SH2 mutants of *c-src* that are host dependent for transformation are *trans*-dominant inhibitors of mouse cell transformation by activated *c-src*

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The *c-src* gene encodes a membrane-associated protein-tyrosine kinase, pp60^{c-src}, whose substrates and regulators have not been identified. In an effort to obtain mutants that might assist the search for proteins that interact with pp60^{c-src}, we have generated by site-directed mutagenesis two alleles of chicken *c-src* that are host dependent for transformation and inhibit transformation of mouse cells by activated *c-src* in a *trans*-dominant manner. These alleles, named M6 and M9, encode nonconservative changes within the highly conserved FLVRES sequence in the *src* homology-2 (SH2) region of pp60^{c-src}, as well as an activating change, Y527F, near the carboxyl terminus. M6 and M9 transform chicken embryo fibroblasts (CEF) more efficiently than the parental allele (Y527F *c-src*), but fail to transform mouse NIH-3T3 cells. The product of M6-*src* is less stable than activated pp60^{c-src} in NIH-3T3 cells and shows decreased protein tyrosine kinase activity on all tested substrates; the product of M9-*src*, however, is stable, has a novel pattern of substrate preference for tyrosine phosphorylation in vitro, and induces a pattern of phosphotyrosine-containing proteins in mouse cells that is similar to that induced by Y527F *c-src*, even though it fails to transform these cells. Both M6 and M9 inhibit transformation of NIH-3T3 cells by activated *c-src* in a dose-dependent manner, as assayed by resistance of M6- or M9-expressing cells to transformation by Y527F *c-src* or by morphological reversion of cells previously transformed by activated *c-src* following introduction of M6 or M9. When reversion occurs, the concentration of protein encoded by the active allele declines, without change in level of *src* mRNA or rate of *src* protein synthesis, implying that the products of M6 and M9, when present at adequate levels, can destabilize transformation-competent *src* protein. These alleles offer new opportunities for interfering with the actions of *src*-related genes and for isolating cellular factors required for the functions of those genes.

[Key Words: SH2 mutants; pp60^{c-src}; M6 and M9 alleles]

Received September 17, 1990; revised version accepted October 17, 1990.

The products of viral and cellular *src* genes, pp60^{v-src} and pp60^{c-src}, are membrane-associated, tyrosine-specific protein kinases whose physiologically relevant effectors and targets have not yet been identified (for reviews, see Jove and Hanafusa 1987; Wyke and Stoker 1987). To obtain mutant *src* proteins that might exhibit altered interactions with other proteins, we and others have recently focused attention upon two components of pp60^{c-src} known as *src* homology regions 2 and 3 (SH2 and SH3; Fig. 1A) (for review, see Pawson 1988). These regions reside outside the kinase domain and are highly conserved among the so-called cytoplasmic protein tyrosine kinases (those confined to the cytoplasmic face of the plasma membrane); in addition, SH2 and SH3 are found

in several other proteins implicated in signal transduction, including phospholipase C γ , GTPase activator protein (GAP), and the product of the *crk* oncogene (Mayer et al. 1988; Stahl et al. 1988; Vogel et al. 1988).

Mutations affecting the SH2- or SH3-coding regions of *src*, *fps*, and *abl* genes have demonstrated that these domains can modulate the enzymatic, structural, and biological properties of cytoplasmic protein tyrosine kinases in complex ways (Bryant and Parsons 1982; Cross et al. 1985; Sadowski et al. 1986; DeClue et al. 1987; DeClue and Martin 1989; Franz et al. 1989; Jackson and Baltimore 1989; Verderame et al. 1989; Wang and Parsons 1989; Hirai and Varmus 1990a,b; O'Brien et al. 1990). We have recently described the production of 34 SH2 and SH3 amino acid substitution and deletion mutants of an activated form of chicken *c-src*. These alleles display a wide range of phenotypes when delivered to chicken embryo fibroblasts (CEFs) in retrovirus vectors:

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Figure 1. Mutations in the FLVRES sequence of chicken *c-src*. (A) Functional domains of pp60^{c-src} comprising a short amino-terminal sequence required for myristylation (Myr); a region (amino acids 8–88) that varies among *src*-like protein kinases; the SH3 and SH2 domains; the kinase domain; and the carboxy-terminal region containing a site for tyrosine phosphorylation (Y527) that negatively regulates kinase activity of pp60. (B) The four principal alleles of chicken *c-src* used in this study (normal *c-src*, Y527F *c-src*, M6, and M9) and the amino acids at the relevant sites in their predicted products. (C) A compilation of FLVRES-like sequences encoded by the genes of human *c-src* (Anderson et al. 1985), mouse *c-src* (Martinez et al. 1987), chicken *c-src* (Takeya et al. 1983), M6 and M9 *src* (Hirai and Varmus 1990a), *Drosophila c-src* (Simon et al. 1985), hydra *c-src* (Barnekow and Muller 1986), human *fgr* (Nishizawa et al. 1986), human *c-yes* (Sukegawa et al. 1987), mouse *c-abl* (Wang et al. 1984), chicken *c-fps* (Roebroek et al. 1985), mouse *lck* (Marth et al. 1985), human *hck* (Quintrell et al. 1987; Ziegler et al. 1987), human *lyn* (Yamanashi et al. 1987), human *fyn* (Kawakami et al. 1986; Semba et al. 1986), bovine GAP (Vogel et al. 1988), bovine phospholipase C (PLC γ ; Stahl et al. 1988), and chicken *crk* (Mayer et al. 1988; B. Mayer, pers. comm.). Nonconservative differences in naturally occurring genes are underlined, and changes in M6 and M9 *src* are P and L, respectively.

