MOLECULAR AND CELLULAR BIOLOGY, Feb. 1988, p. 704–712
0270-7306/88/020704-09$02.00/0

Vol. 8, No. 2

v-*src* Mutations Outside the Carboxyl-Coding Region Are Not Sufficient To Fully Activate Transformation by pp60$^{c\text{-}src}$ in NIH 3T3 Cells

SITA REDDY, PETER YACIUK, THOMAS E. KMIECIK,† PAUL M. COUSSENS,‡ AND DAVID SHALLOWAY*

Department of Molecular and Cell Biology, The Pennsylvania State University, University Park, Pennsylvania 16802

Received 17 September 1987/Accepted 12 November 1987

Previous studies have shown that carboxyl-terminal mutation of pp60$^{c\text{-}src}$ can activate its transforming ability. Conflicting results have been reported for the transforming ability of pp60$^{c\text{-}src}$ mutants having only mutations outside its carboxyl-terminal region. To clarify the effects of such mutations, we tested the activities of chimeric v(amino)- and c(carboxyl)-*src* (v/c-*src*) proteins at different dosages in NIH 3T3 cells. The focus-forming activity of Rous sarcoma virus long terminal repeat (LTR)-*src* expression plasmids was significantly reduced when the v-*src* 3′ coding region was replaced with the corresponding c-*src* region. This difference was masked when the Rous sarcoma virus LTR was replaced with the Moloney murine leukemia virus LTR, which induced approximately 20-fold more protein expression, but even focus-selected lines expressing v/c-*src* proteins were unable to form large colonies in soft agarose or tumors in NFS mice. This suggests that pp60$^{c\text{-}src}$ is not equally sensitive to mutations in its different domains and that there are at least two distinguishable levels of regulation, the dominant one being associated with its carboxyl terminus. v/c-*src* chimeric proteins expressed with either LTR had high in vitro specific kinase activity equal to that of pp60$^{v\text{-}src}$ but, in contrast, were phosphorylated at both Tyr-527 and Tyr-416. Total cell protein phosphotyrosine was enhanced in cells incompletely transformed by v/c-*src* proteins to the same extent as in v-*src*-transformed cells, suggesting that the carboxyl-terminal region may affect substrate specificity in a manner that is important for transformation.

pp60$^{v\text{-}src}$ neoplastically transforms a wide variety of animal cells, but its cellular counterpart pp60$^{c\text{-}src}$ does not, even when expressed at levels 10 times higher than the amount of pp60$^{v\text{-}src}$ required for transformation (9, 12, 22, 26). This may be due to the fact that pp60$^{c\text{-}src}$ has significantly lower protein tyrosine kinase activity than pp60$^{v\text{-}src}$ has (4, 8). In addition to 8 single amino acid substitutions (for Schmidt-Ruppin A [SRA] strain of v-*src*), the two proteins diverge completely just downstream of their catalytic domains, the last 19 amino acids in pp60$^{c\text{-}src}$ being replaced by 12 different amino acids in pp60$^{v\text{-}src}$ (30). It seems likely that the carboxyl-terminal exchange occurred during the transduction of v-*src*, whereas the point mutations occurred during viral evolution (10, 29, 30).

pp60$^{c\text{-}src}$ can be divided functionally into an amino domain, a catalytic domain extending roughly from amino acids 260 to 517, and a carboxyl-terminal regulatory region (1, 17, 33; reviewed by J. Cooper, *in* B. Kemp and P. F. Alewood, ed., *Peptides and Protein Phosphorylation*, in press). The catalytic domain is homologous to similar domains in other tyrosine kinases and protein kinase A (for a review, see reference 7). The modified carboxyl terminus of pp60$^{v\text{-}src}$ is not required for pp60$^{v\text{-}src}$ kinase and transforming activity; its significance is that it disables negative regulation by the pp60$^{c\text{-}src}$ carboxyl terminus (33). Deletion of this region from pp60$^{c\text{-}src}$ stimulates kinase and transforming activities in both NIH 3T3 and chick embryo fibroblasts (CEFs) (2, 24a; P. Yaciuk and D. Shalloway, submitted for publication). Negative regulation by this region is associated with phosphorylation of Tyr-527, a residue 6 amino acids from the carboxyl end (2, 15, 24). However, other regions of the protein must also regulate pp60$^{c\text{-}src}$, since mutants containing Phe in place of Tyr-527 must be expressed at relatively high levels to induce complete transformation and have only 40% of the in vitro kinase activity of pp60$^{v\text{-}src}$ (15).

pp60$^{v\text{-}src}$ tyrosine kinase activity is enhanced by proteolytic separation of its amino and carboxyl regions (1, 17). This suggests that, like protein kinase G (6), the pp60^{src} amino region may modulate the catalytic activity of its carboxyl domain. Whether the amino region mutations found in pp60$^{v\text{-}src}$ can fully activate pp60$^{c\text{-}src}$ in the absence of carboxyl-terminal mutations is unclear, since chimeric v(amino)- and c(carboxyl)-*src* (v/c-*src*) proteins have been shown to cause complete transformation of CEFs (9, 32) but not of NIH 3T3 cells (25). It is not known whether these differences result from the use of different viral strains, expression systems, or cell types.

Here we investigate the activities of chimeric *src* proteins in NIH 3T3 cells in more detail to clarify these disparate results and to better evaluate the functional significance of the v-*src* amino-region mutations. We constructed chimeric v/c-*src* genes containing viral strain SRA, which was used in the CEF experiments, and expressed them with two different transcriptional promoter systems to permit functional comparisons at different protein dosages. Our results indicate that the v-*src* mutations outside the carboxyl-terminal coding region partially activate c-*src* without inducing a completely transformed phenotype in NIH 3T3 cells and that the suppression of transformation by the c-*src* carboxyl-terminal coding region is most evident at lower levels of protein expression. Differences in biological activities between the v-*src* and v/c-*src* proteins did not correlate with in vitro

* Corresponding author.
† Present address: BRI, NCI-FCRF, P.O. Box B, Bldg. 469, Frederick, MD 21701.
‡ Present address: Department of Animal Science, 210-D Anthony Hall, Michigan State University, East Lansing, MI 48824.

specific kinase activity or total cell protein phosphotyrosine but did correlate with differences in Tyr-416 and Tyr-527 in vivo phosphorylation.

