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Site-Directed Mutagenesis of the SH2- and SH3-Coding Domains of c-*src* Produces Varied Phenotypes, Including Oncogenic Activation of p60$^{c\text{-}src}$

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The products of the viral and cellular *src* genes, p60$^{v\text{-}src}$ and p60$^{c\text{-}src}$, appear to be composed of multiple functional domains. Highly conserved regions called *src* homology 2 and 3 (SH2 and SH3), comprising amino acid residues 88 to 250, are believed to modulate the protein-tyrosine kinase activity present in the carboxy-terminal halves of the *src* proteins. To explore the functions of these regions more fully, we have made 34 site-directed mutations in a transformation-competent c-*src* gene encoding phenylalanine in place of tyrosine 527 (Y527F c-*src*). Twenty of the new mutations change only one or two amino acids, and the remainder delete small or large portions of the SH2-SH3 region. These mutant alleles have been incorporated into a replication-competent Rous sarcoma virus vector to examine the biochemical and biological properties of the mutant proteins after infection of chicken embryo fibroblasts. Four classes of mutant proteins were observed: class 1, mutants with only slight differences from the parental gene products; class 2, mutant proteins with diminished transforming and specific kinase activities; class 3, mutant proteins with normal or enhanced specific kinase activity but impaired biological activity, often as a consequence of instability; and class 4, mutant proteins with augmented biological and catalytic activities. In general, there was a strong correlation between total kinase activity (or amounts of intracellular phosphotyrosine-containing proteins) and transforming activity. Deletion mutations and some point mutations affecting residues 109 to 156 inhibited kinase and transforming functions, whereas deletions affecting residues 187 to 226 generally had positive effects on one or both of those functions, confirming that SH2-SH3 has complex regulatory properties. Five mutations that augmented the transforming and kinase activities of Y527F c-*src* [F172P, R175L, Δ(198–205), Δ(206–226), and Δ(176–226)] conferred transformation competence on an otherwise normal c-*src* gene, indicating that mutations in SH2 (like previously described lesions in SH3, the kinase domain, and a carboxy-terminal inhibitory domain) can activate c-*src*.

The product of the cellular proto-oncogene c-*src* is a 60-kilodalton phosphoprotein (p60$^{c\text{-}src}$) that harbors a protein kinase activity specific for tyrosine residues, bears myristic acid at its amino terminus, and associates with cellular membranes (for reviews, see references 21 and 54). Although the normal function of p60$^{c\text{-}src}$ is not known, the protein can induce neoplastic transformation if mutated to augment its intrinsic kinase activity (3, 5, 25, 31, 39). Among the several mutations that activate the oncogenic properties of p60$^{c\text{-}src}$, the best understood are those that alter or remove a tyrosine residue near the carboxy terminus (Y527); phosphorylation of this residue by an unidentified kinase normally restrains the kinase activity of pp60$^{c\text{-}src}$ (5, 6).

Largely on the basis of studies of pp60$^{v\text{-}src}$, the protein-tyrosine kinase activities of *src* proteins appear to be located in the carboxy-terminal halves of these proteins, between sequences upstream of the ATP-binding site (centered at K295) and the negative regulatory region that includes Y527 (1, 19, 30). Other functionally significant regions of *src* proteins have been suggested by site-directed mutagenesis. These include a six-amino-acid signal for myristylation of the amino terminus (8, 24, 36, 37) and regions in the amino-terminal halves of the proteins that (in conjunction with myristic acid) are required for membrane localization (7, 8, 12, 13, 23, 30, 44; J. Kaplan, H. E. Varmus, and J. M. Bishop, Mol. Cell. Biol., in press).

Nucleotide sequencing of many genes encoding protein-tyrosine kinase has suggested additional roles for portions of *src* proteins. For example, residues 10 to 80 are the most variable among the seven proto-oncogenic proteins closely related to pp60$^{c\text{-}src}$ (42; J. A. Cooper, *in* B. Kemp and P. F. Alewood, ed., *Peptides and Protein Phosphorylation*, in press). Two regions between this variable domain and the kinase domain have been conserved during metazoan evolution among protein-tyrosine kinases that are not transmembrane proteins (15, 16, 35, 42, 45; Cooper, in press). One of these, called *src* homology 2 (SH2; residues 140 to 250), is found in all such kinases; the other, SH3 (residues 88 to 139), is absent from a few, such as that encoded by the v-*fps* gene. The first evidence that these regions could alter the function of p60^{src} came from mutations in the SH2 region of p60$^{v\text{-}src}$ that inactivate its transforming activity or affect the morphology of transformed cells (2, 7, 9, 27, 40, 53). Interest in the SH2-SH3 regions of *src* proteins and their close relatives has intensified recently because of several unexpected findings. (i) A naturally occurring mutation that deletes a single amino acid from the most highly conserved region of SH2 in pp60$^{v\text{-}src}$ and a linker insertion mutation that affects a nearby region of v-*fps* protein both produce proteins that exhibit host-dependent transformation (10, 50). (ii) Mutations in c-*src* that affect residues 90 to 95 in the SH3 domain of pp60$^{c\text{-}src}$ or an analogous region of c-*abl* activate their transforming potential (11, 20, 25, 39; M. Fox and J. Brugge, personal communication). (iii) A newly discovered

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TABLE 1. Point and deletion mutations

Mutant	Mutation(s)	Oligomer (oligonucleotide) for site-directed mutagenesis	New restriction site[a]
M1	W119Y	GAATGAGCCAGGTACCAGTCACCTCC	*Kpn*I
M2	W119A	GGGAATGAGCCAGGGCCCAGTCACCTTCCG	*Apa*I
M3	W148Y	CTTCCCAAAGTAGTACTCTTCAGCC	*Sca*I
M4	W148E	CTTCCCAAAGTACTCCTCTTCAGCC	*Sca*I
M5	F172Y	CTCTCCCGGACCAAGTACGTACCCCGGGGGTTTTC	*Sna*BI
M6	F172P	CCCGGACCAAGGGGGTTCCCCGGG	*Sty*I
M7	H201R	GATCTTGTAGCGCTTCACATTG	*Hae*II
M8	H201L	CGGATCTTGTAAAGCTTCACATTG	*Hin*dIII
M9	R175L	GTCTCGCTCTCGAGGACCAAGAAGG	*Xho*I
M10	L226R	GGAGTAGTAGGCCACGCGTTGCTGCAGGCTGC	*Mlu*I
X1	Y92L	GTCCGGGACTCGAGGTCGTAGGAGAG	*Xho*I
X2	Q109E	TGTTGACAATCTCGAGGCGTTCTCCT	*Xho*I
X3	T125E	CCGTCTGTCCTGTCTCGAGGGAATGAGCC	*Xho*I
X4	I143L, Q144E	CCACTCTTCAGCCTCGAGGGAGTCTGAGGGC	*Xho*I
X5	R156L	CGCTCGGACTCGAGACGAGTGATC	*Xho*I
X6	P165L	CCCCGGGGGTTCTCGAGGTTGAGCAGCAGC	*Xho*I
X7	S187E	GTCAAAGTCAGAAACCTCGAGGCAATAGGCAC	*Xho*I
X8	N198E	CTTGTAGTGCTTCACCTCGAGCCCCTTGGC	*Xho*I
X9	R205L, K206E	CCGCTGTCCAGCTCGAGGATCTTGTAGTGC	*Xho*I
X10	V227E	GGAGTAGTAGGCCTCGAGCTGCTGCAGGC	*Xho*I
D1	Δ(93–108)		
D2	Δ(109–124)		
D3	Δ(125–143)		
D4	Δ(144–156)		
D5	Δ(157–165)		
D6	Δ(166–175)		
D7	Δ(176–186)		
D8	Δ(187–197)		
D9	Δ(198–205)		
D10	Δ(206–226)		
D11	Δ(93–143)		
D12	Δ(144–175)		
D13	Δ(176–226)		
D14	Δ(93–226)		

[a] Generated by site-directed mutagenesis.

viral oncogene, the *gag-crk* gene of CT10 avian sarcoma virus, encodes a protein with an SH2-SH3 region but no kinase domain, yet it induces tyrosine phosphorylation in infected cells (32). (iv) Sequences closely related to SH2 and SH3 have been identified in some cellular proteins with proposed roles in signal transduction, including phospholipase C-γ (47) and GTPase activator protein (52).

