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Biochemistry

Characterization of avian and viral p60^{src} proteins expressed in yeast

(tyrosine protein kinase/yeast expression plasmid)

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ABSTRACT **Avian and viral p60^{src} proteins were expressed from a galactose-inducible promoter in the yeast *Saccharomyces cerevisiae*. Both the viral and cellular src proteins produced in yeast cells were myristoylated at their amino termini, as is the case for src proteins expressed in chicken embryo fibroblasts. The viral src protein produced in yeast autophosphorylated at tyrosine-416 *in vivo* and had approximately the same level of *in vitro* kinase activity as p60$^{v\text{-}src}$ expressed in Rous sarcoma virus-transformed cells. Unlike p60$^{c\text{-}src}$ expressed in chicken cells, which is phosphorylated on tyrosine *in vivo* almost exclusively at tyrosine-527, p60$^{c\text{-}src}$ expressed in yeast was phosphorylated 2.5–3 times more at tyrosine-416 than at tyrosine-527. The specific activity of the p60$^{c\text{-}src}$ produced in yeast was 2.5–5.0 times higher than that of p60$^{c\text{-}src}$ overexpressed from a retroviral vector in chicken cells, implicating the altered state of *in vivo* phosphorylation in modulation of the *in vitro* kinase activity. The expression of p60$^{v\text{-}src}$ substantially slowed down the growth of the yeast cells, suggesting that phosphorylation of yeast proteins essential for cell growth may have interfered with their proper functioning.**

p60$^{v\text{-}src}$, the transforming protein of Rous sarcoma virus (RSV) and p60$^{c\text{-}src}$, its cellular homolog, are tyrosine protein kinases modified by myristoylation at their amino termini and localized to the cellular plasma membrane (1–6). Structural and functional studies of these two proteins have shown that the *in vitro* tyrosine protein kinase activity of p60$^{v\text{-}src}$ is at least 10-fold greater than that of p60$^{c\text{-}src}$ and that p60$^{c\text{-}src}$ does not transform cells even when expressed to the level of p60$^{v\text{-}src}$ in RSV-transformed cells (7).

Several investigators have suggested that the tyrosine protein kinase activity of p60$^{c\text{-}src}$ is suppressed by phosphorylation at a carboxyl-terminal tyrosine residue (tyrosine-527), which is present in p60$^{c\text{-}src}$ but is absent from the sequence of the v-src protein (8–11). This hypothesis is supported by experiments showing an increase in the *in vitro* kinase activity of p60$^{c\text{-}src}$ when orthovanadate (a phosphatase inhibitor) was included in the cell lysis buffer (10) or when p60$^{c\text{-}src}$ was extracted from cells treated with orthovanadate (12) and an elevation of p60$^{c\text{-}src}$ kinase activity upon phosphatase treatment *in vitro* (13). Furthermore, single amino acid substitutions in p60$^{c\text{-}src}$ that activate its tyrosine kinase activity and convert it to a transforming protein result in a shift of the phosphorylation site to tyrosine-416 (14, 15). Activation of p60$^{c\text{-}src}$ kinase activity by the middle-sized tumor antigen of polyomavirus also results in a shift of the site of *in vivo* phosphorylation of p60$^{c\text{-}src}$ from tyrosine-527 to tyrosine-416, the major site of p60$^{v\text{-}src}$ tyrosine phosphorylation (16).

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To further study the characteristics of the transforming p60$^{v\text{-}src}$ and nontransforming p60$^{c\text{-}src}$ proteins, we have expressed them in the yeast *Saccharomyces cerevisiae*. In particular, we investigated the fidelity of post-translational modification (as compared with p60^{src} expressed in virally infected chicken cells) and the ability of the yeast cells to produce enzymatically active protein.

MATERIALS AND METHODS

Plasmid Constructions, Yeast Strains, and Yeast Transformation. For the expression of v-src and c-src proteins in yeast cells, the plasmid YEp51 (a gift from J. Broach, Princeton University; ref. 17) was digested with *Sal* I, made flush with T4 DNA polymerase, and was then digested with *Bam*HI (Fig. 1). p5H, containing the c-*src* gene (15), and pN4, carrying the v-*src* gene (7), were digested with *Nco* I, made flush with T4 DNA polymerase, and then digested with *Bgl* II (a linker insertion at an *Nru* I site downstream of the src termination codon). The *Nco* I(blunt)–*Bgl* II fragments were isolated from preparative agarose gels and ligated to the *Sal* I(blunt)–*Bam*HI fragment of YEp51 using T4 ligase. Standard procedures were used throughout the construction and analysis of recombinant DNAs (18).