MATERIALS AND METHODS

Plasmid constructions. Plasmids pRS2, pMv*src*, and pMc*src* have been previously described (12, 25). Other plasmids were constructed by standard recombinant DNA techniques and verified by restriction map analysis (20). *Bam*HI-*Bgl*II fragments (3.1 kilobase pairs [kbp]) encoding the v/c-*src* sequences from pBB4 and pPB5 (9) (gifts from H. Hanafusa) and a 7-kbp *Bam*HI-*Bam*HI c/v-*src* fragment from pRS3 (26) were separately inserted at the unique *Bgl*II site in expression vector pEVX (16) to make pMBB4, pMPB5, and pMRS13, respectively.

pRS4 has been previously described (33). pRS18 was made by replacing the 282-base-pair (bp) *Bgl*I-*Hin*dIII pRS4 fragment containing the *src* 3′ coding sequence with a 462-bp *Bgl*I-*Hin*dIII pMBB4 fragment containing the c-*src* 3′ coding sequences. pRS20 was created from pRS18 in a two-step process by replacing the 820-bp *Bgl*II-*Hpa*I fragment containing sequences downstream from the $pp60^{src}$ termination codon with a 2.4-kbp pBB4 *Bgl*II-*Nae*I fragment containing a Rous sarcoma virus long terminal repeat (RSV LTR) and *gag-env* sequences and then by deleting the 934-bp *gag-env* *Xho*I-*Sal*I fragment. pRS21 was constructed by introducing an 84-bp pMPB5 *Pst*I-*Hin*dIII fragment encoding the 19 carboxyl-terminal amino acids of $pp60^{c\text{-}src}$ between the homologous *Pst*I site and the downstream *Hin*dIII site in pRS4.

pRv*src*A, pRc*src*, pRBB4, and pRPB5 were constructed by inserting the approximately 2-kbp *Nco*I-*Bgl*II fragments containing the *src* coding sequences from pMv*src*, pM5 HHB5, pMBB4, and pMPB5, respectively, between the homologous *Nco*I and *Bgl*II sites of pRS21. pRcv*src*A was constructed by replacing the 3-kbp pRc*src* *Bgl*I-*Bgl*I fragment containing the c-*src* 3′ coding region with the homologous pRv*src*A fragment.

Biological assays. NIH 3T3 cells were grown, transfected, and tested for anchorage-independent growth and tumorigenicity in adult NFS mice as previously described (12). All plasmids were linearized at unique *Nru*I sites located outside the eucaryotic regions shown in Fig. 1 before transfection. Quantitative focus-forming activities were determined in transfections with 20 μg of calf thymus carrier DNA plus 0.002 μg (for *src* expression plasmids containing the Moloney murine leukemia virus [MoMLV] LTR) or 0.1 μg (for *src* expression plasmids containing the RSV LTR) of linearized plasmid DNA per 60-mm tissue culture plate. Pilot experiments showed that these amounts induced numbers of foci in the linear response range of 0 to 100 per plate.

NIH(pMv*src*/focus)A and NIH(pMc*src*/focus)B focus-selected cell lines have been previously described (12). All other focus-selected cell lines were cylinder cloned. Mass cultures of G418-coselected cells expressing $pp60^{src}$ were created by cotransfecting 0.003 pmol of the *src* plasmids with either 0.015 or 0.03 pmol of pSV2*neo* (28) and pooling approximately 50 to 100 G418-resistant colonies as previously described (33).

Biochemical analyses. $pp60^{src}$ was immunoprecipitated in antibody excess with monoclonal antibody 327 (which reacts with $pp60^{c\text{-}src}$ and $pp60^{v\text{-}src}$) (19) or EC10 (which reacts only with $pp60^{v\text{-}src}$ and chicken but not rodent $pp60^{c\text{-}src}$) (23) from cells labeled for 16 h with $^{32}P_i$ or for 48 h with [^{35}S]methionine as previously described (33). Expression level and specific protein kinase activity measurements, partial *Staphylococcus aureus* V8 protease digests, and tryptic phosphopeptide analyses were performed as previously described (15). Total cell phosphoamino acid content was measured as described by Cooper et al. (3) by 2-h acid hydrolysis after 16 h of $^{32}P_i$ metabolic labeling. Relative $pp60^{src}$ in vivo turnover rates were determined by comparing the amounts of radioactivity present in the $pp60^{src}$ immunoprecipitated from lysates containing equal amounts of trichloroacetic acid-precipitable radioactivity (after correction for endogenous $pp60^{c\text{-}src}$) after short (4-h) and long (25-h) [^{35}S]methionine-labeling periods as described by Yaciuk and Shalloway (33).

RESULTS

We have previously shown that the frequency of focus formation induced by transfection of NIH 3T3 cells with pRS2, a plasmid expressing a chimeric v/c-*src* protein containing the 431 amino residues of Schmidt-Ruppin D (SRD) $pp60^{v\text{-}src}$ and 93 carboxyl amino acids of $pp60^{c\text{-}src}$ under transcriptional control of the RSV LTR (Fig. 1), is 200-fold lower than that of p*src*11, a v-*src* expression plasmid of similar construction (25). This result was in contrast with those of Iba et al. (9) and Wilkerson et al. (32), who found that a retrovirus expressing a similar chimeric protein but with the SRA $pp60^{v\text{-}src}$ sequence had the same transforming activity as that of RSV in CEFs. To be certain that the reduced transforming activity of pRS2 relative to that of p*src*11 was not due to differences in the region downstream from the *src* termination codon, we constructed plasmids pRS20 and pRS18, which, respectively, have an RSV LTR (identical to that found in p*src*11) or a simian virus 40 early region polyadenylation site immediately downstream from the v/c-*src* coding sequence (Fig. 1A). Both constructs had very low focus-forming activity (Table 1). In addition, pRS21, a v/c-*src* plasmid in which just the last 12 codons of v-*src* were replaced by the 19 carboxyl-terminal codons of c-*src*, also had low focus-forming activity. In contrast, as previously shown (33), pRS4, a plasmid of parallel construction having a randomly altered v-*src* carboxyl-coding region, had high focus-forming activity.

To see whether these differences in the v/c-*src* chimera transforming potential observed by us and by Iba et al. (9) were due to the use of different viral strains, we inserted the v-*src* and v/c-*src* sequences from the vectors used by Iba et al. (9) into vectors providing an upstream RSV LTR and a downstream simian virus 40 polyadenylation site (Fig.1B) and compared their abilities to induce foci after transfection into NIH 3T3 cells (Table 1). In agreement with our previous results, we found that the focus-forming activity of the v/c-*src* plasmid pRBB4, which like pRS2 has its chimeric crossover at codon 431, was 100-fold lower than that of pRv*src*A, a parallel SRA v-*src* plasmid. The focus-forming activity of plasmid pRPB5, which like pRS21 has its chimeric crossover at codon 515, was higher but was still four times lower than that of pRv*src*A. pRcv*src*A, the inverse c/v-*src* chimera (encoding the amino region of $pp60^{c\text{-}src}$ and carboxyl region of $pp60^{v\text{-}src}$) with crossover at codon 431, induced foci with about the same efficiency as that of pRv*src*A.

To test the transforming potentials of chimeric *src* genes when expressed from a more effective murine promoter, plasmids pMBB4, pMPB5, and pMRS13, which use MoMLV LTRs, were constructed (Fig. 1C). These plasmids induced foci with high efficiencies similar to that of pMv*src*, a previously described SRA v-*src* plasmid of similar construction (12) (Table 1). This was about 20-fold higher than

