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# pp60$^{c\text{-}src}$ Tyrosine Kinase, Myristylation, and Modulatory Domains Are Required for Enhanced Mitogenic Responsiveness to Epidermal Growth Factor Seen in Cells Overexpressing c-*src*

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**In previous studies examining the potential role of pp60$^{c\text{-}src}$ in cellular proliferation, we demonstrated that C3H10T1/2 murine embryo fibroblasts overexpressing transfected chicken genomic c-*src* displayed an epidermal growth factor (EGF)-induced mitogenic response which was 200 to 500% of the response exhibited by parental control cells (Luttrell et al., Mol. Cell. Biol. 8:497–501, 1988). In order to examine specific structural and functional requirements for pp60$^{c\text{-}src}$ in this event, 10T1/2 cells were transfected with chicken c-*src* genes encoding pp60$^{c\text{-}src}$ deficient in tyrosine kinase activity (pm430), myristylation, (pm2A), or a domain hypothesized to modulate the interaction with substrates or regulatory components (dl155). Neomycin-resistant clonal cell lines overexpressing each of the mutated c-*src* genes were assayed for EGF mitogenic responsiveness by measuring [$^3$H]thymidine incorporation into acid-precipitable material or into labeled nuclei. The results were compared with those obtained with lines overexpressing the cDNA form of wild-type (wt) c-*src* or control cells transfected with the neomycin resistance gene only. As previously described for cells overexpressing wt genomic c-*src* (Luttrell et al., 1988), clones overexpressing wt cDNA c-*src* also exhibited enhanced EGF mitogenic responses ranging from approximately 300 to 400% of the control cell response. In contrast, clones overexpressing unmyristylated, modulation-defective, or kinase-deficient c-*src* not only failed to support an augmented response to EGF but also exhibited EGF responses lower than that of the control cells. Furthermore, there were no significant differences in the mitogenic responses to 10% fetal calf serum among any of the cells tested. These results indicate that pp60$^{c\text{-}src}$ can potentiate mitogenic signaling generated by EGF but not by all growth factors. This potentiation requires the utilization of pp60$^{c\text{-}src}$ myristylation, and modulatory and tyrosine kinase domains and can be mediated by cDNA-encoded as well as by genome-encoded wt pp60$^{c\text{-}src}$.**

Cellular *src* is the prototype member of a family of membrane-associated, nonreceptor tyrosine kinase-encoding genes, including *fyn*, *yes*, *hck*, *lck*, *fps*, and *fgr* (J. A. Cooper, *in* B. Kemp and P. F. Alewood, ed., *Peptides and Protein Phosphorylation*, in press). The 60-kilodalton product of c-*src*, pp60$^{c\text{-}src}$, is found in virtually all higher eucaryotic tissues; however, its function(s) and structural requirements in cellular physiology are unknown. The most striking feature of c-*src* is its propensity toward oncogenicity when subjected to relatively minor genetic alterations (22, 40; Cooper, in press; J. T. Parsons and M. J. Weber, Curr. Top. Microbiol. Immunol., in press). The most noted example of this process resulted in the generation of v-*src*, the potent transforming gene of Rous sarcoma virus (47). v- and c-*src* differ only at scattered single amino acids (eight differences between Schmidt-Ruppin A v-*src* and chicken c-*src*) and at the 19 carboxyl-terminal amino acids in pp60$^{c\text{-}src}$, which are replaced with 12 unrelated amino acids in v-*src* (47). Recently, several groups have shown that pp60$^{c\text{-}src}$ transforming potential is activated following the replacement or loss by truncation of a single amino acid residue, tyrosine-527, the major site of tyrosine phosphorylation in pp60$^{c\text{-}src}$ (8, 23, 36, 40). In addition, the transforming activity of the polyomavirus middle-T antigen is inseparable from its ability to bind and posttranslationally activate pp60$^{c\text{-}src}$ (7, 10, 11). These and related findings indicate that deregulated or activated pp60$^{c\text{-}src}$ dramatically affects cellular proliferation.

Several findings support the possible role of unaltered pp60$^{c\text{-}src}$ in normal cellular growth regulation. In response to platelet-derived growth factor, Swiss 3T3 cells contain pp60$^{c\text{-}src}$ which exhibits rapid transient increases in amino-terminal tyrosine and serine phosphorylations and specific activity (16, 37), while pp60$^{c\text{-}src}$ from serum-, phorbol ester-, and platelet-derived growth factor-treated chicken cells displays increases in amino-terminal serine phosphorylation (48). Additionally, NIH 3T3 cells transfected with avian c-*src* contain pp60$^{c\text{-}src}$ with novel amino-terminal threonine phosphorylations which appear during mitosis (9). The growth factor-induced changes seen in pp60$^{c\text{-}src}$ hint at its involvement in mediating extracellular growth signals, an activity which appears to occur at several different points in the cell cycle.

In previous studies directed toward better understanding the possible involvement of pp60$^{c\text{-}src}$ in the regulation of cellular proliferation, we reported an EGF-induced mitogenic hyperresponsiveness in 10T1/2 murine fibroblasts overexpressing avian c-*src*. While the number and affinity of EGF receptors as well as the morphology and generation times of these cells appeared to be normal, their EGF-induced mitogenic response was 200 to 500% of that displayed by untransfected or *neo*-only transfected 10T1/2 cells (27). These findings suggest that pp60$^{c\text{-}src}$ can potentiate the mitogenic effect of EGF and perhaps influence EGF-induced signal transduction in fibroblasts. To further characterize the possible involvement of pp60$^{c\text{-}src}$ in this event, we have transfected into 10T1/2 cells c-*src* genes mutated at sites predicted

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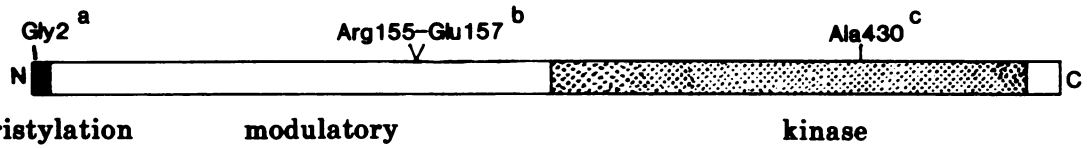


FIG. 1. Domains and mutations in pp60$^{src}$ characterized in this study. Mutations: a, pm2A (Gly-2 to Ala); b, dl155 (Arg-155–Arg-156–Glu-157 deleted); c, pm430 (Ala-430 to Val).

to be critical for c-*src* function. These studies represent a unique structural and functional analysis of c-*src* by using EGF mitogenic responsiveness in transfected cells as a biological assay for c-*src* activity.