The yeast expression plasmids were propagated in *Escherichia coli* (HB101) and were transformed into the yeast strain W303-1A (haploid, *leu*$^-$, *ura*$^-$, *trp*$^-$, *his*$^-$, *ade*$^-$) by the method of Beggs (19). The parent strain was grown in YPD [2% (wt/vol) Bacto-peptone, 1% yeast extract, and 2% (wt/vol) dextrose]. *Leu*$^+$ transformants were selected on minimal media plates containing 0.67% yeast nitrogen base without amino acids, 2% (wt/vol) dextrose, 1.5% (wt/vol) agar, and all required amino acids except leucine. The *src*-expressing yeast were grown in synthetic medium containing 2% (wt/vol) raffinose and 0.67% yeast nitrogen base without amino acids.

Immunoblotting and *in Vitro* Kinase Assays. For the induction of p60^{src}, the yeast transformants were grown to 10^7 cells per ml (100–200 ml of culture), pelleted, resuspended in synthetic medium containing 0.67% yeast nitrogen base and 2% (wt/vol) galactose, and incubated in a shaker at 30°C for 2.5 hr. Cells were pelleted and resuspended in 0.5–1.0 ml of RIPA buffer (10 mM Tris·HCl, pH 7.4/1 mM EDTA/150 mM NaCl/1% Triton X-100/1% sodium deoxycholate/0.1% NaDodSO$_4$/1 mM phenylmethylsulfonyl fluoride/100 kallikrein-inactivating units of aprotinin per ml). An equal volume of RIPA-soaked glass beads was added to each sample, and yeast were lysed by Vortex mixing for 6 min at 4°C. Each lysate was then immunoprecipitated with a monoclonal antibody (mAb) against p60^{src} (mAb 327, a gift from J. Brugge, State University of New York, Stony Brook; ref. 20). One-half of each precipitate was subjected to electrophoresis on 8.5% NaDodSO$_4$/polyacrylamide gels and transferred to

Abbreviations: RSV, Rous sarcoma virus; CEF, chicken embryo fibroblasts; mAb, monoclonal antibody.

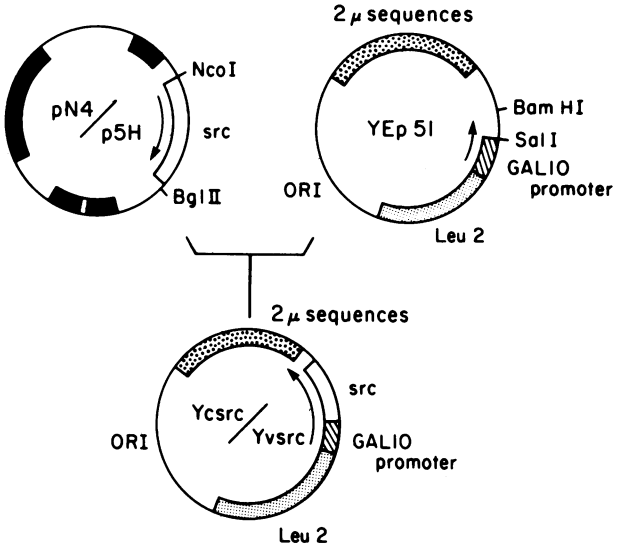

FIG. 1. Construction of the *src*-expressing yeast vectors, Ycsrc and Yvsrc. The solid regions represent sequences encoding viral replicative functions, present in the plasmids from which *src* sequences were excised. Present in the yeast expression vector YEp 51 are a *Gal 10* promoter (hatched), 2-μ plasmid circle sequences (large dots), a pBR322 origin of replication (ORI), and the *leu2* gene (small dots), able to complement the defect in *leu*$^-$ auxotrophic yeast strains.

nitrocellulose filters by electrophoresis at 50 V for 10 hr. Following blocking in phosphate-buffered saline (137 mM NaCl/3 mM KCl/8 mM $Na_2HPO_4 \cdot 7\ H_2O$/1.5 mM KH_2PO_4, pH 7.4) containing 0.0005% Tween 20 and 0.5% gelatin, the filters were incubated with a 1:100 dilution of mAb 327, washed in phosphate-buffered saline with Tween 20, then incubated with 10 μCi (1 Ci = 37 GBq) of ^{125}I-labeled sheep anti-mouse antibody, and finally washed for 2 hr. For quantitation, the p60^{src} bands were excised from the filters, and radioactivity was measured in a γ counter. The remaining one-half of each immunoprecipitate was assayed for *in vitro* kinase activity essentially as described (21).