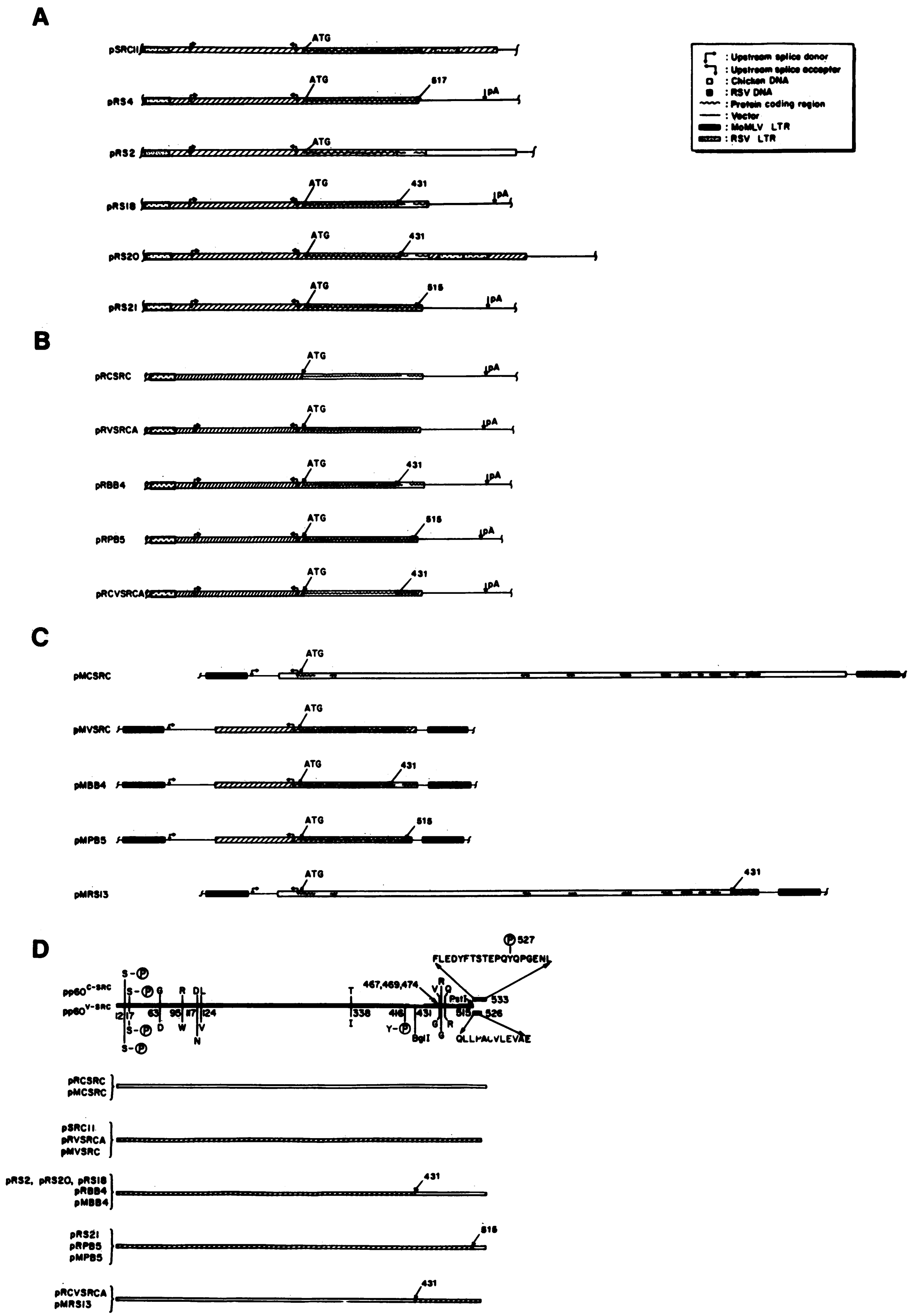
A
pSRC11
pRS4
pRS2
pRS18
pRS20
pRS21
ATG
517
431
515
pA
: Upstream splice donor
: Upstream splice acceptor
: Chicken DNA
: RSV DNA
: Protein coding region
: Vector
: MoMLV LTR
: RSV LTR
B
pRCSRC
pRVSRCA
pRBB4
pRPB5
pRCVSRCA
C
pMCSRC
pMVSRC
pMBB4
pMPB5
pMRS13
D
pp60c-src
pp60v-src
FLEDYFTSTEPQYQPGENL
QLLPALVLEVAE
527
467,469,474
PstI
BglI
533
526
pRCSRC
pMCSRC
pSRC11
pRVSRCA
pMVSRC
pRS2, pRS20, pRS18
pRBB4
pMBB4
pRS21
pRPB5
pMPB5
pRCVSRCA
pMRS13

TABLE 1. Biological activities of chimeric *src* proteins

src plasmid	Transfected gene[a]	Foci/pmol[b]	Cell line[c]	Colony formation in soft agarose[d]	No. of mice with tumors/ no. tested[e]	Latency (days)
Control			NIH 3T3	−	0/4	
RSV LTR SRD						
p*src*11	v-*src*	1.2×10^4 (0.08)				
pRS4	v-*src* (altered COOH)	1.3×10^4 (0.09)				
pRS2	v/c-*src* (431)	0.5×10^2 (0.0003)				
pRS18	v/c-*src* (431)	0.5×10^2 (0.0003)				
pRS20	v/c-*src* (431)	0.5×10^2 (0.0003)				
pRS21	v/c-*src* (515)	0.5×10^2 (0.0003)				
RSV LTR SRA						
pRv*src*A	v-*src*	0.6×10^4 (0.04)				
pRc*src*	c-*src*	<0.5 ($<10^{-5}$)				
pRBB4	v/c-*src* (431)	0.5×10^2 (0.0003)				
pRPB5	v/c-*src* (515)	0.2×10^4 (0.01)				
pRcv*src*A	c/v-*src* (431)	0.5×10^4 (0.04)				
MoMLV LTR SRA						
pMv*src*	v-*src*	1.4×10^5 (1)	NIH(pMv*src*/focus)A	+++	4/4	7–9
pMc*src*	c-*src*	5.4×10^3 (0.04)	NIH(pMc*src*/focus)B	+	0/4	—
pMBB4	v/c-*src* (431)	1.6×10^5 (1)	NIH(pMBB4/focus)A	+	1/4	>21
			NIH(pMBB4/focus)B	+	0/4	—
pMPB5	v/c-*src* (515)	2.0×10^5 (1.4)	NIH(pMPB5/focus)A	+	1/4	>21
			NIH(pMPB5/focus)B	+	0/4	—
pMRS13	c/v-*src* (431)	1.0×10^5 (0.7)	NIH(pMRS13/focus)C	+++	4/4	7–9
			NIH(pMRS13/focus)D	+++	4/4	7–9

[a] Numbers in parentheses identify the amino acid positions of the chimeric crossovers.
[b] Focus-forming activities were determined as geometric averages of three experiments with duplicate plates as described in Materials and Methods. Numbers in parentheses give foci/pmol relative to the value for pMv*src*.
[c] All cell lines were cylinder cloned from foci. NIH(pMv*src*/focus)A and NIH(pMc*src*/focus)B have been previously characterized (12).
[d] Phenotypes displayed: +++, >30% of the cells formed colonies >0.1 mm; +, <10% of the cells formed colonies <0.002 mm; −, no growth in soft agarose.
[e] In vivo tumorigenicity was tested in adult NFS mice by subcutaneous injection of 10^6 cells. Mice were monitored for 8 weeks.

the focus-forming efficiency of pRv*src*A. While the v-*src* and v/c-*src* plasmids induced foci of similar morphology, cells in 10 to 40% (in different experiments) of the foci induced by c/v-*src* plasmids were highly rounded, a phenomenon previously observed in some foci induced by c-*src* containing a Tyr 527 → Phe mutation (15) (Fig. 2). As previously reported (12), the MoMLV LTR c-*src* expression plasmid pMc*src* produced diffuse foci of unique morphology at low frequency.