Taken together, these results suggest that SH2 and SH3 may be important for regulation of kinase activity and for interaction with cellular proteins that serve as substrates or regulators of *src*-related kinases. To explore this possibility, we have constructed a set of *src* alleles that bear point mutations or deletions in SH2-SH3. These mutations have been made in a transformation-competent form of c-*src* encoding phenylalanine in place of Y527. Since c-*src* has little intrinsic transforming ability, the use of an activated form of c-*src* has been necessary to detect mutations which diminish the transforming or enzymatic activity of $pp60^{src}$. As Y527F c-*src* is a less potent transforming gene than the multiply mutant v-*src* gene, we have also been able to identify mutations which enhance c-*src* transforming potential. By introducing the mutant alleles into chicken cells in a retrovirus vector, we have found that relatively subtle lesions in SH2 and SH3 can have profound effects on the stability or specific kinase activity of $pp60^{src}$, with patterns that imply the existence of positive and negative regulatory domains. Moreover, several SH2 mutations that enhance the activity of Y527F c-*src* are sufficient to convert normal c-*src* into a transforming gene.

MATERIALS AND METHODS

Cells, viruses, and plasmids. Chicken embryo fibroblasts (CEF) were prepared from 11-day-old embryos derived from C/O chickens (obtained from SPAFAS, Inc., Norwich, Conn.). pM5HHB5 carrying the transformation-competent and kinase-active Y527F chicken *src* gene (28) was provided by D. Shalloway. An adaptor plasmid, Cla12Nco, and a Rous sarcoma virus (RSV) proviral vector, RCAS, (18) were provided by S. Hughes. High-titer viral stocks of wild-type RSV B31 (50), a strain rescued from a rat line transformed with B77 RSV, were generated from CEF.

Construction of mutant proviral plasmids. The *Nco*I-*Bgl*II fragment of pM5HHB5 was inserted into the cloning vector pGC1 (33), and the *Eco*RI-*Bam*HI fragment of this recombinant plasmid, containing the entire coding sequence of c-*src*, was inserted into M13mp18 (mp18 Y527F c-*src*). Point mutations were then introduced into mp18 Y527F c-*src* DNA by the oligonucleotide-directed mutagenesis method (29, 34). Oligomers used in the mutagenesis procedure and new restriction sites which made it easy to screen mutant clones are shown in Table 1. After confirmation of mutations by nucleotide sequence analysis (43), the *Nco*I-*Bam*HI fragments of the resultant 20 mutants (10 M and 10 X mutants)

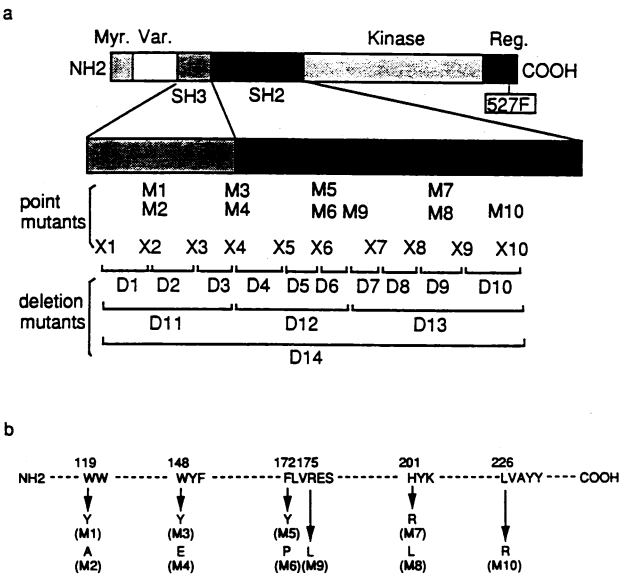

FIG. 1. Schematic positions of mutations. (a) Parental Y527F c-*src* and all mutants. Myr., Domain for myristylation; Var., variable domain; Kinase, protein-tyrosine kinase domain; Reg., negative regulatory domain. (b) Genetic exchanges in M mutants. The numbers of residues of mutations are given at the top. Conserved amino acid residues surrounding mutations are also listed.

were introduced into pCla12Nco. Since M9 and all 10 X mutants have an *Xho*I site in the same frame, 14 deletion mutants were constructed by joining *Xho*I ends (Table 1; Fig. 1). All these mutants were finally inserted into an RSV proviral vector, RCAS. Since RCAS is a helper-independent retroviral vector which contains an intact and nonpermuted copy of an avian leukosis virus genome, there is no need for a helper cell or helper virus. The normal c-*src* (N) and the parental Y527F c-*src* (A) genes were also inserted into the RCAS vector for use as controls.

Transfection and biological analyses. The cells were transfected with proviral plasmid DNA as described elsewhere (14). After high levels of reverse transcriptase activity appeared in the supernatant, culture media were harvested as viral stocks. Reverse transcriptase activity was measured as described by Houts et al. (17). Titers of virus stocks were determined by focus assay in monolayer culture of CEF (48). Secondary CEF infected with these virus stocks were also assayed for anchorage-independent growth (22).

Immunoprecipitation and $p60^{src}$ equilibrium level. Fully infected CEF were labeled with [^{35}S]methionine for 12 h in 2 ml of methionine-free Dulbecco modified Eagle medium containing 10% dialyzed fetal calf serum and 200 μCi of [^{35}S]methionine. Labeled cells were lysed in RIPA buffer (150 mM NaCl, 20 mM Tris [pH 7.2], 0.1% sodium dodecyl sulfate [SDS], 1% sodium deoxycholate, 1% Triton X-100). Labeling efficiency was determined from the ratio of trichloroacetic acid (TCA)-precipitable radioactivity to total cell protein in each lysate (28). Immunoprecipitation of $p60^{src}$ from lysates containing equal amounts of TCA-precipitable radioactivity was performed with an excess of monoclonal antibody 2-17 (obtained from Microbiological Associates, Bethesda, Md.), which recognizes residues 2 to 17 of v-*src* and c-*src*. Immune complexes were adsorbed to Formalin-fixed *Staphylococcus aureus* (Pansorbin; Calbiochem-Behring, La Jolla, Calif.), and labeled protein in the complexes was resolved by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and analyzed by autoradiography. The amounts of $p60^{src}$ per milligram of total cell protein were determined from the scintillation counts of gel bands and efficiency of metabolic labeling (28).

In vitro protein kinase assay and specific kinase activity. Immune complexes from lysates containing equal amounts of TCA-precipitable radioactivity obtained as described above were incubated in kinase buffer (20 mM Tris [pH 7.2], 10 mM $MgCl_2$, 0.1% Triton X-100) for 10 min at room temperature with or without 2 μg of acid-denatured rabbit muscle enolase (Sigma Chemical Co., St. Louis, Mo.) and 1 μCi of [γ-^{32}P]ATP (3000 Ci/mmol) (4). After electrophoresis (10% SDS-PAGE), the amounts of [^{32}P]$p60^{src}$ and [^{32}P] enolase radioactivity were determined by scintillation counting of excised bands. The specific kinase activities for autophosphorylation and enolase phosphorylation were calculated in conjunction with the determined amount of exogenous $p60^{src}$ after correction for any detectable endogenous $p60^{c\text{-}src}$ (28).