The mutations that we introduced into c-*src* were directed toward three functional domains which have been delineated through extensive mutational analysis of v-*src*. Thus, our understanding of these regions is mainly in the context of the viral gene and its product. The first domain, responsible for membrane attachment, resides within the N-terminal seven amino acids of pp60$^{v\text{-}src}$ (21, 35). Mutation of sequences encoding glycine-2 prevents myristylation at the N-terminus of nascent *src* molecules, rendering them cytoplasmic (6). Although unmyristylated pp60$^{v\text{-}src}$ is kinase active, it lacks transforming ability, presumably due to its failure to phosphorylate critical membrane-associated substrates (5, 19, 39). The second domain spans most of the amino-terminal half of v-*src* and is thought to modulate its activity. Mutations of v-*src* between amino acids 143 and 169 within this region, such as the deletion of residues 155 to 157, result in kinase-active, membrane-attached products with diminished transforming ability, suggesting that at least part of the modulatory domain mediates interactions between pp60$^{v\text{-}src}$ and substrates or regulatory components (13, 38, 50). The third domain, required for tyrosine kinase activity, is highly conserved in all members of the *src* family and maps to residues 260 to 516 in pp60$^{c\text{-}src}$ (3; Cooper, in press). Mutations within the highly conserved Ala-Pro-Glu sequence (residues 430 to 432), including pm430, abolish not only the phosphotransferase activity but also the transforming ability of pp60$^{v\text{-}src}$, indicating the importance of tyrosine kinase activity in pp60$^{v\text{-}src}$ function (4, 31).

Homology between the viral and cellular *src* genes is sufficient for prediction of the phenotype of c-*src* mutations based on the results of identical mutations in v-*src* (50; Cooper, in press; Parsons and Weber, in press). For our studies of the cellular gene, the three mutations we have chosen are a Gly-to-Ala change at the myristylation site (pm2A) (19); a deletion of Arg-155–Arg-156–Gly-157 in the modulatory domain (dl155) (50); and an Ala-to-Val change in the kinase domain (pm430) (4) (Fig. 1). Here we demonstrate the ability of overexpressed cDNA-encoded pp60$^{c\text{-}src}$ to mediate EGF mitogenic hyperresponsiveness in murine fibroblasts, supporting our original observations made with overexpressed genome-encoded pp60$^{c\text{-}src}$. In addition, we show that this ability requires the functional integrity of domains mediating pp60$^{c\text{-}src}$ membrane attachment, interaction with cellular components, and tyrosine kinase activity.

## MATERIALS AND METHODS

**DNA and transfections.** Specific mutations in chicken c-*src* cDNA were generated by oligonucleotide-directed mutagenesis in M13 (40, 50). In order to express the mutated genes in 10T1/2 cells, an expression vector was generated by replacing the *Sal*I-*Bgl*II v-*src*-containing fragment from the Moloney murine leukemia virus long terminal repeat (LTR)-driven pMv-*src* plasmid (18) (provided by D. Shalloway) with a *Sal*I-*Bgl*II fragment containing chicken cDNA c-*src* from the p5H plasmid (25) (provided by H. Hanafusa). The

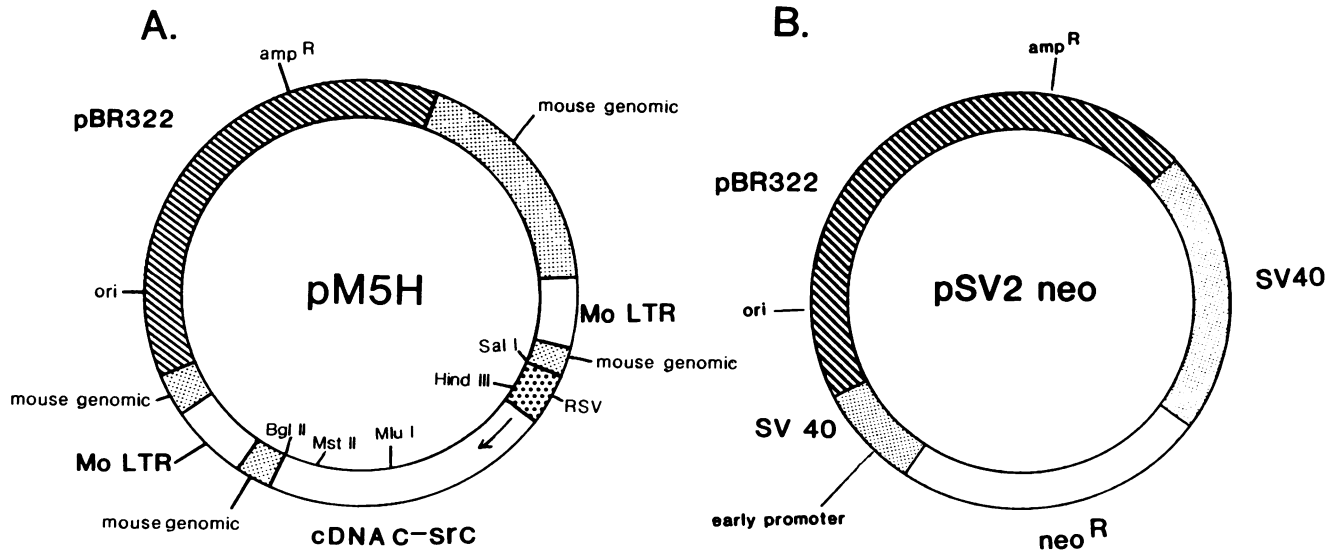


FIG. 2. Plasmids used in transfections of C3H10T1/2 cells. (A) pM5H (see Materials and Methods) was used as a wt c-*src* expression vector and as a parent plasmid into which fragments containing mutated c-*src* fragments were ligated. (B) pSV2neo (37) was used in cotransfections to confer G418 resistance to transfectants. Abbreviations: Mo, Moloney murine leukemia virus; RSV, Rous sarcoma virus; SV40, simian virus 40.

