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Overexpression of the c-*src* protein does not induce transformation of NIH 3T3 cells

(oncogene/recombinant gene/cellular transforming gene)

DAVID SHALLOWAY, PAUL M. COUSSENS, AND PETER YACIUK

Molecular and Cell Biology Program, The Pennsylvania State University, University Park, PA 16802

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ABSTRACT **NIH 3T3 mouse cells were transfected with plasmids that induce efficient expression of either (*i*) the Rous sarcoma virus v-*src* gene, (*ii*) the chicken c-*src* gene, or (*iii*) a recombinant gene combining the 5′ portion of c-*src* with the 3′ end of v-*src*. Focus formation in tissue culture and formation of large colonies in soft agar did not occur in cells transfected with c-*src*. Cells transfected with c-*src* expression plasmids did not form foci but were isolated using a coselectable biological marker. They display morphological and substrate-independent growth characteristics intermediate between those of normal and v-*src*-transformed mouse cells, and lysates from these cells have enhanced *in vitro* tyrosine kinase activity. Transfection with the c-*src*–v-*src* recombinant induced focus formation with an efficiency similar to that obtained with a v-*src* expression plasmid. These results imply that v-*src*-induced transformation does not result just from overexpression of an essentially normal cellular protein but, at least in part, depends on the mutations distinguishing the cellular and viral proteins.**

Neoplastic transformation by $pp60^{v\text{-}src}$ appears to be correlated with its tyrosine kinase activity (for review, see ref. 1). This activity has been assayed by *in vitro* phosphorylation of anti-v-*src* tumor-bearing rabbit (TBR) antisera (2–5), by *in vitro* phosphorylation of specific substrates by either immunopurified or biochemically purified v-*src* protein (3, 4, 6–10), and by *in vivo* comparisons of tyrosine phosphorylation patterns between normal and v-*src*-transformed cells (3) and between temperature-sensitive mutant transformed cells at permissive and nonpermissive temperature (11).

The tyrosine kinase activity is also present in the cellular homolog $pp60^{c\text{-}src}$, which is highly conserved in evolution and is expressed at low levels in normal cells (1, 12). Transformation due to v-*src* probably results from perturbation of a normal growth or developmental regulatory pathway mediated by c-*src*. Such perturbation could be due to the high expression levels of v-*src* relative to c-*src*, to mutational differences, or both. The comparative study of $pp60^{c\text{-}src}$ and $pp60^{v\text{-}src}$ function may allow identification of the differences critical for transformation, but such studies have been hampered by the low levels of endogenous c-*src* expression.

To determine whether v-*src*-induced transformation is the result of high levels of v-*src* expression (relative to c-*src*), of functional differences between the v- and c-*src* proteins, or both, we have transfected NIH 3T3 mouse cells with plasmids that induce expression of c-*src*, v-*src*, and a c-*src*–v-*src* recombinant. Comparison of the effects of expression of these *src* genes shows that there are differences between the c- and v-*src* genes that are important for transformation.

MATERIALS AND METHODS

Plasmids. Plasmid pCS12.13 [and five other plasmids differing in the location of the simian virus 40 (SV40) promoter relative to c-*src* exon 1] was generated by a multistep cloning procedure. The resultant plasmid contains the DNA segments described in the Fig. 1*a* legend ligated together as follows (proceeding counterclockwise from the top): (*i*) the c-*src* *Hin*dIII site at location 9.5 kilobase pairs (kbp) (all c-*src* map locations refer to figure 1 in ref. 13) was filled in with DNA polymerase Klenow fragment (New England Biolabs) and blunt-end ligated to the filled-in pBR322 *Eco*RI site; (*ii*) the pBR322 *Pvu* II site was ligated to the *Pvu* II site proximal to the late side of the SV40 origin; (*iii*) the SV40 origin–Eco-*gpt*–SV40 early region–SV40 origin DNA segment is as described for plasmid pSV3-*gpt* by Mulligan and Berg (14); (*iv*) the following Eco-*gpt*–SV40 early region–SV40 origin segment is a direct repeat of that in *iii*; (*v*) the *Hin*dIII site on the early side of the SV40 origin was filled in and ligated to a *Sal* I linker (Collaborative Research, Waltham, MA); and (*vi*) the *Sal* I site was ligated to a *Sal* I site provided by a linker attached to *Bal* 31 (Bethesda Research Laboratories) trimmed c-*src* DNA derived from λ phage λCS3 (13). (Plasmid pCS1B.13 containing the *Bal* 31 trimmed, *Sal* I-linked c-*src* DNA was donated by A. D. Zelenetz and G. M. Cooper.) Plasmid pCS12.13 was linearized for transfection by cutting at the unique *Pvu* I site in the pBR322 segment or at the unique *Bss*HII site at c-*src* location 6.1 kbp (1.3 kbp downstream from the c-*src* termination codon).