Immunoblotting. Phosphotyrosine-containing proteins were detected by a method described previously (26). Lysates from cells grown in the presence of 50 μM sodium orthovanadate were equalized for protein concentration, loaded onto 10% polyacrylamide gels, and transferred to nitrocellulose. Filters were blocked with bovine serum albumin and then probed with affinity-purified rabbit anti-phosphotyrosine antibody (generously provided by Morris White, Harvard Medical School, Boston, Mass., and Jean Wang, University of California, San Diego, La Jolla, Calif.) followed by alkaline phosphatase-coupled second antibody (Promega Biotec, Madison, Wis.).

RESULTS

Generation of mutants of Y527F c-*src*. Twenty point mutations were introduced into the transformation-competent Y527F c-*src* gene by the oligonucleotide-directed mutagenesis method. All oligomers used for mutagenesis and the mutations generated are summarized in Table 1.

The 10 M mutations were designed to change a highly conserved amino acid in SH2 or SH3. Each mutation also formed a new restriction enzyme cleavage site to simplify detection of mutant clones (Table 1; Fig. 1). The mutations in mutant pairs M1 (mutation W119Y) and M2 (mutation W119A), M3 (W148Y) and M4 (W148E), M5 (F172Y) and M6 (F172P), and M7 (H201R) and M8 (H201L) introduced either a conservative or radical amino acid change at a given position. For example, F172 of c-*src* protein was replaced by a related amino acid, Y, in M5 (F172Y) and by a chemically different amino acid, P, in M6 (F172P). Similarly, the substitutions in M1 (W119Y), M3 (W148Y), and M7 (H201R) represent relatively minor chemical changes, whereas the substitutions in M2 (W119A), M4 (W148E), M8 (H201L), M9 (R175L), and M10 (L226R) are more dramatic (Fig. 1).

The 10 X mutations were designed to produce new *Xho*I restriction sites at many positions in SH3 and SH2 and were used to test the effects of mutations that encode amino acid residues less well conserved than those affected by the M series of mutations. Since M9 (R175L) and all 10 X mutants have an *Xho*I site in the same frame, various deletion mutations were constructed by joining *Xho*I ends. Thus, ten D mutants (D1 to D10), each of which sequentially lacks approximately 10 codons, three larger deletion mutants (D11 to D13), and a deletion mutant [D14; Δ(93–226)] which lacks most of the SH2-SH3 region all rejoin the 5′ and 3′ coding region of *src* in frame. D11 [Δ(93–143)] lacks almost all of the

SH3 region (residues 88 to 139), whereas D12 [Δ(144–175)] and D13 [Δ(176–226)] each lack about half of SH2. All resultant mutants were inserted into the proviral form of the helper-independent RSV vector, RCAS (18), for biological and biochemical studies.

Strategy for characterization of mutant proteins. The 34 mutant alleles of Y527F c-*src* and positive (A [Y527F c-*src*]) and negative (N [c-*src*]) control alleles were tested for a variety of biological and biochemical properties. The general plan of the analysis is described here, and the results are presented in greater detail in subsequent sections.

(i) Biological characterization. All mutant DNAs and both negative and positive control DNAs were transfected into CEF. After two passages, the cells appeared to be fully infected and expressed high levels of the viral structural protein $Pr76^{gag}$ and $p60^{src}$ (data not shown). CEF were infected with stocks containing equal amounts of reverse transcriptase activity, and the transforming activities were determined by measurement of focus formation in monolayer cultures and colony formation in soft agar (Table 2) and by examination of cell morphology (Fig. 2). Results at 41°C were similar to those at 37°C, implying that the lesions were not overtly temperature sensitive. Mutant *src* genes varied by a factor of 40 in their abilities to induce foci and agar colonies; a few mutants caused fusiform rather than round cell transformation (Fig. 2). By all criteria, v-*src* had a higher level of transforming activity than Y527F c-*src* (Table 2; Fig. 2).

(ii) Levels of p60 and protein stabilities. To examine the production of $p60^{src}$ in the fully infected cells, equivalent counts per minute of radioactive proteins from lysates of [^{35}S]methionine-labeled cells were precipitated with monoclonal antibody 2-17, which recognizes residues 2 to 17 of $p60^{src}$ (Fig. 3; Table 2). Some mutations in the SH2 region affected the accumulation of radiolabeled p60 over a 40-fold range, although metabolic labeling may have slightly overestimated the differences of p60 levels. Since the labeling period was only 12 h, it was insufficient in some cases to reach steady-state values. Therefore, we also measured p60 levels for several mutants (M6, M9, X2, X9, D4, D9, D11, and D14) by immunoblotting with ^{125}I-protein A and obtained results that do not differ significantly from those based on metabolic labeling (data not shown).

To explain the differences in the abundance of p60, the relative stabilities of several mutant proteins were determined by pulse-chase experiments (Fig. 4). In particular, some deletion mutant proteins (D4, D5, D6, D7, D11, D12, D13, and D14) were significantly less stable than parental A protein, which showed an apparent half-life of approximately 16 h (Fig. 4; Table 2).

(iii) Tyrosine kinase activities of mutant *src* proteins. The protein kinase activities of mutant proteins on an exogenous substrate were first examined by adding acid-denatured enolase and [γ-^{32}P]ATP to immunoprecipitates of *src* proteins (Fig. 3). Nearly identical values were obtained for autophosphorylation of *src* proteins in the immune complexes (Fig. 3; other data not shown). The total kinase activities varied by a factor of 60; after correction for the abundance of p60, specific kinase activities were found to vary by a factor of about 15 (Table 2).

To obtain an independent measure of the kinase activities of mutant proteins, the levels of tyrosine phosphorylation in vivo were analyzed by transfer of proteins from vanadate-treated infected cells to nitrocellulose and detection with two antiphosphotyrosine sera (Fig. 5; Table 2). Several cultures were also tested without exposure to vanadate; under these conditions, we could detect differences in the levels of tyrosine phosphorylation only in a 60-kilodalton protein, presumably $p60^{src}$, but could not detect differences in other proteins. Thus the levels of phosphotyrosine-containing proteins were too low in the absence of the phosphatase inhibitor to provide useful information.

Classification of mutants. As a result of these assays, we were able to place the mutants into four classes: class 1, mutants with no significant changes from parental Y527F c-*src*; class 2, mutants with reduced transforming and specific kinase activities; class 3, mutants with poor transforming activity despite normal or enhanced specific kinase activity, usually an apparent result of diminished protein stability; and class 4, mutants with augmented transforming activity, augmented kinase activity, or both.

Class 1 mutants. A substantial number of mutants showed no differences or only relatively small differences from the parental allele, Y527F c-*src*. (Normalized values of 0.5 to 1.5 were considered to represent such relatively small differences [Table 2].) Class 1 mutant alleles were produced by all types of mutation, including conservative and nonconservative mutations at highly conserved residues (mutant M1 [mutation W119Y], M2 [W119A], M4 [W148E], M5 [F172Y], M8 [H201L], and M10 [L226R]), mutations at less conserved sites (X1 [Y92L] and X9 [R205L, K206E]), and deletion mutants {D1 [Δ(93–108)] D5 [Δ(157–165)], and D6 [Δ(166–175)]}.

