues 304 and 306 produced a host cell-dependent phenotype. An insertion which removed the major tyrosine autophosphorylation site (Tyr-416) greatly reduced transformation of rat-2 cells, a property not previously observed with other mutations at this site. We conclude that mutations at certain sites within *src* result in conditional phenotypes. These sites may represent regions important in interactions with host cell components.

The product of the Rous sarcoma virus (RSV) *src* gene, pp60^{v-src}, is a protein-tyrosine kinase associated with the plasma membrane and cytoskeleton. The *v-src* protein sequence contains five distinct regions (17, 37). Amino acids 1 to 7 at the N terminus (region I) specify the addition of the fatty acid myristate to pp60^{v-src}; myristylation of pp60^{v-src} is required for association with cellular membranes and transformation (7, 18, 21). Region II, including amino acids 8 to 87, is *src* specific. Region III encompasses the region encoding amino acids 88 to 259 and includes the SH2 domain defined by Sadowski et al. (30); this region is believed to have a regulatory function (9, 16, 17, 24, 26, 33, 37). The catalytic kinase domain (region IV) extending from amino acids 260 to 514 is conserved among all the protein-tyrosine kinases (11, 36). Within the kinase domain are found an invariant lysine residue (Lys-295) required for ATP binding and transformation (20) and the major tyrosine phosphoacceptor site (Tyr-416). The C-terminal 12 amino acids (515 to 526) of *v-src* (region V) represent the portion of the *v-src* coding sequence which is distinct from the *c-src* sequence.

Although the structure of pp60^{v-src} has been extensively characterized, little is known about the host cell proteins with which pp60^{v-src} interacts. A number of cellular substrates for the protein-tyrosine kinase activity of pp60^{v-src} have been identified, but to date none of these substrate proteins has been shown to play a role in the transformation process (4, 5, 17). Most of these cellular substrates have been shown to be extensively phosphorylated at tyrosine in nontransformed cells expressing myristylation-defective pp60^{v-src} (19), indicating that their phosphorylation is insuf-

ficient for transformation. In addition, the cellular proteins responsible for targeting of pp60^{v-src} to the inner surface of the plasma membrane and focal adhesion plaques (28) have not been identified. Although the transmembrane protein-tyrosine kinases are known to be regulated by extracellular ligands, the nature of the factors which regulate the activity of the non-transmembrane kinases remains unclear. As an initial step in identifying the targets and regulators of pp60^{v-src}, we have sought to define regions of the protein which are important in its interaction with host cell components. As previously discussed (9, 39), viral mutants whose transforming capacity depends on the host cell type may result from mutations in the transforming protein which affect its interaction with host cell proteins. We therefore isolated host-dependent transformation mutants of *v-src* which transform avian cells, but which do not transform mammalian cells.

We describe here the results of linker insertion-deletion mutagenesis of the *v-src* gene. These studies were undertaken with two objectives: first, to define in greater detail important functional regions of the molecule; and second, to generate host-dependent transformation mutants of the *v-src* gene. Linkers were inserted into the *v-src* coding sequence, resulting in two- or four-amino-acid insertions, and deletion mutants were generated by eliminating the sequences between linkers inserted at two different sites. The mutated *v-src* genes were expressed in both avian and mammalian cells by the use of two different expression vectors. We report here the properties of the mutants generated by this approach, including both host range mutants and other conditional transformation mutants. To begin to probe the molecular basis for the transformation phenotype of the mutants, we examined the expression and in vitro kinase activity of the mutant pp60^{v-src} proteins in infected chicken

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FIG. 1. Construction of the recombinant plasmid pIRLv-*src*. The 881NC/482 plasmid, which contains a permuted RSV genome with the *ClaI* *v-src* insert, is shown on the upper right. The pIRL19 vector, into which the *ClaI* *v-src* fragment was cloned, is shown in the upper left. The resultant clone, pIRLv-*src*, is shown at the bottom. LTR, Long terminal repeat; kb, kilobases; bp, base pairs.

embryo fibroblasts (CEF). The results of these studies indicate that in general the effects of the mutations on the ability of *v-src* to transform CEF parallel their effects on the kinase activity of pp60^{*v-src*}. In a subsequent paper (J. E. De Clue, E. Liebl, and G. S. Martin, manuscript in preparation), we will describe in detail the characterization of mammalian cells expressing the mutant proteins and the properties of the host range mutants isolated in this study.

MATERIALS AND METHODS

Recombinant DNA methods. The dodecameric *XhoI* linker (CTCGAGCTCGAG) was obtained from International Biotechnologies, Inc. (New Haven, Conn.). The dodecameric *JS4* linker (CGACCTCGAGGT) was a gift of J. Stone. Manipulations were done by standard methods (23) with minor modifications. Ligations were done at 4°C overnight or at room temperature for 4 to 6 h. Restriction endonucleases were purchased from New England BioLabs, Inc. (Beverly, Mass.), and digestions were done as specified by

the manufacturer. Small- and large-scale plasmid preparations were done by the alkaline-sodium dodecyl sulfate lysis method (23). Linkers were phosphorylated with polynucleotide kinase from New England BioLabs. DNA bands were isolated from agarose gels either by elution onto NA-45 paper (Schleicher & Schuell, Inc., Keene, N.H.) or by adsorption to activated glass (Glassmilk; Bio 101) and subsequent elution. Secondary structure predictions were done by the method of Garnier et al. (12), using the PC/Gene software package from Intelligenetics.

Isolation of *v-src* linker-insertion mutants. The mutagenesis protocol was based on the procedure described by Stone et al. (34; personal communication). Cesium chloride-banded pIRLv-*src* (see Results and Fig. 1) was subjected to partial digestion with one of nine different restriction enzymes containing sites in the *v-src* coding sequence. Samples taken at various times during the digestion were analyzed by agarose gel electrophoresis. DNA from time points containing abundant amounts of linear (singly cut) pIRLv-*src* was

pooled. DNA from these pooled samples was treated with calf intestinal phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) and ligated to phosphorylated *XhoI* linkers. The ligation products were digested with an excess of *XhoI* and subjected to agarose gel electrophoresis. The linear DNA was eluted, redigested with an excess of *XhoI* enzyme, and precipitated. The mutated pIRLv-*src* molecules were recircularized by ligation with T4 DNA ligase (New England BioLabs).