Some mutant proteins show reduced kinase activity and transforming capacity, others have enhanced activities, and others are unstable (Hirai and Varmus 1990a).

Because earlier reports from our laboratory (Varmus et al. 1981; Verderame et al. 1989) and others (Sadowski et al. 1986; DeClue and Martin 1989) suggested that some SH2 mutations are associated with host dependence for transformation, we also introduced many of our mutant alleles into NIH-3T3 mouse fibroblasts with a murine retrovirus vector (Hirai and Varmus 1990b). Several of

the SH2 and SH3 mutant genes preferentially transformed CEF or NIH-3T3 cells, generally with corresponding differences in kinase activity, implying that the putative regulatory domains influenced the behavior of pp60^{src} by interacting with host proteins that differed significantly between avian and mammalian host cells.

In our previous survey of mutants, two alleles, called M6 and M9 (Fig. 1B), displayed particularly dramatic host dependence for transformation (Table 1). Both transformed CEFs more efficiently than the parental allele, Y527F *c-src*; in fact, the activating mutation Y527F was not required for transformation of CEFs when the M6- or M9-specific SH2 mutations were present in chicken *c-src* (Hirai and Varmus 1990a). On the other hand, we failed to observe transformation of NIH-3T3 cells with either M6 or M9, even when we selected colonies of infected cells with the highest attainable levels of mutant pp60 (Hirai and Varmus 1990b).

Interestingly, both of these alleles encode nonconservative amino acid changes (F172P or R175L) in the two invariant residues in the FLVRES motif, a sequence extraordinarily well conserved in all SH2-bearing proteins (Fig. 1C). We have consequently examined the behavior of M6- and M9-encoded proteins more closely. Although both kinases are relatively inactive *in vitro* when immunoprecipitated from mouse cells, the M9 kinase induces a pattern of phosphotyrosine-containing proteins in NIH-3T3 cells similar to that induced by Y527F *c-src*, despite its inability to cause neoplastic transformation in these cells; when immunoprecipitated from mouse cells, the M9 kinase exhibits a novel spectrum of substrate preference *in vitro*. The M6 kinase is relatively inactive on all substrates when synthesized in mouse cells, although both M6 and M9 kinases are more active than that encoded by Y527F *c-src* when produced in CEFs. Moreover, both M6 and M9 can interfere with transformation of NIH-3T3 cells by activated *c-src* in a dose-dependent manner *in trans*. Inhibition of transformation is accompanied by destabilization of transformation-competent *src* protein. Thus, simple alterations in the FLVRES sequence can dramatically affect the behavior of *src* protein in different cellular environments, providing materials for novel approaches to the functions of the *src* gene family.

Results

Proteins encoded by M6 and M9 behave differently in avian and mammalian host cells

To assess the basis of the host dependence of the M6 and M9 alleles for transformation, we performed a direct comparison of the proteins encoded by these two mutants, by the activated parental allele (Y527F *c-src*), and by normal chicken *c-src*, in CEF and NIH-3T3 cells infected by avian and murine retrovirus vectors (Fig. 2; Table 1). As reported in our earlier surveys, the [³⁵S]methionine-labeled proteins encoded by both M6 and M9 accumulate in CEFs to higher concentrations in a 12-hr labeling period than do those encoded by normal and activated *c-src* (Fig. 2A, left), apparently reflecting prolonged half-lives (Hirai and Varmus 1990a). In NIH-3T3

Table 1. Biological and biochemical activities of M6 and M9 alleles of *c-src*

src allele in virus vector	Transforming activity ^a						Specific kinase activity ^c							
	focus- forming activity		colonies in soft agar		pp60 level ^b		enolase		angiotensin		casein		poly[Glu-Tyr]	
	CEF	3T3	CEF	3T3	CEF	3T3	CEF	3T3	CEF	3T3	CEF	3T3	CEF	3T3
None	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
<i>c-src</i>	<0.1	<0.1	<0.1	<0.1	2.1	2.0	0.1	0.1	0.2	0.2	0.2	0.2	0.1	0.1
Y527F <i>c-src</i>	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
M6	1.9	<0.1	1.7	<0.1	2.6	0.3	1.8	0.3	1.5	0.2	1.7	0.3	2.0	0.2
M9	1.5	<0.1	2.1	<0.1	4.0	0.9	1.2	0.2	1.4	0.5	1.3	0.7	1.6	1.1

RSV and MLV-neo vectors carrying the indicated alleles of chicken *c-src* were used to infect CEF and NIH-3T3 cells, respectively, as described in Materials and methods.

^aInfected cells were tested for transformation competence by focus formation and soft agar cloning assays as described previously (Hirai and Varmus 1990b). Results for these and other properties were normalized to 1.0 for the activated allele Y527F *c-src*.