Plasmid pRS3 was also constructed by a multistep procedure. It is identical to pCS12.13 except that the c-*src* region between the *Bgl* I site (c-*src* map location, 4.4 kbp) and the *Sac* I site (at c-*src* map location 4.9 kbp, 11 bp downstream from the c-*src* termination codon) was replaced by the *Bgl* I/*Sac* I Rous sarcoma virus (RSV) circular form DNA fragment (described in Fig. 1*b*) derived from Schmidt–Ruppin (SR)-D v-*src* expression plasmid p*src*11 (donated by A. D. Zelenetz and G. M. Cooper). The c-*src* and v-*src* *Bgl* I sites are homologous sites at the sequences coding for amino acid 431 in both genes (15–18). The inserted v-*src* fragment contains the v-*src* coding sequence downstream from the *Bgl* I site, the downstream long-terminal repeat, and circularly permuted DNA from the 5′ end of RSV down to the *Sac* I site that is ≈120 bp upstream of the *gag* coding region.

The structure of the recombinant plasmid was verified by restriction enzyme cleavage site mapping. Because of the 64-fold degeneracy of the *Bgl* I recognition sequence (due to 3 arbitrary nucleotides in the sticky ends), the v-*src* *Bgl* I sticky end can only ligate with the homologous c-*src* *Bgl* I sticky end from the corresponding point in the c-*src* sequence. Plasmid pRS3 was linearized for transfection by cutting at the unique *Bss*HII site at c-*src* location 6.1 kbp.

Tissue Culture and Biological Assays. NIH 3T3 mouse cells were grown and assayed in Temin's modified Eagle's medium (M. A. Bioproducts, Walkersville, MD) supplemented with 10% calf serum. This medium was supplemented with mycophenolic acid (25 μg/ml), aminopterin (2 μg/ml), xan-

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Abbreviations: RSV, Rous sarcoma virus; SV40, simian virus 40; TBR, tumor-bearing rabbit; bp, base pair(s); kbp, kilobase pair(s).