A portion of the ligation mix was used to transform competent *Escherichia coli* DH5 (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). Ampicillin-resistant colonies were analyzed for linker insertions by digestion of plasmid DNA with *ClaI* (which removes *v-src* from pIRLv-*src*) and *XhoI*. A large majority of the colonies screened (typically 85%) were found to be mutated. The location of the inserted linkers was determined by double digestions of the mutant DNA with *XhoI* and one of a number of enzymes with a single site in pIRLv-*src* (e.g., *EcoRI*, *HindIII*, *MluI*) (Fig. 1), using known mutants generated with different enzymes as standards.

The mutants were designated with a letter and number code based on that used by Stone et al. (34). The first letter, S, designates *v-src*; the second letter denotes the restriction enzyme used to generate the mutant (A, *AluI*; D, *DpnI*; H, *HpaII* [for SHX1 to SHX11] and *HaeIII* [for SHX12 to SHX21]; N, *NlaIV*; P, *PvuII*; R, *RsaI*; S, *SmaI*; T, *ThaI*); the third letter, X, shows the introduced *XhoI* site; and the number denotes the site in the *v-src* coding sequence which was mutated. The precise location of the insertions is shown in Table 2; when necessary the site of the mutation (the residue preceding the inserted linker) is included in brackets after the mutant designation.

Insertion of mutant *v-src* genes into retroviral expression vectors. The effects of the mutations were analyzed by expressing the mutant *v-src* genes in the Moloney murine leukemia virus-based vector fpGV-1 (27) and in the avian vector RCAN (15). The *ClaI* *v-src* fragment was subcloned from pIRLv-*src* into *ClaI*-digested fpGV-1 and then subcloned again from the latter plasmid into *ClaI*-digested RCAN. To eliminate the vector from which the *v-src* fragment was to be cloned, we digested the donor DNA with an enzyme with a site or sites in the donor vector, but not in the *v-src* insert. When subcloning from the pIRLv-*src* vector, *PvuI*, which has a single site in pIRL19, was used, while *HindIII*, which has two sites in fpGV-1, was used to eliminate fpGV-1. The orientation of *v-src* fragments cloned into fpGV1 or RCAN was determined by digestion with an enzyme which cuts within the vector sequences and either *XhoI* (which cuts at the linker) or *NcoI* (which cuts at codon 1 in *src*).

Construction of *v-src* deletion mutants. Deletion mutants were generated by ligating DNAs derived from different in-frame insertion mutants. fpGV-1 plasmids donating the 5' or 3' portion of *v-src* were digested with *XhoI*, which cuts at the unique site of linker insertion, and *BglII*, which cuts fpGV-1 once, within the Tn5 transposon sequences. Digested DNAs were then ligated and used to transform *E. coli*. This strategy depends on the fact that to recircularize and restore kanamycin resistance, a plasmid must contain both the *XhoI*-*BglII* and the *BglII*-*XhoI* fragments. Subsequent digestion with *ClaI* revealed those plasmids which contained deletions in *v-src*. Deletion mutants were designated XD1 to XD6 (Table 1).

Transfection of avian and mammalian fibroblasts. CEF and rat-2 cells (35) were cultured as described previously (9).

TABLE 1. Mutants created by deletion of *v-src* sequences

Mutant	Donor of 5' <i>XhoI</i> site	Donor of 3' <i>XhoI</i> site	Amino acids deleted	Amino acids inserted ^a
XD1	SHX1	SHX3	15-168	RPRG
XD2	SHX3	SHX4	169-174	RPRG
XD3	SHX4	SHX5	175-262	RPRG
XD4	SHX2	SPX1	77-225	DLE
XD6	SRX1	SHX4	149-174	SRG

^a Amino acids encoded by the joined half-linkers.

One day prior to transfection, cells were plated at 36°C at a density of 10⁶/100-mm dish. The next day, the cells were transfected with 10 µg of purified plasmid DNA by the Polybrene-dimethyl sulfoxide method (22). Three days after the CEF cultures were transfected, the cells were trypsinized and replated. Typically, two passages posttransfection were required to achieve a high level of infection, and on day 9 the cells were plated for radiolabeling, virus harvests, and photomicroscopy. Two days after transfection of the rat-2 line, cultures were trypsinized and replated into two plates. The cells were maintained in growth medium containing 250 µg of G418 (geneticin; GIBCO Laboratories, Grand Island, N.Y.) per ml. Rat-2 cells were fed at 3- or 4-day intervals, and 12 or 13 days after replating, the cells were scored for transformation.

Radiolabeling and virus harvests. CEF were plated in 35-mm wells for radiolabeling as described previously (9). The next day, the cells were labeled for 18 h with [³⁵S]methionine in a medium containing 2.6 mg of methionine per liter (20% of the level in growth medium); after 18 h the cells were lysed. For virus harvests, infected CEF were plated at 2.5 × 10⁶/100-mm dish. Three days later, virus was harvested, clarified by centrifugation, and frozen.

Immunoprecipitation and kinase assays. Immunoprecipitation procedures were as described previously (9). For immunoprecipitation of ³⁵S-labeled proteins, portions of lysates containing equal numbers of acid-precipitable radioactivity were compared. Tumor-bearing rabbit serum and a goat anti-p27^{gag} serum (Research Resources, National Cancer Institute, Bethesda, Md.) were used in excess to immunoprecipitate pp60^{v-src} and Pr76^{gag}, respectively.

For immune complex kinase assays, one-half of the mock-labeled lysates was immunoprecipitated with 10 µl of tumor-bearing rabbit serum. The immune complexes were collected on 20 volumes of 10% fixed *Staphylococcus aureus* and washed as described previously (9). The kinase reaction buffer used for analyses of pp60^{v-src} contained 5 mM MgCl₂ and 5 mM MnCl₂. The protein concentration of duplicate portions of the mock-labeled lysates was determined by the bicinchoninic acid assay (31) with reagents from Pierce Chemical Co. (Rockford, Ill.). To calculate the specific activities of the mutant pp60^{v-src} molecules, the values obtained for total kinase activity (kinase activity per microgram of cellular protein) were divided by the amount of radiolabel in pp60^{v-src} immunoprecipitated from parallel [³⁵S]methionine-labeled cultures. The values obtained for total kinase activity and specific activity were both normalized to the values obtained for cells expressing wild-type Schmidt-Ruppin RSV subgroup A (SR-A) pp60^{v-src}.