resulting cDNA c-*src* expression vector, pM5H (Fig. 2), was used for transfections of wild-type (wt) c-*src* and as a parent plasmid into which gene fragments containing the c-*src* mutations were ligated from M13. Specifically, the 3′ half of *src* (from *Mlu*I to *Mst*II) and the 5′ half of *src* (from *Hin*dIII to *Mlu*I) were used to introduce the pm430 and the dl155 mutations, respectively, into pM5H. A *Sal*I-*Mlu*I fragment containing the pm2A mutation was used to replace the homologous region in pJHc, a vector virtually identical to pM5H. Each of the four plasmids, pM5H (encoding wt $pp60^{c\text{-}src}$), pM430 (encoding kinase-deficient pm430 $pp60^{c\text{-}src}$), pM2A (encoding unmyristylated pm2A $pp60^{c\text{-}src}$), and pM155 (encoding modulatory-defective dl155 $pp60^{c\text{-}src}$), identical except for the specified mutations, were cotransfected with the pSV2neo plasmid (Fig. 2) (45) into passage 22 10T1/2 cells by the calcium phosphate precipitate method (49, 51). Transfected G418-resistant colonies were cultured in Dulbecco modified essential medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 0.4 mg of Genticin (GIBCO, Grand Island, N.Y.) per ml and screened immediately for expression of chicken $pp60^{c\text{-}src}$ by the Western immunoblotting technique (data not shown) with immunoprecipitates prepared with the avian $pp60^{c\text{-}src}$-specific monoclonal antibody (MAb) EC10, whose epitope requires an intact region containing $pp60^{c\text{-}src}$ residues 28 to 38 (33, 34). All cells were maintained at subconfluence and used for experimentation prior to passage 8 posttransfection.

**$pp60^{c\text{-}src}$ kinase assays.** Immunoprecipitations and kinase assays were performed essentially as described before (29). Briefly, cells were lysed in RIPA buffer (150 mM NaCl, 1% deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate [SDS], and 50 mM Tris [pH 7.2]) and immunoprecipitated with the rodent or avian $pp60^{src}$-specific MAb GD11, whose epitope requires an intact region containing $pp60^{c\text{-}src}$ residues 92 to 128 (33, 34). Immune complexes were washed twice in RIPA containing 1 M NaCl, once in HO buffer (100 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 1 mM EDTA, 50 mM Tris [pH 7.2]), and once in phosphate-buffered saline (150 mM NaCl, 10 mM phosphate, pH 7.0) and then incubated for 15 min at 25°C in 20 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)]–10 mM $MnCl_2$–1 μM (10 μCi) [γ-$^{32}$P]ATP (7,000 Ci/mmol; New England Nuclear [NEN], Boston, Mass.)–5 μg of enolase in a final volume of 30 μl. Reaction products were analyzed by electrophoresis through sodium dodecyl sulfate–7.5% polyacrylamide gels and autoradiography and quantitated by laser densitometry.

**Western immunoblots.** Western immunoblots were prepared essentially as described by Kanner et al. (20). $pp60^{c\text{-}src}$ immunoprecipitates (prepared with MAb GD11) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose (Schleicher & Schuell, Keene, N.H.) for 3 h at 80 V in 1.4% glycine–0.3% Trizma base–20% methanol. Blots were incubated for 3 h in blocking solution (3% instant nonfat dry milk [generic], 0.5% bovine serum albumin [Sigma], 0.1% Tween 20 [Sigma], 0.5 M NaCl, 0.5% Nonidet P-40 [Sigma], 50 mM Tris [pH 7.2]) and for an additional 2 h in blocking solution plus $^{125}$I-labeled MAb 327 (1 μCi/μg) (26). Blots then underwent three 10-min washes in blocking solution, were allowed to air dry, and were then exposed to Kodak X-OMAT RP film. The level of $pp60^{c\text{-}src}$ was quantitated by laser densitometric analysis of autoradiographs.

**$pp60^{c\text{-}src}$ subcellular distribution.** The subcellular distribution of $pp60^{c\text{-}src}$ was determined as described by Krueger et al. (24), by preparing cytosolic and membrane plus nuclear fractions from cells which had been hypotonically swollen in 10 mM Tris (pH 7.4)–1 mM $MgCl_2$, homogenized, and centrifuged at 100,000 × *g* for 30 min at 4°C. The resulting supernatant and pellet fractions were adjusted to equal volumes in electrophoresis sample buffer and analyzed directly for $pp60^{c\text{-}src}$ content by Western immunoblotting.

**Growth factor treatment and measurements of DNA synthesis.** To measure incorporation of [$^3$H]thymidine into acid-precipitable material, density-arrested cells plated in 35-mm dishes were serum starved in DMEM plus 0.1% bovine serum albumin (fraction V) for 24 h and refed with starvation medium containing either EGF (Collaborative Research, Bedford, Mass.) (30 ng/ml) or 10% FCS. After 20 h, cells were labeled with 1 μCi of [*methyl*-$^3$H]thymidine (20 Ci/mmol; NEN) in starvation medium at 37°C for 30 min, washed, scraped, and incubated for 30 min at 4°C in STE (0.15 M NaCl, 50 mM Tris [pH 7.2], 1 mM EDTA) and 10% trichloroacetic acid (TCA). TCA precipitates were filtered on GF/A glass fiber filters (Whatman, Inc., Clifton, N.J.), rinsed extensively with 6% TCA, digested in NCS tissue solubilizer (Amersham Corp., Arlington Heights, Ill.), and quantitated by liquid scintillation counting. Cells in duplicate unlabeled plates were counted after stimulation, and counts per minute incorporated were represented per $10^5$ cells. To quantitate percentages of labeled nuclei, cells were starved, EGF stimulated, and labeled for 1 h as described above, fixed for 30 min in cold methanol-acetic acid (3:1), washed with cold methanol, coated with Kodak NTB2 emulsion-water (1:1), and exposed for 72 h at 10°C. Autoradiographic plates were developed with Kodak D-19 and fixed, and nuclei were quantitated by scoring fields of approximately 200 cells in pairs of duplicate plates.

