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Molecular Cloning of the Phenylalanine Ammonia Lyase Gene from *Rhodosporidium toruloides* in *Escherichia coli* K-12

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A genomic library of *Rhodosporidium toruloides* DNA was constructed in bacteriophage λ1059. Recombinant phage containing phenylalanine ammonia lyase (PAL) gene sequences were identified by using ^{32}P-labeled cDNA to partially purified PAL mRNA. The PAL gene was subcloned on an 8.5-kilobase *Pst*I DNA restriction fragment into pUC8 to generate the recombinant plasmid pHG2. A restriction map of the PAL gene, together with its flanking regions, was constructed. Northern hybridization analysis of *R. toruloides* RNA with a restriction fragment encoding part of the PAL gene indicates that PAL mRNA is 2.5 kilobases in length. A single-stranded DNA hybridization probe was constructed and used to quantitate PAL mRNA levels in *R. toruloides* grown under different physiological conditions. PAL mRNA levels paralleled changes in functional PAL mRNA and antigen. These data are consistent with control of PAL expression being at the level of transcription.

Phenylalanine ammonia lyase (PAL; EC 4.3.1.5), which occurs in plants (3), yeasts (24), fungi (30), and streptomycetes (10), catalyzes the nonoxidative deamination of L-phenylalanine to *trans*-cinnamic acid (16). A dehydroalanine moiety acts as a prostetic group (16). The enzyme has a potential role in the treatment and diagnosis of phenylketonuria (2) and has industrial applications in the synthesis of L-phenylalanine from *trans*-cinnamic acid (34). The enzyme in plants is involved in flavanoid biosynthesis and is induced by illumination (29). In gherkin and mustard seedlings induction is the result of activation of a constitutive pool of inactive enzyme (3). In other botanical species the enzyme is synthesized de novo in response to illumination (29). In gherkins, apples, sweet potatoes, and sunflowers, PAL is also regulated by a specific inactivating system (32).

In *Rhodosporidium toruloides* phenylalanine can act as the sole source of carbon, nitrogen, and energy. As PAL catalyzes the initial reaction in the catabolism of the amino acid, the enzyme plays a key role in regulating phenylalanine metabolism. In *R. toruloides* PAL is induced by the presence of L-phenylalanine or L-tyrosine (24). Growth with glucose or with ammonia and glucose together causes repression of PAL synthesis (24). Work in this laboratory has shown that induction of PAL activity is the result of de novo synthesis of the enzyme rather than activation of an inactive precursor or a decrease in the rate of PAL degradation (14). Glucose represses PAL synthesis but has no effect upon the stability of the enzyme, whereas ammonia prevents uptake of phenylalanine and so may repress enzyme synthesis through inducer exclusion (14). In vitro translation of mRNA from *R. toruloides* grown under different physiological conditions showed that phenylalanine, ammonia, and glucose regulate PAL synthesis by adjusting the level of functional PAL mRNA (13).

In eucaryotic cells the concentration of functional mRNA can be regulated at the levels of transcription (4, 22, 35), processing of nuclear RNA into functional mRNA (1), and degradation of mRNA (15, 22). This report describes the isolation of the gene encoding PAL from *R. toruloides* and its use in studying mechanisms by which functional PAL mRNA is controlled. The results indicate that glucose, phenylalanine, and ammonia regulate the PAL gene at the level of transcription. The gene was present as a single copy in the *R. toruloides* genome, and PAL is synthesized from a monocistronic mRNA of 2.5 kilobases (kb).

MATERIALS AND METHODS

Microbial strains and plasmids. Microbial strains and plasmids used in this study are listed in Table 1.

Media. Minimal media used for growth of yeast were described by Gilbert and Tully (14). *Escherichia coli* Q358, Q359, and JM83 were cultured in L broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl). Media were solidified with the addition of 2% (wt/vol) Bacto-Agar (Difco Laboratories). Ampicillin was added to 100 μg/ml. Strain JM101 was grown in 2YT medium (1.6% tryptone, 1% yeast extract, 0.5% NaCl).