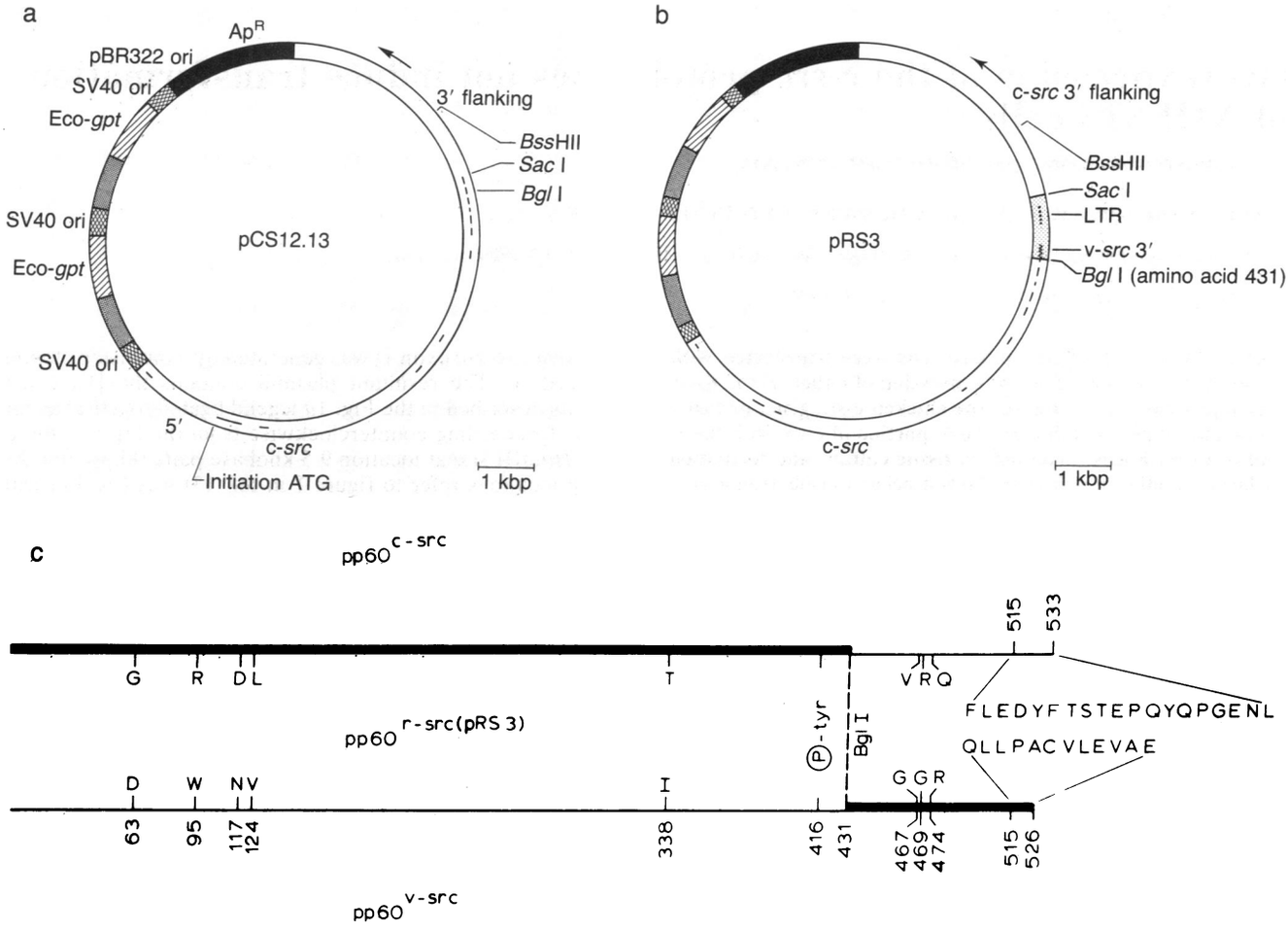

FIG. 1. Expression plasmids. (*a*) c-*src* expression plasmid pCS12.13. Plasmids were constructed by a multistep cloning procedure from pBR322, SV40, bacterial (Eco-*gpt*), and chicken c-*src* DNA. Counterclockwise from top, they contain DNA sequence from (*i*) pBR322 (*Eco*-RI/*Pvu* II fragment) containing ampicillin-resistance (Ap^R) gene and origin of replication, (*ii*) SV40 origin–Eco-*gpt*–SV40 early region DNA derived from the *Pvu* II/*Bam*HI fragment of pSV2, (*iii*) a direct repeat of region *ii*, except beginning at the *Hpa* II site near the SV40 origin, (*iv*) the SV40 origin region (*Hpa* II/*Hin*dIII fragment) containing the 72-bp tandem repeat (enhancer) and early promoter, and (*v*) the chicken c-*src* locus running from a *Bal* 31-generated location at −3660 bp to the *Hin*dIII site at 9490 bp (see figure 1 in ref. 13 for coordinates). Locations of exons determined by heteroduplex analysis and sequencing are indicated by the lines in the c-*src* region. SV40 promoter is upstream from exon 1. Termination codon is 11 bp before the *Sac* I site. Total length, ≈20,500 bp. (*b*) Recombinant-*src* expression plasmid pRS3. A DNA fragment from circular SR-D RSV from the v-*src* *Bgl* I site through the downstream long-terminal repeat (LTR) to the first *Sac* I site in *gag* (255 bp from the beginning of U_3) was inserted into pCS12.13 between the homologous c-*src* *Bgl* I site and the *Sac* I site 11 bp downstream from the c-*src* termination codon. The resultant plasmid pRS3 codes for a recombinant protein that (assuming similarity between the SR-A and SR-D v-*src* sequences) is identical to $pp60^{c\text{-}src}$ except for substitutions at amino acid positions 467, 469, 474, and the extensive mutation at the carboxyl terminus. Total length, ≈21,000 bp. Both plasmids were linearized prior to transfection by digestion at their unique *Bss*HII sites or at the unique *Pvu* I site in pCS12.13. (*c*) Approximate predicted $pp60^{r\text{-}src}$ amino acid sequence generated by pRS3. pRS3 encodes a recombinant-*src* protein composed of chicken c-*src* and SR-D v-*src* sequences. Since the SR-D v-*src* sequence is not known, the predicted pRS3 amino acid sequence has been approximated using the SR-A v-*src* sequences. The positions of the amino acid differences between $pp60^{c\text{-}src}$ and $pp60^{v\text{-}src}$ (SR-A) are shown using one-letter amino acid symbols. The protein sequence regions coded by pRS3 are indicated by heavy line. The position of the unique *Bgl* I site used for the recombination is conserved in c-*src*, SR-A v-*src*, and Pr-C v-*src* and is found at the same location in restriction maps of SR-D v-*src* (unpublished data).