## RESULTS

**Generation of cell lines overexpressing wt or mutated c-*src*.** Clonal cell lines overexpressing wt or mutated c-*src* were derived by cotransfecting C3H10T1/2 cells with the cDNA c-*src*-containing plasmid pM5H or its mutant-containing derivatives together with the pSV2neo plasmid (Fig. 1 and 2), and relative levels of c-*src* expression were initially analyzed by using the avian $pp60^{c\text{-}src}$-specific MAb EC10, as described in Materials and Methods. Multiple neomycin-resistant cell lines which overexpressed each form of *src* were used for subsequent characterizations. These cells retained normal morphology and saturation density when maintained at low passage and subconfluence. For comparison with the avian *src*-containing cotransfected lines, a nonclonal mixture of G418-resistant cells (NM cells) transfected solely with pSV2neo was also generated. Table 1 lists representatives of each cell type and their properties, which are described in detail below.

**Characterization of overexpressed $pp60^{c\text{-}src}$.** To determine the relative levels of expression and the specific kinase activities of the wt and structurally altered $pp60^{c\text{-}src}$ produced in each clone, cell lysates adjusted to equal protein were immunoprecipitated with the avian and murine c-*src*-reactive MAb, GD11. Immunoprecipitates were analyzed either by the Western immunoblotting technique or by the in vitro immune complex kinase assay (Fig. 3). Levels of introduced $pp60^{c\text{-}src}$ in different cotransfected cell lines ranged from 2- to 34-fold over the endogenous level found in parental 10T1/2 or NM cells (Fig. 3B, Table 1). Although we generated multiple clones which expressed pm2A or pm430 c-*src* at high levels (approximately 20-fold higher than endogenous levels), we were unable to obtain clones ex-

TABLE 1. Characteristics of individual transfected cell lines

| Cell line | Transfected DNA | Transfected c-*src* phenotype | Relative expression[a] | Sp act[b] | | pp60$^{c\text{-}src}$ distribution (M:C, % of total)[c] | Increase in EGF response[d] (fold) |
|---|---|---|---|---|---|---|---|
| | | | | Auto | Enolase | | |
| 10T1/2 | None | None | 1 | 1 | 1 | 76:24 | 4.7 |
| NM | pSV2neo | None | 1 | 1 | 1 | 92:8 | 4.9 |
| 5Hb10 | pM5H + pSV2neo | wt | 23.8 | 1.4 | 0.8 | 93:7 | 20.4 |
| H13 | pM5H + pSV2neo | wt | 3.3 | ND[e] | ND | ND | 14.9 |
| 5Hb7 | pM5H + pSV2neo | wt | 2.0 | 1.4 | 1.9 | ND | 13.4 |
| 430-7 | pM430 + pSV2neo | Kinase deficient | 1.98 | <0.02 | 0.01 | 96:4 | 2.2 |
| 430-4 | pM430 + pSV2neo | Kinase deficient | 12.8 | 0.13 | 0.08 | 80:20 | 2.5 |
| 430-15 | pM430 + pSV2neo | Kinase deficient | 4.3 | <0.01 | 0.06 | ND | 3.2 |
| 2A-5 | pM2A + pSV2neo | Unmyristylated | 33.7 | ND | ND | 6:94 | 1.7 |
| 2A-2 | pM2A + pSV2neo | Unmyristylated | 8.6 | 0.7 | 0.4 | 10:90 | 2.5 |
| 2A-7 | pM2A + pSV2neo | Unmyristylated | 5.3 | 2.3 | 1.1 | ND | 1.7 |
| 155-9 | pM155 + pSV2neo | Altered modulation | 8.5 | 0.6 | 0.5 | 98:2 | 2.0 |
| 155-16 | pM155 + pSV2neo | Altered modulation | 2.3 | 0.8 | 1.9 | ND | 3.0 |

[a] pp60$^{c\text{-}src}$ was quantitated in each cell line by laser densitometry of Western immunoblot autoradiographs prepared from MAb GD11 immunoprecipitates as described in Materials and Methods. Values were normalized to the level of endogenous pp60$^{c\text{-}src}$ found in NM cells.

[b] pp60$^{c\text{-}src}$ specific activity values represent the extent of autophosphorylation or phosphorylation of the exogenous substrate enolase, normalized to the amount of pp60$^{c\text{-}src}$ present in the immune complex. The amount of pp60$^{c\text{-}src}$ was determined by Western immunoblotting, and phosphotransferase activities were measured by the in vitro immune complex kinase assay, each performed as described in the legend to Fig. 3. Values thus obtained were corrected for the contribution of endogenous pp60$^{c\text{-}src}$. Specific activities are expressed as the specific activity of overexpressed pp60$^{c\text{-}src}$ divided by the specific activity of endogenous pp60$^{c\text{-}src}$ from NM cells.

[c] pp60$^{c\text{-}src}$ subcellular distribution was quantitated by laser densitometry of Western immunoblot autoradiographs of cell fractions prepared as described in Materials and Methods. Values are expressed as percentage of total cellular pp60$^{c\text{-}src}$ in each fraction. M, Membrane-nuclear fraction; C, cytosolic fraction.

[d] EGF response values ([$^3$H] cpm [EGF stimulated]/[$^3$H] cpm [nonstimulated]) from a representative experiment performed with each cell line by the acid precipitation technique. Values indicate fold increase in EGF response in EGF-stimulated cultures. Each experiment was done with triplicate plates of both EGF-stimulated and nonstimulated cells.