Chemicals. Methylmercuric(I)hydroxide was purchased from the Alpha Chemical Company. [8-^{3}H]adenine (25 Ci/mmol), [α-^{32}P]dATP (410 Ci/mmol), L-[^{35}S]methionine (1,120 Ci/mmol), in vitro packaging kit, and rabbit reticulocyte lysate (N.90) were obtained from Amersham International. Reverse transcriptase was from J. Beard, Life Sciences, Inc. 5-Bromo-4-chloro-indoyl-β-D-galactoside, isopropyl-β-D-thiogalactoside, low-melting-point agarose, restriction enzymes, S1 nuclease, T4 DNA ligase, and vanadyl complex RNAase inhibitor were from Bethesda Research Laboratories. Formamide was obtained from Fluka G. Novozyme 234 was purchased from Novozyme Products, Ltd. X-Omat RP X-ray film, DX-80 developer, and FX-40 fixer were from Eastman Kodak Co. Oligodeoxythymidylic acid cellulose type 7 and oligodeoxythymidylic acid primer were obtained from P-L Biochemicals, Inc. BA85 nitrocellulose filters were from Schleicher & Schuell, Inc. Donkey anti-rabbit immunoglobulin G was from Wellcome Reagents,

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TABLE 1. Microbial strains and plasmids used in this work

Strain, plasmid, or phage	Genotype or phenotype	Source (reference)
R. toruloides IFO0559	Prototrophic	Osaka, Japan (5)
E. coli		
Q358	*hsdR* 80^r *supE*	(23)
Q359	*hsdR* 80^r *supE* P2	(23)
JM83	*ara* Δ(*lac-pro*)*rpsL thi* φ80d *lacI^{q}ZM15*	(26)
JM101	Δ(*lac-pro*)*supE thi*/F *lacI^{q}ZM15 traD pro$^+$*	(26)
GWI	*dam$^-$*	L. Clarke
Bacteriophage		
M13mp10	*lacZ*	(29)
M13mp11	*lacZ*	(29)
λ1059		(23)
Plasmid		
pUC8	Amp^r *lacZ*	(26)
pUC9	Amp^r *lacZ*	(26)
pUC12	Amp^r *lacZ*	(26)
pUC13	Amp^r *lacZ*	(26)

Ltd. All other chemicals were from Sigma Chemical Co. or BDH.

Isolation and purification of RNA. Polyadenylic acid [poly(A)]-containing RNA was extracted from *R. toruloides* as described by Gilbert et al. (13). PAL mRNA was purified by sucrose gradient centrifugation (28), followed by agarose gel electrophoresis in the presence of methylmercuric hydroxide (23).

Cell-free translation and immunoprecipitation of PAL. Cell-free translations in a mRNA-dependent translation system derived from rabbit reticulocyte lysates, as well as subsequent immunoprecipitation, were as described by Gilbert et al. (13). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was as described by Laemmli (21).

Construction of phage library of *R. toruloides* DNA. A phage library of *R. toruloides* DNA was constructed in bacteriophage λ1059 essentially as described by Karn et al. (20).

^{32}P-labeling of nucleic acid. cDNA was synthesized from 1-μg portions of mRNA as described by Emtage et al. (11). The specific activities of the product was ca. 10^8 cpm/μg of DNA. Double-stranded DNA was labeled by nick translation to a specific activity of 1×10^8 to 4×10^8 cpm/μg DNA (23).

Screening of the λ library for the PAL gene. For screening the library, recombinant phage were grown on *E. coli* to a density of 100 plaques per cm^2. Phage DNA was transferred to nitrocellulose as described by Benton and Davis (6). Hybridization of the filters to the probe, subsequent washing, and autoradiography of the filters were done as described by Maniatis et al. (23).

Hybrid-selected translation. Plasmids were immobilized on nitrocellulose as described by Parnes et al. (27). Incubation of filters with yeast poly(A)-containing RNA, washing of filters, and elution of RNA were as described by Maniatis et al. (23).

DNA isolation. (i) *R. toruloides* DNA. Cells of *R. toruloides* (20 g [wet wt]) grown to mid-log phase were harvested at 4°C, washed in 20 ml of water, and incubated for 30 min at room temperature in 45 mM EDTA–300 mM 2-mercaptoethanol. The cells were pelleted, suspended in 65 mM sodium phosphate (pH 4.8) containing 1 M sorbitol, 25 mM EDTA, and 8 mg of Novozyme 234 per ml, and incubated overnight at 30°C to form protoplasts.