^bLevels of pp60^{src} were determined as shown in Fig. 2.

^cThe specific kinase activities on the four indicated substrates were determined in immunoprecipitates formed with monoclonal antibody 327 as described in Materials and methods. Results of the transformation assays were reported previously (Hirai and Varmus 1990a,b) and are included here for comparison with the biochemical results, some of which are derived from repetition of experiments described in earlier papers.

cells, however, the M6 protein was less abundant and the M9 protein was no more abundant than protein encoded by Y527F *c-src* (Fig. 2A, right; Hirai and Varmus 1990b). (Similar results were obtained by the immunoblot procedure with *src*-specific antiserum; data not shown.)

As measured in a conventional immune complex assay with acid-treated enolase or pp60, itself, as substrates, the kinase activities of the mutant proteins also exhibited host dependence (Fig. 2B; Hirai and Varmus 1990a,b). Thus, both M6 and M9 proteins showed higher specific activities than did pp60^{Y527F c-src} when immunoprecipitated from CEFs, but much lower specific activities when precipitated from NIH-3T3 cells (Table 1).

The M9 kinase made in mouse cells has unusual substrate specificity in vitro and induces abundant phosphotyrosine

To explore the apparent deficiencies in specific kinase activity of M6 and M9 proteins synthesized in mouse cells, we used additional substrates [enolase, angiotensin, casein, and poly(Glu-Tyr)] in the in vitro kinase assay (Table 1) and examined the pattern of phosphotyrosine-containing proteins in cells expressing the mutant alleles (Fig. 3).

When produced in CEF, the M6 kinase shows a 1.5- to 2-fold higher specific activity than does the product of activated *c-src* on all four substrates tested; when made in NIH-3T3 cells, however, the specific activity is reduced three- to fivefold on all four substrates (Table 1). The M9 kinase is also slightly more active than activated *c-src* protein on all substrates after synthesis in CEFs. The M9 kinase from mouse cells is more selective in the use of substrates, showing strongly diminished activity with enolase (see Fig. 2B), twofold reduction with angiotensin, 30% reduction with casein, and slightly increased activity when compared with pp60^{Y527F c-src} on poly(Glu-Tyr).

As another measure of the kinase activity of the proteins encoded by M6 and M9, we used the immunoblot procedure with anti-phosphotyrosine antibodies to examine the abundance of phosphotyrosine-containing proteins in lysates of CEF and NIH-3T3 cells expressing these and control alleles (Fig. 3). As anticipated from results shown in Table 1 and Figure 2, both M6 and M9 alleles induced in CEFs an intensified version of the same pattern of phosphotyrosine proteins observed in cells transformed by Y527F *c-src*. Also, as expected from the low concentration and specific kinase activity of M6 protein in NIH-3T3 cells, little or no increase in phosphotyrosine-containing proteins was observed when mouse cells expressing M6 were compared with cells expressing normal *c-src*. Surprisingly, however, the M9 allele induced a pattern of phosphotyrosine proteins in NIH-3T3 cells with only a few differences from that induced by the transforming allele, Y527F *c-src*. Similar findings were observed with three anti-phosphotyrosine antibodies and with multiple clones and mass cultures of NIH-3T3 cells expressing M9-*src* or Y52F *c-src*, but we have not yet established the identities of those proteins that account for the few differences in the two patterns. However, the phosphotyrosine-containing proteins detected in M9-expressing NIH-3T3 cells are apparently not sufficient for transformation, although we cannot exclude a requirement for them.

The findings shown in Figure 3 emphasize the unusual nature of the protein encoded by the M9 allele. Although the M9 protein is more active than pp60^{Y527F c-src} in chicken cells and although it is stably expressed and able to induce widespread tyrosine phosphorylation in mouse cells, it still fails to cause morphological transformation of mouse cells.

M6 and M9 alleles inhibit transformation of 3T3 cells in trans

Since the M6- and M9-specific mutations in the FLVRES

Figure 2. The products of M6 and M9 alleles in CEF and NIH-3T3 cells. (A) The accumulation of [³⁵S]methionine-labeled pp60^{src} after 12 hr of metabolic labeling of CEF or G418-resistant NIH-3T3 cells infected with RSV or MLV-*neo* vectors carrying the indicated alleles. Proteins immunoprecipitated from half of each lysate with mAb 2-17 (see Materials and methods) were separated by electrophoresis in a 10% SDS-PAGE. (B) Immunoprecipitates from the remaining half lysate were tested for protein kinase activity by incubation with acid-denatured enolase and [γ -³²P]ATP before SDS-PAGE. The positions of enolase and autophosphorylated pp60 are indicated. Uninfected cells and c-*src*- or Y527F c-*src*-infected cells are included as controls.

sequence inhibited in *cis* the competence of Y527F c-*src* to transform NIH-3T3 cells, it seemed possible that the M6 and M9 alleles might also exhibit a *trans*-dominant effect upon the transformation competence of activated c-*src*.