thine (250 μg/ml), adenine (25 μg/ml), and thymidine (10 μg/ml) to make Eco-*gpt*-selective medium (19).

Transfection of NIH 3T3 cells was initiated by plating cells at a density of 5×10^5 cells per 60-mm culture dish. After overnight incubation at 37°C, the cells were inoculated with linearized DNA and treated with 15% (vol/vol) glycerol as described by Copeland and Cooper (20). Media were changed at 2- to 3-day intervals, and foci of transformed cells were counted 12–14 days after exposure to DNA. pCS12.13-transfected cells were selected for Eco-*gpt* expression using selective medium as described by Mulligan and Berg (14). Transfected cells were biologically cloned by end-point dilution in multiwell plates to generate cell lines NIH-(pCS12.13).1 and NIH(pCS12.13).3.

Colony formation in soft agarose by transfected NIH 3T3 cell lines was assayed by plating 3×10^5 cells in 4 ml of medium containing 0.25% agarose over a base layer of 3 ml of medium containing 0.5% agarose. Colonies were counted after 7–14 days of incubation at 37°C. Cell lines were passaged in selective medium but were "weaned" into normal medium ≈7 days prior to soft agarose assays. Cells were weaned by plating into normal medium containing xanthine (250 μg/ml)/adenine (25 μg/ml)/thymidine (10 μg/ml) 3 days prior to shifting them to normal medium. This procedure was required because direct transfer to normal medium led to cell death, presumably because at least one of the toxins in the selective medium (mycophenolic acid and aminopterin) has a longer biological half-life than at least one of the above supplements.

Immune Complex Kinase Assay. Cell lysis and immune

complex kinase assays were carried out essentially as described by Collett and Erikson (2) except that protein A beads (Pharmacia) were substituted for protein A-containing *Staphylococcus aureus* and adsorption times were increased to 45 min at 4°C. Protein kinase activity was assayed by adding 0.2–1.0 μM [γ-^{32}P]ATP (>400 Ci/mmol; 1 Ci = 37 GBq; ICN) to the phosphorylation reaction mixture and incubating for 10 min at room temperature.

RESULTS

c-*src* expression plasmids (Fig. 1*a*) were created by a multistep cloning procedure in which the SV40 origin region containing the 72-bp tandem repeat enhancer and early promoter was ligated upstream of exon 1 (noncoding) of the c-*src* gene (13, 15, 21, 22). The entire c-*src* coding sequence plus 4.5 kbp of downstream flanking sequence was included. Parker *et al.* (22) have shown that flanking sequences further downstream from this point do not hybridize to c-*src* mRNA, implying that the c-*src* polyadenylylation signal is included in these plasmids. The plasmids also contained two copies of the Mulligan and Berg (14) SV40 origin–Eco-*gpt* sequence for biological selection of stably transfected cells.

