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Expression of Rous Sarcoma Virus Transforming Protein pp60$^{v\text{-}src}$ in *Saccharomyces cerevisiae* Cells

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The Rous sarcoma virus (RSV) pp60$^{v\text{-}src}$ protein was expressed in *Saccharomyces cerevisiae* cells either from a plasmid vector carrying the v-*src* gene or in yeast cells containing a single-copy v-*src* gene chromosomally integrated. In both yeast strains, v-*src* gene transcription is regulated by the galactose-inducible *GAL10* promoter. Growth in galactose-containing medium resulted in constitutive expression of pp60$^{v\text{-}src}$ in the integrated strain and transient expression of higher levels of pp60$^{v\text{-}src}$ in the plasmid-bearing strain. The concentration of pp60$^{v\text{-}src}$ in the plasmid-bearing strain at its peak of expression was approximately threefold lower than that found in RSV-transformed mammalian cells. pp60$^{v\text{-}src}$ synthesized in yeast cells was phosphorylated in vivo on sites within the amino and carboxyl halves of the molecule. In immune complex kinase assays, the yeast pp60$^{v\text{-}src}$ was autophosphorylated on tyrosine and was able to phosphorylate exogenous substrates such as casein and enolase. The specific activity of pp60$^{v\text{-}src}$ synthesized in yeast cells was approximately 5- to 10-fold higher than that made in mammalian cells. Induction of pp60$^{v\text{-}src}$ caused the death of the plasmid-bearing yeast strain and transient inhibition of growth of the single-copy strain. Concomitantly, this induction resulted in high levels of tyrosine phosphorylation of yeast cell proteins. This indicates that pp60$^{v\text{-}src}$ functions as a tyrosine-specific phosphotransferase in yeast cells and suggests that hyperphosphorylation of yeast proteins is inimical to cell growth.

Investigations of the transforming proteins of oncogenic retroviruses and cellular growth hormone receptors have led to the identification of a new class of protein phosphotransferases. The enzymes in this group can be distinguished from other protein kinases according to several criteria, most notably by their specificity for tyrosine residues (26, 27). The precise function of these enzymes has yet to be elucidated, but the evidence that the transforming protein of many oncogenic retroviruses and cellular growth hormone receptors carries this activity implicates tyrosine kinases in the regulation of cellular morphology and growth control.

Rous sarcoma virus (RSV) has proven to be a model system for investigations of oncogenic transformation by this class of retroviruses. The transforming protein of RSV is a 60-kilodalton (kDa) protein that possesses an intrinsic tyrosine-specific phosphotransferase activity (2). The tyrosine kinase activity of the protein is believed to be required for oncogenic transformation since mutant viruses, which possess defective phosphotransferase activity, are defective for transformation (26, 35). While many cellular proteins are phosphorylated on tyrosine after transformation by RSV, it is not known which cellular substrates are involved in mediating the phenotypic changes that occur after transformation by RSV. The characterization of the enzymatic activity of this enzyme and other tyrosyl kinases has been limited by the small quantities of enzyme available for analysis. The v-*src* gene product has been expressed at high levels in bacteria (11, 13, 19); however, many aspects of the expression of the v-*src* gene product in this system have limited the uses of the protein that is expressed. Most of the v-*src* gene product produced in *Escherichia coli* aggregates in insoluble precipitates which cannot be solubilized except with strong denaturing agents that destroy the enzymatic activity of the enzyme. In addition, while the v-*src* gene product expressed in bacteria possesses tyrosine kinase activity, the specific activity of the enzyme is very low compared with the protein expressed in animal cells and the enzyme is not autophosphorylated in vitro or in vivo (11; R. L. Erikson, personal communication). Thus, the use of the v-*src* gene product expressed in bacteria has been limited to the isolation of large quantities of antigen to produce antibodies against pp60$^{v\text{-}src}$.

We have cloned the v-*src* gene into a plasmid which allows the expression of the v-*src* gene in *Saccharomyces cerevisiae* to determine whether yeast cells provide a better environment for the expression of the tyrosine kinase activity of pp60$^{v\text{-}src}$. In this study we demonstrate that pp60$^{v\text{-}src}$ expressed in yeast cells is phosphorylated in vivo and possesses tyrosine kinase activity in vivo and in vitro. Thus pp60$^{v\text{-}src}$ produced in yeast cells appears to be much better suited for analysis than pp60$^{v\text{-}src}$ that has been expressed in procaryotic cells.

MATERIALS AND METHODS

Bacterial and yeast strains. The *E. coli* strains used in this study were DH1 (F$^-$ *recA1 endA1 gyrA96 thi-1 hsdR17* [r_k^- m_k^+] *supE44* λ^-), HB101 (F$^-$ *hsdS20* [r^- m^-] *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44*), and GM33 (*dam-3*) (18). The yeast strains used were Y294 (*MAT*α *leu2-3,112 ura3-52 his3*Δ *trp1* Gal$^+$ [cir$^+$]), Y406 (*MAT*α *leu2-3,112 ura3-52 trp1 HIS3*::*GAL10*–v-*src* Gal$^+$), JR212-10A (*MAT*α *leu2*), and JR212-10B (*MATa leu2 trp1 ura3-52 HIS3*::*GAL10*–v-*src*). We constructed strain Y406 by transforming strain Y294 to histidine prototrophy by using *Bam*HI-digested pHIS3-SRC DNA (see Fig. 1 and below [23]). Strains JR212-10A and JR212-10B are sister spore clones derived from a diploid formed between strain Y406 and strain DC04 (MATa *leu2-04 ade1*) (3). Bacterial