Expression of src Proteins and *in Vivo* Labeling. Chicken embryo fibroblasts (CEF) were labeled with [^{32}P]orthophosphate and [^{35}S]methionine as described (17, 21). For labeling yeast with [^{32}P]orthophosphate, yeast were grown in synthetic medium containing 2% (wt/vol) raffinose and induced with 2% (wt/vol) galactose. The yeast were incubated in phosphate-free synthetic medium (22) for 1 hr, then labeled with 3 mCi of [^{32}P]orthophosphate per ml of phosphate-free medium for an additional hour, and lysed. Yeast proteins were labeled with [^{35}S]methionine as with phosphate except yeast were starved and labeled in medium containing 2% (wt/vol) galactose, the appropriate amino acids, and 0.67% yeast nitrogen base without amino acids and without ammonium sulfate. Labeling was for 10 min with 1 mCi of [^{35}S]methionine per ml. [^{35}S]Methionine-labeled yeast cells were lysed in NaOH and 2-mercaptoethanol, and proteins were precipitated with 50% (wt/vol) trichloroacetic acid. Trichloroacetic acid precipitates were resuspended in 4% (wt/vol) $NaDodSO_4$ with 5 min of boiling, diluted into RIPA buffer without $NaDodSO_4$, and immunoprecipitated with mAb 327.

[^{3}H]Myristate labeling of yeast proteins was done essentially as described (23), including the pretreatment of yeast cells with cerulenin to inhibit fatty acid biosynthesis (23).

Peptide Mapping. V8 protease mapping was done as described (24). Chymotryptic digestion was done *in situ* as was described for tryptic digestion (24). Digested peptides were separated in two dimensions as described by Neil *et al.* (25) except that electrophoresis was for 45 min at 600 V and that chromatography was for 5.5 hr. For cyanogen bromide cleavage analysis, preparative gel bands containing p60^{src} were washed in 10% (vol/vol) methanol, lyophilized to dryness, and then incubated in 70% (vol/vol) formic acid containing cyanogen bromide at 50 mg/ml for 30 min at room temperature as described (8). Intact gel bands were then washed with water, lyophilized to dryness, and loaded directly onto an 18.75% $NaDodSO_4$/polyacrylamide gel containing 6 M urea (26). Following electrophoresis, urea was removed from the gel by extensive washing with 5% (vol/vol) methanol, 5% (vol/vol) acetic acid, and 5% (vol/vol) glycerol, and the gel was then dried under high heat and vacuum.

Yeast Growth Curves. Yeast were grown overnight in synthetic medium containing either 2% (wt/vol) galactose or 2% (wt/vol) raffinose, diluted to 10^6 cells per ml in galactose-containing medium, and monitored for growth by measuring OD_{600}.

RESULTS

Construction of the src-Expressing Plasmids and Transformation of Yeast. We constructed p60$^{v\text{-}src}$- and p60$^{c\text{-}src}$-expressing plasmids by ligating the sequences encoding the src proteins into a yeast–*E. coli* shuttle vector (YEp 51) containing a galactose-inducible promoter (*Gal 10*), a 2-μ plasmid sequence for replication in yeast, an origin of replication for propagation in *E. coli*, and the *leu2* gene for selection in *leu*$^-$ auxotrophic yeast (Fig. 1). The coding sequences of p60$^{c\text{-}src}$ were derived from a plasmid, p5H, that encodes the equivalent of a cDNA copy of the c-*src* gene (15). p60$^{v\text{-}src}$ coding sequences were derived from pN4, a plasmid described (7). Since there are *Nco* I sites at the start codons of both the v-*src* and c-*src* sequences, it was possible to place the *Gal 10* promoter directly adjacent to the src coding sequences, facilitating expression of these proteins (17). The *src*-encoding yeast expression plasmids, Ycsrc and Yvsrc, were transformed into the haploid yeast strain W303-1A (*leu*$^-$, *ura*$^-$, *ade*$^-$, *his*$^-$, *trp*$^-$), and colonies were selected from synthetic media plates and cultured in medium containing 2% (wt/vol) raffinose. The production of src proteins was induced by incubation of the yeast for 2.5 hr in medium containing 2% (wt/vol) galactose. The yeast-expressed c-src and v-src proteins will hereafter be referred to as yeast p60$^{c\text{-}src}$ and yeast p60$^{v\text{-}src}$, respectively.