RESULTS

Generation of linker insertion-deletion mutants of *v-src*. The procedure used to generate *v-src* mutants was similar to

that used by Stone et al. (34) to mutate the *v-fps* oncogene. In this procedure, 6- or 12-base-pair *XhoI* linkers are inserted at a variety of restriction enzyme sites, resulting in two- or four-amino-acid insertions, as well as the generation of a novel *XhoI* site, which serves as a marker for the mutation.

To facilitate the isolation of *v-src* mutants, we transferred the *v-src* coding sequences to a small (2.2-kilobase) pBR322-derived vector, pIRL19 (29). A 1.9-kilobase *Clal* fragment isolated from the plasmid 881NC/482 (13, 14), a derivative of the SR-A clone described by DeLorbe et al. (10), was cloned into the unique *Clal* site in pIRL19 (Fig. 1). The resulting clone, termed pIRLv-*src*, comprises 4,138 base pairs, of which 1,578 make up the coding sequences of *v-src*.

A total of 46 insertion mutants were derived by partial digestion of pIRLv-*src* with nine different restriction enzymes (Table 2). Eight of the enzymes yield blunt double-stranded molecules, which were mutated with a blunt, self-complementary dodecameric linker (CTCGAGCTC GAG). Following digestion with *XhoI*, this linker produced two-amino-acid insertions (or a one-amino-acid deletion with a three-amino-acid insertion). Eleven mutants were produced from pIRLv-*src* cut with the enzyme *HpaII*. This enzyme, which cleaves DNA to yield a 3' G-C overhang, was used in conjunction with a partially self-complementary decanucleotide (CGACCCTGAGGGT) to generate four-amino-acid insertions (Table 2). In addition, five different deletion mutants were generated by deleting segments between *XhoI* linker insertions in the same reading frame (Table 1).

To analyze the transformation potential of the *v-src* mutants in mammalian and avian cells, we cloned the wild-type and mutant *v-src* *Clal* fragments into retroviral vectors. For expression in mammalian cells, the Moloney murine leukemia virus-derived vector fpGV-1 (27) was used (Fig. 2). This vector contains a selectable marker, the Tn5 neomycin phosphotransferase gene, which provides resistance in bacterial cells to kanamycin and in eucaryotic cells to neomycin (G418). The *v-src* *Clal* fragments were cloned into the unique *Clal* site located at the 3' end of fpGV-1, so that the splice acceptor site within the *v-src* fragment lay 5' to the coding sequences, resulting in the expression of *v-src* from a subgenomic mRNA. For expression in CEF, *v-src* *Clal* fragments were cloned into the avian expression vector RCAN (15) (Fig. 2). This vector contains an intact, nonpermuted copy of an avian leukosis virus genome with a unique *Clal* site. A spliced subgenomic mRNA is produced which encodes pp60^{v-src}.

Transformation of rat-2 cells by mutant *v-src* genes. Rat-2 cells were transfected with fpGV-1 plasmids, and stable transfectants were selected with G418. Neo^r rat-2 cell clones derived from cultures transfected with fpGV-1 were uniformly flat and nonrefractile, like the parental rat-2 line. Upon transfection of rat-2 with the wild-type *v-src*-containing fpGV-1 construct, approximately 55% of the colonies displayed alterations in morphology and a loss of density inhibition of growth, characteristic of *v-src*-transformed fibroblasts. Of 51 fpGV-1 derivatives encoding linker insertion-deletion mutant *v-src* genes, 21 retained the ability to transform rat-2 cells (Table 2; Fig. 3). The majority of these (15 of 21) bear mutations in the amino-terminal 200 amino acids of pp60^{v-src}. However, several mutants with alterations in region III displayed a reduced frequency of transformation (insertions after amino acids 138, 148, 152, and 203), while others were entirely defective (SPX1[225], SHX13[228], XD4 [dl77-225], and XD6 [dl49-174]). In the great majority of cases (24 of 27), mutations in the kinase

domain (region IV; amino acids 260 to 513) abolished transformation (Table 2; Fig. 3). Two of the three transformation-competent mutants with mutations in the kinase domain (SHX6[229] and SNX3[300]) contained insertions just C terminal to the conserved lysine residue (Lys-295) (Table 2; Fig. 3). The third transformation-competent mutant with a mutation in the kinase domain, SRX4, encoded an insertion after amino acid 356. Mutations in the last 12 amino acids of pp60^{v-src} did not abolish transformation.

Transformation of CEF by *v-src* mutants. CEF were transfected with the RCAN-*v-src* plasmids. After two or three passages, the cells became fully infected and expressed abundant levels of viral structural proteins (e.g., Pr76^{gag}; see Fig. 6 and Table 3) and pp60^{v-src}. CEF infected with the RCAN vector with no insert (Fig. 4) resembled uninfected CEF; the cells remained in a monolayer and were uniformly nonrefractile. Cells infected with the wild-type *v-src*-containing RCAN virus (Fig. 4) displayed a transformed morphology, characterized by rounding, piling of cells on one another, and a generally disorganized pattern of growth.

The properties of the *v-src* mutants expressed in CEF were in general similar to those observed in rat-2 cells, but some significant differences were noted (Table 2). As previously observed in the transfected rat-2 cells, mutations in the amino-terminal *src*-specific domain (amino acids 8 to 87) did not affect the transforming capacity of *v-src*, while most insertions in the kinase domain abolished transformation. Mutations affecting SH2 resulted in a variety of alterations in the transformation potential of *v-src*. Four mutants with mutations in this domain (two insertions, SPX1[225] and SHX13[228], and two deletions, XD4 [dl77-225] and XD6 [dl49-174]) previously identified as transformation defective in rat-2 cells were nevertheless able to transform CEF. All these mutants, as well as one (SDX1[152]) which transformed rat-2 cells at a reduced frequency, produced a fusiform morphology in CEF that was distinct from that induced by the wild type. Another mutant with a mutation in the SH2 domain (SDX2[203]) produced a typical rounded shape in CEF cultured at 36°C (Fig. 5), yet when these cells were cultured at 41.5°C, they were fusiform. These results indicate that the SH2 domain exerts a strong influence on the transforming activity of *v-src*. Mutations in this region are capable of conferring a host-dependent phenotype on *v-src* and of altering the morphology of transformation in CEF.