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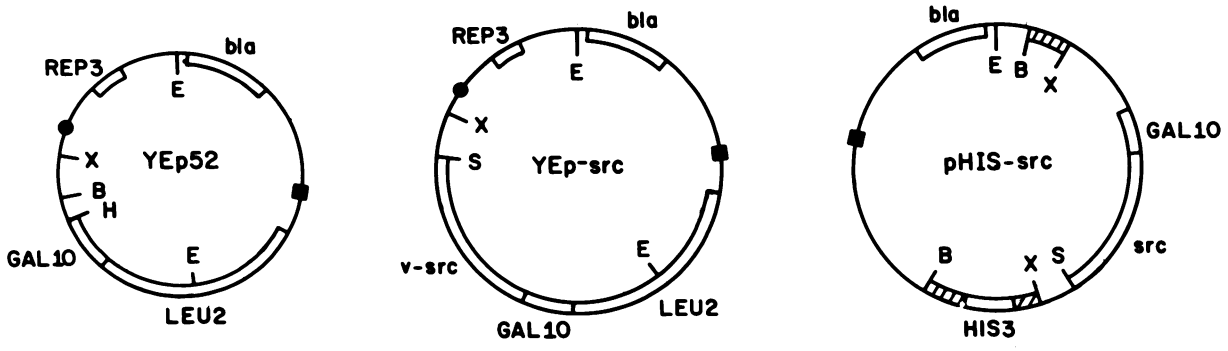

FIG. 1. Plasmids used in the study. The compositions of various plasmids used in this study are shown, indicating the location of various genes, as well as the ColE1 origin of replication (■) and the 2μm circle origin of replication (●). Yeast sequences spanning the *HIS3* gene are indicated by hatched lines. Restriction site designations: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; S, *Sal*I; and X, *Xba*I.

cultures were routinely grown in Luria broth. Yeast strains were grown either in synthetic complete (SC) medium, consisting of 0.67% yeast nitrogen base supplemented with amino acids, purines, and pyrimidines as described previously (30) or in rich medium (YEP), consisting of 2% Bacto-Peptone (Difco Laboratories) and 1% yeast extract. Media contained 2% galactose, 2% raffinose, or 2% glucose as the carbon source as indicated.

Plasmids. The plasmids used in this study are shown in Fig. 1. Plasmid pASV carries a fragment spanning the RSV v-*src* gene cloned into pBR322 and was provided by Steve Hughes (10). The yeast expression vector YEp52 carries the inducible *GAL10* yeast promoter and sequences for selection and propagation in both yeast cells and *E. coli* (4).

The v-*src* coding region was inserted into plasmid YEp52 under control of the *GAL10* promoter as follows. YEp52 DNA was isolated from strain GM33, digested with *Hin*dIII, treated with DNA polymerase Klenow fragment plus deoxynucleoside triphosphates to render the ends flush, and then digested with *Bcl*I. Concurrently, plasmid pASV was digested with *Nco*I, treated with Klenow fragment, and then digested with *Bgl*II. The two samples were mixed and incubated with T4 DNA ligase, and then the ligated mixture was used to transform *E. coli* HB101 to ampicillin resistance. The resulting transformants were screened by colony hybridization with ^{32}P-labeled *src* DNA as a probe, and one positive colony that had the restriction pattern shown in Fig. 1 was retained. This plasmid was designated YEp-src.

Plasmid pHIS3-SRC was constructed by converting the *Eco*RI site within the *LEU2* gene of YEp-src to an *Xba*I site by using a synthetic *Xba*I octanucleotide linker. The resulting *Xba*I fragment spanning the *GAL10*–v-*src* fusion was then cloned into the *Xba*I site adjacent to the *HIS3* gene in plasmid pMM62 (20).

Enzymes. Restriction enzymes, T4 DNA ligase, and DNA polymerase I Klenow fragment were obtained from New England BioLabs and used according to the supplier's instructions.

Antibodies. TBR serum was obtained from rabbits bearing tumors induced by RSV as described previously (5). Monoclonal antibody 327 was obtained from mice immunized with gel-purified v-*src* gene product that was extracted from *E. coli* cells carrying a *lac-src* fusion gene (16). SRC-3 antiserum was obtained from rabbits immunized with the same antigen by the method of Gilmer and Erikson (12). Anti-pp60$^{v\text{-}src}$ SRC-3 antibodies were isolated by affinity purification with the *lac-src* fusion gene product bound to nitrocellulose filters by the method of Smith and Fisher (31). Antiserum to mouse immunoglobulins prepared by Dako Laboratories was obtained from Accurate Chemical and Scientific Corp.

Animal cells. RSV-3T3 cells were prepared by transformation of BALB/c-3T3 cells with the Schmidt-Ruppin D strain of RSV.

Cell lysis and immunoprecipitations. (i) Zymolyase method. Yeast cells were incubated for 30 min in 30 mM dithiothreitol (DTT), washed in water, and incubated for 30 min in 1.1 M sorbitol containing 1 μg of 60-kDa zymolyase per ml (Miles Laboratories). The cells were then washed in 1.1 M sorbitol, lysed in RIPA buffer (50 mM Tris [pH 7.2]–150 mM NaCl–1% sodium deoxycholate–0.1% sodium dodecyl sulfate [SDS]–1% Triton X-100) and clarified at 49,000 × *g* for 30 min.

(ii) Glass bead method. Cells were suspended in RIPA buffer containing 0.5-volume glass beads and vortexed vigorously for 10 min. Lysates were then clarified as described above.

Protein concentrations were determined by the Lowry method (17). Lysates were incubated with antibody for 45 min, and immune complexes were adsorbed to Formalin-fixed *Staphylococcus aureus* for 20 min. The bacteria were washed three times with RIPA buffer and then either suspended in Laemmli sample buffer (14) for direct analysis of the immunoprecipitates or washed with 0.1 M NaCl–10 mM Tris hydrochloride, pH 7.4, to assay protein kinase activity.

Immunoprecipitated proteins were eluted from the immunoadsorbent in SDS-sample buffer at room temperature (14) and analyzed on 7.5% SDS-polyacrylamide gels. Under these conditions only the disulfide bonds between the two heavy chains of immunoglobulin G are reduced. Lightning-Plus intensifying screens (Du Pont Co.) were used to enhance detection of ^{32}P and ^{125}I. Quantitation of protein phosphorylation and ^{125}I binding was performed by determining the counts per minute in the excised protein bands from gels or nitrocellulose by scintillation spectroscopy.

Protein kinase assays. Washed immune complexes were incubated with 10 mM Tris hydrochloride (pH 7.4), 5 mM $MgCl_2$, 1 μM unlabeled ATP, and 5 μCi of [γ-^{32}P]ATP (carrier-free, ICN Pharmaceuticals, Inc.) for 10 min at 22°C (16). The reaction was terminated by washing the immune complexes with 1 ml of RIPA buffer. The reaction products were incubated in Laemmli sample buffer and subjected to electrophoresis on SDS-polyacrylamide gels.