Analysis of p60^{src} Production in Yeast. To examine the p60^{src} proteins produced by the yeast transformants, we labeled galactose-induced yeast *in vivo* with [^{32}P]orthophosphate and immunoprecipitated the src proteins from yeast cell lysates. As shown in Fig. 2, a mAb against p60^{src} specifically immunoprecipitated ^{32}P-labeled p60 from the p60$^{v\text{-}src}$- and p60$^{c\text{-}src}$-producing yeast. Digestion of p60$^{v\text{-}src}$ and p60$^{c\text{-}src}$ from CEF with V8 protease initially produced two fragments of 34 kDa (V1) and 26 kDa (V2) (slightly smaller in the case of v-src). Further digestion of fragment V1 produced two smaller fragments, V3 and V4. V8 protease digestion of yeast p60$^{c\text{-}src}$ and p60$^{v\text{-}src}$ proteins labeled *in vivo* with [^{32}P]orthophosphate produced cleavage patterns identical to those of p60s produced in CEF (Fig. 2).

The yeast proteins were also labeled *in vivo* with [^{35}S]methionine and analyzed by V8 digestion. Although the V1 and V2 fragments were clearly present (Fig. 3), further digestion revealed the absence of methionine-labeled V3 and V4 fragments. Since the only methionine present in the amino acid sequences encoding the overlapping V3 and V4 fragments is the initiator methionine (8, 9), this suggested that the initiator methionine was cleaved off in yeast, as it is in CEF, to allow for myristoylation at glycine-2 (27). Since cleavage of methionine-1 by an aminopeptidase and *N*-myristoylation are not obligatorily coupled (28), we tested the possibility of myristoylation directly by labeling the yeast carrying the src

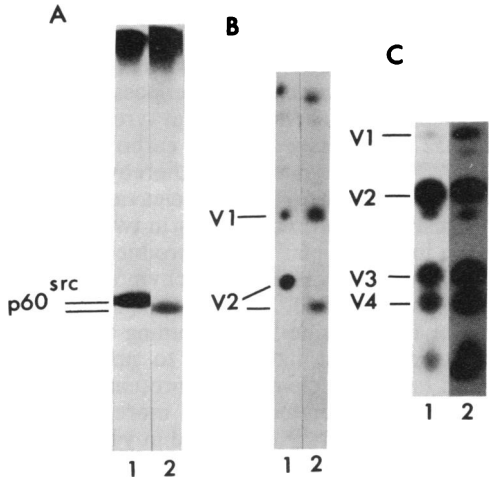

FIG. 2. Analysis of $p60^{v\text{-}src}$ and $p60^{c\text{-}src}$ produced by yeast labeled *in vivo* with [^{32}P]orthophosphate. (*A*) c-src-expressing yeast (lane 1) and v-src-expressing yeast (lane 2) were lysed in RIPA buffer and immunoprecipitated with mAb 327 (against $p60^{src}$). (*B*) ^{32}P-labeled c-src (lane 1) and v-src (lane 2) proteins from yeast were excised from preparative gels and digested with V8 protease. V1 and V2 were the primary cleavage fragments. Undigested p60 can be seen at the top of the gel. (*C*) V8 maps of ^{32}P-labeled $p60^{c\text{-}src}$ produced in yeast (lane 1) and in CEF (lane 2). V3 and V4 fragments were derived by further cleavage of V1. Exposure of gel in *A* was for 3 hr with an intensifying screen. Gels in *B* and *C* were exposed overnight with an intensifying screen.

proteins with [^{3}H]myristate. Both v-src and c-src proteins were labeled with myristate, as in CEF (Fig. 3).