Protoplasts were pelleted at 1,200 × *g* for 5 min and suspended in 20 ml of 0.15 M NaCl–0.1 M EDTA (pH 8.0). Proteinase K (50 μg/ml) and SDS (1%) were added, and the cells were incubated for 2 h at 37°C, after which time 90% lysis had occurred. The suspension was extracted once with chloroform-isoamyl alcohol (24:1 [vol/vol]). Two volumes of ethanol was layered over the aqueous phase, and the DNA was spooled. After the DNA was redissolved in 20 ml of 50 mM Tris-hydrochloride (pH 7.4), RNase A (DNase free) was added to a final concentration of 80 μg/ml, and the solution was incubated at 37°C for 2 h. After the DNA solution was dialyzed for 16 h at 4°C against 200 volumes of 50 mM Tris-hydrochloride (pH 7.4), the aqueous phase was extracted twice with phenol and once with chloroform and made 0.1 M with NaCl, and the DNA was spooled as described above. The DNA pellet was redissolved in 10 mM Tris-hydrochloride (pH 7.4)–1 mM EDTA to a final concentration of 1 mg/ml.

(ii) Plasmid DNA. *E. coli* plasmids were purified from 500 ml of L broth cultures containing 100 μg of ampicillin per ml by "Brij lysis" and subsequent CsCl density centrifugation (7). The rapid boiling method of Holmes and Quigley (18) was employed for small-scale plasmid isolation for screening purposes.

General DNA recombinant procedures. Restriction endonucleases and DNA ligase were used according to the instructions of the manufacturer. Transformation of *E. coli* was performed as described by Cohen et al. (8). Hybridization conditions for either Southern blots of DNA gels or Northern blots of RNA gels were as described in screening the λ phage bank for the PAL gene. All recombinant DNA work was done under category I containment.

Electrophoresis of nucleic acid. DNA was electrophoresed in agarose gels (0.8 to 1.5%) by using a Tris-borate-EDTA buffer (25). λ digested with *Hin*dIII or *Hin*dIII-*Eco*RI and φX174 RF digested with *Hae*III were used as molecular weight standards. DNA was transferred to nitrocellulose as described by Southern (31).

For analytical electrophoresis RNA was denatured at 60°C for 15 min in 20 mM 4-morpholinepropanesulfonic acid (MOPS; pH 7.0)–5 mM sodium acetate–1 mM EDTA–2.2 M formaldehyde–50% formamide (vol/vol). Poly(A)-containing RNA (2.5 μg/cm slot) was electrophoresed for 16 h at 20 mA in 1% gels. Gel buffer was as described above, except formamide was omitted. Reservoir buffer consisted of 20 mM MOPS (pH 7.0), 5 mM sodium acetate, and 1 mM EDTA. After the gel was stained with ethidium bromide, RNA was transferred to nitrocellulose as described by Thomas (33).

PAL activity. PAL catalytic and antigenic activity were determined as described by Gilbert and Jack (12).

Quantitation of PAL mRNA. A single-stranded probe which hybridized to PAL mRNA was synthesized from a 0.6-kb *Sal*I-*Sst*I DNA restriction fragment encoding part of the PAL gene as described by Durnham and Palmiter (9). In hybridization reactions ca. 1,000 cpm of PAL cDNA was added per reaction. Hybridization and S1 nuclease conditions were as described previously (9), except 4 U of the nuclease was optimal for digestion of single-stranded DNA while maintaining DNA-RNA hybrids intact.