Immunoblot assay. The immunoprecipitated proteins were subjected to electrophoresis on SDS-polyacrylamide gels, transferred to nitrocellulose, and incubated with affinity-

purified SRC-3 antibodies and ^{125}I-labeled protein A as described previously (32).

Phosphoamino acid analysis. Yeast cells were lysed by the RIPA method, and proteins were extracted from the clarified lysates by phenol extraction and trichloroacetic acid precipitation as described by Sefton et al. (28). Proteins were hydrolyzed in 6 N distilled HCl for 1.75 h, and the hydrolysate was analyzed by two-dimensional paper electrophoresis. The first dimension was carried out at pH 3.5 for 2.5 h, and the second dimension was carried out at pH 1.9 for 4 h. The positions of the markers phosphoserine, phosphothreonine, and phosphotyrosine were determined with ninhydrin. The analysis was quantitated by counting the radioactivity at the positions of each marker phosphoamino acid by scintillation spectroscopy.

RESULTS

Yeast cells containing *GAL10*–v-*src* express active pp60$^{v\text{-}src}$ protein. The expression of the RSV *src* gene was examined in yeast cells by cloning the *src* gene into the yeast expression plasmid YEp52. This plasmid carries the yeast *LEU2* and *REP3* genes and the 2μm circle replication origin to allow selection and autonomous propagation in yeast cells, as well as the *bla* gene and the ColE1 origin from pBR322 to allow selection and propagation in *E. coli*. Insertion of the v-*src* coding region immediately downstream from the *GAL10* promoter in YEp52 provided a means of regulating its expression in yeast cells, since the *GAL10* promoter is very active in strains growing on galactose and essentially completely inactive in strains growing on glucose. *GAL10*–v-*src* fusion was introduced into yeast in two different ways. For strain Y294[YEp-src], plasmid YEp-src was used to transform strain Y294 to leucine prototrophy. In this strain the fusion persists as an autonomously replicating, multicopy plasmid. As a control, strain Y294 was transformed with the vector plasmid YEp52 to generate strain Y294[YEp52]. The second v-*src*-bearing strain (Y406) was constructed by using site-directed integrative transformation to insert the *GAL10*–v-*src* fusion into a nonessential site adjacent to the *HIS3* gene. Thus, in strain Y406 the *GAL10*–v-*src* fusion is stably integrated as a single copy on chromosome 15.

To assay the expression of the v-*src* gene product, lysates from strain Y294[YEp-src] or Y294[YEp52] were incubated with antiserum to pp60$^{v\text{-}src}$ obtained from rabbits bearing tumors induced by RSV (TBR serum [5]). The immune complexes were adsorbed to Formalin-fixed *Staphylococcus* bacteria, washed with detergent-containing buffers, and then incubated with [γ-^{32}P]ATP and $MgCl_2$. The phosphorylated protein products were analyzed on SDS-polyacrylamide gels (Fig. 2). pp60$^{v\text{-}src}$ has previously been shown to phosphorylate immunoglobulin heavy chain in this kinase assay (9, 15). In TBR serum immunoprecipitates from Y294[YEp-src] cells grown in glucose, a low level of immunoglobulin heavy chain phosphorylation was detected. After 6 h of induction in galactose, the levels of activity increased 10- to 20-fold. No immunoglobulin phosphorylation was detected in lysates of Y294[YEp52] or Y294[YEp-src] cells immunoprecipitated with normal rabbit serum.

The time course of induction of pp60$^{v\text{-}src}$ was examined under several different conditions of cell growth and cell lysis. Figure 3 shows the pattern of v-*src* induction with galactose in both Y406 and Y294[YEp-src] cells pregrown on the nonrepressing carbon source raffinose. We assayed the levels of pp60$^{v\text{-}src}$ in an immunocomplex kinase reaction with

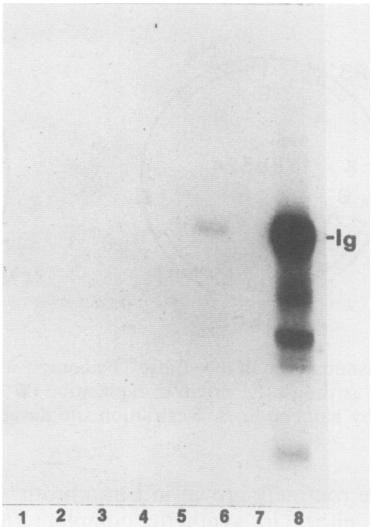

FIG. 2. Detection of pp60 in Y294[YEp-src] yeast cultures. Stationary cultures of Y294[YEp52] (lanes 1 to 4) and Y294[YEp-src] (lanes 5 to 8) cells grown in SC medium (minus leucine) plus 2% glucose were diluted 1:5 in SC medium (minus leucine) containing either 2% glucose (lanes 1, 2, 5, and 6) or 2% galactose (lanes 3, 4, 7, and 8). Lysates were prepared after 6 h of growth by the DTT-zymolyase method. After clarification, 1 ml of lysate containing 1 mg of protein was incubated with 5 ml of normal rabbit serum (lanes 1, 3, 5, and 7) or TBR serum (lanes 2, 4, 6, and 8). The immunoprecipitates were assayed for the phosphorylation of TBR immunoglobulin as described in Materials and Methods.

monoclonal antibody directed against pp60$^{v\text{-}src}$. pp60$^{v\text{-}src}$ was autophosphorylated in this reaction (Fig. 3). In Y294[YEp-src] cells (panel B), the highest levels of v-*src*-specific kinase activity were observed 2 to 4 h after induction with galactose. The v-*src*-specific kinase activity then decreased continuously, such that only 1 to 5% of the peak activity was detected at 20 h postinduction.

The pattern of v-*src* induction, after the addition of galactose to raffinose-grown Y406 cells, was different from that of the Y294[YEp-src] cells. In Y406 cells, pp60$^{v\text{-}src}$ induction was much slower, reaching a plateau level at 20 h postinduction (Fig. 3A). This level was then maintained as long as galactose was present in the medium (data not shown). The steady-state activity detected in Y406 cells was 25% of the level detected during the peak of v-*src* expression in the Y294[YEp-src] cells.