Analysis of $p60^{src}$ Kinase Activity. Because using yeast as an expression system would be of particular value if authentic, enzymatically active $p60^{src}$ molecules were produced, we determined the specific activity of the src proteins present in the yeast lysates. Total cell lysates from $p60^{c\text{-}src}$- and $p60^{v\text{-}src}$-producing yeast and lysates from CEF infected with retroviruses overexpressing either $p60^{v\text{-}src}$ or $p60^{c\text{-}src}$ proteins were immunoprecipitated with mAb 327. The amount of protein in one-half of each immunoprecipitate was then quantitated by immunoblotting. Fig. 4 shows the results of a representative immunoblotting experiment. The remaining half of each lysate was assayed for *in vitro* kinase activity

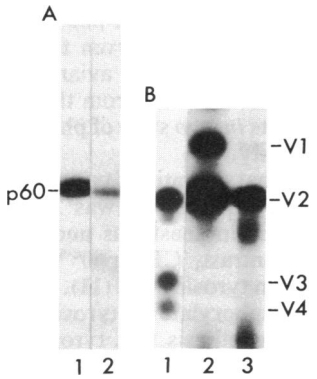

FIG. 3. Cleavage of the initiator methionine and myristoylation of src proteins. (*A*) Lanes: 1, $p60^{c\text{-}src}$ produced in yeast, labeled with [^{3}H]myristate, and immunoprecipitated with mAb 327; 2, $p60^{v\text{-}src}$ labeled with [^{3}H]myristate. Prior to labeling, fatty acid biosynthesis was inhibited with cerulenin. Autoradiograms were exposed for 10 days. (*B*) V8 analysis of labeled yeast proteins. $p60^{c\text{-}src}$ protein was labeled with [^{32}P]orthophosphate (lane 1) or [^{35}S]methionine (lanes 2 and 3) and cleaved with 25 ng (lane 2) or 250 ng (lanes 1 and 3) of V8 protease per gel slot. Peptide fragments are identified.

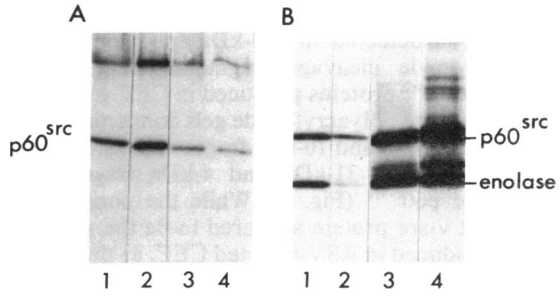

FIG. 4. Immunoblot and kinase assay. (*A*) $p60^{src}$ proteins from yeast (c-src, lane 1; v-src, lane 3) and virally-infected CEF (p5H c-src, lane 2; pN4 v-src, lane 4) were immunoprecipitated with mAb 327 and resolved by electrophoresis on an 8.5% gel. Proteins were blotted onto nitrocellulose and probed with mAb 327 and ^{125}I-labeled sheep anti-mouse IgG antibody. Exposure was for 30 hr without a screen. (*B*) Half of each sample blotted in *A* was assayed for *in vitro* kinase activity using enolase as an exogenous substrate. Yeast c-src protein (lane 1), CEF c-src protein (lane 2), yeast v-src protein (lane 3), and CEF v-src protein (lane 4).

using enolase as an exogenous substrate (Fig. 4). In several experiments (Table 1), we found that the specific activity of the yeast $p60^{c\text{-}src}$ protein (as determined by ^{32}P in enolase/^{125}I in the $p60^{src}$ bands on the immunoblot) was 2.5–5.0 times higher than that of $p60^{c\text{-}src}$ produced in CEF. The specific activity of the yeast $p60^{v\text{-}src}$ protein was comparable to that of $p60^{v\text{-}src}$ produced in CEF (Table 1 and Fig. 4).

Phosphorylation Sites of p60 Produced in Yeast. $p60^{v\text{-}src}$ and $p60^{c\text{-}src}$ produced in CEF are phosphorylated *in vivo* on tyrosine at different sites in their carboxyl-terminal regions (tyrosine-416 in the case of $p60^{v\text{-}src}$ and tyrosine-527 on $p60^{c\text{-}src}$) (11, 14). Given that a number of reports have suggested that phosphorylation of avian $p60^{c\text{-}src}$ at tyrosine-527 may suppress its tyrosine protein kinase activity and that the $p60^{c\text{-}src}$ produced in yeast was of higher specific activity than that produced in CEF, we determined the tyrosine phosphorylation sites of the src proteins produced in yeast.