Five plasmids of this type were created that differed only in the location of the SV40 promoter relative to c-*src* exon 1 and were transfected into NIH 3T3 mouse cells. No foci were observed in multiple transfection experiments so ≈30 colonies of transfected cells per plate selected for Eco-*gpt* expression were grown in mass culture in selective medium and analyzed for immune complex phosphorylation of cross-reactive TBR anti-v-*src* IgG using antibody excess. Cells expressing Eco-*gpt* activity from any of the five plasmids showed enhanced *in vitro* kinase activity. c-*src* kinase activity levels ranged from ≈5% to ≈60% of the v-*src* kinase activity levels. (Direct comparison of the v-*src* and overexpressed chicken c-*src* levels with the endogenous mouse c-*src* level cannot be made because of possible differences in the antiserum cross-reactivity between species. It is also possible that the extent of c-*src* overexpression is understated because of lack of complete v-*src* antisera cross-reactivity with chicken c-*src*.) Those transfected with plasmid pCS12.13 had the highest *in vitro* kinase activity (Fig. 2) and were biologically cloned to generate cell lines NIH(pCS12.13).1 and NIH-(pCS12.13).3. *In vitro* kinase activity levels in both biological clones were ≈80% of the v-*src* level. Lysates from *in vivo* ^{32}P-labeled NIH(pCS12.13).1 and NIH(pCS12.13).3 cells were immunoprecipitated using v-*src* TBR antisera (data not shown) and displayed the presence of enhanced levels of a 60-kDa phosphoprotein expressed at ≈2/3 the level of pp60$^{v\text{-}src}$ in p*src*11 transformed cells. [Only a very weak mouse c-*src* band was observed, <25% of the NIH-(12.13).3 c-*src* band. This was probably due to poor serum cross-reactivity with mouse c-*src*, and cannot be used for quantitative comparison with the endogenous level.]

Multiple transfection experiments with plasmid pCS12.13 have shown no focus-forming activity (<2 foci per pmol of DNA compared with 2 × 10^4 foci per pmol for the v-*src* expression plasmid p*src*11). However, the morphological characteristics of the genetically transformed c-*src* overexpresser cells from both biological clones are intermediate between those of normal NIH 3T3 cells and v-*src*-transformed cells (Fig. 3*a*). While there are minor morphological differences between the NIH(pCS12.13).1 and NIH(pCS12.13).3 cells, they are both more rounded and refractile than NIH 3T3 cells, although not as much as the v-*src*-transformed cells. Furthermore, they form small colonies in soft agarose with reasonable efficiency, a characteristic that is intermediate between that of normal and v-*src*-transformed cells (Fig. 3*b*; Table 1).

Control transfections were carried out with a plasmid containing the SV40–Eco-*gpt* tandem repeat region of pCS12.13 but not containing any *src* sequences. This plasmid also induced no foci. Coselected cells containing this plasmid show no morphological alteration and no enhanced ability to grow in soft agarose. Similarly, control experiments showed that coselected pp60$^{v\text{-}src}$ overexpresser cells (selected after cotransfection with the Eco-*gpt* expression plasmid) and pp60$^{v\text{-}src}$ overexpresser cells selected on the basis of focus formation have similar morphological and anchorage-independent growth characteristics.