The time course of v-*src* induction in cells which had been grown on medium containing ethanol as a carbon source was similar to that found in cells grown on raffinose-containing medium. However, the induction in cells which had been grown in glucose-containing medium was slower, showing a peak 6 to 8 h after the addition of galactose (data not shown). This retarded induction is consistent with a previous observation on the repression by glucose of galactose-inducible genes (1).

During the preliminary characterization of these cells, two different protocols were used to lyse yeast cells before immunoprecipitation. Under one condition, the yeast cells were incubated with 30 mM DTT before incubation with zymolyase to digest the cell wall. The cell membrane was then solubilized with the immunoprecipitation detergent lysis buffer (RIPA). The other method involved vigorous vortexing of the yeast cells with RIPA buffer in the presence

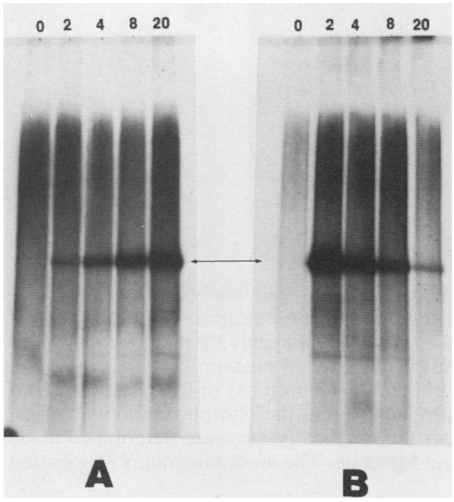

FIG. 3. Time course of pp60$^{v\text{-}src}$ induction in Y294[YEp-src] and Y406 cells. (A) Strain Y406 cells from an overnight culture grown in YEP medium containing 2% raffinose were diluted to 3×10^6 cells per ml in YEP medium plus 2% galactose and grown at 30°C for 0, 2, 4, 8, and 20 h (lanes 1 to 5, respectively). (B) Similarly, strain Y294[YEp-src] cells from an overnight culture grown in SC medium (minus leucine) plus 2% raffinose were diluted to 3×10^6 cells per ml and grown at 30°C for 0, 2, 4, 8, and 20 h (lanes 1 to 5, respectively). For both panels the 20-h cultures were diluted after 8 h of growth to prevent the cells from reaching the stationary phase during this growth period. Lysates were prepared from these cells by the RIPA-glass bead method, 1 ml of clarified lysate containing 1 mg of protein was incubated with TBR serum, and the immunoprecipitates were assayed to determine the extent of immunoglobulin phosphorylation as described in Materials and Methods. The arrow designates pp60$^{v\text{-}src}$. In this experiment the strain was resistant to growth inhibition by pp60$^{v\text{-}src}$ (Fig. 7; see also Discussion).

of glass beads. The DTT-zymolyase method yielded more protein from the yeast cells than the RIPA-glass bead method, especially if the cells were grown to stationary phase (in which case, the RIPA-glass bead method was almost totally ineffective). However, at least 50% of the pp60$^{v\text{-}src}$ was cleaved to several lower-molecular-size species (50 to 53 kDa) under these conditions (data not shown). The relative levels of v-*src* kinase activity detected in lysates at different periods after induction were not affected by the lysis conditions.

In summary, our comparison of the various conditions used during this study for the growth and lysis of the yeast cells has indicated that the conditions found to be most ideal for producing high levels of the v-*src* gene product are growth of Y294(YEp-src) cells in raffinose to mid-log phase followed by induction for 3 h with galactose. Cell lysis was best accomplished by vigorous agitation with glass beads in detergent-containing buffers. The use of cell-wall-disruptive enzymes is expensive and time-consuming and leads to the generation of v-*src* cleavage products.

pp60$^{v\text{-}src}$ is phosphorylated and phosphorylates other cellular proteins in yeast cells. To examine the phosphorylation of pp60$^{v\text{-}src}$ in yeast cells, Y294[YEp-src] cells were labeled with $^{32}P_i$ for 4 h after galactose induction. The v-*src* gene products were immunoprecipitated from cell lysates with monoclonal antibody to pp60$^{v\text{-}src}$ (Fig. 4A). The major protein that was specifically immunoprecipitated from the galactose-induced yeast cells comigrated with the pp60$^{v\text{-}src}$ marker from RSV-transformed mouse cells. The peptide analysis shown in panel B shows that both the 34-kDa amino-terminal V1 fragment and the 26-kDa carboxyl-terminal V2 fragment of the yeast pp60$^{v\text{-}src}$ (lane 1) were radiolabeled with ^{32}P. This pattern of phosphorylation is similar to that detected on the pp60$^{v\text{-}src}$ protein isolated from mammalian cells (lane 5). In avian and mammalian cells, pp60$^{v\text{-}src}$ is phosphorylated on serine 17 (22), presumably by cyclic AMP-dependent protein kinase (8), and on tyrosine 416 by an autophosphorylation event (21, 30). Further peptide analysis is required to determine whether the precise sites of phosphorylation are the same as those phosphorylated in animal cells.