Cyanogen bromide cleavage of either ^{32}P-labeled $p60^{c\text{-}src}$ or $p60^{v\text{-}src}$ proteins produces a 31-kDa fragment derived from the amino terminus of the src protein (phosphorylated on serine) and a 10-kDa fragment containing tyrosine-416 (if this residue is phosphorylated). If $p60^{c\text{-}src}$ is phosphorylated on

Table 1. Specific activity of $p60^{src}$ proteins

Exp.	Protein	Relative values: Amount of $p60^{src}$ protein*	Kinase level†	Specific activity‡
1	Yeast $p60^{c\text{-}src}$	1.0	1.0	1.0
	CEF $p60^{c\text{-}src}$	0.8	0.2	0.25
	Yeast $p60^{v\text{-}src}$	0.25	1.4	5.6
	CEF $p60^{v\text{-}src}$	0.6	2.9	4.8
2	Yeast $p60^{c\text{-}src}$	1.0	1.0	1.0
	CEF $p60^{c\text{-}src}$	0.9	0.2	0.2
	Yeast $p60^{v\text{-}src}$	0.5	3.2	6.4
	CEF $p60^{v\text{-}src}$	1.3	6.4	5.0
3	Yeast $p60^{c\text{-}src}$	1.0	1.0	1.0
	CEF $p60^{c\text{-}src}$	2.3	0.9	0.4
4	Yeast $p60^{c\text{-}src}$	1.0	1.0	1.0
	CEF $p60^{c\text{-}src}$	2.9	0.7	0.2

*$p60^{src}$ bands detected with mAb 327 and ^{125}I-labeled anti-mouse IgG antibody were cut from the immunoblotted nitrocellulose filters, and radioactivity was measured with a γ counter. Values are corrected for background counts.

†*In vitro* phosphorylated enolase bands were excised from the $NaDodSO_4$/polyacrylamide gels, and radioactivity was measured by Cerenkov counting in a scintillation counter.

‡Relative ^{32}P counts/relative ^{125}I counts.

tyrosine-527, as is characteristic of $p60^{c\text{-}src}$ produced in CEF, the ^{32}P label is detected in a 4-kDa fragment. When the cyanogen bromide cleavage fragments from ^{32}P-labeled $p60^{c\text{-}src}$ and $p60^{v\text{-}src}$ proteins produced in CEF were resolved on high-percentage polyacrylamide gels containing 6 M urea, the predicted 31-kDa and 10-kDa fragments were seen in the case of $p60^{v\text{-}src}$ and 31-kDa and 4-kDa fragments were prominent for $p60^{c\text{-}src}$ (Fig. 5). While the phosphorylation state of yeast v-src protein appeared to be the same as that of $p60^{v\text{-}src}$ produced in RSV-infected CEF, as determined by cyanogen bromide cleavage (Fig. 5), yeast c-src protein was phosphorylated predominantly on the 10-kDa cleavage fragment characteristic of tyrosine-416 phosphorylation. In several experiments, the ratio of 10-kDa/4-kDa phosphorylation for cyanogen bromide-cleaved yeast $p60^{c\text{-}src}$ was ≈2.5:1. Because the same ratio of tyrosine-416/tyrosine-527 phosphorylation was observed following labeling times of 1–5 hr, it is likely that the src proteins were labeled to equilibrium (data not shown).

Although the major site of $p60^{c\text{-}src}$ carboxyl-terminal phosphorylation in CEF has been unambiguously determined (11), the 4-kDa cyanogen bromide fragment does contain an additional tyrosine at residue 519, which is present in $p60^{c\text{-}src}$ but not in $p60^{v\text{-}src}$. To rule out the possibility that yeast $p60^{c\text{-}src}$ protein was phosphorylated at tyrosine-519, and not at tyrosine-527, we cleaved the V2 carboxyl-terminal fragment of $p60^{c\text{-}src}$ with chymotrypsin, thereby separating these two tyrosines, and resolved the cleavage fragments by electrophoresis and chromatography in two dimensions. As can be seen in Fig. 5, $p60^{c\text{-}src}$ overproduced in CEF, phosphorylated *in vivo*, and cleaved with chymotrypsin, produced one major spot (Fig. 5*C*). This spot migrated as would be expected for the tyrosine-527-containing peptide (11). The minor spots are most likely due to nonspecific protein degradation (since we cleaved the protein for 18 hr *in situ* rather than digesting eluted protein more briefly). Chymotryptic cleavage of $p60^{c\text{-}src}$ expressed in yeast produced two readily detectable ^{32}P-labeled spots, one migrating similarly to the tyrosine-527-containing spot of $p60^{c\text{-}src}$ produced in CEF, the other major spot (spot 2) migrating more slowly in both dimensions (Fig. 5). Mixing of chymotryptic fragments from $p60^{c\text{-}src}$ produced in yeast and in CEF (equal cpm) identified the minor spot in the yeast $p60^{c\text{-}src}$ digest (spot 1) as a tyrosine-527-containing peptide (Fig. 5*D*). When chymotryptic fragments of $p60^{v\text{-}src}$ produced in RSV-infected cells were mixed with chymotryptic fragments from the yeast $p60^{c\text{-}src}$, spot 2 was identified as the tyrosine-416-containing fragment (data not shown).