Several mutations close to the ATP-binding site within the kinase domain resulted in a conditional transformation phenotype. The mutant SHX7, which contains an insertion after amino acid 304, was found to transform CEF despite being transformation defective in rat-2 cells. CEF transformed by SHX7-*v-src* resembled cells transformed by the wild-type gene product (Fig. 4); this was in contrast to CEF cells transformed by the other host-dependent mutants. A second insertion in this region, SHX15, with an insertion after amino acid 306, resulted in a disorganized growth pattern in infected CEF but had little effect on the morphology of individual cells. Additional conditional mutants were isolated in the same region of *v-src*; both SHX6[299] and SNX3[300] elicited a temperature-sensitive transformation of CEF (Fig. 5; Table 2). At 36°C, CEF expressing these mutants exhibited a fully rounded shape, while at 41.5°C, the cells reverted to a nontransformed morphology (Fig. 5). These mutants also were temperature sensitive for transformation of rat-2 cells (data not shown).

Another mutation, SRX5[415], which contains an insertion that deletes the major tyrosine phosphoacceptor site (tyrosine 416) within pp60^{v-src} also resulted in a conditional

TABLE 2. Transformation of rat-2 cells and CEF by *v-src* insertion-deletion mutants

Mutant	Residue preceding mutation	Amino acids inserted	Rat-2 cell transformation ^a	CEF transformation ^b	Virus titer (FFU/ml) ^c
SNX1	10	SS	0.99	+++	2.9×10^5
SHX1	14	RPRG	0.97	+++	2.5×10^5
SNX2	52	LE	0.99	+++	2.7×10^5
SHX12	55	RA	0.97	+++	3.0×10^4
SAX1	59	LE	0.90	+++	4.0×10^4
SHX2	76	DLEV	0.92	+++	3.0×10^5
STX1	138	RA	0.65	++	1.5×10^4
SRX1	148	<i>dIY::SRD</i>	0.76	++	7.0×10^4
SDX1	152	<i>dII::TRV</i>	0.37	++F	5.0×10^4
SHX3	168	RPRG	1.06	+++	2.3×10^5
SSX1	168	<i>dIR::PRG</i>	0.98	+++	1.0×10^5
SHX4	174	RPRG	1.12	++	8.0×10^4
SDX2	203	<i>dII::TRV</i>	0.77	+++	1.1×10^5
(41.5°C)				++F	5.0×10^4
SAX2	206	LE	0.94	+++	3.0×10^5
SPX1	225	LE	0.01	++F	4.5×10^4
SHX13	228	RA	0.01	++F	1.0×10^5
STX2	259	RA	1.01	+++	3.0×10^4
SHX5	263	RPRG	$<2.9 \times 10^{-3}$	—	$<5 \times 10^0$
SSX2	263	<i>dIR::PRG</i>	$<2.9 \times 10^{-3}$	—	$<5 \times 10^0$
SAX3	272	LE	$<2.9 \times 10^{-3}$	—	2.0×10^2
SHX14	293	RA	$<2.9 \times 10^{-3}$	—	$<5 \times 10^0$
SHX6	299	DLEV	0.96	+++	3.0×10^5
(41.5°C)				—	7.0×10^2
SNX3	300	LE	0.97	+++	3.5×10^5
(41.5°C)				—	5.0×10^2
SHX7	304	TSRS	$<2.9 \times 10^{-3}$	++	1.2×10^5
SHX15	306	RA	$<2.9 \times 10^{-3}$	+	1.4×10^4
SAX4	316	LE	$<2.9 \times 10^{-3}$	—	$<5 \times 10^0$
SHX8	317	RPRG	$<2.9 \times 10^{-3}$	—	$<5 \times 10^0$
SAX5	321	LE	$<2.9 \times 10^{-3}$	—	$<5 \times 10^0$
SRX2	325	<i>dIY::SRD</i>	$<2.9 \times 10^{-3}$	—	$<5 \times 10^0$
SRX3	339	<i>dIY::SRD</i>	$<2.9 \times 10^{-3}$	—	$<5 \times 10^0$
SRX4	356	<i>dIY::SRD</i>	1.04	+++	4×10^5
SAX6	362	LE	$<2.9 \times 10^{-3}$	—	$<5 \times 10^0$
SHX16	375	RA	$<2.9 \times 10^{-3}$	—	$<5 \times 10^0$
SHX17	390	RA	$<2.9 \times 10^{-3}$	—	2×10^1
SRX5	415	<i>dIY::SRD</i>	0.13	+++	3.0×10^5
SHX18	437	SS	$<2.9 \times 10^{-3}$	—	$<5 \times 10^1$
SHX9	437	RPRG	$<2.9 \times 10^{-3}$	—	$<5 \times 10^0$
SAX7	454	LE	$<2.9 \times 10^{-3}$	—	$<5 \times 10^0$
SHX19	459	SS	$<2.9 \times 10^{-3}$	—	$<5 \times 10^0$
SHX10	459	RPRG	$<2.9 \times 10^{-3}$	—	$<5 \times 10^0$
SNX4	461	SR	$<2.9 \times 10^{-3}$	—	$<5 \times 10^0$
SHX11	473	RPRG	$<2.9 \times 10^{-3}$	—	$<5 \times 10^0$
SHX20	506	LE	$<2.9 \times 10^{-3}$	—	$<5 \times 10^0$
SRX6	509	<i>dIY::SRD</i>	$<2.9 \times 10^{-3}$	—	2.5×10^1
SHX21	514	RA	0.90	+++	7.0×10^4
(41.5°C)				±	5.0×10^2
SPX2	515	LE	0.97	++	5×10^5
XD1	<i>dI15-168</i>		$<2.9 \times 10^{-3}$	±F	3×10^1
XD2	<i>dI169-174</i>		0.93	+++	1.4×10^5
XD3	<i>dI175-262</i>		$<2.9 \times 10^{-3}$	—	$<5 \times 10^0$
XD4	<i>dI77-225</i>		$<2.9 \times 10^{-3}$	++F	2×10^5
XD6	<i>dI149-174</i>		$<2.9 \times 10^{-3}$	++F	5×10^5
Vector alone			$<2.5 \times 10^{-4}$	—	$<5 \times 10^0$
(41.5°C)				—	$<5 \times 10^0$
Wild-type <i>v-src</i>			1.00	+++	9×10^5
(41.5°C)				+++	1.1×10^6

^a Frequency of transformation by fpGV-1-*v-src* plasmids as a fraction of the value obtained with wild-type *v-src* (0.55).^b Morphological transformation of infected CEF. F, Fusiform. +++, Morphology indistinguishable from CEF infected with wild-type *v-src*-RCAN; ++, morphology as wild type but less than 100% transformation; +, morphological transformation less distinct than wild type; ±, morphology much less distinct than wild type and/or minority of cells transformed.^c Titer of RCAN-*v-src* constructs in CEF. Assays were done at 36°C except when indicated. FFU, Focus-forming units.