Expression of the RSV *src* gene product in chicken and mammalian cells causes a 7- to 10-fold increase in phosphorylation of tyrosine residues on cellular proteins (28). Multiple cellular proteins have been shown to contain elevated levels of phosphotyrosine (27, 35). To determine whether pp60$^{v\text{-}src}$ expressed in yeast cells phosphorylates yeast cell proteins on tyrosine, we examined the levels of phosphotyrosine in yeast cell proteins after induction of pp60$^{v\text{-}src}$ in Y294[YEp-src] cells. Table 1 and Fig. 5 show the levels of phosphoserine, phosphothreonine, and phosphotyrosine in induced and uninduced cells. Phosphotyrosine was undetectable in uninduced Y294[YEp-src] cells, indicating levels lower than 0.01 to 0.02% phosphotyrosine. In the induced cells, the percentage of phosphotyrosine rose to 4% of the total phosphoamino acids incorporated in proteins. It is unlikely that the phosphorylated residues from pp60$^{v\text{-}src}$ itself could account for this high level of phosphorylation since the levels of pp60$^{v\text{-}src}$ are very low in the Y294[YEp-src] cells. In addition, we observed multiple phosphotyro-

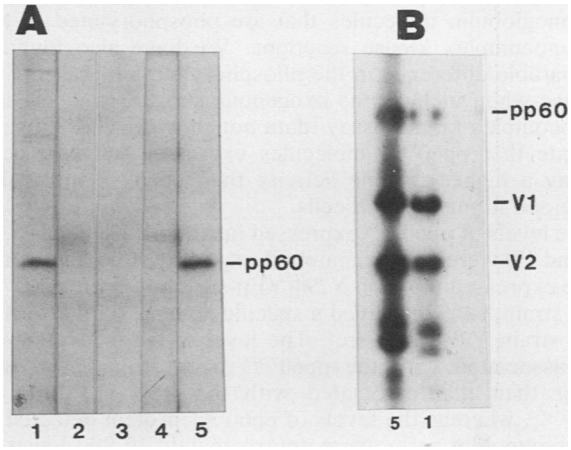

FIG. 4. Analysis of the phosphorylation of pp60$^{v\text{-}src}$ in Y294[YEp-src] cells. (A) A stationary-phase culture of Y294[YEp-src] cells grown in 2% raffinose was diluted 1:10 into 10 ml of phosphate-depleted medium containing 1 mCi of $^{32}P_i$ and either 2% galactose (lanes 1 and 2) or 2% raffinose (lanes 3 and 4). The cells were grown for 4 h, and the lysates were prepared by the glass bead method and immunoprecipitated with either monoclonal antibody 327 plus rabbit anti-mouse immunoglobulin (lanes 1 and 3) or rabbit anti-mouse immunoglobulin alone (lanes 2 and 4). A ^{32}P-labeled pp60$^{v\text{-}src}$ marker was included on this gel as a marker (lane 5). (B) The 60-kDa proteins from lanes 1 and 5 from the gel shown in panel A were excised and subjected to electrophoresis on a 12.5% SDS-polyacrylamide gel in the presence of 50 ng of *Staphylococcus* V8 protease (Miles Scientific) by the method of Cleveland et al. (7). The difference in mobility of the V3 and V4 fragments in the RSV-3T3 and YEp-src pp60$^{v\text{-}src}$ molecules is due to differences in the structure of the v-*src* gene encoded by the Schmidt-Ruppin A (cloned in yeast plasmid) and D (integrated in RSV-3T3 cells) strains of RSV.

sine-containing proteins by immunoblotting extracts of induced Y294[YEp-src] cells with antiphosphotyrosine antibody (data not shown). Thus, the high levels of phosphotyrosine most likely result from pp60$^{v\text{-}src}$-mediated phosphorylation of yeast cell proteins.

The specific activity of pp60$^{v\text{-}src}$ made in yeast cells is higher than that of pp60^{src} made in mammalian cells. To compare the relative levels of pp60$^{v\text{-}src}$ protein and the specific activity of tyrosine phosphorylation by pp60$^{v\text{-}src}$ expressed in yeast cells and RSV-transformed mammalian cells, pp60$^{v\text{-}src}$ was immunoprecipitated from lysates containing different concentrations of protein by using either a monoclonal antibody or an affinity-purified rabbit antibody to pp60$^{v\text{-}src}$. One-half of the immunoprecipitate was assayed to determine the extent of autophosphorylation, and one-half was analyzed to determine the levels of pp60$^{v\text{-}src}$ by an immunoblot assay. Yeast strains were pregrown in medium containing raffinose as a carbon source, and lysates were prepared from Y294[YEp-src] cells after 3 h of growth in galactose and from Y406 cells after 20 h of growth in galactose. Lysates were also prepared from RSV-transformed mouse 3T3 cells. Results of this analysis are shown in Fig. 6.

The immunoblots shown in the upper panel of Fig. 6A indicate that the RSV-3T3 cells (lanes 1, 3, 6, and 9) contain approximately threefold higher levels of pp60$^{v\text{-}src}$ protein than the YEp-src yeast cells (lanes 2, 4, 7, 10, and 12). In contrast, the levels of pp60$^{v\text{-}src}$ autophosphorylation detected in the same immunoprecipitates (Fig. 6A, lower panel) were threefold higher in the YEp-src yeast cells (lanes 2, 4, 7, 10, and 12) than in the RSV-3T3 cells (lanes 1, 3, 6, and 9). The 90-kDa phosphoprotein detected in SRC-3 antibody immunoprecipitates is derived from the SRC-3 immunoglobulin molecules that are phosphorylated in the immunocomplex kinase reactions. We have also found a comparable difference in the phosphorylation of casein and enolase when included as exogenous substrates in the immunocomplex kinase assay (data not shown). These results indicate that pp60$^{v\text{-}src}$ molecules expressed in yeast cells display a higher specific activity than pp60$^{v\text{-}src}$ molecules expressed in mammalian cells.

The levels of pp60$^{v\text{-}src}$ expressed in strain Y406 (lanes 5, 8, 11, and 13) were approximately three- to fourfold lower than those expressed in strain Y294[YEp-src]; however, pp60$^{v\text{-}src}$ from strain Y406 displayed a specific activity similar to that from strain Y294[YEp-src]. The level of autophosphorylation associated with the pp60$^{v\text{-}src}$ from strain Y406 was higher than that associated with the RSV-3T3 form of pp60$^{v\text{-}src}$, whereas the levels of pp60$^{v\text{-}src}$ protein detected in the immunoblot assay were approximately 10-fold lower in strain Y406 than in the RSV-3T3 cells (lanes 6 and 8).

TABLE 1. Phosphoamino acid content of Y294[YEp-src] and Y294[YEp-52]

Strain and carbon source	% Phosphoamino acid[a]		
	pser	pthr	ptyr
Y294[YEp-52]			
Raffinose	90.8	9.2	<0.1
Galactose	91.4	8.6	<0.1
Y294[YEp-src]			
Raffinose	89.2	10.8	<0.1
Galactose	85.2	10.3	4.5

[a] The percentages of phosphoserine (pser), phosphothreonine (pthr), and phosphotyrosine (ptyr) in Y294[YEp-52] and Y294[YEp-src] cells were determined as described in the legend to Fig. 5.