[e] ND, Not done.

pressing dl155 c-*src* more than 8.5-fold higher than the endogenous level, perhaps reflecting a toxicity effect or the low stability of the dl155 variant protein. Indeed, a decreased half-life of the product of dl155 viral *src* has been reported (50), suggesting that this deleted region of both v-*src* and c-*src* may be critical for interaction with putative stabilizing components.

As calculated from levels of expression and phosphotransferase activity, the relative specific kinase activity of overexpressed wt, pm2A, and dl155 c-*src* was found to be similar to that of endogenous murine pp60$^{c\text{-}src}$ (Fig. 3A, Table 1). The twofold differences in specific kinase activity seen in Table 1 are within the range of accuracy and reproducibility of the immune complex kinase assay. In contrast, pm430-encoded specific kinase activity was markedly reduced to approximately 1 to 10% of the wt level (Table 1). This observation is in agreement with previous studies on the pm430 mutation in v-*src* (4).

To verify the predicted subcellular distributions of the various overexpressed c-*src* products, cells were separated into cytoplasmic and membrane plus nuclear fractions by differential centrifugation as described in Materials and Methods. Relative pp60$^{c\text{-}src}$ levels in the resulting fractions were then quantitated by the Western immunoblotting procedure (Fig. 4, Table 1). wt transfected pp60$^{c\text{-}src}$ showed a distribution similar to that of endogenous pp60$^{c\text{-}src}$ from 10T1/2 parental cells or NM cells, with 76% or more found in the membrane plus nuclear fraction. pm430- and dl155-

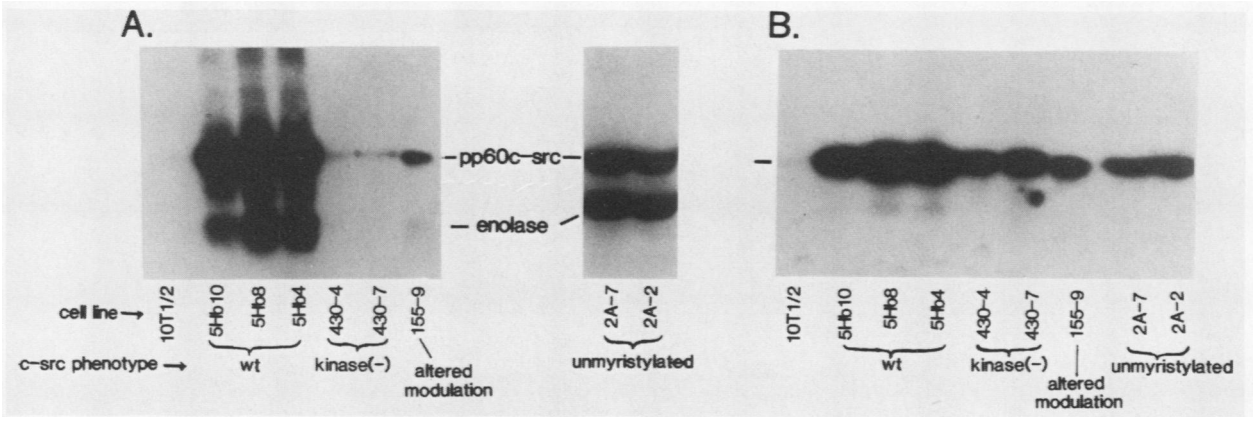


FIG. 3. Activity and expression levels of overexpressed pp60$^{c\text{-}src}$. The expected phenotypes of pp60$^{c\text{-}src}$ in representative cell clones are indicated. (A) In vitro immune complex kinase activity. Cell lysate samples, each containing 250 μg of total protein, were immunoprecipitated with pp60$^{src}$-specific MAb GD11 and assayed for phosphotransferase activity, with enolase added as an exogenous substrate as described in Materials and Methods. Autoradiograph exposure time was 30 min. (B) pp60$^{c\text{-}src}$ expression level. Samples from the same lysates as in panel A, each containing 750 μg of total protein, were immunoprecipitated with MAb GD11 and analyzed by the Western immunoblot technique with $^{125}$I-labeled MAb 327 as a probe. Autoradiograph exposure time was 16 h. Kinase(−), kinase deficient.

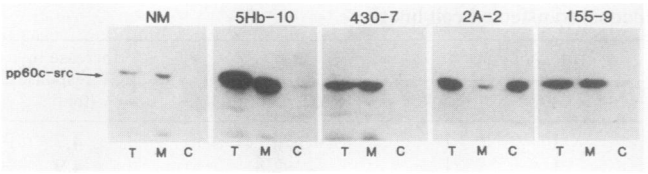


FIG. 4. Subcellular distributions of the overexpressed c-*src* products. Cell lysates obtained by hypotonic disruption were adjusted to equal protein concentration and centrifuged to obtain cytosolic and membrane-nuclear fractions. Both fractions were brought to equal volume in sample buffer, and the amount of $pp60^{c\text{-}src}$ was quantitated by Western immunoblotting. Equal cell amounts were loaded into each lane. Lanes: T, total cellular $pp60^{c\text{-}src}$ (membrane-nuclear fraction plus cytosolic fraction loaded together); M, membrane-nuclear fraction; C, cytosolic fraction.

encoded $pp60^{c\text{-}src}$ also exhibited subcellular localizations similar to that of endogenous $pp60^{c\text{-}src}$, demonstrating that membrane binding is independent of both kinase activity and the presence of a critical portion of the modulatory domain. In contrast, due to its lack of amino-terminal myristylation, approximately 90% of pm2A $pp60^{c\text{-}src}$ was found in the cytosolic fraction. This observation is consistent with earlier studies on the pm2A mutation in v-*src* (19).

The relative changes in kinase activities and membrane localizations of the pm430, pm2A, and dl155 c-*src* products closely resembled those of v-*src* products bearing the same mutations (4, 19, 50), highlighting the structural and functional similarities between the two genes. Disruption of the membrane-binding domain in pm2A c-*src* resulted in the appearance of a cytoplasmic yet kinase-active product, while the carboxyl-terminal pm430 mutation resulted in a kinase-deficient yet membrane-bound $pp60^{c\text{-}src}$. However, because the product of dl155 c-*src* retained nearly normal membrane-binding and phosphotransferase abilities, assessment of its putative functional defect (i.e., deficient interaction with cellular components) required the in vivo proliferation assays described below. The only functional characteristic of the modulatory domain determined thus far has been its requirement for v-*src*-mediated transformation.