Degradation of PAL mRNA. *R. toruloides* grown in phenylalanine or glucose-phenylalanine medium was labeled

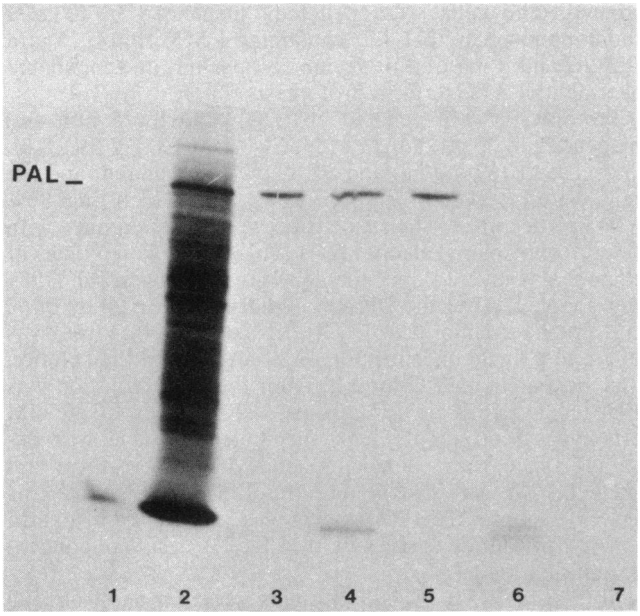

FIG. 1. Flurogram of an SDS-polyacrylamide gel of in vitro translation products. Plasmids were immobilized on nitrocellulose and used to select specific mRNAs, which were eluted and used to direct in vitro translation in a rabbit reticulocyte lysate. Lanes: 1, 1 μl of lysate without exogenous RNA; 2, 5 μl of lysate incubated with poly(A)-containing RNA from phenylalanine minimal medium-grown cells; 3, the product of immunoprecipitation with PAL antiserum of the translation products in lane 2; 4, 1 μl of lysate incubated with RNA which was hybrid-selected by pHG2; 5, the product of immunoprecipitation with PAL antiserum of the translation products in lane 4; 6, 1 μl of lysate incubated with RNA which was hybrid-selected by pUC8; 7, the product of immunoprecipitation with PAL antiserum of the translation products in lane 6. The 10% SDS-polyacrylamide gel was flurographed and exposed for 16 h at −70°C. The position of purified PAL is indicated on the gel.

with [8-^{3}H]adenine as described previously (13). Cells were rapidly harvested, washed in 0.15 M NaCl, and suspended in media containing 5 mM nonradioactive adenine. At regular intervals cells were removed, the RNA was extracted (13), and ^{3}H-PAL mRNA was quantitated by hybridization to PAL cDNA as described above. The degradation rate of the message was determined from the decrease in ^{3}H-PAL mRNA per μg of total RNA with time.

Partial proteolysis in SDS-polyacrylamide gels. Limited proteolysis was performed as described by Gilbert et al. (13).

RESULTS

Isolation of the PAL gene. A clone bank of *R. toruloides* DNA was constructed in bacteriophage λ1059. A total of 120,000 recombinants were isolated. A small portion of the bank was screened for the presence of the PAL gene by using ^{32}P-labeled cDNA prepared from partially purified PAL mRNA. One recombinant phage, which hybridized to cDNA prepared from partially purified PAL mRNA but not to control mRNA from *R. toruloides* grown in glucose-ammonia medium (where no translatable PAL mRNA can be detected [13]), was selected for further study. DNA extracted from this recombinant was digested with restriction enzymes, and the resultant fragments were hybridized to PAL cDNA. Results indicated that only part of the PAL gene had been cloned, as DNA fragments which hybridized to the probe were too small to encode the whole gene. A 0.9-kb *Sal*I DNA restriction fragment, which hybridized to PAL cDNA, was ^{32}P-labeled by nick translation and used to screen the rest of the clone bank for the presence of the PAL gene. DNA was extracted from 6 of 30 recombinants which hybridized to the probe. Digestion of the phage DNA with *Pst*I produced an 8.5-kb restriction fragment, common to five of the six phage, which hybridized to the probe. The DNA restriction fragment was cloned into pUC8 to give recombinant plasmid pHG2.

To investigate whether pHG2 contains the PAL gene, pHG2 and pUC8 were immobilized on nitrocellulose and incubated with total poly(A)-containing mRNA. mRNA which specifically hybridized to the plasmids was eluted and employed in cell-free translation in a rabbit reticulocyte lysate. Results showed (Fig. 1) that material eluted from filters containing pUC8 alone did not direct cell-free synthesis of any protein. This indicates that pUC8 does not select any specific mRNA. Material eluted from filters containing pHG2 directs the in vitro synthesis of a polypeptide which comigrates with native PAL on SDS-polyacrylamide gels. The protein gives a similar spectrum of peptides compared with native PAL, when partially digested with chymotrypsin and V8 protease. These data show that the in vitro translation product produced by mRNA which hybridizes to pHG2 is PAL, and therefore, the plasmid pHG2 contains PAL gene sequences.