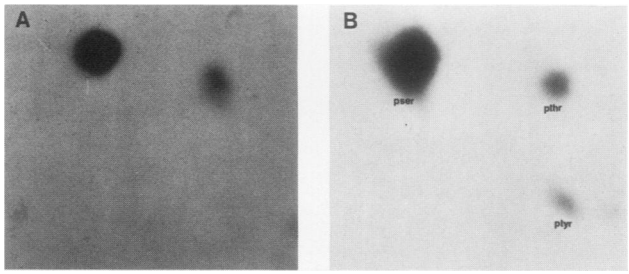

FIG. 5. Phosphoamino acid analysis of Y294[YEp-src] yeast cultures. A stationary-phase culture of Y294[YEp-src] cells grown in SC medium (minus leucine) plus 2% raffinose was diluted 1:10 into 10 ml of phosphate-depleted medium (24) containing 1 mCi of $^{32}P_i$ per ml and either 2% raffinose (A) or 2% galactose (B). Cells were lysed in RIPA buffer, and the phosphoamino acid contents of the proteins from the clarified lysate were determined as described in Materials and Methods. The autoradiogram was exposed for 12 h.

A comparison of the levels of pp60$^{v\text{-}src}$ immunoprecipitated from the lysates with either the monoclonal 327 antibody or the affinity-purified rabbit antibodies indicated that SRC-3 antibodies more efficiently immunoprecipitated

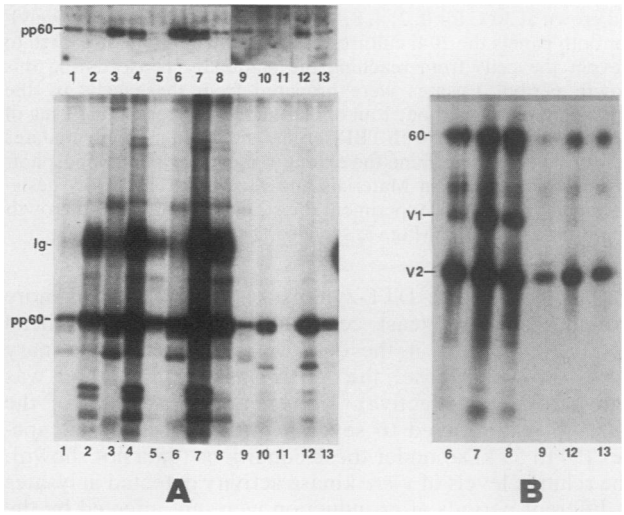

FIG. 6. Specific activity of pp60$^{v\text{-}src}$ expressed in yeast and mammalian cells. (A) Strains Y406 and Y294[YEp-src] were grown overnight in SC medium (minus leucine) plus 2% galactose and SC medium (minus leucine) plus 2% raffinose, respectively. Both cultures were diluted 1:10 into fresh SC medium (minus leucine) plus galactose and grown for 3 h. Lysates were prepared from the Y406 cells (lanes 5, 8, 11, and 13), Y294[YEp-src] cells (lanes 2, 4, 7, 10, and 12), and RSV-3T3 cells (lanes 1, 3, 6, and 9), by using the RIPA-glass bead method for the yeast cells and RIPA buffer alone for the animal cells. Portions (1 ml) of lysate containing 0.3 mg (lanes 1 and 2), 1 mg (lanes 3 to 5 and 9 to 11), or 3 mg (lanes 6 to 8, 12, and 13) of protein were incubated with either anti-mouse immunoglobulin plus monoclonal antibody 327 (lanes 9 to 13) or SRC-3 affinity-purified rabbit immunoglobulin to pp60$^{v\text{-}src}$ (lanes 1 to 8). One-half of the immunoprecipitate was assayed for pp60$^{v\text{-}src}$ protein by the immunoblot procedure described in Materials and Methods (upper panel), and one-half was assayed for autophosphorylation of pp60$^{v\text{-}src}$ as described in Materials and Methods (lower panel). Radioactivity in each pp60$^{v\text{-}src}$ band was determined by scintillation spectroscopy. (B) pp60$^{v\text{-}src}$ bands from panel A (lanes 6 to 9, 12, and 13) were excised and subjected to electrophoresis on a 12.5% SDS-polyacrylamide gel in the presence of 50 ng of *Staphylococcus* V8 protease as described in the reference 7.