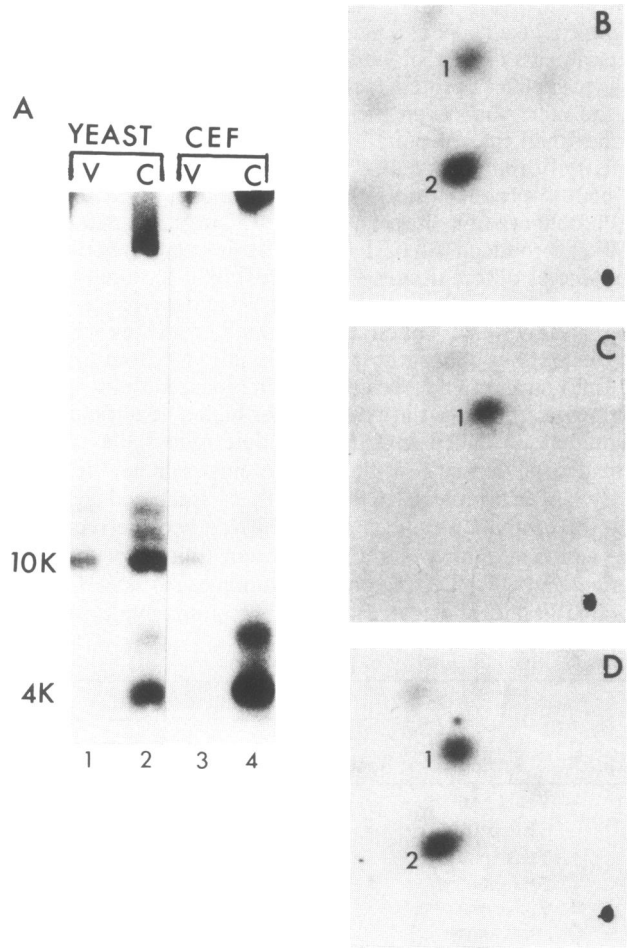

FIG. 5. Analysis of carboxyl-terminal phosphorylation sites of src proteins. (*A*) Gel slices containing $p60^{v\text{-}src}$ (lanes 1 and 3) and $p60^{c\text{-}src}$ (lanes 2 and 4) labeled with [^{32}P]orthophosphate in yeast cells and in CEF were cleaved *in situ* with cyanogen bromide and resolved by $NaDodSO_4$/PAGE using an 18.75% gel containing 6 M urea. The 10-kDa fragment contains tyrosine-416, and the 4-kDa peptide contains tyrosine-527. (*B–D*) Gels slices containing the V2 fragment of ^{32}P-labeled src proteins were incubated with chymotrypsin, and the eluted peptides were separated in two dimensions by electrophoresis at pH 8.9 followed by chromatography. Samples were spotted at the dot in the lower right-hand corner. (*B*) $p60^{c\text{-}src}$ expressed in yeast. (*C*) $p60^{c\text{-}src}$ from CEF. (*D*) Mix of equal number of counts of chymotrypsin-cleaved yeast $p60^{c\text{-}src}$ and CEF $p60^{c\text{-}src}$. Exposure was for 8 days with an intensifying screen.

Growth of Yeast Expressing src Proteins. In the course of studying $p60^{src}$-expressing yeast, we noticed that yeast carrying the v-*src* expression plasmid grew very poorly in the galactose induction medium, yet grew well in raffinose-containing medium.

To examine this phenomenon further, we grew individual yeast colonies for ≈12 hr in galactose-containing medium, diluted them to a density of 10^6 cells per ml, and monitored their growth by determining OD_{600} every 1.5–2 hr. Following dilution, the difference in the growth rates of yeast expressing $p60^{v\text{-}src}$, $p60^{c\text{-}src}$, and YEp 51 (vector alone) was immediately apparent, with the v-src yeast doubling in ≈5.5 hr, the c-src yeast doubling every 4 hr, and the YEp 51-carrying yeast doubling every 3 hr.