Some class 1 alleles had minor but reproducible effects that may be functionally important. For example, M5 (F172Y) showed a consistent decline in transforming and specific kinase activities, although another mutation at the same position (in mutant M6 [F172P]) enhanced both activities. M4 (W148E) showed a modest decrease in transforming activity despite a mild increase in specific and total kinase activity; a mutant produced by yet another change at the same position (M3 [W148Y]) had impaired kinase activity and more significantly impaired transforming activity than M4 (W148E) had.

Class 2 mutants. Several mutants showed decreased transforming and specific kinase activities. These lesions support the conventional claim that the protein kinase activity of $p60^{src}$ is an important determinant of oncogenic activity, and they indicate regions of the primary amino acid sequence that may have roles in maintaining kinase activity. However, class 2 mutations map at various positions within the SH3 and SH2 domains. Point and deletion mutations in the SH3 region had especially strong effects: X2 (Q109E), X3 (T125E), and X4 (I143L, Q144E) showed marked negative effects on biological activity and were found to exhibit loss of both specific kinase activity and protein stability. Similar effects on biological and kinase activities were caused by deletion mutations in SH3 {mutants D2 [Δ(109–124)] and D3 [Δ(125–143)]}, suggesting that SH3 might normally have a positive effect on kinase activity. The following mutants resulting from point mutations elsewhere in the mutagenized region showed similar though milder changes: M3 (W148Y), X5 (R156L), X6 (P165L), X7 (S187E), and X8 (N198E). However, except for D4 [Δ(144–156)], mutants resulting from deletion mutations in regions overlying these point mutations did not fall into class 2.

Class 3 mutants. Mutations that impaired biological activity without causing significant loss of catalytic activity could be among the most interesting, since they might be deficient in interactions with substrate proteins or improperly localized in the cell. Unfortunately, most mutants in this class are large-deletion mutants that showed reductions in protein

TABLE 2. Biological and biochemical activities of mutant proteins in infected CEF

Mutant	Mutation	Growth in soft agar[a,b,c,d]	Focus-forming activity[b,c,d]	p60		Kinase activity		In vivo phospho-tyrosine[e]	Class[f]
				Level[c,d,g]	Half-life[d,h]	Total[c,d,i]	Specific[c,d,j]		
—[k]		<0.1	<0.1	<0.1	NT	<0.1	<0.1	0	
RSV	B77-*src*	2.5	1.9	1.0	NT	2.4	2.4	4	
N	c-*src*	<0.1	<0.1	2.1	L	0.2	0.1	1	
A	Y527F	1.0	1.0	1.0	L	1.0	1.0	4	
M1	W119Y	0.9	1.0	1.0	NT	0.9	0.9	4	1
M2	W119A	1.5	1.2	1.0	NT	0.9	0.9	4	1
M3	W148Y	0.3	0.4	0.7	NT	0.4	0.6	2	2
M4	W148E	0.8	0.6	1.2	NT	1.7	1.4	2	1
M5	F172Y	0.5	0.7	0.9	NT	0.6	0.7	3	1
M6	F172P	1.9	1.7 (f)[l]	2.6	L	4.7	1.8	4	4
M7	H201R	0.3	0.3	0.8	NT	0.7	0.9	2	3
M8	H201L	0.6	0.5 (f)	1.8	NT	1.6	0.9	2	1
M9	R175L	1.5	2.1	4.0	L	4.8	1.2	4	4
M10	L226R	1.2	1.0	1.4	NT	1.8	1.3	4	1
X1	Y92L	0.7	0.7	1.0	NT	0.9	0.9	3	1
X2	Q109E	0.1	0.1	0.2	S	0.1	0.2	1	2
X3	T125E	0.1	<0.1	0.6	S	0.2	0.3	1	2
X4	I143L, Q144E	0.3	0.2	0.7	NT	0.2	0.3	2	2
X5	R156L	0.4	0.5 (f)	1.0	NT	0.4	0.4	3	2
X6	P165L	0.2	0.4 (f)	1.9	NT	1.0	0.5	2	2
X7	S187E	0.2	0.6 (f)	0.7	NT	0.3	0.4	3	2
X8	N198E	0.2	0.2 (f)	1.0	NT	0.3	0.3	2	2
X9	R205L, K206E	0.7	0.6 (f)	2.1	NT	1.5	0.7	3	1
X10	V227E	1.2	1.4	0.8	NT	2.2	2.7	3	4
D1	Δ(93–108)	1.0	1.1	1.0	NT	0.7	0.7	4	1
D2	Δ(109–124)	0.2	0.1	1.0	NT	0.2	0.2	1	2
D3	Δ(125–143)	0.2	0.4	0.8	M	0.3	0.4	3	2
D4	Δ(144–156)	0.1	0.1 (f)	0.3	M	0.1	0.4	1	2
D5	Δ(157–165)	0.8	0.6 (f)	0.8	M	1.2	1.5	3	1
D6	Δ(166–175)	0.7	0.4 (f)	0.6	M	0.8	1.4	2	1
D7	Δ(176–186)	0.4	0.4 (f)	0.8	M	0.9	1.1	1	3
D8	Δ(187–197)	1.3	1.0 (f)	1.3	M	3.0	2.3	3	4
D9	Δ(198–205)	1.5	1.5 (f)	2.2	L	5.7	2.6	4	4
D10	Δ(206–226)	1.1	1.3 (f)	1.9	L	4.9	2.6	4	4
D11	Δ(93–143)	<0.1	0.1	0.3	M	0.3	0.9	1	3
D12	Δ(144–175)	<0.1	<0.1	0.2	M	0.4	1.8	0	3
D13	Δ(176–226)	0.9	0.7 (f)	0.8	M	2.4	3.0	2	4
D14	Δ(93–226)	<0.1	<0.1	0.1	S	0.3	3.3	0	3

[a] Only colonies larger than 1 mm in diameter were counted.
[b] Transforming activities per unit of virus-fraction-containing medium with an equal amount of reverse transcriptase activity.
[c] Data are normalized to those of p60 carrying Y527F (A).
[d] Data are averages of the results of three independent experiments.
[e] Total phosphotyrosine-containing proteins in the lysates (footnote *g*) judged by color intensities in Western blots (immunoblots) probed with two polyclonal antiphosphotyrosine antisera (see Materials and Methods and Fig. 4). Scores were judged as 0, 1, and 2 in each of the two blots and added to compute total scores.
[f] For definitions of mutant classes, see Results.
[g] [^{35}S]methionine radiolabel in p60 from lysates containing an equal amount of TCA-precipitable radioactivity following a 12-h labeling of infected CEF.
[h] Computed from the results of pulse-chase experiments (see Materials and Methods and the legend to Fig. 4) and listed as follows: S, less than 6 h; M, 6 to 12 h; L, 12 to 24 h; NT, not tested.
[i] Total immune-complex kinase activity of p60 from the lysates (footnote *g*), measured with enolase as substrate.
[j] Specific activity of p60 kinase, obtained by dividing total kinase activity (footnote *i*) by the level of p60 (footnote *g*).
[k] —, Uninfected CEF.
[l] (f), Infected cells were fusiform.

stability and hence in abundance of p60^{src} and total kinase activity {D11 [Δ(93–143)], D12 [Δ(144–175)], and D14 [Δ(93–226)]}. However, at least one point mutant (M7 [H201R]) and one small-deletion mutant {D7 [Δ(176–186)]} showed impaired transforming activity without appreciable protein instability or loss of kinase activity as measured in vitro; on the other hand, these mutants induced less intracellular phosphotyrosine than did alleles with normal transforming activities. Other point and deletion mutants that had destabilized p60^{src} also exhibited diminished specific kinase activity {e.g., X2 [Q109E], and D4 [Δ(144–156)]}.