Multiple cell lines were biologically cloned from foci induced by the MoMLV LTR *src* plasmids and were tested for anchorage-independent growth and in vivo tumorigenicity in adult NFS mice. Lines expressing v/c-*src* proteins grew poorly in soft agarose compared with lines expressing v-*src* or c/v-*src* and were only weakly tumorigenic or nontumorigenic in adult NFS mice (Fig. 2; Table 1).

To test the hypothesis that the different focus-forming activities of plasmids containing the RSV and the MoMLV LTRs were due to different induced expression levels (not observable in focus-selected cells, Table 2), we measured the average $pp60^{src}$ equilibrium levels in G418-resistant populations of cells which had been cotransfected with pSV2*neo* (which confers G418 resistance) and the *src* expression plasmids. In separate experiments, the plasmids were cotransfected in 10:1 and 5:1 (*neo*:*src*) molar ratios so that essentially all the cells which integrated the *src* DNAs were represented in the G418-selected populations. Average $pp60^{src}$ expression levels were measured by immunoprecipitation in antibody excess from [^{35}S]methionine-labeled cells (Fig. 3). Cultures cotransfected with RSV LTR plasmids had about 20-fold less $pp60^{c\text{-}src}$ expression than those cotransfected with the MoMLV LTR plasmids. The $pp60^{src}$ expression levels induced by the MoMLV LTR plasmids varied: the v-*src* and c/v-*src* proteins were expressed at the lowest level, whereas 3 to 4 times more v/c-*src* proteins and approximately 20 times more $pp60^{c\text{-}src}$ were expressed (Fig. 3).

The specific activities of phosphorylation of enolase by the *src* proteins were compared after immunoprecipitation with monoclonal antibody 327. The v/c-*src*, c/v-*src*, and v-*src*

FIG. 1. *src* expression plasmids. In panels A through C, only eucaryotic coding and control regions are shown. Chicken DNA sequences are from the c-*src* locus (27). The exon positions and the splice donor and acceptor sites which are predicted to be used in mRNA leader region processing are shown. Numbers above the lines indicate the codons at which the c-*src* and v-*src* sequences are ligated. pA indicates the position of the simian virus 40 early region polyadenylation site. (A) RSV LTR SRD *src* plasmids. Preliminary results indicate that SRA v-*src* and SRD v-*src* differ in six codons (S. Reddy and D. Mazzu, unpublished data). The dark band at the 3′ end of the pRS4 coding sequence represents a random sequence encoding nine arbitrarily chosen amino acids (33). (B) RSV LTR SRA *src* plasmids. (C) MoMLV LTR SRA *src* plasmids. (D) $pp60^{src}$ amino acid sequences encoded by chimeric plasmids. The sequences of $pp60^{c\text{-}src}$ (30) and SRA $pp60^{v\text{-}src}$ (SRA) (5) are compared by the single-letter amino acid code. Restriction sites used for the chimeric constructions are indicated. Ⓟ, Site of phosphorylation; ▨, v-*src* coding region; ▭, c-*src* coding region.

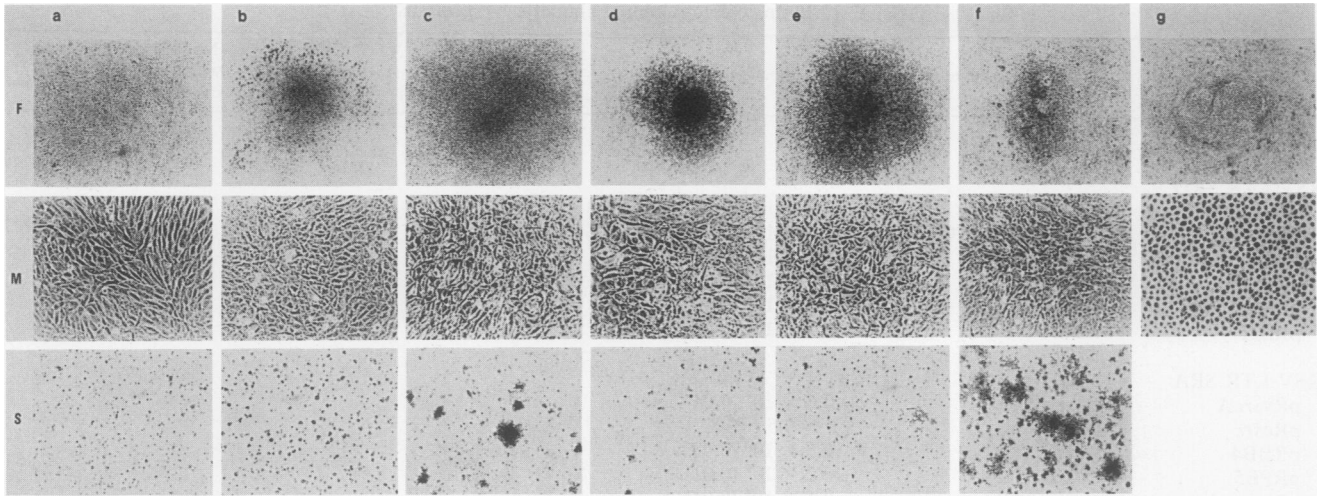

FIG. 2. Biological characteristics of chimeric-*src* expresser cells. (F) Foci formed in monolayer culture by NIH 3T3 cells 12 days after transfection with no plasmid (a), pMc*src* (b), pMv*src* (c), pMBB4 (d), pMPB5 (e), pMRS13 (f), and pMRS13 (g) (a mosaic focus). (M) Cell morphology of focus-selected lines grown in monolayer culture. (S) Colony formation of focus-selected lines 14 days after suspension in medium containing 0.3% soft agarose.

proteins had similar specific kinase activities, which were about 10-fold higher than that of c-*src*, when the proteins were immunoprecipitated from focus-selected lines or G418-selected cultures transfected with plasmids containing either the RSV or MoMLV LTRs (Table 2). In vivo total cell protein phosphotyrosine in the focus-selected cell lines expressing the v-*src*, c/v-*src*, and v/c-*src* proteins was five to nine times higher than that in normal NIH 3T3 cells, whereas the amount in lines overexpressing pp60$^{c\text{-}src}$ was only two times higher (Table 2). Thus, neither the in vitro nor the in vivo phosphorylating activities of the chimeric proteins correlated with their different transforming abilities.

The relative half-lives of both v/c-*src* and c/v-*src* chimeras were determined by comparing the extent of pp60^{src} labeling after brief (4-h) and long-term (24-h) cell incubation with [^{35}S]methionine. The chimeric v/c-*src* proteins were at least as, if not more, stable than pp60$^{v\text{-}src}$ was (Fig. 4), indicating that their poor transforming activity was not due to biological instability.

The in vivo phosphorylation states of the chimeric *src* proteins were determined by tryptic phosphopeptide mapping. As expected, pp60$^{v\text{-}src}$ was phosphorylated predominantly on Ser-17 and Tyr-416, whereas pp60$^{c\text{-}src}$ was phosphorylated on Ser-17 and Tyr-527. The v/c-*src* chimeric proteins were phosphorylated at Ser-17 and to significant extents at both Tyr-416 and Tyr-527. The ratios among phosphates in the peptides containing Tyr-527 and Tyr-416 ranged from 0.4 to 1.1 in seven cell lines expressing v/c *src* chimeras. Similar phosphorylation patterns were observed whether the proteins were expressed by the RSV or the MoMLV LTR (Fig. 5).