DISCUSSION

We have produced both the cellular and viral src proteins in the yeast *S. cerevisiae*. While the post-translational modifications and enzymatic activities of $p60^{v\text{-}src}$ protein produced in yeast were indistinguishable from those of $p60^{v\text{-}src}$ produced in RSV-infected CEF, the avian cellular src protein produced in yeast was different from that produced in CEF with regard to both its *in vivo* state of phosphorylation and its *in vitro* kinase activity.

The observation that the ratio of tyrosine-416/tyrosine-527 phosphorylation of yeast $p60^{c\text{-}src}$ was >1 strongly suggests that $p60^{c\text{-}src}$ produced in yeast was underphosphorylated at tyrosine-527; by contrast, CEF $p60^{c\text{-}src}$ is phosphorylated stoichiometrically on tyrosine-527 (11). The known ability of $p60^{c\text{-}src}$ to autophosphorylate at tyrosine-416 *in vitro* (14), coupled with the hypothesis that tyrosine-527 phosphorylation suppresses the kinase activity of $p60^{c\text{-}src}$, suggests a model to explain the ratio of tyrosine-416/tyrosine-527 phosphorylation seen on yeast $p60^{c\text{-}src}$. If $p60^{c\text{-}src}$ produced in CEF were rapidly phosphorylated on tyrosine-527 by a cellular tyrosine kinase, its activity would be suppressed, and little tyrosine-416 phosphorylation would be observed. However, if there were no endogenous yeast tyrosine kinase capable of phosphorylating tyrosine-527, tyrosine-416 phosphorylation could occur by autophosphorylation. In addition, if yeast $p60^{c\text{-}src}$ molecules could transphosphorylate other $p60^{c\text{-}src}$

molecules at tyrosine-527, but this phosphorylation were less favorable than tyrosine-416 autophosphorylation, a ratio of tyrosine-416/tyrosine-527 phosphorylation >1 would be observed.

In support of this model, a kinase-negative mutant $p60^{c\text{-}src}$ protein was not phosphorylated at either tyrosine-416 or tyrosine-527 in yeast, yet was well phosphorylated at tyrosine-527 in CEF (unpublished data), suggesting that yeast does not have an endogenous tyrosine kinase capable of tyrosine-527 phosphorylation. The analysis of the state of phosphorylation of yeast $p60^{c\text{-}src}$ is, however, complicated by the difficulty in determining the rate of phosphate turnover given the large pools of phosphate and by the fact that some population of the $p60^{c\text{-}src}$ protein may not be phosphorylated at all.

The hypothesis that tyrosine-527 phosphorylation may contribute to the suppression of $p60^{c\text{-}src}$ kinase activity (8–11) is consistent with the elevation of yeast $p60^{c\text{-}src}$ *in vitro* kinase activity relative to that of $p60^{c\text{-}src}$ overexpressed in CEF, since in yeast $p60^{c\text{-}src}$ appears to be underphosphorylated at tyrosine-527.

We observed removal of the initiator methionine and myristoylation of the cellular and viral src proteins in yeast as has been seen in CEF (5, 6, 27, 28). The finding of a myristoyltransferase activity in yeast by Towler and Glaser (23) makes it likely that the [^{3}H]myristate label was incorporated into the src proteins expressed in yeast in the form of myristate. The presence of an aminopeptidase and a myristoyltransferase in yeast that recognizes src protein implies that the enzymatic machinery and processing signals necessary for proper myristoylation have been evolutionarily conserved.

The "slow growth" phenotype of the yeast expressing $p60^{v\text{-}src}$ supports the notion that the $p60^{v\text{-}src}$ produced in yeast is enzymatically more active than the $p60^{c\text{-}src}$ protein that slows down growth only slightly. It is also an interesting possibility that $p60^{v\text{-}src}$ and $p60^{c\text{-}src}$ proteins have different substrate specificities in yeast and that $p60^{v\text{-}src}$ is phosphorylating and thereby inhibiting the proper functioning of some yeast protein essential for cell growth. While several reports have suggested that yeast does have endogenous tyrosine kinase activity (29, 30), it has been difficult to identify any endogenous yeast proteins phosphorylated on tyrosine (31). The relevance of tyrosine phosphorylation to the control of yeast growth, therefore, awaits the identification of endogenous yeast tyrosine kinases and their substrates.

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