Cells expressing M6 or M9 are resistant to transformation We first asked whether the production of M6- or M9-encoded *src* proteins rendered NIH-3T3 cells resistant to transformation by the activated c-*src* allele, Y527 c-*src*, present in a superinfecting MLV vector (Table 2). Clones of NIH-3T3 cells were derived after infection with MLV-*gpt* vectors carrying the following c-*src* al-

leles; M6, M9, normal c-*src*, activated c-*src*, and two nontransforming alleles with deletions encompassing part or all of the FLVRES sequence [residues 93–224 (D14) and residues 166–175 (D6)]. Most clones used for the superinfection experiment contained amounts of pp60 equal to or greater than that determined previously to be just over the threshold required for transformation of NIH-3T3 cells by Y527F c-*src* (denoted 1.0 in Table 2; see Hirai and Varmus 1990b). However, the highest observed levels of M6 protein and of protein encoded by the D14 mutant were only about half this value.

Following superinfection with MLV-*neo* carrying activated c-*src*, G418-resistant colonies were observed for their morphologic phenotype. From microscopic evaluation of several hundred colonies from each superinfected culture, it was apparent that the cell lines expressing M6 and M9 were relatively resistant to transformation by activated c-*src* (Table 2). Whereas MLV carrying activated c-*src* transformed approximately two-thirds (60–72%) of control cells (normal NIH-3T3 cells or cells expressing normal c-*src* or the two deletion mutants), less than one third (28–31%) of the superinfected cells expressing M6 or M9 were transformed. When the experiment was repeated with lines containing two- or threefold higher levels of M9-encoded protein, the resistance to transformation increased slightly, with only 19% of the G418-resistant colonies being transformed at the highest level. In contrast, cells expressing more normal pp60^{c-*src*} were transformed with equal or slightly higher efficiencies. Superinfection of cells already transformed by Y527F c-*src* produced, as expected, antibiotic-resistant colonies that were nearly uniformly transformed.

Figure 3. Phosphotyrosine-containing proteins from CEF and NIH-3T3 cells expressing M6 and M9 alleles. Lysates of uninfected CEF and NIH-3T3 cells and cells infected with RSV or MLV-*neo* vectors bearing the indicated alleles of c-*src* were subjected to SDS-PAGE, transferred to nitrocellulose, and probed with affinity-purified, polyclonal anti-phosphotyrosine antibody (provided by J. Wang; see Materials and methods). Uninfected and c-*src*- or Y527F c-*src*-infected cells are included as controls. Molecular size standards are indicated in kilodaltons.

Table 2. *M6 and M9 alleles confer resistance to transformation*

		<i>src</i> allele present before superinfection						
		none	<i>c-src</i>	Y527F <i>c-src</i>	M6	M9	D6	D14
(A) $0.1 \leq \text{pp60} \leq 1$	pp60 level	0.1	1.0	1.0	0.5	1.0	1.0	0.5
	neo ^R clones	477	449	423	391	416	425	432
	transformed	286	324	408	109	129	298	273
	transformed (%)	60	72	96	28	31	70	63
(B) $\text{pp60} = 2.0$	neo ^R clones		380	404		387	412	
	transformed		292	402		93	327	
	transformed (%)		77	>99		24	79	
(C) $\text{pp60} = 3.0$	neo ^R clones		430	415		417		
	transformed		345	414		79		
	transformed (%)		80	>99		19		

Clones of NIH-3T3 cells infected previously with MLV-gpt vectors carrying the indicated alleles of *c-src* were superinfected with an MLV-neo vector containing Y527F *c-src*. The levels of pp60 expressed (relative to a level of protein, 1.0, the minimum sufficient for transformation by Y527F *c-src*) are listed for each experiment. The indicated number of G418-resistant (neo^R) colonies, selected as described in Materials and methods, were scored by microscopy for morphological transformation. The 0.1 pp60^{src} level in cells infected with MLV-gpt without a *src* allele (experiment A) was endogenous mouse pp60^{c-src}. Codons 166–175 are deleted from Y527F *c-src* in the D6 allele, and codons 93–226 are deleted from Y527F *c-src* in D14.

Introduction of M6 or M9 reverts transformed cells to normal morphology The results of the superinfection experiment presented in Table 2 suggested that the M6- and M9-encoded proteins could prevent transformation by activated *c-src* in a dose-dependent manner. To corroborate this finding with a different experimental strategy, we asked whether the M6 and M9 alleles could revert the morphological phenotype of cells transformed by activated *c-src* (Table 3). We therefore superinfected NIH-3T3 lines containing different amounts of pp60^{Y527F c-src} with MLV-neo vectors carrying the same set of *src* alleles tested previously for their ability to confer resistance to transformation. Antibiotic-resistant colonies were then examined for morphologic evidence of partial or complete reversion, as manifested by flattening of the cells and loss of refractility (Fig. 4).