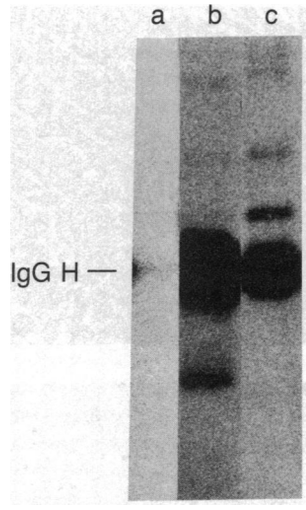

FIG. 2. Immune complex kinase assay. Cell lysates prepared from normal NIH 3T3 mouse cells (lane a), cells transformed with v-*src* DNA (lane b), or cells transfected with c-*src* expression plasmid pCS12.13 (lane c) and selected for stable Eco-*gpt* expression were assayed for *in vivo* kinase activity. Lysates from ≈10^6 mass culture cells that had been grown from ≈30 independent foci (v-*src*) or colonies (c-*src*) were used for each assay. Samples were analyzed by electrophoresis on 10% polyacrylamide gels containing 0.8% *N,N'*-methylenebis-acrylamide using the buffer system described by Laemmli (23). The phosphorylated IgG heavy chain (IgG H) was visualized by autoradiography (12 hr exposure with an intensifying screen). Lanes a, b, and c are nonadjoining lanes cut from the same autoradiograph. Densitometer tracings show the ratio of signal in the three lanes to be 1:210:95.

To determine whether functional differences between c-*src* and v-*src* could be localized to a specific region of the gene sequence, the 3′ end of the c-*src* gene in plasmid pCS12.13 was replaced with the corresponding end of the v-*src* gene in plasmid p*src*11 to generate plasmid pRS3 (Fig. 1*b*). The replacement took place beginning at a *Bgl* I site (corresponding to amino acid position 431), which lies in a region of sequence common to both v- and c-*src* (15–18). This site is 15 amino acids downstream from the *src* phosphotyrosine residue. The recombination results in replacement of 102 amino acids at the COOH end of pp60$^{c\text{-}src}$ with 95 amino acids from the COOH end of pp60$^{v\text{-}src}$. Most of these amino acids are identical: comparison of the SR-A v-*src* (16, 17) and c-*src* (15) sequences implies that the proteins encoded by pCS12.13 and pRS3 differ only at the immediate carboxyl terminus [19 amino acids in pCS12.13 c-*src* are replaced by 12 different amino acids in pRS3 recombinant-*src* (r-*src*)] and at isolated amino acids at positions 467, 469, and 474. Since the strain of *src* used (SR-D) has not been sequenced itself, it is possible that other differences between c-*src* and r-*src* exist. But, the conservation of the sequence at the immediate carboxyl terminus in the two sequenced v-*src* strains (Pr-C and SR-A) (16–18) suggests that the same sequence is found in our cloned SR-D r-*src* sequence. Transfection of this plasmid into NIH 3T3 cells caused efficient focus formation (10^4 foci per pmol), an enhancement of 5 ×