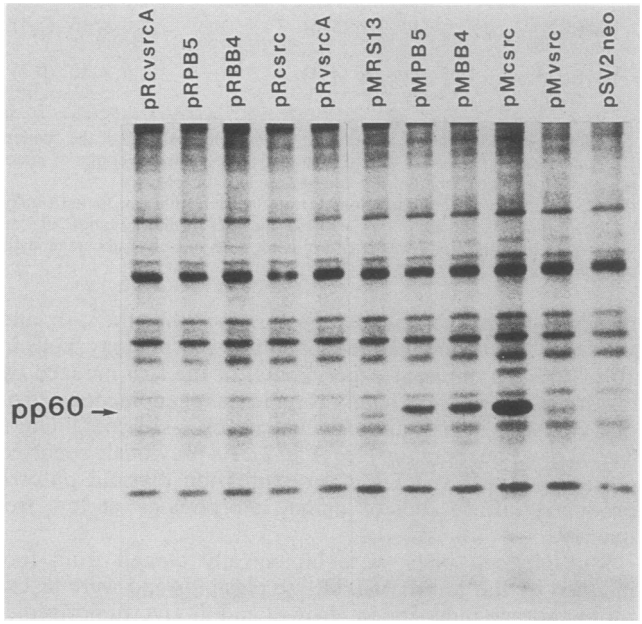

FIG. 3. Differences in pp60^{src} expression levels induced by RSV and MoMLV LTRs. G418-selected mass cultures of NIH 3T3 cells which had been cotransfected with pSV2*neo* and RSV LTR *src* or MoMLV LTR *src* expression plasmids were labeled for 48 h with [^{35}S]methionine; immunoprecipitates with monoclonal antibody EC10 from lysates containing equal trichloroacetic acid-insoluble radioactivities were prepared and analyzed on gels (10% polyacrylamide) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography as described in Materials and Methods. The cotransfected *src* plasmids are indicated above each lane, except that pSV2neo denotes a control line transfected with pSV2*neo* alone. Faint pp^{60src} bands in the lysates from cells transfected with RSV LTR plasmids were visible in longer exposures. Because these signals were weak, expression level values are only approximate. The relative pp60^{src} levels determined by scintillation counting of excised bands were as follows: NIH(pMv*src*, pSV2*neo*/mc)K, 1; NIH(pMc*src*, pSV2*neo*/mc)K, 18; NIH(pMBB4, pSV2*neo*/mc)K, 4; NIH(pMPB5, pSV2*neo*/mc)K, 3; NIH(pMRS13, pSV2*neo*/mc)K, 1.1; NIH (pRv*src*A, pSV2*neo*/mc)K, 0.6; NIH(pRc*src*, pSV2*neo*/mc)K, 0.7; NIH(pRBB4, pSV2*neo*/mc)K, 0.7; NIH(pRPB5, pSV2*neo*/mc)K, 0.4; and NIH(pRcv*src*A, pSV2*neo*/mc)K, 0.8. Exposure time was 2 days.

DISCUSSION

Replacement of the carboxyl region of pp60$^{c\text{-}src}$ with that of pp60$^{v\text{-}src}$ activated pp60$^{c\text{-}src}$ transforming activity in NIH 3T3 cells, whereas replacement of the carboxyl region of pp60$^{v\text{-}src}$ with that of pp60$^{c\text{-}src}$ reduced pp60$^{v\text{-}src}$ transforming activity. In contrast, replacement of the carboxyl region of pp60$^{v\text{-}src}$ with a randomly selected sequence did not affect transforming activity. These effects, which show that the v-*src* modifications which are most important for transfor-

TABLE 2. Biochemical characteristics of chimeric *src* proteins

Cell line	Enolase-specific kinase activity[a]	% Total (relative) cell protein phosphotyrosine[b]
NIH3T3	NM[c] (1)	0.025 (1)
NIH(pRv*src*A/focus)A	1.0 (2)	
NIH(pRPB5/focus)A	0.9 (0.4)	
NIH(pRPB5/focus)B	1.1 (2)	
NIH(pRcv*src*A/focus)A	0.8 (2)	
NIH(pRcv*src*A/focus)B	0.9 (3)	
NIH(pMv*src*/focus)A	1.0 (2)	0.17 (7)
NIH(pMc*src*/focus)B	0.08 (20)	0.06 (2.4)
NIH(pMBB4/focus)A	0.8 (2)	ND[d]
NIH(pMBB4/focus)E	1.0 (3)	0.22 (9)
NIH(pMPB5/focus)A	1.3 (2)	ND
NIH(pMPB5/focus)B	0.9 (7)	0.2 (8)
NIH(pMRS13/focus)C	0.8 (2)	ND
NIH(pMRS13/focus)D	1.0 (2)	0.12 (5)
NIH(pMv*src*, pSV2*neo*/mc)K	1.0 (0.7)	
NIH(pMc*src*, pSV2*neo*/mc)K	0.1 (13)	
NIH(pMBB4, pSV2*neo*/mc)K	0.8 (3)	
NIH(pMPB5, pSV2*neo*/mc)K	0.8 (2)	
NIH(pMRS13, pSV2*neo*/mc)K	1.6 (0.8)	

[a] Enolase-specific kinase activities were determined from the amounts of ^{32}P incorporated into the enolase bands, the amounts of ^{35}S-pp60^{src} in the immunoprecipitated pp60^{src} bands, and the specific activities of metabolic [^{35}S]methionine labeling as described in Materials and Methods. Relative expression levels are given in parentheses.

[b] Values are averages of two experiments. Numbers in parentheses give amounts relative to that in NIH 3T3 cells.

[c] NM, Not measurable.

[d] ND, Not done.

mation are those in the carboxyl-terminal region and that the specific c-*src* carboxyl-terminal region is required for suppression of transformation, were more evident at the lower levels of expression induced by RSV LTRs in rodent cells.

Focus-forming activity was less sensitive to suppression by the pp60$^{c\text{-}src}$ carboxyl region than was anchorage-independent growth or in vivo tumorigenicity. Its suppression was masked at the high expression levels induced by MoMLV LTRs: both MoMLV LTR v/c-*src* and MoMLV LTR c/v-*src* chimeric genes had high focus-forming activities. However, even focus-selected lines expressing v/c-*src* proteins at high levels did not induce anchorage-independent growth or in vivo tumorigenicity.

The suppressive effect of the pp60$^{c\text{-}src}$ carboxyl terminus on focus-forming activity could be modulated by mutations in other regions of the protein. Replacing the 12 carboxyl-terminal amino acids of pp60$^{v\text{-}src}$ with those of pp60$^{c\text{-}src}$ was sufficient to suppress focus formation 250-fold when SRD v-*src* was used (plasmid pRS21) but only 4-fold when SRA v-*src* was used (plasmid pRPB5) (Table 1). (Preliminary results indicate that SRA and SRD v-*src*s differ in six codons [S. Reddy and D. Mazzu, unpublished data].) For SRA v-*src*, significantly more suppression was caused by also restoring the mutated residues at positions 467, 469, and 474 to their pp60$^{c\text{-}src}$ forms (plasmid pRBB4). Thus, the effects of separated mutations on pp60$^{c\text{-}src}$ transforming activity are nonadditive.