Using a transformed cell line with levels of activated *c-src* protein just above the threshold value of 1.0, we observed reversions of 35% and 40%, respectively, of the colonies obtained after infection with MLV-neo carrying M6 and M9 alleles. In contrast, <10% of the colonies in any of the other cultures displayed loss of the transformed phenotype by morphologic criteria, and these changes tended to be less pronounced than in the cells exposed to M6 and M9 viruses. When the experiment was performed with cells containing higher levels of pp60^{Y527F c-src}, the frequency of reversion was reduced to 17% and 21%, respectively, with activated *c-src* protein at the 2.0 level (controls 5% or less), and to apparently insignificant numbers (both M6 and M9 alleles at 4%) at the 3.0 level (controls 2% or less). Thus, in the reversion test, as well as in the test of resistance to transformation, both the M6 and M9 alleles appeared to interfere with transformation by activated *c-src* in a dose-dependent fashion. We conclude from these experiments that the M6 and M9 mutants suppress transformation by Y527F *c-src* in a *trans*-dominant negative manner.

Inhibition of transformation by M6 or M9 destabilizes protein encoded by a transforming allele of c-src

To define more rigorously the role of *src* protein concentrations in the observed *trans*-dominant effects, we repeated the reversion analysis with an activated *c-src* protein that can be distinguished physically from the products of M6 and M9. In previous experiments we showed that the D11 deletion mutant of Y527F *c-src*, lacking amino acids 93–143, can transform NIH-3T3 cells with efficiencies similar to those observed for the parental allele, even though it has poor transforming capacity in CEFs, where the protein is relatively unstable (Hirai and Varmus 1990a,b). Because the product of the D11 mutant is ~5 kD smaller than pp60^{c-src}, it is readily separated from the products of M6 and M9 mutants by polyacrylamide gel electrophoresis (PAGE).

A clone of NIH-3T3 cells transformed by MLV-gpt carrying the D11 allele was superinfected with MLV-neo vectors containing M6 or M9 alleles. In addition, cells expressing M6 or M9 alleles were superinfected with MLV-gpt carrying the D11 allele. Several clones of cells displaying either a persistently transformed or morphologically flat phenotype, like those illustrated in Figure 4, were isolated and expanded for biochemical tests. Immunoprecipitation after metabolic labeling for 12 hr with [³⁵S]methionine showed that levels of M6 and M9 proteins were modestly but consistently higher in flat than in transformed cells (Fig. 5A). Levels of the active 55-kD protein encoded by the D11 allele, on the other hand, were dramatically decreased in the morphologically normal cell lines. [A variable amount of a smaller protein was also observed in lines expressing M6 or M9; it has not been proved, but is assumed that this is a degradation product of *src* protein, similar to 52-kD product of pp60^{v-src} described previously (Levinson et al. 1980)].

We performed two kinds of experiments to show

Table 3. M6 and M9 alleles of c-src can revert NIH-3T3 cells transformed by activated c-src

		src allele in superinfecting virus						
		none	c-src	Y527F c-src	M6	M9	D6	D14
(A) pp60 = 1.0	neo ^R clones	920	427	398	382	405	411	393
	transformed	860	391	390	227	224	377	367
	partially flat	28	27	6	89	107	21	17
	flat	4	12	2	45	57	8	4
	reverted (%)	3	9	2	35	40	7	5
(B) pp60 = 2.0	neo ^R clones	880	452	423	403	391	428	415
	transformed	802	428	420	326	301	409	400
	partially flat	4	16	2	48	59	14	12
	flat	4	8	1	21	23	5	3
	reverted (%)	1	5	1	17	21	4	4
(C) pp60 = 3.0	neo ^R clones	960	431	407	377	386	402	423
	transformed	956	420	406	361	369	395	415
	partially flat	4	8	1	11	10	5	7
	flat	0	3	0	5	7	2	1
	reverted (%)	<1	2	<1	4	4	2	2

NIH-3T3 cells transformed by infection with an MLV-gpt vector carrying Y527F c-src and expressing three levels of pp60 (1.0 for experiment A, 2.0 for experiment B, and 3.0 for experiment C) were superinfected with MLV-neo vectors carrying the indicated alleles of c-src (see legend to Table 2). G418-resistant colonies were scored by light microscopy for partial or complete restoration of the normal NIH-3T3 morphological phenotype.

whether the unexpectedly low level of D11 src protein in flat cells was caused by regulation of mRNA levels, src protein synthesis, or protein stability. First, we measured steady-state concentrations of src mRNA in transformed and flat cells (Fig. 6). Radiolabeled probes for the bacterial genes (*neo* and *gpt*) present in the two MLV vectors distinguished between the vector-length RNAs that serve as mRNAs for D11 protein and for M6 or M9 proteins. [The probes also detect smaller spliced or alternatively initiated mRNAs; these are more abundant from the MLV-*neo* vector, which contains an internal herpes simplex virus thymidine kinase (HSVtk) promoter.] We observed little or no differences in the abundance of D11 src mRNA in coinfecting lines that did and did not display a transformed phenotype. Thus, we cannot explain by changes in mRNA concentration the marked decline in D11 src protein in flat cell lines, whether obtained by reversion or by resistance to transformation. Likewise, similar amounts of M6 and M9 src mRNAs were found in all clones, although we might not have detected the relatively subtle differences required to explain the differences in M6 and M9 protein levels seen in Figure 5A.