**Mitogenic responsiveness in cells overexpressing normal and mutated c-*src*.** We showed previously that C3H10T1/2 cells overexpressing wt genomic avian c-*src* displayed greater mitogenic responsiveness to EGF than parental 10T1/2 cells (27). To determine whether this effect was reproducible with the use of a different c-*src* expression plasmid and an intronless form of avian c-*src*, pM5H-transfected cell lines overexpressing wt cDNA c-*src* were tested for their mitogenic responsiveness to EGF. Density-arrested cells were serum starved and treated with EGF, and their incorporation of [$^3$H]thymidine into TCA-precipitable material was measured as described in Materials and Methods. As shown in Table 1, three clonal cell lines overexpressing wt cDNA c-*src* exhibited responses that ranged from 270 to 430% of those of the parental 10T1/2 or NM cell lines. Figure 5 depicts the average counts of [$^3$]thymidine incorporated per min (per $10^5$ cells) into these cell lines in multiple experiments and allows comparison with averages obtained for NM cells and cell lines expressing mutated forms of c-*src*. As calculated from the values in Fig. 5, the average response

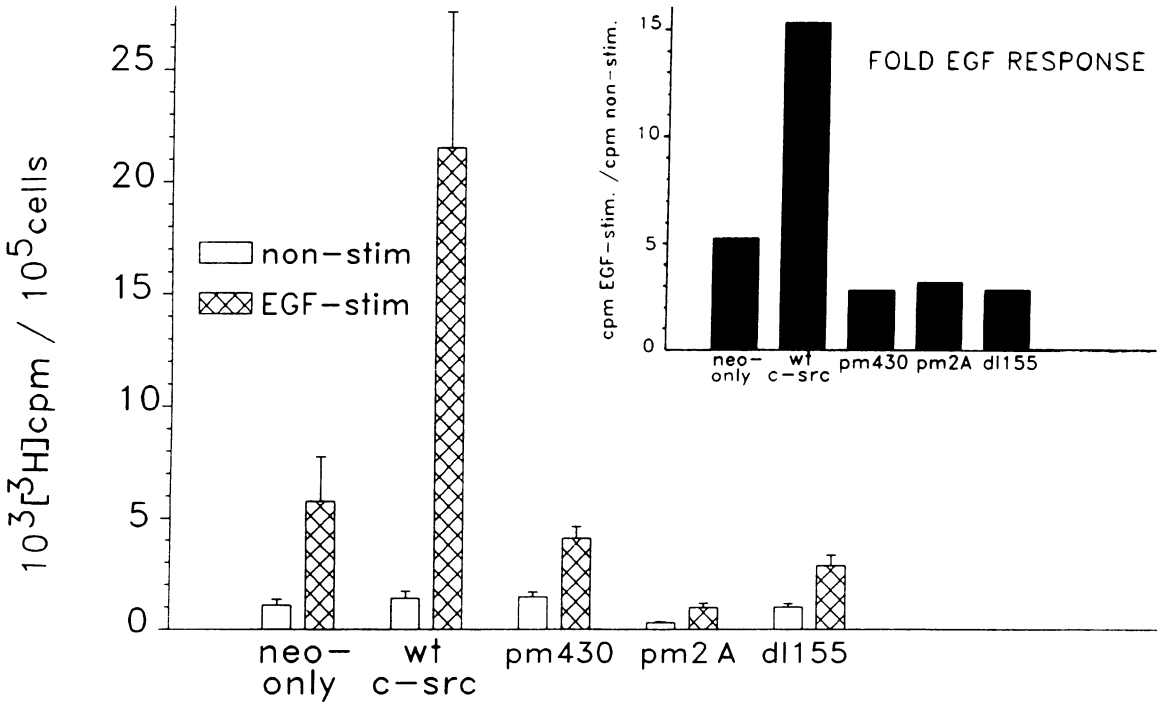


FIG. 5. EGF-induced DNA synthesis in cells overexpressing wt and mutated c-*src*. Confluent cells were serum starved, stimulated with EGF for 20 h, and assayed for the incorporation of [$^3$H]thymidine into TCA-precipitable material as described in Materials and Methods. The bars labeled neo-only represent the mean values ± SEM of four experiments performed with NM cells. The bars labeled wt c-*src*, pm430, pm2A, and dl155 represent the mean value ± SEM of 8, 10, 4, and 5 experiments, respectively, performed on the cell lines listed in Table 1. Each experiment was done on triplicate sets of EGF-stimulated (cross-hatched bars) and nonstimulated (open bars) dishes of cells. Values for EGF-stimulated cells were divided by the corresponding values for nonstimulated cells to obtain the fold increase in EGF response after stimulation for each cell type (inset).

TABLE 2. Comparison of the EGF mitogenic response as measured by [$^3$H]thymidine-labeled nuclei and acid precipitation assays[a]

| Cell line | Mean % LN ± SEM[b] | | | Mean TCA-precipitated DNA ($^3$H cpm) ± SEM[c] | | |
|---|---|---|---|---|---|---|
| | NS | EGF | Ratio, EGF/NS (fold stimulation) | NS | EGF | Ratio, EGF/NS (fold stimulation) |
| NM (*neo* only) | 0.33 ± 0.02 | 2.56 ± 0.54 | 7.7 | 2,200 ± 450 | 9,700 ± 1,300 | 4.5 |
| H13 (wt c-*src*) | 0.31 ± 0.03 | 3.86 ± 0.22 | 12.5 | 2,700 ± 580 | 29,500 ± 4,300 | 11.1 |
| 430-4 (kinase deficient) | 0.27 ± 0.04 | 1.02 ± 0.06 | 3.8 | 1,000 ± 40 | 5,000 ± 700 | 4.8 |
| 2A-5 (unmyristylated) | 0.28 ± 0.01 | 1.73 ± 0.48 | 6.2 | 4,400 ± 430 | 4,500 ± 150 | 1.0 |
| 155-9 (altered modulation) | 0.49 ± 0.08 | 1.48 ± 0.81 | 3.0 | 2,000 ± 280 | 5,200 ± 980 | 2.6 |

[a] Confluent cells were serum starved, not stimulated (NS) or stimulated with EGF, and labeled with [$^3$H]thymidine. Fraction of labeled nuclei (LN) and amount of TCA-precipitable material were quantitated as described in Materials and Methods.