Class 4 mutants. Mutations that enhanced protein kinase activity, biological activity, or both were encountered with surprising frequency despite the use of an activated parental allele, Y527F c-*src*. Two of the enhancing mutations (in M6 [F172P] and M9 [R175L]) caused amino acid substitutions in the most highly conserved sequence in SH2, the FLVRES sequence at residues 172 to 177. Both mutations raised

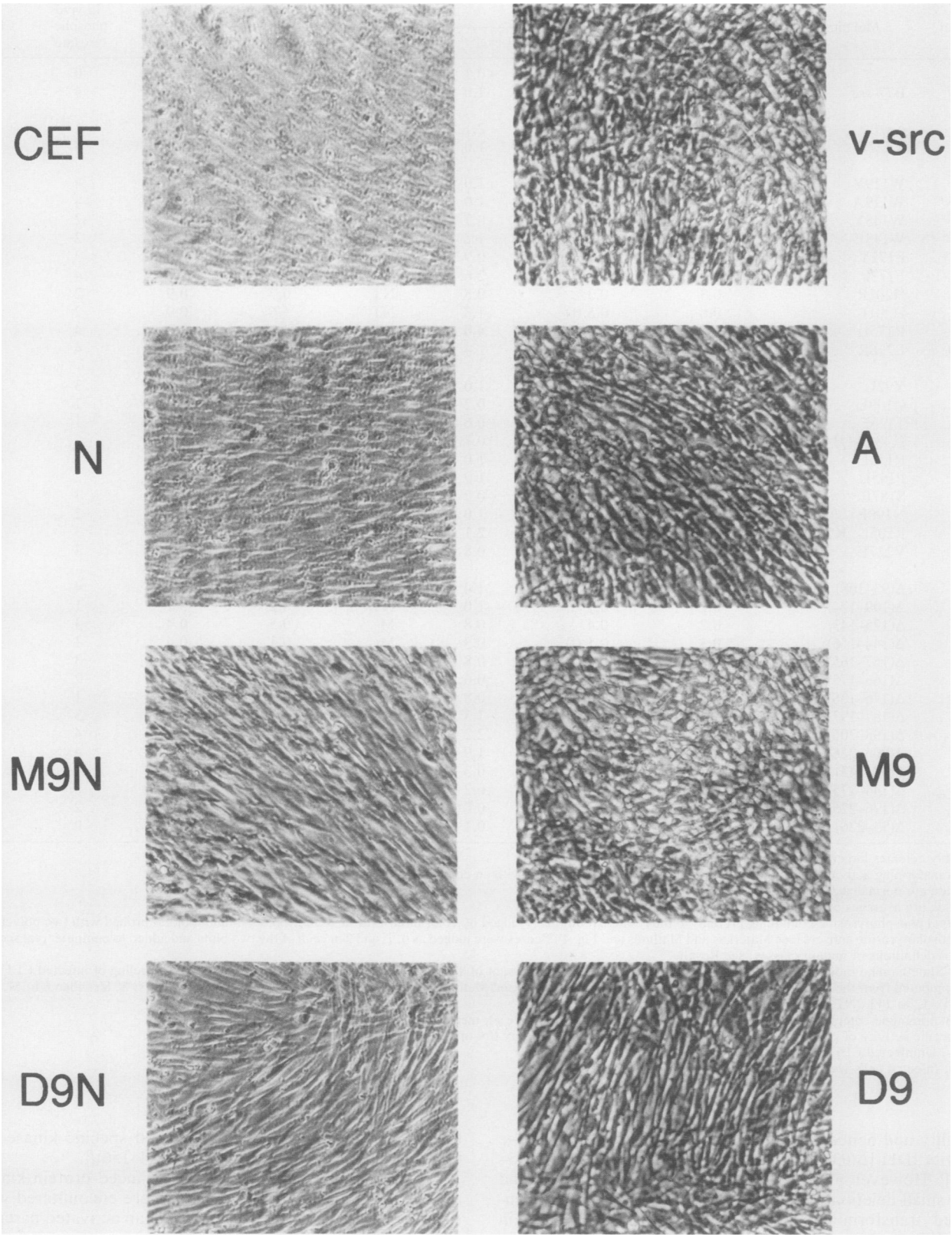

FIG. 2. Photographs of infected cells in monolayer culture. CEF fully infected with viruses bearing the indicated *src* alleles (Tables 2 and 3) were plated at a density of 2×10^6 per 100-mm dish 3 days before photomicroscopy.

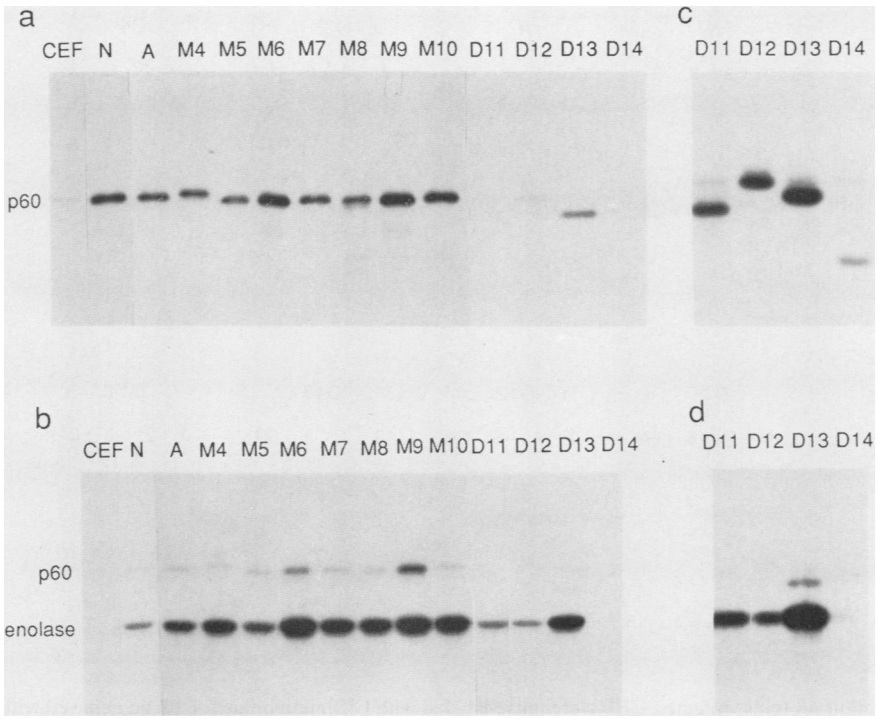

FIG. 3. Levels of mutant *src* proteins and their kinase activities in vitro. (a) Metabolic labeling. Fully infected CEF were labeled with [^{35}S]methionine for 12 h. Lysates containing equal amounts of TCA-insoluble radioactivity were immunoprecipitated with monoclonal antibody 2–17, and half of the immunoprecipitates were analysed by 10% SDS-PAGE and autoradiographed for 12 h. (b) Immune complex kinase activity. The remaining half of the immunoprecipitates obtained in panel a were incubated with 2 μg of acid-denatured enolase and 1 μCi of [γ-^{32}P]ATP, and the products were separated by 10% SDS-PAGE. (c and d) Lanes D11 to D14 in the autoradiogram shown in panels a and b were exposed for 72 h.

transforming titers 1.5- to 2-fold above those seen with Y527F c-*src*, and both enhanced the stability and specific kinase activity of p60^{src}; however, M6 (F172P) had a greater effect on kinase activity and M9 (R175L) had a more marked effect on protein stability. Notably, M6 (F172P) produced a fusiform transformed phenotype in CEF, a characteristic previously observed with a deletion mutant of v-*src* that lacked codon 172 (50, 51). More modest but similar effects were observed in a mutant produced by a mutation at a less well conserved residue (X10 [V227E]). Some mutants produced by deletion mutations that mapped near these substitutions {D8 [Δ(187–197)], D9 [Δ(198–205)], D10 [Δ(206–226)], and D13 [Δ(176–226)]} also enhanced the activity of p60, although cells transformed by these mutants were less refractile than cells transformed by Y527F c-*src* (Fig. 2). Deletions that removed parts of the FLVRES sequence {mutants D6 [Δ(166–175)] and D7 [Δ(176–186)]} did not enhance the biological and biochemical properties of p60^{src}.