FIG. 2. Expression vectors used in analysis of *v-src* mutants. The mammalian fpGV-1 vector is shown at the top, while the avian expression vector RCAN is depicted at the bottom. The 1.9-kilobase (kb) *ClaI* *v-src* fragment is shown at the positions of the unique *ClaI* sites into which the fragments were cloned. The sizes of the vectors plus *src* inserts are given. For clarity, the polyadenylation site in fpGV-1, which is actually within the long terminal repeat (LTR), is shown at the right; the vector does not contain any bacterial sequences outside the retrovirus genome. S.A., Splice acceptor site; S.D., splice donor site; P.L., polylinker. Symbols: $\odot$, ColE1 origin of replication; $\square$, simian virus 40 origin of replication; $\boxplus$, Tn5 transposon sequences.

phenotype. This mutant, which displayed a greatly reduced transformation potential in rat-2 cells, nevertheless was fully transforming in CEF at 36°C (Fig. 5). Following a shift to 41.5°C, SRX5-infected CEF were found to be somewhat less transformed, although this effect was variable and the cells never reverted to a completely normal phenotype (Fig. 5). Thus, transformation by this mutant is host dependent and partially temperature dependent. One other temperature-dependent mutant, SHX21, was isolated (Fig. 5). The insertion in SHX21 lies after amino acid 514, in the extreme carboxy terminus of pp60^{*v-src*}.

To confirm the properties of *v-src* mutants in CEF, *v-src*-containing RCAN viruses were harvested from cultures of infected CEF and tested for focus induction on indicator CEF monolayers. Without exception, RCAN derivatives encoding transformation-competent *v-src* mutants yielded virus stocks which contained 10⁴ to 10⁶ focus-forming units per ml (Table 2). In contrast, RCAN viruses encoding transformation-defective mutant *v-src* genes formed no foci, or very few foci. The titer of wild-type *v-src*-RCAN virus was not affected when the assay was conducted at 41.5°C (Table 2). In contrast, the titers of the temperature-sensitive mutants (SDX2[203], SHX6[299], SNX3[300], and SHX21[514]) were reduced 100- to 1,000-fold (relative to the titer obtained at 36°C) when assayed at

41.5°C. In all cases, foci were induced with single-hit kinetics with respect to the inoculated dose.

Expression of viral proteins in mutant-infected CEF. To examine the expression of viral proteins in infected CEF, we immunoprecipitated Pr76^{*gag*} and its cleavage products with anti-p27^{*gag*} antiserum from lysates of [³⁵S]methionine-labeled cells (Fig. 6B, odd-numbered lanes; and Table 3). In almost every case, the expression of Pr76^{*gag*} in CEF infected with mutant *v-src*-RCAN viruses was within 25% of the level of parallel cultures infected with the unmodified RCAN vector, indicating that the cultures were fully infected. To examine the expression of pp60^{*v-src*} in the infected cultures, we immunoprecipitated the protein from lysates of [³⁵S]methionine-labeled cells with a tumor-bearing rabbit serum which immunoprecipitates both viral structural proteins and pp60^{*v-src*} (Fig. 6B, even-numbered lanes; Table 3). The level of expression of pp60^{*v-src*} encoded by many of the mutants was equal to that observed with the wild-type virus (Table 3); this was the case with both transformation-competent and transformation-defective mutants. In some cases, reduced levels of pp60^{*v-src*} were detected; pulse-chase experiments indicated that this was due to decreased stability of the mutant proteins (data not shown).

Kinase activity of mutant pp60^{*v-src*} molecules. The kinase activity of the mutant pp60^{*v-src*} molecules expressed in CEF was determined by the immune complex kinase assay (Fig. 6A; Table 3). Lanes 1 to 10 of Fig. 6A represent the phosphorylation of immunoglobulin heavy chain in immunoprecipitates prepared in parallel with the immunoprecipitates from [³⁵S]methionine-labeled cells shown in Fig. 6B. Lane 1 represents cells infected with RCAN vector alone, lane 10 shows cells infected with the wild-type *v-src*-RCAN construct, and lanes 2 to 9 show cells infected with a series of RCAN viruses with mutant *v-src* genes. The in vitro immune complex kinase assay may not always accurately reflect the ability of pp60^{*v-src*} to phosphorylate exogenous substrates. Nevertheless, the kinase activity per microgram of cellular protein (Table 3) correlated very well with the ability to transform CEF. Thus, mutants which induced kinase activities in excess of 3 to 4% of the wild-type level were in general transforming for CEF, while mutants which induced kinase activities below this level were nontransforming. (There were a few minor exceptions to this rule; for example, CEF expressing SHX21[514]-*v-src* at 41.5°C contained over 10% of the kinase activity of cells expressing wild-type *v-src*.) These data confirm that transformation by *v-src* depends on pp60^{*v-src*} kinase activity and that the level of kinase activity in wild-type SR-A *v-src*-transformed CEF far exceeds the threshold required for transformation.

To investigate the effects of the individual mutations on the specific activity of the pp60^{*v-src*} kinase, we calculated the relative specific activity of pp60^{*v-src*} molecules using values obtained for the level of expression of pp60^{*v-src*} in parallel [³⁵S]methionine-labeled cultures. Mutants with insertions within the first 76 amino acids and at the extreme carboxy terminus retained 50 to 110% of the activity of the wild type. Most insertions and deletions in the SH2 domain reduced the relative specific activity of pp60^{*v-src*} two- to sixfold, while mutants bearing insertions in the kinase domain that abolished transformation in most cases retained only 2% or less of the wild-type activity. These data indicate that the effects of the insertion-deletion mutations on the transforming capacity of *v-src* bear a strong relation to their effects on the in vitro kinase activity of pp60^{*v-src*}.