The levels of *src* proteins expressed with the MoMLV LTR were inversely correlated with their transforming activities: lines expressing v-*src* and the c/v-*src* chimera had the least pp60^{src}, whereas those expressing v/c-*src* proteins had three to four times more pp60^{src} (Fig. 3). We have previously noted that c-*src* and nontransforming *src* mutants are expressed at higher levels than that of v-*src* (12, 15), even when the genes are expressed with otherwise identical vectors. These differences are partly due to different protein stabilities (Fig. 4) but must also reflect additional mechanisms, such as cytotoxicity from highly expressed transforming *src* proteins (e.g., see reference 31) or by cellular down-regulation of their expression to avoid toxicity.

The masking of differences between the functional activities of the mutants when expressed from the MoMLV LTRs was not due to dose-dependent differences in their phosphorylation states or kinase activities. It can be simply explained by a model wherein the *src* functional differences appear as differences in the threshold levels of expression required for transformation and wherein the observed transforming activities (e.g., of focus formation) are proportional to the fractions of transfected cells which express each mutant at levels above their thresholds (Fig. 6). This is consistent with the finding by Jakobovits et al. (11) that rat fibroblasts have distinct thresholds for v-*src* transformation. The MoMLV LTR, which induces about 20-fold higher expression levels,

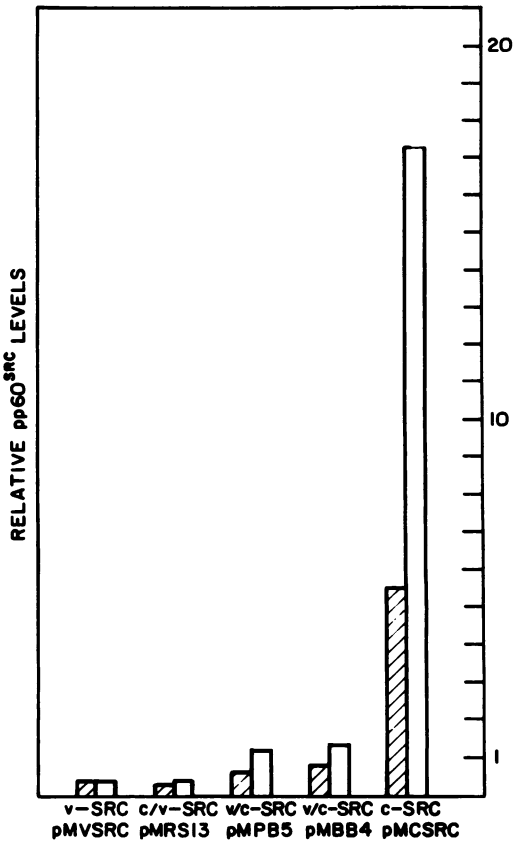

FIG. 4. Relative in vivo stabilities of viral, cellular, and chimeric pp60^{src} proteins. pp60^{src} overexpresser cell lines NIH(pMvsrc/focus)A, NIH(pMRS13/focus)C, NIH(pMPB5/focus)A, NIH(pMBB4/focus)A, and NIH(pMc*src*/focus)B were labeled with [^{35}S]methionine for short (4-h) and long (24-h) periods relative to the pp60$^{v\text{-}src}$ apparent half-life (~8 h [8]), and [^{35}S]pp60^{src} radioactivities as fractions of total cell trichloroacetic acid- precipitable ^{35}S radioactivities were determined by immunoprecipitation in antibody excess with monoclonal antibody 327. Fractions after short- (▨) and long-term (▭) labeling are plotted relative to the values for pp60$^{v\text{-}src}$, which were set to 1 to fix normalization. Relative in vivo half-lives determined from the fraction ratios (see reference 33) were as follows: pp60$^{v\text{-}src}$ (pMvsrc), 1; pp60$^{c/v\text{-}src}$ (pMRS13), 1.7; pp60$^{v/c\text{-}src}$ (pMPB5), 1.9; pp60$^{v/c\text{-}src}$ (pMBB4), 1.6; and pp60$^{c\text{-}src}$ (pMcsrc), 3.2.