To examine the rate of src protein synthesis in flat and transformed lines, proteins were labeled with [³⁵S]methionine for 1 hr, rather than 12 hr, and subjected to immunoprecipitation and PAGE as before (Fig. 5B). Under these conditions, the amounts of labeled D11 protein are very similar in cell lines with either phenotype, indicating that altered rates of synthesis do not account for depressed accumulation during a prolonged labeling period. As expected, the rates of synthesis of M6 and M9 proteins also appear nearly invariant among the lines. We conclude that the reduction in the active D11 protein can be ascribed to protein instability in the presence

of a critical level of M6 and M9 protein and that reduced levels of the transformation-competent protein contribute to the dominant-negative effect.

Discussion

Amino acid substitutions in an especially well-conserved portion of the SH2 domain of a transformation-competent mutant of chicken pp60^{c-src} are encoded by alleles M6 and M9 that are host dependent for transformation and capable of inhibiting transformation of NIH-3T3 cells by activated c-src. It is striking that the proteins encoded by M6 and M9 behave differently, both biochemically and biologically, in the environments of CEF and in an established line of mouse fibroblasts and interfere in *trans* with the oncogenic function and stability of a nearly identical transforming protein in the mouse cells. Indeed, it seems very likely that the phenomena of host dependence and dominant-negative behavior are closely related, perhaps mediated through interactions between the src gene products and cellular protein(s).

The FLVRES motif that has been altered by mutation in the M6 and M9 alleles does not vary at all between mammalian and avian versions of c-src (Fig. 1C), and there are few amino acid differences throughout the SH2 regions; hence, the greater activity exhibited in CEF by the products of these alleles cannot be attributed simply to their avian origin. This is supported by studies of other alleles of chicken c-src, most with mutations in SH3, that encode proteins more active in mouse NIH-3T3 cells than in chicken embryo cells (Hirai and Varmus 1990b). (The D11 mutant used for the experiment shown in Figs. 5 and 6 is an example of such a host-dependent allele.) Nevertheless, it will be of ob-

Figure 4. M6 and M9 alleles can revert the phenotype of NIH-3T3 cells transformed by Y527F *c-src*. MLV-*neo* vectors carrying M6 or M9 were used to infect NIH-3T3 cells transformed by threshold levels of pp60^{Y527F}*c-src*. After selection with G418, colonies with reverted and persistently transformed phenotypes were photographed under light microscopy at 100 $\times$ (bottom). As controls, uninfected cells and colonies of cells infected with the indicated *c-src* alleles are also shown (top). (For further details, see Table 3.)

vious interest to make lesions that are analogs of those in M6 and M9 in mammalian *c-src* genes to look for host-dependent effects.

We have generated and initially tested all of our SH2 and SH3 mutants in the background of a *c-src* gene activated by the Y527F mutation, and we have yet to ask whether the mutation at position 527, normally a site for an inhibitory tyrosine phosphorylation, is required for the *trans*-dominant effect. Similarly, we do not know which regions of the proteins encoded by M6 and M9 must be retained for this effect; additional mutations that remove the variable, SH3, and kinase regions or more precisely, inactivate the catalytic function of M6- or M9-encoded pp60, will need to be tested to address this question. Although preliminary experiments (H. Hirai, unpubl.) indicate that the M6 and M9 proteins are associated with membranous components of NIH-3T3 cells, more rigorous localization of the proteins and mutagenesis to eliminate membrane association will be required to judge the effects of the intracellular position of pp60 on the phenotypes that we have observed.

Reversion and prevention of Y527F *c-src*-mediated transformation by M6 and M9 are incomplete and dose dependent, suggesting that a critical ratio of the products of the dominant-negative and activated allele is necessary for the nontransformed phenotype (Tables 2 and 3) and for destabilization of the transforming protein (Figs. 5 and 6). This result is consistent with the idea that two closely related proteins are competing for some limited host factor essential for transformation by *src*. However, we have yet to examine carefully the possibility that the M6 and M9 alleles can interfere with *c-src* functions other than transformation by the Y527F allele, with other oncogenes or proto-oncogenes in the *src* gene

family, or with other proteins containing SH2 domains. We have thus far been unable to revert the phenotype of NIH-3T3 cell lines transformed by *v-src*, polyoma middle T antigen, or *v-Ha-ras* by superinfection with vectors carrying M6 or M9 (H. Hirai, unpubl.), but we cannot say whether these failures should be attributed to the relative strengths of these oncogenes (compared with Y527F *c-src*), to unfavorable ratios of mutant to transforming proteins in the cell lines, or to an inherent inability of the M6 or M9 genes to act as dominant-negative alleles in these settings.

Although M6 and M9 are the first alleles of *src* to display dominant-negative phenotypes, several oncogenes and related genes have recently been reported to have a variety of *trans*-inhibitory effects. (1) Most closely related to the phenomena described here is the dose-dependent inhibition of p21^{v-Ki-ras}-mediated transformation of rat cells by overexpression of a human cDNA clone encoding the 21-kD product of *Krev-1* (*rap1A*), a gene belonging to the *ras* family (Kitayama et al. 1989). (2) Inherited alleles of the mouse *c-kit/W* gene with mutations in the kinase domain can produce abnormal phenotypes in the presence of a normal allele (Reith et al. 1990). (3) Mutant alleles of the p53 locus, a tumor suppressor gene, appear to contribute to oncogenesis or transformation by interference with normal p53 (Finlay et al. 1989). (4) The product of the *v-erbA* oncogene blocks the normal repressive function of the thyroid hormone-responsive transcription factor encoded by the *c-erbA* proto-oncogene (Damm et al. 1989).