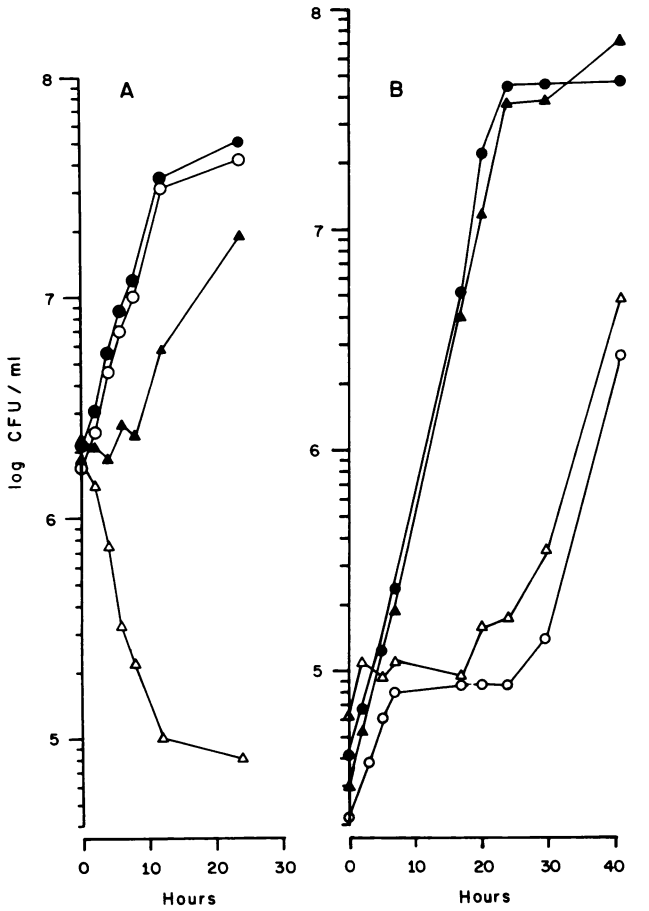

FIG. 7. Analysis of the growth of yeast cells containing multiple or single copies of *GAL10*–v-*src*. (A) Strains Y294[YEp52] (circles) and Y294[YEp-src] (triangles) were pregrown at 30°C in SC medium (minus leucine) plus 2% raffinose to approximately 2×10^6 cells per ml. Galactose was added to 2%, and the cultures were incubated at 30°C with aeration. Samples were removed at various times, cell counts were determined in a hemacytometer, and appropriate dilutions were plated on YEP plates containing 2% glucose. After 3 days of growth at 30°C, colonies were replicated onto SC medium (minus leucine) plates, and the colonies were scored for leucine prototrophy after an additional 1 day of growth at 30°C. From these data the number of viable cells (closed symbols) and the number of viable plasmid-bearing cells (open symbols) per milliliter were calculated and are plotted versus time after the addition of galactose. (B) Strains lacking v-*src* (filled symbols) and those carrying a single integrated copy of v-*src* (open symbols) were grown overnight in SC medium plus 2% raffinose. Samples of each culture were diluted to approximately 10^4 cells per ml in the same medium, and the cultures were incubated with aeration at 30°C for 5 h. Galactose was then added to each culture to a concentration of 2%. Incubation was continued, and samples were removed periodically to determine viable cell counts by plating appropriate dilutions on YEPD plates. The number of viable cells per milliliter is plotted versus time after the addition of galactose. Strains: Y294 (▲), JR212-10A (●), Y406 (○), and JR212-10B (△).

pp60$^{v\text{-}src}$ from the yeast cells; however, the relative levels of pp60$^{v\text{-}src}$ protein detected in the immunoblots and the level of autophosphorylation were similar when either reagent was used to immunoprecipitate pp60$^{v\text{-}src}$.

Figure 6B shows a partial proteolytic cleavage profile of the 60-kDa protein phosphorylated in the assay shown in the lower panel of Fig. 6A. In the monoclonal antibody immunoprecipitates, the predominant site of phosphorylation on all forms of pp60$^{v\text{-}src}$ was in the V2 fragment, which represents the carboxy-terminal 26 kDa of pp60$^{v\text{-}src}$. This fragment contains the previously identified site of autophosphorylation (tyrosine 416) (21, 30). However, in the SRC-3 antibody immunoprecipitates, phosphorylation was detected in both the amino-terminal 34-kDa fragment (V1) and in V2. Both V1 and V2 were found to contain phosphotyrosine (data not shown). The V1/V2 phosphorylation ratio on pp60$^{v\text{-}src}$ molecules expressed in yeast cells was slightly higher than that for pp60$^{v\text{-}src}$ from mammalian cells.

Hyperexpression of v-*src* is inimical to yeast cell growth. The rapid decrease in v-*src* expression after 4 to 6 h of induction suggested the possibility that hyperexpression of the protein might have a toxic effect on yeast growth. To examine this possibility, we monitored the growth of Y294[YEp-src] after induction with galactose. Strains Y294[YEp-src] and Y294[YEp52] were grown to mid-exponential phase in SC medium containing raffinose as the carbon source and lacking leucine, to select for retention of the plasmids. Raffinose does not induce expression from galactose-regulated promoters but does not prevent rapid induction of such promoters upon addition of galactose. Galactose was added to both cultures, and samples were removed at various times, counted, and plated onto solid YEPD medium to determine the viable-cell count. Colonies obtained on YEPD plates were replica plated onto medium lacking leucine to determine the number of cells that retained the plasmid. Results from this experiment are presented in Fig. 7A, which shows viable-cell counts and the concentration of plasmid-bearing cells in both strains as a function of time following the addition of galactose. Strain Y294[YEp52] exhibited continued exponential growth after addition of galactose, and the proportion of plasmid-bearing cells remained essentially constant at approximately 80% over the course of the experiment. In contrast, strain Y294[YEp-src] exhibited a substantial lag in increase in cell count after addition of galactose, concomitant with a significant drop in the number of viable plasmid-bearing cells. We interpret these results to indicate that high-level expression of pp60$^{v\text{-}src}$ in this strain is lethal. We suspect that the increase in cell number at later times is due to lysis of plasmid-bearing cells, liberating sufficient leucine to allow some growth of non-plasmid-bearing cells.

In Fig. 7B we show similar growth curves for strains Y406 and JR212-10B, each of which contains a single copy of *GAL10*–v-*src* integrated into chromosome 15, and strains Y294 and JR212-10A, *src*-free strains closely related to the *src*-bearing strains. Both *src*-free strains showed completely normal growth profiles after addition of galactose, exhibiting a mean doubling time of approximately 2.5 h. In contrast, both *GAL10*–v-*src* strains exhibited a nearly complete cessation of growth several hours after the addition of galactose. Visual inspection of these cells did not reveal any specific cell cycle arrest associated with the inhibition of growth. Using different induction schemes, we found that the extent of growth inhibition can vary. However, we confirmed the growth-inhibitory effect of single-copy v-*src* expression by segregation analysis. In crosses of Y406 to other Gal$^+$ strains, we found 2:2 segregation of galactose sensitivity that was 100% linked to the integrated v-*src* (data not shown).

Approximately 20 h after cessation of growth, both cultures showed a restitution of apparently normal growth (Fig. 7B). This could result from recovery of cells from *src*-induced inhibition or from continuous growth of a resistant subpopulation of cells originally present in the culture. To

address this question, samples of strain Y406 or JR212-10B obtained at the end of growth in the induced cultures were removed, diluted 1:1,000 into YEPD medium, and grown overnight to allow deinduction of v-*src*. This conditioned culture was used as the starting material for the growth regimen described in Fig. 7B. In this case no lag in growth of the strain upon addition of galactose was observed. Thus, resistance to v-*src* induction persists after expression ceases, either as the result of acquisition of a suppressor mutation or through extended persistence of an epigenetic change. To distinguish between these possibilities, we crossed cells from the conditioned culture to a naive strain containing single-copy v-*src*. After sporulation and dissection of such diploids, resistance segregated 2:2. Thus, resistance acquired upon exposure to galactose can result from a single Mendelian alteration. These observations suggest that hyperexpression of $pp60^{v\text{-}src}$ in yeast cells is lethal but that these cells can become desensitized or resistant to moderate levels of the protein. We cannot provide a quantitative estimate as to the threshold level of v-*src* expression necessary for lethality with our current data.