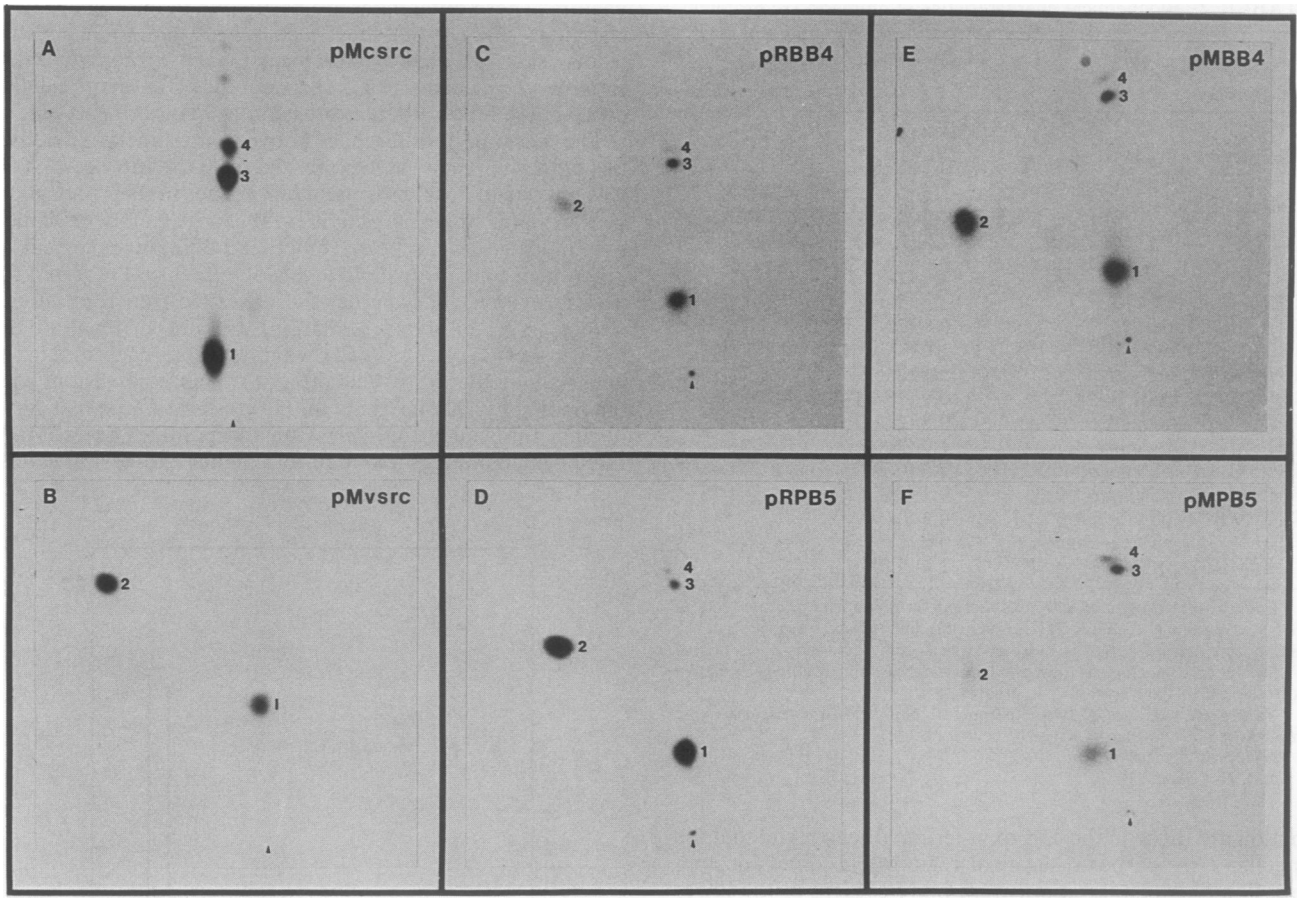

FIG. 5. Tryptic phosphopeptide maps of chimeric v/c-*src* proteins. pp60^{src} was immunoprecipitated from $^{32}P_i$-labeled cell lysates with monoclonal antibody EC10, digested with trypsin, and separated by electrophoresis in the horizontal dimension (with the anode to the left) and chromatography in the vertical direction, as described in Materials and Methods. Origins are indicated with arrowheads. Spot numbers (peptides): 1, Ser 17; 2, Tyr 416; 3 and 4, Tyr 527. Cell lines: A, NIH(pMc*src*/focus)B; B, NIH(pRBB4, pSV2*neo*/cos)K; C, NIH(pMBB4, pSV2*neo*/cos)K; D, NIH(pMv*src*/focus)A; E, NIH(pRPB5, pSV2*neo*/cos)K; and F, NIH(pMPB5, pSV2*neo*/cos)K. Approximately 500 dpm were spotted on each plate. Exposure time was 3 days.

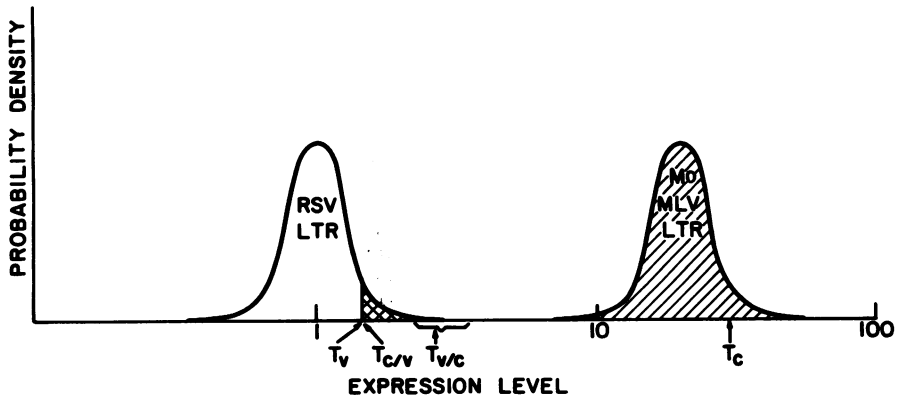

FIG. 6. Threshold model for focus-forming activities of pp60^{src} mutants. Because of differences in integration sites, integrated plasmid copy numbers, etc., plasmid transfection will generate a distribution of initial expression levels in the cell population. We assume that this distribution depends only on the transcriptional promoter-enhancer (LTR) of the plasmid and not on the transcribed gene. (Subsequent cell-mediated reductions in the expression levels of potentially toxic genes such as v-*src* do not affect the argument.) For simplicity, the distribution has been assumed to be log normal (omitting the large number of cells which take up no DNA at all). The mean of the MoMLV LTR distribution is set 20 times higher than that of the RSV LTR, in accord with the experimental data. Threshold levels for focus formation by pp60$^{v\text{-}src}$ (T_v), pp60$^{c/v\text{-}src}$ ($T_{c/v}$), pp60$^{v/c\text{-}src}$ ($T_{v/c}$), and pp60$^{c\text{-}src}$ (T_c) have been set by using the focus-forming data of Table 1 and noting that the number of foci induced by a plasmid is predicted to be proportional to the area under its distribution curve that is to the right of the threshold level for the gene being expressed. For example, the focus-forming activities of plasmids pRv*srcA* and pMv*src* are proportional to the areas of the cross-hatched and hatched areas, respectively.

saturates the assay by causing even weakly active mutants to induce foci in most of the transfected cells.

Saturation may account for the fact that differences in focus-forming efficiencies of the v/c-*src* chimeras and v-*src* were not detected in prior experiments with RSV vectors and CEFs (9, 32). The RSV LTR may saturate the focus-forming assay in CEFs (about five times more $pp60^{v\text{-}src}$ is expressed in RSV-infected CEFs than in MoMLV LTR *src* plasmid-transformed NIH 3T3 cells [J. Brugge, personal communication]). Saturation of the focus-forming assay may also account for the reported transforming potential of single point mutations present outside the carboxyl-terminal regulatory domain in $pp60^{c\text{-}src}$ (14, 18). However, this does not explain the observation that v/c-*src* genes also induce both extensive anchorage-independent growth and tumors in chickens (H. Hanafusa, personal communication) or that direct injection of the chimeric plasmids used in this study (pMBB4, pMPB5, and pMRS13) into newborn chicks causes tumors with frequencies similar to that induced by injection of pMv*src* (D. Robinson, P. Luciw, P. Yaciuk, D. Shalloway, H. Hanafusa, L. Crittendon, and H.-J. Kung, manuscript in preparation). Thus, species-specific differences must also be involved; this phenomenon has previously been observed with some v-*src* mutants which transform chicken but not rat cells (21).

It is interesting that whereas both the v/c-*src* and c/v-*src* proteins had high overall in vitro and in vivo protein kinase activities, the activities of the v/c-*src* proteins were not sufficient to induce complete transformation. In this regard these proteins are similar to nonmyristylated, nontransforming pp60v-*src* mutants, which also have high kinase activities (13). The $pp60^{c\text{-}src}$ carboxyl terminus may block interactions with specific cellular components without suppressing activity in general. The altered phosphorylation states of the $pp60^{c/v\text{-}src}$ proteins themselves, which were phosphorylated on both Tyr-416 and Tyr-527 (Fig. 5), may reflect this. We are not certain whether individual *src* molecules were phosphorylated simultaneously on both residues, but no mobility shifts (as would be expected for doubly phosphorylated peptides) were observed in *S. aureus* V8 Cleveland digests of these proteins (data not shown). It is possible that the tyrosine phosphorylation alternates between residues 416 and 527 in a time-dependent manner as the cell moves through a cycle of division. If so, it will be interesting to determine the time periods during which these changes occur.

ACKNOWLEDGMENTS

We thank P. J. Johnson and D. Greenspan for expert technical assistance, H. Hanafusa for plasmids pBB4 and pPB5, J. Brugge for monoclonal antibody 327, and S. Parsons for monoclonal antibody EC10.

This work was supported by Public Health Service grant CA32317 from the National Cancer Institute and a Research Career Development Award (to D.S.) from the National Institutes of Health.