Introduction of class 4 mutations into normal c-*src*. To determine whether the lesions that enhance the activity of Y527F c-*src* were sufficient to convert wild type c-*src* into a transforming gene, we transferred several class 4 mutations {in M6 [F172P], M9 [R175L], D9 [Δ(198–205)], D10 [Δ(206–226)], and D13 [Δ(176–226)]} into a normal c-*src* allele (see Materials and Methods). In addition, the large-deletion mutant D14 [Δ(93–226)] was moved into normal c-*src*. The biological and biochemical attributes of proteins encoded by these constructs were then assayed as described above for mutants of Y527F c-*src* (Fig. 2 and 6; Table 3; other data not shown).

All of the mutations that enhanced the activity of Y527F c-*src* produced significant levels of transforming activity in the normal c-*src* background; transforming activities ranged from 20 to 70% of those seen with Y527F c-*src*-encoded p60^{src} (Table 2). In all cases, the proteins were as abundant as Y527F c-*src*-encoded p60^{src}, and the specific kinase activities were very similar to that of protein activated by the change at codon 527. The large-deletion mutant D14N [Δ(93–226)] was unstable and transformation defective compared with normal c-*src*, but the deletion did not augment the specific kinase activity as it did in Y527F c-*src* (Table 3).

DISCUSSION

We have constructed and characterized a substantial set of substitution and deletion mutations that affect the SH2 and SH3 regions of p60^{src}, portions of the protein believed to modulate its kinase activity or affect its interactions with other proteins.

We chose to construct our mutations in a parental allele of c-*src* with a single lesion (Y527F) known to increase the intrinsic protein-tyrosine kinase activity of p60^{src} and to convert c-*src* into a transforming gene (3, 28, 38, 41). Because the activation of Y527F c-*src* depends on one mutation rather than multiple mutations, as in the known alleles of v-*src* (25), we hoped that the genetic background would be especially sensitive to second-site mutations that might diminish or augment biological or biochemical characteristics of encoded proteins. The results imply that this was so: many of the changes we made, even relatively subtle ones, produced an altered phenotype, and we have observed several examples of lesions that augment and several that impair *src* activity. Furthermore, those that make Y527F c-*src* a stronger transforming allele are also able to activate

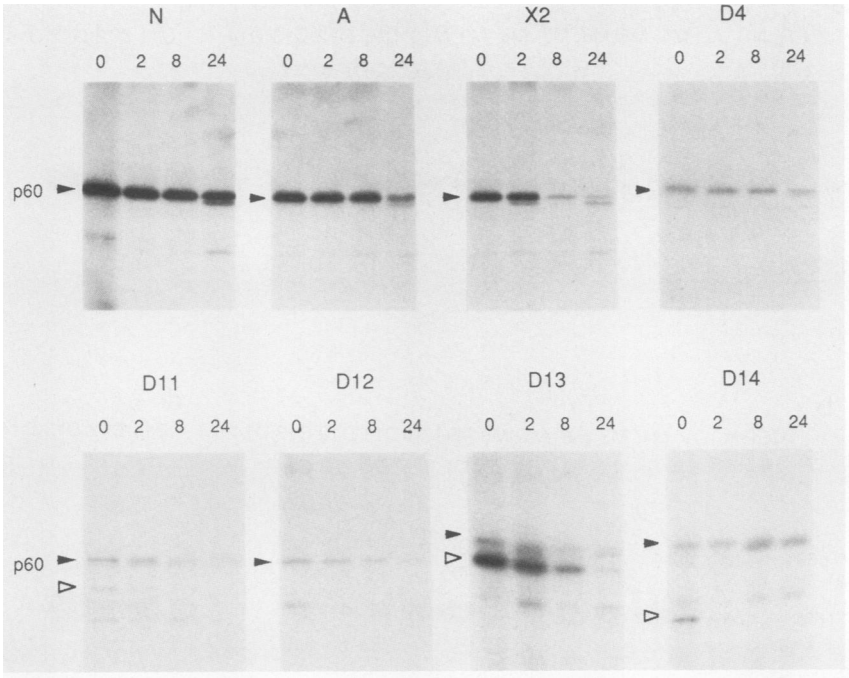

FIG. 4. Stability of mutant proteins. Infected CEF were pulse-labeled with [^{35}S]methionine for 1 h and chased with an excess of unlabeled methionine for increasing lengths of time. Immunoprecipitation was performed as described in the legend to Fig. 3. The time of chase (0, 2, 8, or 24 h) is indicated above the corresponding lanes. The position of p60 is indicated by an closed arrowhead. The positions of mutant proteins with mobilities different from that of p60^{src} are indicated by open arrowheads. The half-lives of p60^{src} are calculated and listed in Table 2.

normal c-*src* and thus act independently of the Y527F mutation.

Although our mutants exhibited diverse characteristics, we have been able to categorize them into four classes: class 1, those without major alterations in any of the properties we measured; class 2, those with diminished protein kinase activity and associated loss of transforming activity; class 3, those with normal or near-normal kinase activity but impaired transforming capacity because of instability or other problems; and class 4, those with increased catalytic or biological activity or both.

Among the most striking mutations in our collection are those that enhance the transforming and enzymatic properties of Y527F c-*src*. These mutations are located in two portions of SH2, the highly conserved FLVRES sequence at

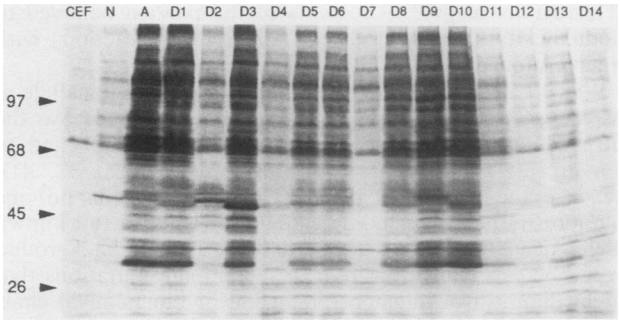

FIG. 5. Phosphotyrosine-containing proteins in infected cell lysates. Lysates from fully infected CEF grown in the presence of 50 μM sodium orthovanadate were equalized for protein concentration, separated by 10% SDS-PAGE, transferred to nitrocellulose, and probed with affinity-purified antiphosphotyrosine antibody. Molecular sizes are given in kilodaltons.