Association of mutant pp60^{*v-src*} molecules with cellular proteins pp50 and pp90. pp60^{*v-src*} transiently associates with

FIG. 3. Positions of linker insertion-deletion mutations and their effects on transformation of rat-2 cells. The sites of the mutations are shown on a schematic map of the *v-src* coding sequence. For the linker insertion mutations, the positions of the residues preceding the insertions are given within the circles; below these are listed the names of the mutants and the amino acids inserted. The deletions are drawn in as bars, with the positions of the first and last amino acids deleted at the ends. Those mutations which do not abolish transforming function in rat-2 cells (as judged by the transfection assay; see text and Table 2) are drawn above the bar. Mutations listed below the bar result in a transformation-defective phenotype in rat-2 cells. Mutations denoted by triangles transform rat-2 cells but at a reduced frequency.

two cellular phosphoproteins, pp50 and pp90, prior to its association with cellular membranes (1). This association can be detected in immunoprecipitates of pp60^{v-src} from [³⁵S]methionine-labeled cells (Fig. 6B). In several cases, pp60^{v-src} molecules encoded by transformation-defective *v-src* genes have been shown to be more extensively associated with the pp50-pp90 complex (37). We noted that most insertions in the region from residues 263 to 362 resulted in a strong association of pp60^{v-src} with the complex (Table 3). Two temperature-sensitive mutants with mutations in this region (SHX6[299] and SNX3[300]) showed a stronger association at 41.5°C than at 36°C. These mutations may prevent the release of pp60^{v-src} from the complex by disrupting determinants which specify interactions of pp60^{v-src} with these proteins.

DISCUSSION

Effects of mutations within different regions of *v-src*. The construction and analysis of 46 insertion mutants and 5 deletion mutants spanning the *v-src* coding sequence permitted a systematic comparison of the effects of mutations in different regions of pp60^{v-src}. The effects of an insertion on transforming activity were clearly dependent on the site of insertion and the domain of the protein which was altered. Mutations within the region between amino acids 8 and 77 (region II), as well as a mutation in the extreme carboxy terminus (region V), had little effect on the transforming or kinase activities of pp60^{v-src}. Mutations within region III resulted in different phenotypes depending on the site of the mutation. Insertions in the region 138 to 152 and after amino acid 203 reduced the frequency of transformation in rat-2 cells, while other insertions (after 168, 174, 206, and 259) had

little effect on transformation. Insertions after amino acids 225 and 228 and the deletions XD4 (dl177–225) and XD6 (dl149–174) produced a host-dependent phenotype.

With few exceptions, mutations throughout the kinase domain, encompassing amino acids 260 to 514, abolished transforming ability and kinase activity, indicating that transformation and enzymatic activity require the entire kinase domain to be intact. Mutations in the kinase domain also affected the formation or stability or both of the complex of pp60^{v-src} with the cellular phosphoproteins pp50 and pp90. Most (but not all) of the insertions between residues 263 and 362 led to accumulation of the pp60-pp50-pp90 complex. In contrast, most of the mutations in the region 375 to 509 led to decreased levels of the complex. These findings confirm and extend previous reports that deletions or point mutations in *src* can affect the formation or dissociation of the complex (1, 17, 37).

Conditional mutations in region III. The sequences of region III have previously been proposed to represent a modulatory domain (6, 16, 17, 24, 26, 30, 33, 37). A number of *v-src* mutants have been described with deletions or alterations in this region which exhibit altered transforming capacity or kinase activity. The SR-A *v-src* deletion mutant NY320, which lacks amino acids 149 to 169, was found to induce a fusiform morphology in infected CEF (6). The RSV Prague A strain *v-src* mutants CH119 and CH120 lack amino acids 173 to 227 and 169 to 225, respectively, and both induce a temperature-sensitive transformation of CEF (2). Further mutagenesis of the Prague A strain *v-src* (26) has defined amino acids 149 to 172 as essential for transformation by that gene. In addition, mutations in the corresponding region of *c-src* at amino acids 95 and 96 can activate

FIG. 4. Phase-contrast photomicrographs of CEF infected with host-dependent transformation mutants. Fully infected CEF were plated at a density of $2 \times 10^6/100\text{-mm}$ dish 3 days prior to photomicroscopy. All cells were cultured at 36°C . WT, wild-type.

transforming capacity (17). We have previously shown that a mutation in the SH2 region of *v-fps* induces a host-dependent phenotype, arguing that this region of the *v-fps* protein may direct its interaction with cellular components (9).

The sequences of region III, in particular the SH2 segment or B-C regions (residues 140 to 250) and the SH3 segment or A region (residues 88 to 140), show marked homology with the sequences of other members of the protein-tyrosine kinase family that are membrane associated but not transmembranous (24, 30, 33). A phosphatidylinositol-specific phospholipase C, C-148, has recently been shown to contain a large region of homology with region III of the *src* gene (33). The sequences within phospholipase C that are related to region III may serve a regulatory function in this enzyme. The recently described *v-crck* gene, which is expressed as a fusion to viral *gag* sequences, also contains blocks of homology to region III (24). Expression of this gene elevates the phosphorylation of cellular proteins on tyrosine, despite

the fact that the *crck* gene does not itself encode a kinase domain; this suggests that these sequences can activate cellular protein-tyrosine kinases, perhaps by titration of a cellular inhibitor (24). These findings support the idea that region III has a regulatory function.

Two linker insertions within SH2 resulted in host-dependent transformation and a fusiform morphology in CEF. The insertions in the mutants SPX1 (after residue 225) and SHX13 (after residue 228) lie in a region which is highly conserved among the cytoplasmic protein-tyrosine kinases. Secondary structure prediction (12) suggests that the segment from 223 to 231 forms an extended region, which might be a site at which $\text{pp60}^{\text{v-src}}$ interacts with host cell protein(s).