LITERATURE CITED

1. **Brugge, J. S., and D. Darrow.** 1984. Analysis of the catalytic domain of phosphotransferase activity of two avian sarcoma virus-transforming proteins. J. Biol. Chem. **259**:4550–4557.
2. **Cartwright, C. A., W. Eckhart, S. Simon, and P. L. Kaplan.** 1987. Cell transformation by $pp60^{c\text{-}src}$ mutated in the carboxy-terminal regulatory domain. Cell **49**:83–91.
3. **Cooper, J. A., B. M. Sefton, and T. Hunter.** 1983. Detection and quantification of phosphotyrosine in proteins. Methods. Enzymol. **99**:387–402.
4. **Coussens, P. M., J. A. Cooper, T. Hunter, and D. Shalloway.** 1985. Restriction of the in vitro and in vivo tyrosine protein kinase activities of $pp60^{c\text{-}src}$ relative to $pp60^{v\text{-}src}$. Mol. Cell. Biol. **5**:2753–2763.
5. **Czernilofsky, A. P., A. D. Levinson, H. E. Varmus, J. M. Bishop, E. Tischer, and H. Goodman.** 1983. Corrections to the nucleotide sequence of the *src* gene of Rous sarcoma virus. Nature (London) **301**:736–738.
6. **Flockhart, D. A., and J. D. Corbin.** 1982. Regulatory mechanisms in the control of protein kinases. Crit. Rev. Biochem. **12**: 133–186.
7. **Hunter, T., and J. A. Cooper.** 1985. Protein-tyrosine kinases. Annu. Rev. Biochem. **54**:897–930.
8. **Iba, H., F. R. Cross, E. A. Garber, and H. Hanafusa.** 1985. Low level of cellular protein phosphorylation by nontransforming overproduced $p60^{c\text{-}src}$. Mol. Cell. Biol. **5**:1058–1066.
9. **Iba, H., T. Takeya, F. R. Cross, T. Hanafusa, and H. Hanafusa.** 1984. Rous sarcoma virus variants that carry the cellular *src* gene instead of the viral *src* gene cannot transform chicken embryo fibroblasts. Proc. Natl. Acad. Sci. USA **81**:4424–4428.
10. **Ikawa, S., K. Hagino-Yamogishi, S. Kawai, T. Yamamoto, and K. Toyoshima.** 1986. Activation of the cellular *src* gene by transducing retrovirus. Mol. Cell. Biol. **6**:2420–2428.
11. **Jakobovits, E. G., J. E. Majors, and H. E. Varmus.** 1984. Hormonal regulation of the Rous sarcoma virus *src* gene via heterologous promoter defines a threshold dose for cellular transformation. Cell **38**:757–765.
12. **Johnson, P. J., P. M. Coussens, A. V. Danko, and D. Shalloway.** 1985. Overexpressed $pp60^{c\text{-}src}$ can induce focus formation without complete transformation of NIH 3T3 cells. Mol. Cell. Biol. **5**:1073–1083.
13. **Kamps, M. P., J. E. Buss, and B. M. Sefton.** 1986. Rous sarcoma virus transforming protein lacking myristic acid phosphorylates known polypeptide substrates without inducing transformation cell. Cell **45**:105–112.
14. **Kato, J. Y., T. Takeya, C. Gvandori, H. Iba, J. B. Levy, and H. Hanafusa.** 1986. Amino acid substitutions sufficient to convert the nontransforming $p60^{c\text{-}src}$ protein to a transforming protein. Mol. Cell. Biol. **6**:4155–4160.
15. **Kmiecik, T. E., and D. Shalloway.** 1987. Activation and suppression of $pp60^{c\text{-}src}$ transforming ability by mutation of its primary sites of tyrosine phosphorylation. Cell **49**:65–73.
16. **Kriegler, M., C. F. Perez, C. Hardy, and M. Botchan.** 1984. Transformation mediated by SV40 T antigens: separation of the overlapping SV40 early genes with a retroviral vector. Cell **38**: 483–491.
17. **Levinson, A. D., S. A. Courtneidge, and J. M. Bishop.** 1981. Structural and functional domains of the Rous sarcoma virus transforming protein ($pp60^{src}$). Proc. Natl. Acad. Sci. USA **78**: 1624–1628.
18. **Levy, J. B., H. Iba, and H. Hanafusa.** 1986. Activation of the transforming potential of $p60^{c\text{-}src}$ by a single amino acid change. Proc. Natl. Acad. Sci. USA **83**:4228–4232.
19. **Lipsich, L. A., A. J. Lewis, and J. S. Brugge.** 1983. Isolation of monoclonal antibodies that recognize the transforming proteins of avian sarcoma viruses. J. Virol. **48**:352–360.
20. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
21. **Oppermann, H., A. D. Levinson, and H. E. Varmus.** 1981. The structure and protein kinase activity of proteins encoded by non conditional mutants and back mutants in the *src* gene of avian sarcoma virus. Virology **108**:47–70.
22. **Parker, R. C., H. E. Varmus, and J. M. Bishop.** 1984. Expression of v-*src* and chicken c-*src* in rat cells demonstrates qualitative differences between $pp60^{v\text{-}src}$ and $pp60^{c\text{-}src}$. Cell **37**: 131–139.
23. **Parsons, S. J., D. J. McCarley, C. M. Ely, D. C. Benjamin, and J. T. Parsons.** 1984. Monoclonal antibodies to Rous sarcoma virus $pp60^{src}$ react with enzymatically active cellular $pp60^{src}$ of avian and mammalian origin. J. Virol. **51**:272–82.
24. **Piwnica-Worms, H., K. B. Saunders, T. M. Roberts, A. E. Smith, and S. H. Cheng.** 1987. Tyrosine phosphorylation regulates the biochemical and biological properties of $pp60^{c\text{-}src}$. Cell

49:75–82.

24a. **Reynolds, A. B., J. Vila, T. J. Lansing, W. M. Potts, M. J. Weber, and J. T. Parsons.** 1987. Activation of the oncogenic potential of the avian cellular src protein by specific structural alteration of the carboxyl terminus. EMBO J. 8:2359–2364.

25. **Shalloway, D., P. M. Coussens, and P. Yaciuk.** 1984. c-*src* and *src* homolog overexpression in mouse cells, p. 9–17. *In* G. F. Vande Woude, A. J. Levine, W. C. Topp, and J. D. Watson (ed.), Cancer cells, vol. 2. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

26. **Shalloway, D., P. M. Coussens, and P. Yaciuk.** 1984. Overexpression of the c-*src* protein does not induce transformation of NIH 3T3 cells. Proc. Natl. Acad. Sci. USA 81:7071–7075.

27. **Shalloway, D., A. D. Zelenetz, and G. M. Cooper.** 1981. Molecular cloning and characterization of the chicken gene homologous to the transforming gene of Rous sarcoma virus. Cell 24: 531–541.

28. **Southern, P. J., and P. Berg.** 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. J. Mol. Appl. Genet. 1:327–341.

29. **Swanstrom, R., R. C. Parker, H. E. Varmus, and J. M. Bishop.** 1983. Transduction of a cellular oncogene: the genesis of Rous sarcoma virus. Proc. Natl. Acad. Sci. USA 80:2519–2523.

30. **Takeya, T., and H. Hanafusa.** 1983. Structure and sequence of the cellular gene homologous to the RSV *src* gene and the mechanism for generating the transforming virus. Cell 32:881–890.

31. **Tarpley, W. G., and H. M. Temin.** 1984. The location of v-*src* in a retrovirus vector determines whether the virus is toxic or transforming. Mol. Cell. Biol. 4:2653–2660.

32. **Wilkerson, V. W., D. L. Bryant, and J. T. Parsons.** 1985. Rous sarcoma virus variants that encode *src* proteins with an altered carboxy terminus are defective for cellular transformation. J. Virol. 55:314–321.

33. **Yaciuk, P., and D. Shalloway.** 1986. Features of the pp60$^{v\text{-}src}$ carboxyl terminus that are required for transformation. Mol. Cell. Biol. 6:2807–2819.