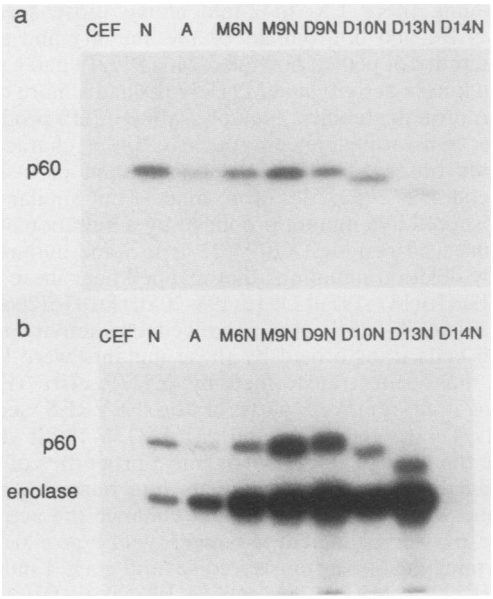

FIG. 6. Biochemical properties of proteins encoded by mutant alleles of c-*src*. (a) Metabolic labeling. Fully infected CEF were labeled with [^{35}S]methionine for 12 h. Lysates containing equal amounts of TCA-insoluble radioactivity were immunoprecipitated with monoclonal antibody 2–17, and half of the immunoprecipitates were analyzed by 10% SDS-PAGE and autoradiographed for 12 h. (b) Total kinase activity. The remaining immunoprecipitates were incubated with 2 μg of acid-denatured enolase and 1 μCi of [γ-^{32}P]ATP and separated by 10% SDS-PAGE. Results are compiled in Table 3.

TABLE 3. Effects of mutations introduced into normal c-src (527Y)

Mutant	Mutation	Growth in soft agar[a,b,c]	Focus-forming activity[b,c]	p60 level[c,d]	Kinase activity		In vivo phosphotyrosine[e]
					Total[c,f]	Specific[c,g]	
—[h]		<0.1	<0.1	<0.1	<0.1	<0.1	0
N	c-src	<0.1	<0.1	2.5	0.3	0.2	1
A	Y527F	1.0	1.0	1.0	1.0	1.0	4
M6N	F172P	0.4	0.4 (f)[i]	1.8	1.6	0.9	3
M9N	R175L	0.6	0.6 (f)	2.4	1.9	0.8	4
D9N	Δ(198–205)	0.7	0.7 (f)	1.7	1.8	1.1	4
D10N	Δ(206–226)	0.5	0.6 (f)	1.9	1.5	0.8	3
D13N	Δ(176–226)	0.3	0.2 (f)	1.1	1.9	1.8	3
D14N	Δ(93–226)	<0.1	<0.1	0.1	<0.1	<0.1	0

[a] Only colonies larger than 1 mm in diameter were counted.
[b] Transforming activities per unit of virus-fraction-containing medium with an equal amount of reverse transcriptase activity.
[c] Data are normalized to those of p60 carrying Y527F (A) and are averages of the results of three independent experiments.
[d] [^{35}S]methionine radiolabel in p60 from lysates containing an equal amount of TCA-precipitable radioactivity following a 12-h labeling of infected CEF.
[e] Total phosphotyrosine-containing proteins in the lysates (footnote *d*) judged by color intensities in Western blots probed with two polyclonal antiphosphotyrosine antisera (see Materials and Methods and Fig. 4). Values are sums of scores from two blots; each blot was scored as 0, 1, or 2.
[f] Total immune complex kinase activity of p60 from the lysates (footnote *d*), measured with enolase as substrate.
[g] Specific activity of p60 kinase, obtained by dividing the total kinase activity (footnote *f*) by the level of p60 (footnote *d*).
[h] —, Uninfected CEF.
[i] (f), Infected cells were fusiform.

positions 172 to 177 and the sequence of residues 198 to 226; they are also capable of converting normal c-*src* genes to active oncogenes which encode proteins with kinase activities similar to those found in the product of Y527F c-*src* (Table 3). Mutations that activate c-*src* and related proto-oncogenes have previously been reported to reside in the kinase domain itself (25, 31), in a putative negative regulatory site (Y527) that is a target for an unknown tyrosine kinase (3, 28, 38, 41), and in the amino terminal portion of SH3 (11, 20, 25, 39). The mechanism(s) by which changes in SH3 or SH2 activate p60 is unknown but could mimic the effects of cellular proteins that normally potentiate the catalytic function of p60^{src} and related kinases (see below).

Unlike mutations that completely inactivate the catalytic potential of p60$^{v\text{-}src}$ (46) or render it unsuitable as a substrate for myristoyl transferase (7, 23), none of our substitution or small-deletion mutations in SH2 or SH3 fully eliminated the transforming activity of Y527F c-*src*. This is consistent with the modulatory role proposed for these regions. Moreover, since the small-deletion mutations (D1 to D10) cover the entire amino acid sequence from residue 93 to residue 226, it is evident that no component of this region is absolutely required for transformation or catalytic function.

We have asked whether our mutants support the thesis that transformation by *src* depends on achieving a certain level of protein-tyrosine kinase in the cell. First, we plotted the total *src* kinase activity (the specific kinase activity determined in vitro for each mutant protein multiplied by the concentration of *src* protein observed in vivo) against the transformation efficiency of each mutant (assayed after infection of CEF with an RSV vector carrying the mutant allele) (Fig. 7a). In general, the correlation was strong: as the

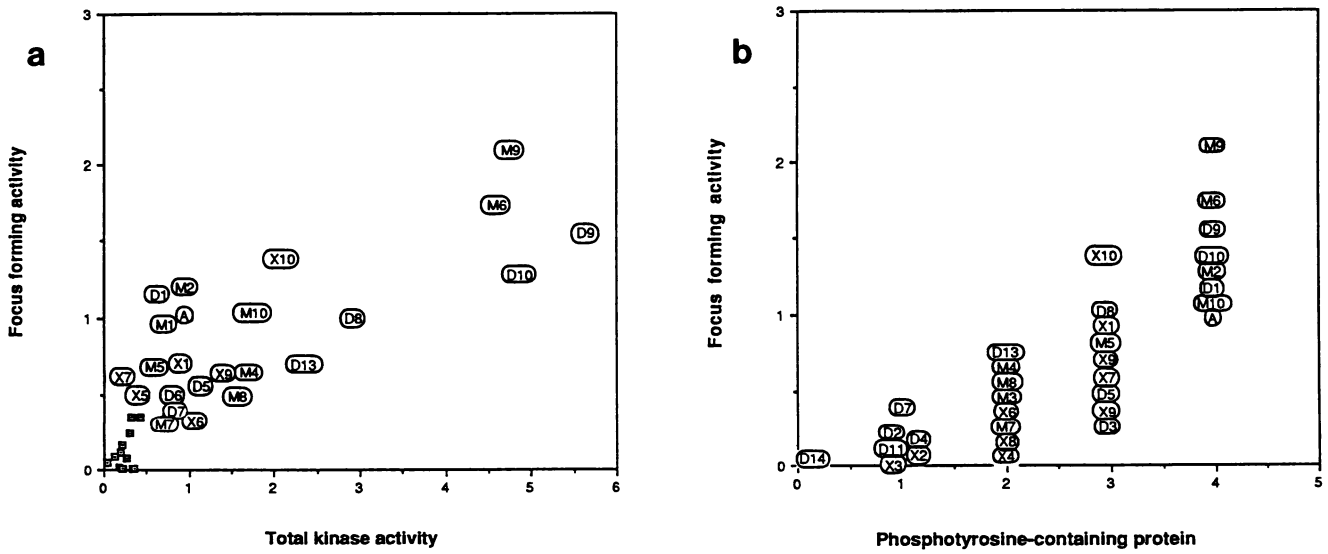

FIG. 7. (a) Correlation between focus-forming activity and total kinase activity. Focus-forming activities and total kinase activities are normalized to those of A (Y527F c-*src*). Symbols: ⊡, mutant whose normalized focus-forming activity was less than 0.4 and whose total kinase activity was less than 0.5. (b) Correlation between focus-forming activity and levels of phosphotyrosine-containing proteins. Levels of phosphotyrosine-containing proteins were calculated as described in Table 2, footnote *e*.

total kinase activity rose, so did the production of transformed foci and agar colonies. A few especially interesting mutants, however, showed poor transforming potential despite near-normal in vitro kinase activity {e.g., X6 [P165L], D7 [Δ(176–186)], and M7 [H201R]}. Such mutants indicate the importance of qualitative as well as quantitative aspects of *src* kinase and might assist in the search for relevant targets for kinase activity, since they may encode proteins with low affinity for those essential substrates.