DISCUSSION

The results presented in this report indicate that yeast cells provide an environment compatible with efficient expression of tyrosine kinase activity of $pp60^{v\text{-}src}$ protein. Our preliminary evidence indicates that the $pp60^{v\text{-}src}$ expressed in yeast cells is more closely analogous to the protein synthesized in animal cells than the v-*src* gene product expressed in bacteria. This conclusion is based on several criteria. (i) The v-*src* gene product expressed in yeast cells is phosphorylated in vivo on sites within the amino and carboxyl halves of the molecule. (ii) Expression of v-*src* in yeast cells results in high levels of total cellular protein phosphorylation on tyrosine. (iii) The yeast cell $pp60^{v\text{-}src}$ protein can autophosphorylate and phosphorylate exogenous substrates in immunocomplex kinase reactions. In contrast, the v-*src* gene product that has been expressed in *E. coli* is not phosphorylated in vivo or in vitro, the specific activity of tyrosine phosphorylation is very low in vitro, and expression of v-*src* does not induce phosphorylation of bacterial-cell proteins on tyrosine (11, 13, 19). In addition, most of the protein present in bacteria is aggregated in insoluble precipitates. The major advantage of the bacterial expression system is that the levels of protein produced in *E. coli* are much higher than in the yeast cells.

The basis for the toxicity of the v-*src* gene product in the Y294[YEp-src] cells is not known. The mere physical presence of the v-*src* gene product in the cell might account for the observed growth inhibition, if the protein perturbed the membrane structure or sterically interfered with one or more essential components of the cell. A more likely possibility is that v-*src*-promoted phosphorylation of some endogenous proteins inhibits their normal function and thereby causes toxicity. Preliminary results from analysis of yeast proteins that react with antiphosphotyrosine antibodies (34) indicates that many different yeast cell proteins are phosphorylated on tyrosine in galactose-grown Y294[YEp-src] cells (data not shown). The failure to observe cell cycle arrest during v-*src*-induced inhibition of growth suggests that inhibition results from interference with some aspect(s) of the general metabolism of the cell rather than with a specific step in progression through the cell cycle. Analysis of the v-*src*-resistant yeast mutants may assist in revealing the particular metabolic processes inhibited by the presence of $pp60^{v\text{-}src}$.

We note from the growth pattern of our single-copy v-*src* strains after induction by galactose that resistant cells rapidly take over the culture. This would suggest either that mutation of any one of a large number of loci yields resistance to v-*src* or that resistant mutants accumulate in the population even when the strain is growing under noninducing conditions. The latter possibility is not unreasonable, since glucose repression of *GAL10*–v-*src* is not absolute (Fig. 2, lane 6). The preexistence of resistant cells in the population of *GAL10*–v-*src*-containing strains could explain the variable level of growth inhibition we observed after induction of such strains with galactose. We noted that the resistant mutants are not merely Gal^- revertants, since the resistant cells that grow in galactose medium are perforce Gal^+. Furthermore, these resistant mutants are not resistant to growth inhibition by *GAL10-RAS2*$^{Val\text{-}19}$, another construct that causes galactose-inducible lethality in yeast cells (M. Fedor-Chaiken and J. Broach, unpublished observations). However, resistance could be due to mutational inactivation of the *GAL10*–v-*src* allele in the strain. Further genetic analysis will clarify this issue.

$pp60^{v\text{-}src}$ molecules expressed in yeast cells appeared to display a higher specific activity than $pp60^{v\text{-}src}$ protein expressed in mouse cells. This difference is not attributable to differences in the kinase activity of the v-*src* gene products encoded by the Schmidt-Ruppin A and D strains of RSV since the specific activities of these two forms of RSV are similar (J. Brugge, unpublished results). The only difference between the yeast and mouse v-*src* gene products detected in this study was that the level of phosphorylation within the amino-terminal region of $pp60^{v\text{-}src}$ in the immunocomplex kinase reactions was greater for the yeast $pp60^{v\text{-}src}$ than for the mouse $pp60^{v\text{-}src}$. This could reflect an alteration in the structure of the $pp60^{v\text{-}src}$ molecules expressed in yeast cells. However, the nature of a structural alteration that could be responsible for the enhanced phosphorylation within this region of $pp60^{v\text{-}src}$ is not known. Further analysis of the precise sites of phosphorylation in vivo may be informative.

No direct evidence convincingly supports the possibility that yeast cells contain endogenous tyrosine kinases. No tyrosine-specific protein kinases have been isolated from yeast cells, and the identification of proteins which are phosphorylated on tyrosine in vivo is hampered by technical difficulties in distinguishing tyrosine phosphorylation from tyrosine adenylylation (29, 33). That is, during acid hydrolysis of adenylated proteins, adenylyltryrosine is converted to phosphotyrosine. Extracts of yeast cells have been shown to phosphorylate endogenous proteins on tyrosine or synthetic copolymers of glutamic acid and tyrosine (6, 25). However, the 100-fold lower specific activity of these extracts compared with animal cell extracts raises questions about the specificity of these phosphorylation events. The observed phosphorylations may reflect the secondary activity of other types of kinases.

Since yeast cells contain at most low levels of tyrosine kinase activity, this organism provides a useful system for the expression of animal cell tyrosine kinase. This will facilitate the study of mutant gene products that would be difficult to analyze in animal cells containing the endogenous kinase itself, as well as numerous other gene products with associated tyrosine kinase activity. While the levels of protein produced in yeast are not as high as in bacterial expression systems, the ease of growing large quantities of yeast cells, compared with animal cells, also makes the yeast system attractive for studies on the functional activity of tyrosine kinases.

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