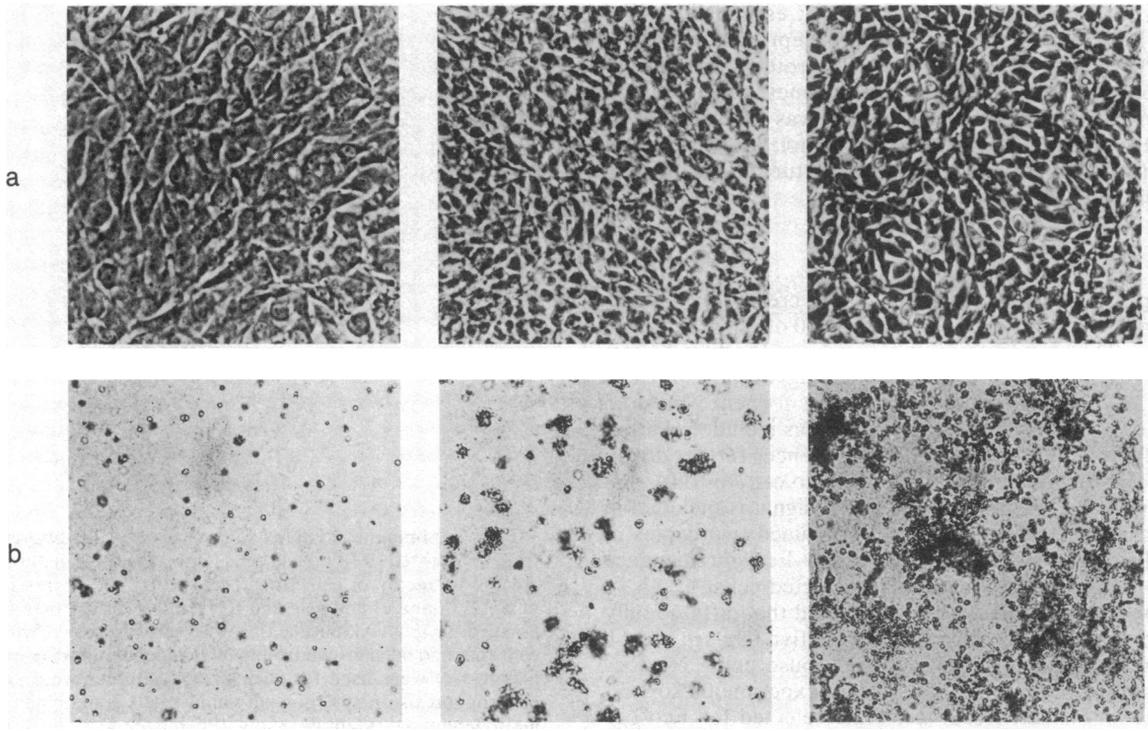

FIG. 3. Biological characterization of c-*src* overexpresser cells. NIH 3T3 cells transfected with plasmid pCS12.13 were selected using the Eco-*gpt* marker and were biologically cloned by end-point dilution. Pictures of one of the biological clones, NIH(pCS12.13).3, are shown. [Cells from an independent biological clone of c-*src* overexpresser cells, NIH(pCS12.13).1, have a similar phenotype.] Left to right, NIH 3T3, NIH(pCS12.13).3, NIH(p*src*11) (v-*src*-transformed cells). (*a*) Cell morphology. (*b*) Growth in semisolid medium. Cells were plated in soft agarose and colonies were photographed after 10 days.

10^3 relative to c-*src* expression plasmid pCS12.13. We suspect that this difference is associated with the difference at the COOH termini of the encoded $pp60^{c\text{-}src}$ and $pp60^{r\text{-}src}$ proteins (Fig. 1*c*). However, in addition to the coding sequence modification, the downstream flanking sequences also differ between pCS12.13 and pRS3 and may play a role in the alteration of functional activity. pRS3 contains 853 bp of RSV downstream flanking sequence including the ≈100-bp direct repeat region, the downstream long-terminal repeat, and some *gag* sequence. These sequences are followed by the c-*src* downstream flanking sequences (beginning at the *Sac* I site 11 bp after the c-*src* termination codon contained in pCS12.13).

DISCUSSION

Expression of significantly enhanced levels of $pp60^{c\text{-}src}$ in NIH 3T3 cells generating *in vitro* kinase activity levels comparable to those of $pp60^{v\text{-}src}$ in transformed cells does not lead to focus formation. This suggests that this normal cellular protein is less effective at inducing transformation than its viral counterpart. While the results provide a rough measure of c-*src* expression, they should be interpreted with care, because the measured activities are dependent on any possible differences in antibody reactivity with proteins from v-*src*, chicken c-*src*, and mouse c-*src*. Indeed, experiments using different TBR antisera yield variable values

Table 1. Comparison of activities of v-*src*, c-*src*, and r-*src* genes and transfected cells

	Focus-forming activity, foci/pmol	% growth in soft agarose (efficiency)	*In vitro* kinase activity (relative)
Plasmid			
p*src*11 (v-*src*)	2×10^4		
pCS12.13 (c-*src*)	<2		
pRS3 (r-*src*)	1×10^4		
Cell line			
NIH 3T3		0%	
NIH(p*src*11) (v-*src*)		≈65% large colonies	1
NIH(pCS12.13).1 (c-*src*)		≈15% small colonies	0.8
NIH(pCS12.13).3 (c-*src*)		≈12% small colonies	0.8

Focus-forming activities were determined from a minimum of two independent transfections of NIH 3T3 cells using triplicate plates. Data were taken using both 0.01 and 0.1 μg of linearized DNA per plate for p*src*11 and pRS3 and using both 0.1 and 1 μg of linearized DNA per plate for pCS12.13. Carrier calf thymus DNA at 20 μg per plate was used in all cases. No foci were seen in the c-*src* transfections. Two biologically cloned v-*src*-transfected cell lines and two biologically cloned pCS12.13-transfected cell lines were assayed for growth in soft agarose. Relative *in vitro* kinase activities were determined by counting phosphorylated IgG bands from immune complex kinase assays on lysates from cloned cells.