DNA enzyme restriction analysis and subcloning of the PAL gene. The position of the PAL gene within the 8.5-kb *Pst*I restriction fragment was determined by digesting pHG2 with *Bcl*I and *Pst*I. The three *R. toruloides* DNA fragments generated (1.5-kb and 0.3-kb *Bcl*I-*Pst*I fragments flanking a 6.7-kb *Bcl*I fragment) were cloned into pUC8 and employed

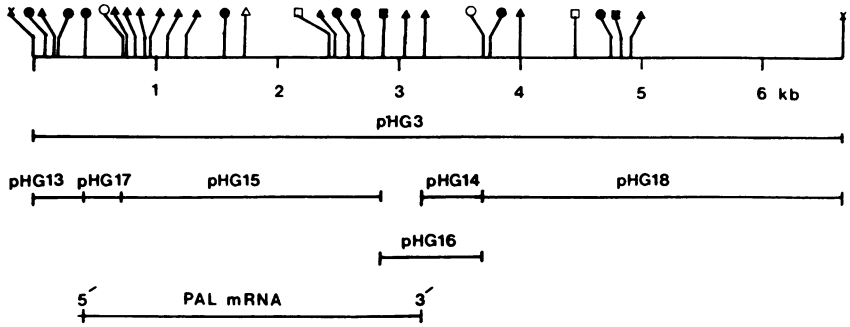

FIG. 2. Restriction map of *R. turuloides* DNA insert in pHG3. The positions of the cleavage sites of *Bam*HI (○), *Sst*I (□), *Sal*I (●), *Bgl*II (■), *Xho*I (▲), *Xba*I (□), and *Bcl*I (×) are shown. The *Eco*RI and *Pst*I sites shown in Fig. 4 are not present in the *R. toruloides* fragment present in pHG3.

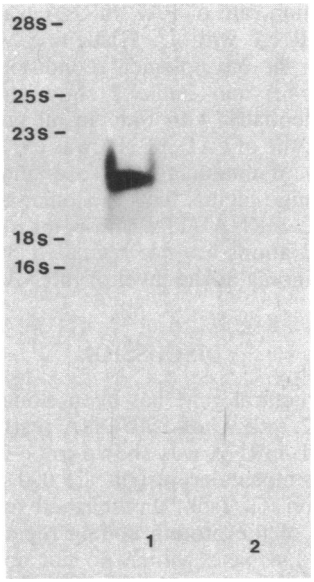

FIG. 3. Size of PAL mRNA. Poly(A)-containing RNA from cells grown on phenylalanine minimal medium (lane 1) and glucose-ammonia minimal medium (lane 2) were subjected to agarose gel electrophoresis under denaturing conditions as described in the text. Each tract contains 3 μg of RNA. The RNA was transferred to nitrocellulose, hybridized with ^{32}P-labeled pHG15, washed, and autoradiographed for 7 h. The molecular weight markers were yeast rRNA (18S, 25S), mammalian rRNA (18S, 28S), and *E. coli* rRNA (16S, 23S).

in hybrid-selected translation of PAL mRNA. Only plasmid pHG3, which contained the 6.7-kb *Bcl*I fragment, selected PAL mRNA, indicating that the PAL gene was contained within this fragment. The cleavage sites of different restriction enzymes were mapped within the 6.7-kb *Bcl*I DNA fragment (Fig. 2). To localize the PAL gene within pHG3, the plasmid was digested with restriction enzymes, and the resultant fragments were cloned into pUC8. The recombinant plasmids were used in hybrid-selected translation of PAL mRNA. Results showed (Fig. 2) that the *Bcl*I-*Sal*I fragment in pHG13, the *Bam*HI-*Xho*I fragment in pHG14, and the *Bam*HI-*Bcl*I fragment in pHG18 did not hybrid-select PAL mRNA. However, the *Bam*HI-*Bgl*II fragments present in pHG15 and pHG16, together with the *Sal*I-*Bam*HI fragment in pHG17, hybridized to PAL mRNA. These data localize the PAL gene to a 2.7-kb *Xho*I-*Sal*I restriction fragment within pHG3 (Fig. 2).