[b] Percentages of labeled nuclei from duplicate pairs of nonstimulated and EGF-stimulated plates. Approximately 200 cells per 35-mm dish were scored.

[c] EGF-induced DNA synthesis measured by the TCA precipitation technique. Counts of $^3$H incorporated per minute per $10^5$ nonstimulated and EGF-stimulated cells are presented.

of the wt c-*src* overexpressers was 290% of the average response of the NM cells (Fig. 5, inset). Although hyperresponsiveness was observed in multiple cell lines overexpressing wt c-*src* (from 2 to 34 times the level detected in parental cells), a strict correlation between the level of transfected wt pp60$^{c\text{-}src}$ and the extent of EGF hyperresponsiveness could not be made. Possible reasons for these findings have been discussed previously (27). These results confirm our original observations of EGF hyperresponsiveness in cells overexpressing genomic c-*src* (27) and support the contention that pp60$^{c\text{-}src}$ can potentiate the EGF signaling process.

To investigate the structural and functional requirements of c-*src* in its apparent potentiation of EGF-induced mitogenesis, cells overexpressing mutated c-*src* were also examined for their levels of [$^3$H]thymidine incorporation in response to EGF treatment. As shown in Table 1 and Fig. 5, none exhibited an augmented EGF response such as that seen in cells overexpressing wt c-*src*. Rather, they appeared to respond less well than the parental 10T1/2 or *neo*-only transfected cells. For example, three clonal lines overexpressing pm430 c-*src* exhibited responses which ranged from approximately 50 to 70% of that of NM cells (Table 1), clearly indicating the requirement for tyrosine kinase activity in the potentiation of EGF responsiveness. Likewise, two cell lines overexpressing dl155 c-*src* showed an EGF response which ranged from 40 to 60% of that of NM cells, suggesting that pp60$^{c\text{-}src}$ must be able to interact with cellular components in order to augment the EGF mitogenic response (Fig. 5, Table 1). However, cells overexpressing pm2A c-*src* also showed reduced DNA synthesis not only after EGF stimulation, but also during serum starvation (Fig. 5), resulting in an EGF response ranging from 40 to 50% of that of NM cells. The observations with pm2A indicate a requirement for membrane localization in the EGF potentiation effect and also suggest a possible increase in serum requirements of pM2A c-*src*-containing cells, which is detectable during serum starvation and not during routine culturing in 10% FCS. The decrease in the fold EGF responsiveness in cells overexpressing each of the mutated forms of c-*src* may reflect inhibition of the normal EGF signaling pathways and is addressed further in the Discussion.

To determine whether the differences in EGF responsiveness were reflected in the actual number of cells responding in a population, labeled nuclei from [$^3$H]thymidine-labeled, fixed, and autoradiographed cells were quantitated (Table 2). The patterns of EGF responsiveness obtained in the labeled-nuclei experiments were similar to those obtained in the acid precipitation experiments. Thus, overexpression of wt c-*src* appeared to increase the number of cells that would normally enter S phase in response to EGF, yet those numbers were substantially decreased in cell lines containing high levels of pp60$^{c\text{-}src}$ deficient in kinase activity, myristylation, or protein interaction.

To determine whether other growth-stimulatory pathways were affected by overexpressed c-*src*, confluent cells were serum starved, treated with 10% FCS as a source of growth-stimulatory agents, and assayed for DNA synthesis by the acid-precipitation method. As shown in Fig. 6, cells overexpressing wt c-*src* showed no significantly greater mitogenic response to FCS (shown as fold increase in DNA synthesis after stimulation) than did NM cells, revealing a predominance of growth factors present in FCS to which wt c-*src* overexpressors did not hyperrespond. Cells overexpressing pm430-, pm2A-, and dl155-encoded c-*src* responded to FCS with a vigor similar to or even greater than that of NM cells, suggesting that their slight EGF hyporesponsiveness did not result from a general deficiency in mitogenic signaling. Thus, the overexpression of wt and mutant pp60$^{c\text{-}src}$ did not appear to influence mitogenic responsiveness to all growth factors, but probably to an as yet undefined subset which includes EGF.

## DISCUSSION

The experiments illustrated in this report demonstrate that overexpression of wt c-*src* encoded by a cDNA expression vector results in augmentation of the EGF mitogenic response in C3H10T1/2 murine fibroblasts in a manner analo-

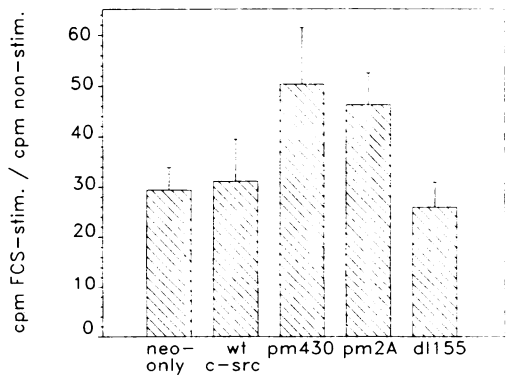


FIG. 6. FCS-induced DNA synthesis in cells overexpressing wt or mutant c-*src*. Confluent cells were serum starved, stimulated with 10% FCS for 20 h, and assayed for DNA synthesis as described in the legend to Fig. 5. Mean values of 10 separate experiments with two to five clones of each cell type are represented as fold increase in response over that in nonstimulated cells ± SEM.