As a second measure of the role of tyrosine phosphorylation in transformation, we asked whether the levels of phosphotyrosine-containing proteins in cells expressing the mutant alleles correlated with the efficiency of transformation (Fig. 7b). Again, in general, the anticipated correlation can be made: cells with little or no increment in phosphotyrosine (values 0 to +1) are unlikely to be transformed, whereas those with high levels of phosphotyrosine (+3 and +4) are likely to be transformed. However, there is considerable variation as well; most obviously, cells with intermediate levels of phosphotyrosine (+2) exhibit a wide range of biological phenotypes. It might be valuable to examine this last group of mutants more closely for induction of particular phosphotyrosine-containing proteins that correlate well with the appearance of the transformed phenotype.

Attempts to explain certain aspects of our mutants are complicated by the use of CEF as host cells. We chose these cells for initial characterization of the mutants because they are the most commonly used tester cells for *src* genes and are conveniently infected by RSV vectors carrying our alleles. However, the cells are extremely difficult to clone; consequently, we were obliged to draw conclusions from mass cultures in which individual infected cells may have contained different amounts of *src* protein. This may help explain why our mutants often show fractional changes in transforming activity, rather than all-or-none changes. If a mutation increases the threshold concentration of $p60^{src}$ required for transformation, some, albeit fewer, cells will contain the necessary concentration above the threshold level. Indeed, when several of the mutant genes described here were expressed in clonal populations of mouse cells, we generally found the clones to be fully transformed or nontransformed, depending on the level of mutant protein; this further work supports the idea that the lesions in SH2 and SH3 affect the threshold dose of $p60^{src}$ required for transformation (H. Hirai and H. E. Varmus, manuscript in preparation).

Our attempt to design mutations on the basis of the relative conservation of sequence among *src* gene family members reflects the difficulty of predicting the behavior of a protein without information about its structure. Thus, the changes introduced at highly conserved residues (mutations producing the M mutant series) had no more dramatic effects than changes introduced without concern for amino acid conservation (mutations producing the X mutant series) (Table 2). Interestingly, different amino acid substitutions at the conserved positions did have different phenotypic effects, although the more radical substitutions did not necessarily have the more dramatic effects. (Compare, for example, M3 and M4 or M7 and M8.) In sum, it is not yet possible to draw major conclusions about the organization or function of SH2 and SH3 from studies of the twenty substitution mutants described here.

Analysis of the consecutive deletion mutations constructed between codons 93 and 226, on the other hand, offers some insight into the arrangement and purpose of this region (Fig. 8). With the exception of D1 [Δ(93–108)],

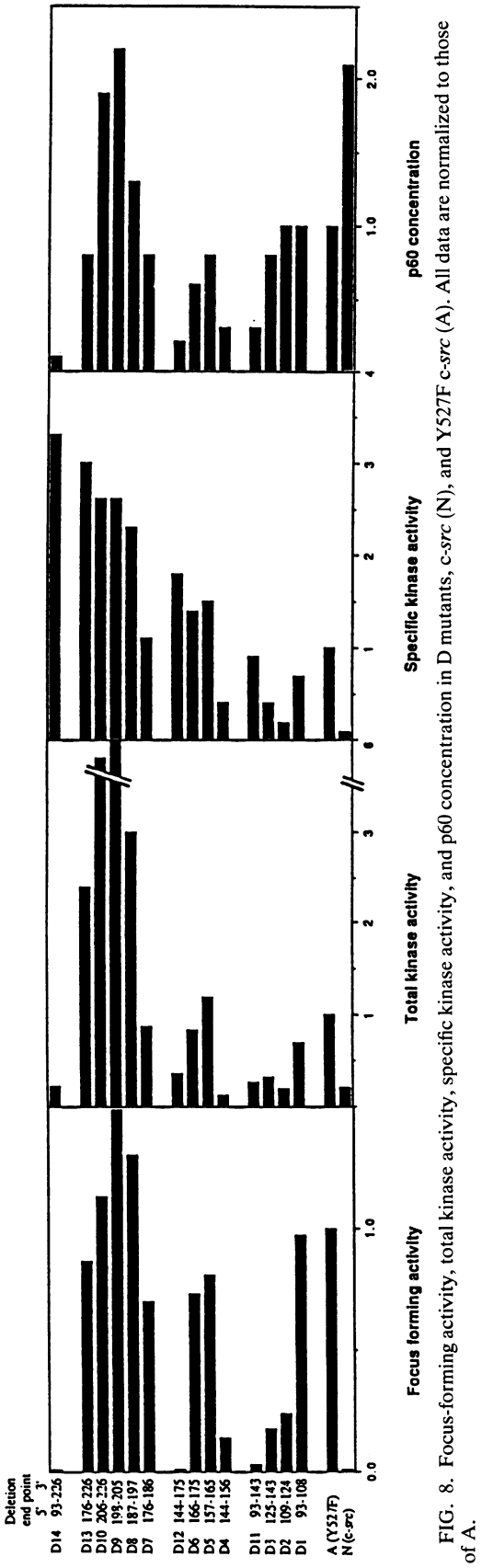

FIG. 8. Focus-forming activity, total kinase activity, specific kinase activity, and p60 concentration in D mutants, c-*src* (N), and Y527F c-*src* (A). All data are normalized to those of A.

deletion mutations in the SH3 domain showed impaired kinase and transforming activities, whereas deletions in the portion closest to the carboxy terminus, residues 187 to 226, showed increases in both activities. These findings imply that part of SH3 normally has a positive effect on the activity of $p60^{src}$ and that part of SH2 has a negative effect. Deletions in the middle of the mutated region are more difficult to evaluate: they raise the specific kinase activity but tend to impair protein stability and consequently biological activity. This pattern is seen more dramatically with the large-deletion mutation D12 [Δ(144–175)].

The amino-terminal portions of SH3 in c-*src* and c-*abl* may normally restrain catalytic activity, since substitutions and small deletions in these regions can convert proto-oncogenes to oncogenes (11, 20, 25, 39). A linker insertion in the carboxy terminus of SH3 in c-*abl* protein can also activate transforming potential (49). Although we found no evidence for activating mutations in the SH3 region, we made no mutations at residues upstream of 92 and we did not test any of our SH3 mutations in the context of normal c-*src*. Thus, the X1 mutant, whose mutation (Y92L) activates normal c-*src* according to Potts et al. (39) but had minimal impact on the behavior of Y527F c-*src* in our experiments (Table 2), was not tested in a c-*src* background, and none of the other reported activating lesions in SH3 was recapitulated in our study.

Because our mutations were generated in activated c-*src* rather than v-*src* or normal c-*src*, it is difficult to compare the biological effects we have observed with the phenotypes of previously reported mutants (2, 7, 27, 40, 53). The problem is obviously compounded by differences in the nature of the mutations (deletion endpoints or substituted residues) and in the assay conditions used to evaluate the phenotype. However, it is notable that two regions of SH2, the FLVRES sequence (residues 172 to 177) and the sequence comprising positions 198 to 226, have previously been implicated in mutations that render genes host dependent for transformation (9, 42, 51). Since we show here that mutations in these regions can activate the biological and catalytic properties of normal c-*src*, it is tempting to suggest that activating mutations in SH2 might frequently be host dependent, implying an altered species-specific interaction between $p60^{src}$ and host proteins. Studies of this possibility are in progress.

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