(66%–80%) for the c-*src*/v-*src* expression ratio depending on which particular antiserum is used. Since the antisera were made against v-*src*-induced rabbit tumors, the results probably overemphasize the differences between v-*src* and chicken c-*src* expression levels and the difference between these and the endogenous mouse c-*src* expression level, and we expect that the higher relative c-*src*/v-*src* values are correct. Accurate quantitative comparisons will only be possible when characterized monoclonal antibodies directed at conserved structural domains are used.

The hypothesis that plasmid pCS12.13 is expressing authentic $pp60^{c\text{-}src}$ is supported by its demonstrated tyrosine kinase activity, the protein molecular weight, and the transfection results with plasmid pRS3. Since the c-*src* sequence shows that there are at least 30 codons in exons 1 and 2 separating the normal c-*src* initiation codon from any potential upstream initiation codon, we know that a protein initiated at such an inappropriate upstream site would have a molecular size of >63 kDa, in contrast with the observed size of 60 kDa. In addition, r-*src* plasmid pRS3 (which contains exactly the same promoter system, 5′ end, and 10 of 11 of the introns found in c-*src* plasmid pCS12.13) generates a functionally active $pp60^{r\text{-}src}$ that induces efficient focus formation (Fig. 1; Table 1). This implies that pRS3 and, by inference, pCS12.13 are being correctly spliced as well as correctly initiated and expressed to generate functional proteins in amounts adequate for transformation. The downstream RSV LTR is not required for transformation, because an additional clone, pRS13, has been constructed from pRS3 by replacing the RSV LTR and downstream flanking sequence with the SV40 early region polyadenylylation site. This clone, which is identical to pCS12.13 and pRS3 in the promoter and c-*src* portions, also causes focus formation (T. Kmiecik, personal communication).

Given that the pRS3 gene is being correctly processed, it is not surprising that it generates a transforming protein, because the protein sequence generated by the correctly spliced gene is predicted to be very similar to that generated by the transforming recovered avian sarcoma virus rASV1441 (24). This virus, isolated by Hanafusa's group, codes for an *in vivo* r-*src* protein that is identical to that of pRS3 except for a substitution at amino acid position 16 and any other possible differences between SR-A and SR-D v-*src* sequences in the COOH-terminal 96 amino acids.

While $pp60^{c\text{-}src}$ overexpression does not lead to focus formation or *in vivo* tumorigenicity, the altered cellular morphology and partial growth in soft agarose of the c-*src* overexpresser cells show that c-*src* is capable of altering at least some cellular biological parameters. Thus, it is possible that $pp60^{c\text{-}src}$ has a decreased transforming potential that might be fully realized at even higher levels of expression. In this regard, we note that our preliminary results with Moloney murine leukemia virus long-terminal repeat c-*src* expression plasmids suggest that very high level $pp60^{c\text{-}src}$ overexpression (10× the level of $pp60^{v\text{-}src}$ in transformed cells) may induce focus formation (although a completely transformed phenotype including anchorage-independent growth is not induced even at these levels). However, the possible role of mutation in these preliminary results has not yet been excluded.

While the pRS3 recombinant data are consistent with the hypothesis that the functional differences between the c- and v-*src* proteins encoded in the COOH-terminal region, it could be that the activation of transforming activity associated with substitution of the 3′ end of v-*src* for that of c-*src* is at least partially associated with the removal of the downstream untranslated c-*src* sequence. This possibility has precedent in the case of c-*fos*, where it has been found that the 3′ untranslated c-*fos* sequence has an inhibitory effect on transformation (25, 26).

Subsequent to submission of this manuscript, Parker *et al.* (27) have reported that rat cells also are not transformed by overexpression of $pp60^{c\text{-}src}$ to levels above that required for $pp60^{v\text{-}src}$-induced transformation. In their system, they also see evidence of some c-*src*-induced morphological alteration but not of limited c-*src*-induced increase in anchorage-independent growth.

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