The explanation of such dominant-negative effects is believed to depend on formation of inactive multimers of mutant and wild-type proteins or on competition between mutant and wild-type proteins for some essential

Figure 5. Trans-dominant inhibition of transformation by M6 or M9 alleles destabilizes active c-src protein in NIH-3T3 cells. Experiments analogous to those described in Tables 2 and 3 were performed with the SH3 deletion mutant, D11 (Y527F c-src lacking codons 93–143), as an active allele in the MLV-*gpt* vector and with M6 or M9 alleles in the MLV-*neo* vector. Flat (F) and transformed (T) cells obtained from these experiments were grown to mass culture, metabolically labeled with [³⁵S]methionine for 12 hr (top) or 1 hr (bottom), and analyzed by immunoprecipitation with mAb 2-17 and SDS-PAGE. Arrows indicate the positions of products of the M6 and M9 alleles (p60) and of the D11 allele (p55). (F) flat cell phenotype; (T) transformed cell phenotype. The order of the alleles over each lane indicates the order in which they were added to produce each cell line.

target (e.g., an enzymatic substrate or binding element; Herskowitz 1987). Thus, the *v-erbA* and *c-erbA* proteins may compete for DNA-binding sites in several enhancer elements, and mutant *kit* proteins may inactivate wild-type proteins when oligomers form in response to physiologic ligands. Because *src* proteins are believed to remain monomeric in cells (Levinson et al. 1980; Brugge 1986), it is likely that the mechanism of interference involves other proteins that interact with pp60^{src}. Many candidates may be considered, including proteins that modify pp60^{src} (e.g., by phosphorylation or myristylation) or serve as substrates for the protein-tyrosine kinase activity of pp60^{src} (for review, see Cooper 1990), factors that may localize pp60^{src} to cell membranes or the cytoskeleton (Resh 1989; Kaplan et al. 1990), proteins (hsp90 and an unidentified protein of 50 kD) known to form a transient complex with pp60^{v-src} (Opperman et al. 1981; Brugge 1986), and other proteins in

the signal transduction pathway containing SH2 domains (Stahl et al. 1988; Vogel et al. 1988).

Because it is likely that the dominant-negative and host-dependent effects of M6 and M9 are related, it may be possible to identify the host component for which activated pp60^{c-src} and the products of M6 and M9 compete by exploiting the host-dependent properties. For example, the relevant gene(s) could be isolated from NIH-3T3 cells transfected with chicken DNA that permit transformation by M6 or M9. Alternatively, it might be possible to modify the kinase activity of M6 or M9 proteins in vitro with extracts of chicken or mouse cells; factors responsible for such modifications might prove to be important for the dominant-negative effects.

We consider it probable that the avian and mammalian protein(s) responsible for the host-dependent and dominant-negative properties of the M6 and M9 alleles are homologous proteins that normally interact with *src* (and perhaps *src*-related) proteins to stabilize them and influence their specific kinase activities. Although *src* proteins with a normal SH2 domain do not usually differentiate between the avian and mammalian homologs of the hypothetical protein(s), the FLVRES mutants encoded by M6 and M9 are able to make this subtle dis-

Figure 6. Reversion of transformation by M6 or M9 alleles does not alter levels of *src* mRNA encoding a transformation-competent protein. Whole-cell polyadenylated RNA was prepared from the cell lines described in the legend to Fig. 5, subjected to electrophoresis in agarose gels, and probed for species containing D11-*src* with a ³²P-labeled probe for *Escherichia coli gpt* (top) and for species containing M6- and M9-*src* with a probe for *neo*^R (bottom); arrows indicate the expected positions of the relevant RNAs. (F) Flat cell phenotype; (T) transformed cell phenotype.

crimination. According to this view, M6 and M9 proteins should interact with the mammalian homolog, but the resulting kinase activity is either low (in the case of M6) or altered with respect to substrate specificity (in the case of M9). Presumably, successful competition for the homolog by M6- or M9-encoded proteins can displace the product of Y527F *c-src* and thereby account for its instability under conditions that produce dominant-negative effects.

Materials and methods

Plasmids

M6 (F172P/Y527F *c-src*) and M9 (R175L/Y527F *c-src*) mutations were generated by site-directed mutagenesis as described (Kunkel 1985). Normal *c-src*, Y527F *c-src*, D6 (del 166–175/Y527F *c-src*), D11 (del 93–143/Y527F *c-src*), and D14 (del 93–226/Y527F *c-src*) were also used in the analyses. All alleles were inserted into a replication-competent Rous sarcoma virus (RSV) vector (RCAS) for infection of CEFs and MLV-derived retroviral vectors, ZAS, carrying a *gpt* marker gene (MLV-*gpt*), and MV7, carrying a *neo* gene (MLV-*neo*), for infection of NIH-3T3 cells (Kunkel 1985; Housey 1988; Hirai and Varmus 1990a,b).