gous to that previously shown for the genomic c-*src* (27). Workers in our laboratory and others have detected c-*src* products with altered electrophoretic mobility in cells transfected with the genomic c-*src* expression vector pMc-*src* (data not shown) or in cells cotransfected with pMc-*src* and a plasmid encoding the 5′ half of polyomavirus large-T antigen (42). Use of the cDNA c-*src* expression vector pM5H, which yielded no size-altered products, ensured that pp60$^{c\text{-}src}$ of normal mobility was responsible for the enhanced EGF response. More importantly, we were able to use the cDNA vector as a parent for expression of mutated forms of the gene. We transfected cells with pm2A, dl155, and pm430 c-*src* DNA in place of wt and observed a striking decrease in EGF responsiveness, in contrast to the enhanced levels seen in cells overexpressing wt c-*src*. Thus, we have clearly demonstrated the requirement for intact myristylation, modulatory, and kinase domains of c-*src* in its potentiation of the EGF mitogenic response. Although the pm2A, dl155, and pm430 mutations have been introduced into v-*src* and shown to ablate its transforming ability, this is the first report of the introduction of these mutations into c-*src* and the use of a biological assay (potentiation of the EGF response) to test their effects. These results support the model that the cellular and viral *src* genes encode structurally and functionally similar proteins, both of which potentiate growth-stimulatory pathways, but which differ in their regulation.

The mechanism by which pp60$^{c\text{-}src}$ influenced the EGF response is unclear, but analysis of variant c-*src* proteins may provide some insights. Expression of the pm2A, dl155, and pm430 c-*src* genes each had similar effects on EGF responsiveness. First, cells overexpressing the mutated genes failed to show the EGF mitogenic hyperresponsiveness seen with overexpression of wt c-*src* whether total DNA synthesized or percentage of responding cells was measured. The mutations apparently altered regions of pp60$^{c\text{-}src}$ which were critical to its growth potentiation activity. The second common effect of the pm2A, pm430, and dl155 mutations was that the cell lines expressing them were less responsive to EGF than were the mock-transfected NM cells. This suggests a partial inhibition of normal EGF signaling due either to the competitive inhibition of endogenous pp60$^{c\text{-}src}$ or to inappropriate interactions or phosphorylations by the mutant pp60$^{c\text{-}src}$. Because the EGF potentiating activity of wt overexpressed pp60$^{c\text{-}src}$ requires intact myristylation, modulatory, and tyrosine kinase domains, pp60$^{c\text{-}src}$ most likely functions by contacting and phosphorylating one or more specific membrane-localized proteins which affect the EGF response.

Although the cells overexpressing wt or mutant c-*src* showed altered mitogenic responsiveness to EGF, their response to 10% FCS was similar to that of NM cells. This explains the ability of these cell lines to grow normally under regular growth conditions in 10% FCS and indicates that pp60$^{c\text{-}src}$ can potentiate signaling pathways triggered by only a subset of the growth factors present in serum. Serum components other than EGF appear to be sufficient for the maximum release of NM or mutant c-*src* overexpresser cell populations from quiescence. The major growth factors (by mass) found in serum include insulin and transferrin (17; Hyclone Laboratories, personal communication), so the ability of these peptides to elicit hyperresponsiveness in wt c-*src* overexpressers is doubtful. Indeed, we have found little if any difference in insulin-stimulated mitogenesis between NM cells and wt c-*src* overexpressers (unpublished observations). Although overexpression of wt pp60$^{c\text{-}src}$ does not affect the EGF concentration required for maximum mitogenic stimulation (27), it may alter the dose requirements for serum and mitogens other than EGF. Therefore, detailed dose-response analyses of these substances are under way.

The specific functional niche occupied by pp60$^{c\text{-}src}$ will remain elusive until the proteins with which it interacts are identified. Although there is no direct evidence for interaction between pp60$^{c\text{-}src}$ and the EGF receptor, activation by EGF seems to elicit changes in pp60$^{c\text{-}src}$ activity, as suggested by the increased phosphorylation of the middle-T antigen in polyomavirus-infected cells treated with EGF (41) and in the rapid and transient increases in autophosphorylation activity of pp60$^{c\text{-}src}$ from EGF-stimulated Swiss 3T3 fibroblasts (27). In addition, in Rat-1 fibroblasts infected with temperature-sensitive LA29 Rous sarcoma virus, pp60$^{v\text{-}src}$ has been found to stimulate the tyrosine phosphorylation of overexpressed human EGF receptor at the permissive but not at the nonpermissive temperature in the absence of EGF (W. Wasilenko and M. Weber, manuscript in preparation). Together, these findings suggest some type of interaction between pp60$^{c\text{-}src}$ and the EGF receptor, providing a possible mode by which overexpressed c-*src* could potentiate the EGF response. It is possible that pp60$^{c\text{-}src}$ and the EGF receptor contact and phosphorylate each other or that they have common substrates which in turn continue propagation of the mitogenic signal. Alternatively, pp60$^{c\text{-}src}$ may influence the EGF response at a site downstream from the EGF receptor. Interestingly, the *src* modulatory domain exhibits sequence similarity with the B region of the v-*crk* oncogene product and the growth-regulatory enzyme bovine brain phospholipase C II, suggesting that these proteins may share protein interaction properties with pp60$^{c\text{-}src}$ (28, 46). These sequence similarities, together with our recent observation that c-*src* overexpressers produce elevated amounts of cyclic AMP in response to epinephrine (unpublished observations), hint at possible G protein involvement with pp60$^{c\text{-}src}$.

Accumulating evidence suggests that pp60$^{c\text{-}src}$ participates in activities as diverse as proliferation (9, 37, 48), differentiation (2, 43, 44), exocytosis (14, 15, 32), and junctional communication (1), depending on the cell type studied. This apparent diversity seems feasible if pp60$^{c\text{-}src}$ functions in a mechanism, such as receptor-mediated signaling, which may be required for each cellular event. Artificially elevating the amounts of pp60$^{c\text{-}src}$ in cells has provided us with a way of amplifying what is perhaps a natural function of endogenous pp60$^{c\text{-}src}$ in dividing cells: propagation of the intracellular signals for certain growth factors.

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