Two deletion mutations affecting SH2 also resulted in a host-dependent phenotype. The mutant XD4, lacking residues 77 to 225, and the XD6 mutant, lacking residues 149 to 174, exhibited similar transformation properties; both induced a fusiform transformation in CEF but did not trans-

form rat-2 cells. In XD6, some of the most highly conserved sequences in SH2 are deleted, while in XD4, the entire region homologous to phospholipase C-148 (including both SH2 and SH3) is deleted. Despite the similarity in their phenotypic effects on CEF and rat-2 cells, these two dele-

tions differed greatly in their effect on the kinase activity of the *v-src* gene product. In XD6-infected CEF, the kinase activity is approximately 20% of the wild-type level. In contrast, XD4 expresses levels of kinase activity in excess of the wild-type levels. This indicates that the fusiform phenotype is not simply a consequence of reduced kinase activity. The finding that XD4 pp60^{v-src}, which contains an extensive deletion (*dl77-225*), exhibits higher kinase activity than XD6 pp60^{v-src}, which contains a smaller deletion (*dl149-174*), suggests that the additional sequences deleted in XD4 normally function to inhibit pp60^{v-src} kinase activity. This is consistent with the finding that mutations in SH3 (the A region) can activate the transforming capacity and kinase activity of pp60^{c-src} (17; J. Brugge, personal communication).

The mutant SRX1, which has an insertion after amino acid 148, is the analog of the *v-fps* mutant RX15m (30), which was found to be devoid of transforming function and to have a greatly decreased kinase activity. By contrast, SRX1-*v-src* was found to transform both CEF and rat-2 cells (the latter at a reduced frequency), and the pp60^{v-src} encoded by SRX1 exhibited a relative specific activity of 30.6%. Thus, the effects of mutations in SH2 of the SR-A *v-src* may differ from those of the analogous mutations in other protein-tyrosine kinases. This may reflect at least in part the greater levels of protein-tyrosine phosphorylation induced by SR-A pp60^{v-src} (5); with SR-A a large decrease in protein kinase activity is necessary to reduce the level of activity below the threshold required for transformation.

Mutations in the kinase domain. A cluster of conditional mutations mapped to a small area four to nine amino acids C terminal to lysine 295. This region precedes a sequence of 21 amino acids (304 to 324) which secondary structure predictions suggest may form a stretch of alpha-helix (data not shown). Two of these mutants (SHX6[229] and SNX3[300]) were found to have a temperature-dependent phenotype in both rat-2 cells and CEF, while SHX7[304] and SHX15[306] exhibited a host-dependent phenotype. The lesion responsible for the temperature-sensitive phenotype of the RSV mutant *tsLA32* was found to be at amino acid 300 (37). The kinase activity of pp60^{v-src} encoded by *tsLA32* is low relative to the wild-type activity and is not significantly temperature sensitive; the same is true for the SNX3 mutant. Thus, this small area appears to be a hot spot for the generation of conditional mutants. It is possible that this region contains determinants involved in the binding of substrates, as they would be expected to lie in close apposition to the ATP molecule.

One insertion mutant in the kinase domain, SRX4, which contains an insertion after amino acid 356, retained full transforming function. Secondary structure predictions (12) suggest that this site lies within a six-amino-acid stretch of extended conformation between two alpha-helical regions. Thus, this insertion may lie in a less rigid, hingelike region and may exert only minimal effects on protein conformation.

FIG. 5. Phase-contrast photomicrographs of CEF infected with temperature-sensitive mutants and grown at 36 or 41.5°C. CEF were transfected with RCAN and derivatives encoding wild-type (WT) or mutant *v-src* genes and passaged at 36°C. Three days before photomicroscopy, the cells were replated and one set was shifted to 41.5°C. The left panel in each row represents cells maintained continuously at 36°C; the right panel represents the same cell type after 3 days at 41.5°C. Row 1, CEF infected with RCAN with no insert; row 2, CEF expressing wild-type *v-src*; rows 3 to 7, CEF expressing mutant *v-src* genes.

FIG. 6. pp60^{v-src} immune complex kinase activity (A) and expression of Pr76^{gag} and pp60^{v-src} (B) in mutant-infected CEF. (A) Phosphorylation of the immunoglobulin heavy chain in immune complexes from mutant-infected CEF. pp60^{v-src} was immunoprecipitated from lysates of mock-labeled CEF and incubated with [γ -³²P]ATP. Radiolabeled proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Autoradiography was for 30 min at room temperature. CEF were infected with RCAN vectors expressing the following *v-src* genes: lane 1, none; lane 2, SAX7[454]; lane 3, SHX8[317]; lane 4, SHX13[228]; lane 5, STX1[138]; lane 6, STX2[259]; lane 7, SDX1[152]; lane 8, SAX1[59]; lane 9, SHX12[55]; lane 10, wild-type SRA. IgG, Immunoglobulin G. (B) Expression of RCAN-encoded Pr76^{gag} and pp60^{v-src} in mutant-infected CEF. Lysates of CEF labeled for 18 h with [³⁵S]methionine were immunoprecipitated with goat anti-p27^{gag} or tumor-bearing rabbit serum. Immunoprecipitates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography (3 days at -70°C). The order of the samples is the same as in panel A; the left lane of each pair is the anti-p27 immunoprecipitate, the right lane the tumor-bearing rabbit serum immunoprecipitate. Numbers on left show molecular weight ($\times 10^3$).

The mutant SRX5[415], which encodes a three-amino-acid insertion and deletes tyrosine 416, was found to transform rat-2 cells at a greatly reduced frequency. However, tyrosine 416 itself is not required for transformation of mammalian cells by *v-src*. A mutant in which tyrosine 416 of *v-src* was changed to phenylalanine was shown to transform NIH-3T3 cells and to encode a fully active kinase (32). (Cross and Hanafusa [8] reported a mutation in which amino acids 412 to 416 were replaced with linker sequences. This mutant was fully transforming in CEF, but its transforming properties in mammalian cells were not reported.) Thus, it is clear that the host-dependent properties of SRX5 do not result simply from a loss of tyrosine 416, but must be related to the amino acid insertion at this site. Strikingly, the *in vitro* kinase activity of SRX5-pp60^{v-src} equaled or exceeded that of the wild type; we have found that this is also true when SRX5-pp60^{v-src} is expressed in rat-2 cells (J. E. DeClue, E. Liebl, and G. S. Martin, unpublished data).