Transfection and biological analysis

Transfection and biological assays for CEFs were performed as described (Hirai and Varmus 1990a). ZAS or MV7 plasmid DNAs were transfected into Ψ 2 packaging cells, with or without pSV2-*neo* DNA, respectively, and transfected cells were selected with medium containing 400 μ g/ml of G418 (GIBCO). Culture media were harvested as virus stocks. NIH-3T3 cells were infected with these viruses and selected with *gpt*-selective medium containing mycophenolic acid (20 μ g/ml), xanthine (250 μ g/ml), and hypoxanthine (14 μ g/ml) for MLV-*gpt* vectors or with G418-containing medium for MLV-*neo* vectors. Biological assays for infected or uninfected NIH-3T3 cells were described previously (Hirai and Varmus 1990b). For superinfection experiments, some cell clones expressing an indicated level of mutant *src* protein were infected with *neo* viruses, and transformed or flat colonies were scored in medium with G418. p60 levels were determined relative to the threshold level for transformation by parental Y527F *c-src* (arbitrarily defined as 1.0; Hirai and Varmus 1990b).

Immunoprecipitation and p60 level

Fully infected CEFs and infected and selected NIH-3T3 cells were labeled with [35 S]methionine in 2 ml of methionine-free, Dulbecco-modified Eagle medium containing 10% dialyzed fetal calf serum and 400 μ Ci of [35 S]methionine. Labeled cells were lysed in RIPA buffer [150 μ M NaCl, 20 μ M 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 and total cell protein in each lysate (Kmiecik et al. 1987). Immunoprecipitation of p60^{c-src} from lysates containing equal amounts of TCA-precipitable radioactivity were performed using an excess of monoclonal antibody (mAb) 2-17 (obtained from Microbiological Associates), which recognizes residues 2-17 of *c-src*. Immune complexes were adsorbed to formalin-fixed *Staphylococcus aureus* (Pansorbin; Calbiochem-Behring), and labeled proteins in the complexes were resolved by 10%

SDS-PAGE and analyzed by autoradiography. The amounts of p60^{c-src} per milligram of total cell protein were determined from the scintillation counts of gel bands and efficiency of metabolic labeling (Kmiecik et al. 1987). The data for levels of p60^{c-src} were confirmed by immunoblotting analysis with 125 I-labeled protein A. Lysates from cells were equalized for protein concentration, loaded onto 10% polyacrylamide gels, and transferred to nitrocellulose. Filters were blocked with milk and probed with mAb 327 (provided by J. Brugge), followed by rabbit anti-mouse immunoglobulin (DAIKO) and 2 μ Ci of 125 I-labeled protein A (38 mCi/ml; Amersham), and analyzed by autoradiography.

In vitro protein kinase assay

Immune complexes from lysates containing equal amounts of TCA-precipitable radioactivity obtained as above were incubated in kinase buffer [20 mM Tris (pH 7.2), 10 mM MgCl₂, 0.1% Triton X-100] for 10 min at room temperature with 2 μ g of acid-denatured rabbit muscle enolase (Sigma), 10 μ g of casein (Sigma), 10 μ g of poly(Glu-Tyr) (Sigma), or 5 μ g of angiotensin (Sigma), and 1 μ Ci [γ - 32 P]ATP (3000 Ci/mmol; Amersham). The amounts of [32 P]enolase and [32 P]casein radioactivity were determined by scintillation counting of excised bands after electrophoresis in 10% SDS-PAGE. For poly (Glu-Tyr) and angiotensin phosphorylation, a part of the kinase reaction mixture was spotted onto DE-81 paper, washed with phosphate buffer, and counted. The specific kinase activities for these exogenous substrates were calculated in conjunction with the determined amount of exogenous pp60^{c-src} after correction for any detectable endogenous pp60^{c-src}.

In vivo phosphotyrosine analysis

Lysates from infected CEF cells and selected NIH-3T3 cells were equalized for protein concentration, separated on a 10% SDS-PAGE, transferred to nitrocellulose, blocked with bovine serum albumin, and then probed with affinity-purified polyclonal antiphosphotyrosine antibody (provided by J. Wang and M. White) or monoclonal antiphosphotyrosine antibody (provided by D. Morrison) followed by alkaline phosphatase-coupled second antibody (Promega).

Northern blotting

Total RNA was prepared from cells according to the method described by Kaufmann and Sharp (1982), and poly(A) RNA was selected by oligo(dT)-cellulose. One microgram of poly(A) RNA was electrophoresed on a 1.1% agarose gel and analyzed by Northern hybridization (Church and Gilbert 1984) with 32 P-labeled *gpt* DNA (0.9-kb *Xho*I–*Hind*III fragment) or *neo* DNA (0.9-kb *Pst*I fragment).

Acknowledgments

We thank D. Shalloway, H. Hanafusa, and S. Hughes for molecular clones; M. White, J. Wang, and D. Morrison for phosphotyrosine antibodies; and D. Morgan, C. Lowell, and D. Kaplan for their helpful discussions and comments on the manuscript. This work was supported by a grant from the National Institutes of Health; H.H. was supported by a grant from the Leukemia Society of America; H.E.V. is an American Cancer Society Research Professor.

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Genes Dev. 1990, **4**:

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