The temperature-sensitive mutant SHX21[514] bears an

insertion located at the junction of regions IV and V. The phenotype of this mutant was somewhat leaky; a full reversion was not achieved following upshift of CEF to 41.5°C . The SHX21 insertion is predicted to fall near the end of an α -helix which contains the last eight amino acids found in both *v-src* and *c-src*. Bryant and Parsons (3) have used deletion and frameshift analysis to show that alterations of the amino acids predicted to make up this α -helix result in a transformation-defective phenotype. A series of deletions in the C terminus of the *v-src* gene of the SR-D strain were constructed by Yaciuk and Shalloway (38), and the effects were measured by transfection of the mutant *src* genes into 3T3 cells. These studies showed that leucine 516, which is present in both *v-src* and *c-src* and which is conserved among other protein-tyrosine kinases, is required for transformation. This leucine would be moved from its normal position by the insertion in SHX21.

Ng and Privalsky (25) have previously suggested that in-frame insertional mutagenesis is of general use in the

TABLE 3. Expression of viral proteins in mutant-infected CEF

Mutant	Site of mutation	Level of Pr76 ^{gag} ^a	Level of pp60 ^{src} ^b	Total src kinase activity ^c	Kinase sp act ^d	pp50-pp90 complex ^e
SNX1	10	0.86	0.93	105.0	112.5	±
SHX1	14	1.27	0.53	40.9	77.9	±
SNX2	52	1.07	0.77	87.0	113.4	—
SHX12	55	0.65	1.21	76.0	63.1	±
SAX1	59	1.28	2.31	187.0	81.0	±
SHX2	76	0.79	1.42	63.4	44.4	±
STX1	138	0.66	0.91	85.6	94.2	±
SRX1	148	0.81	1.24	38.0	30.6	+
SDX1	152	1.30	0.80	14.2	17.8	+
SHX3	168	0.78	0.51	60.1	118.6	±
SSX1	168	1.26	0.83	90.1	108.2	±
SHX4	174	0.34	0.15	3.6	23.8	±
SDX2	203	0.90	0.45	8.5	19.0	+
SAX2	206	0.87	1.52	113.3	71.5	±
SPX1	225	0.95	0.33	5.4	16.3	+
SHX13	228	1.13	0.53	17.2	32.5	±
STX2	259	0.86	1.91	84.2	44.1	+
SHX5	263	1.08	1.09	1.5	1.4	++
SSX2	263	0.95	1.13	1.5	1.3	++
SAX3	272	1.15	0.88	1.6	1.9	++
SHX14	293	1.22	0.22	0.05	0.2	++
SHX6	299	1.22	0.35	5.9	16.5	+
(41.5°C)		0.99	0.38	2.3	6.1	++
SNX3	300	1.37	0.58	4.6	7.9	+
(41.5°C)		1.49	0.38	4.3	11.3	++
SHX7	304	0.83	0.32	31.2	96.7	±
SHX15	306	0.68	0.63	3.2	5.1	++
SAX4	316	0.70	0.28	0.4	1.3	++
SHX8	317	1.07	1.14	0.9	0.8	—
SAX5	321	0.80	0.39	0.8	2.0	++
SRX2	325	1.66	0.18	1.2	6.5	++
SRX3	339	1.17	0.22	0.5	2.1	++
SRX4	356	1.41	0.76	44.2	58.2	+
SAX6	362	1.47	0.24	0.4	1.6	++
SHX16	375	1.78	0.12	0.1	0.7	—
SHX17	390	0.98	0.51	0.4	0.8	—
SRX5	415	0.98	1.45	224.5	154.7	+
SHX18	437	0.66	0.50	1.1	2.1	—
SHX9	437	1.31	0.26	1.9	7.5	±
SAX7	454	1.10	0.64	0.5	0.7	++
SHX19	459	1.07	0.71	2.4	3.4	+
SHX10	459	1.25	0.23	0.3	1.2	—
SNX4	461	1.09	0.33	0.2	0.7	—
SHX11	473	1.32	0.16	0.3	1.8	—
SHX20	506	1.07	0.55	0.4	0.6	—
SRX6	509	1.44	0.12	0.5	4.0	—
SHX21	514	0.82	0.34	34.9	102.1	+
(41.5°C)		1.52	0.47	11.4	24.2	++
SPX2	515	1.42	0.53	40.3	75.7	±
XD1	dl15–168	1.39	0.03	1.8	54.6	++
XD2	dl169–174	1.39	0.47	10.0	21.5	+
XD3	dl175–262	0.83	0.99	3.3	3.4	++
XD4	dl77–225	0.66	0.72	130.6	181.4	—
XD6	dl149–174	1.28	0.41	8.3	20.0	+
RCAN		1.00				—
Wild-type v-src		1.05	1.00	100.0	100.0	±
(41.5°C)		1.22	1.00	100.0	100.0	+

^a [³⁵S]methionine radiolabel in Pr76^{gag} following an 18-h labeling of RCAN-v-src-infected CEF, expressed as a fraction of the radiolabel obtained with CEF infected with the RCAN vector without an insert.

^b [³⁵S]methionine radiolabel in pp60^{v-src} following an 18-h labeling of RCAN-v-src-infected CEF, expressed as a fraction of the radiolabel obtained with CEF infected with the RCAN vector with the wild-type SR-A v-src insert.

^c Total immune complex (immunoglobulin heavy chain) kinase activity of pp60^{v-src} per microgram of cellular protein, expressed as a percentage of the value obtained with wild-type SR-A v-src.

^d Specific activity of the src kinase, obtained by dividing the total immune complex kinase activity (footnote c) by the abundance of pp60^{v-src} (footnote b).

^e The level of the pp60-pp50-pp90 complex was assessed by inspection of autoradiograms of immunoprecipitates prepared with tumor-bearing rabbit serum from [³⁵S]methionine-labeled CEF. The values for the pp50-pp90 complex reflect the ratio of pp50 and pp90 to pp60^{v-src} in the immunoprecipitates.

creation and analysis of conditional mutants. The results described here bear out this suggestion and indicate that both host- and temperature-conditional mutations can be isolated by this type of mutagenesis. Previous studies (17, 37) on mutants of *v-src* and *c-src* have utilized either CEF or mammalian cells as hosts, but generally not both; however, it is clear from the findings reported here that the two cell types may respond differently to mutations in *src*. Host-dependent phenotypes may result from alterations in the ability of the mutant transforming proteins to interact with host cellular components. Characterization of such mutants may provide information about the host components required for transformation.

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