Size of PAL mRNA. Total poly(A)-containing RNA, extracted from *R. toruloides* grown on phenylalanine minimal medium and glucose-ammonia medium, was fractionated under denaturing conditions on agarose gels. The RNA was transferred to nitrocellulose and hybridized with ^{32}P-labeled pHG15. The probe hybridized to an RNA species, 2.5 kb in length, which was present only in RNA extracted from cells grown on phenylalanine minimal medium (Fig. 3).

Orientation of the PAL gene. To orientate the PAL gene within the 6.7-kb *Bcl*I DNA fragment in pHG3, the 0.6-kb *Sal*I-*Sst*I restriction fragment, which encodes part of the PAL gene, was cloned in opposite orientations in M13mp10 (HS1) and M13mp11 (HS2). Recombinant phage were identified by insertional inactivation in the *Sal*I and *Sst*I sites of the *lacZ* gene. Single-stranded DNA was extracted from the phage, immobilized on nitrocellulose, and hybridized to cDNA prepared from poly(A)-containing RNA extracted from yeast cells containing PAL mRNA (phenylalanine minimal medium) and *R. toruloides* containing no PAL mRNA (glucose-ammonia medium). The results showed that cDNA containing no PAL mRNA complementary sequences did not hybridize to either of the phage DNA. cDNA prepared against RNA containing PAL mRNA hybridized only to HS2. HS1 and HS2 were also employed in hybrid-selected translations. HS1 hybrid-selected PAL mRNA, but HS2 did not. These data were consistent with the *Sal*I restriction site being proximal to the 5′ end of the PAL gene (Fig. 2).

Number of PAL gene copies. The 3-kb-*Bam*HI DNA restriction fragment, encoding most of the PAL gene (Fig. 2), was ^{32}P-labeled and used to probe Southern blots of *R. toruloides* DNA digested with different restriction enzymes. The results showed (Fig. 4) that the probe hybridized to single restriction fragments when genomic DNA was digested with enzymes which did not cleave within the 3-kb *Bam*HI fragment. Two restriction fragments hybridized to the probe when *R. toruloides* DNA was digested with *Sst*I, which did cut within the PAL gene. These data are consistent with the suggestion, based on genetic analyses, that only one copy of the PAL gene is present in the *R. toruloides* genome.

PAL mRNA levels in *R. toruloides*. Single-stranded ^{32}P-labeled PAL cDNA was employed in RNA-driven hybridization reactions to determine PAL mRNA levels in *R. toruloides* grown under different physiological condi-

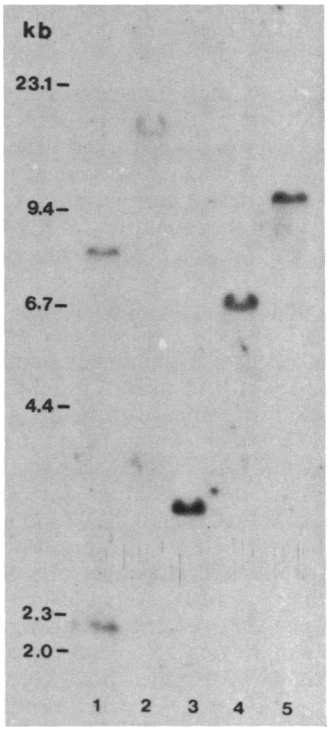

FIG. 4. Autoradiograph of a Southern blot of *R. toruloides* DNA digested with restriction enzymes and probed with pHG4. *R. toruloides* DNA (2 μg) was digested with *Sst*I (lane 1), *Eco*RI (lane 2), *Bam*HI (lane 3), *Bcl*I (lane 4), and *Pst*I (lane 5). The DNA was transferred to nitrocellulose, hybridized to ^{32}P-labeled pHG4, and autoradiographed for 72 h. pHG4 contains the 3-kb *Bam*HI fragment present in pHG3 which encodes most of the PAL gene (Fig. 2). Molecular weight markers were DNA fragments of λ digested with *Hin*dIII.

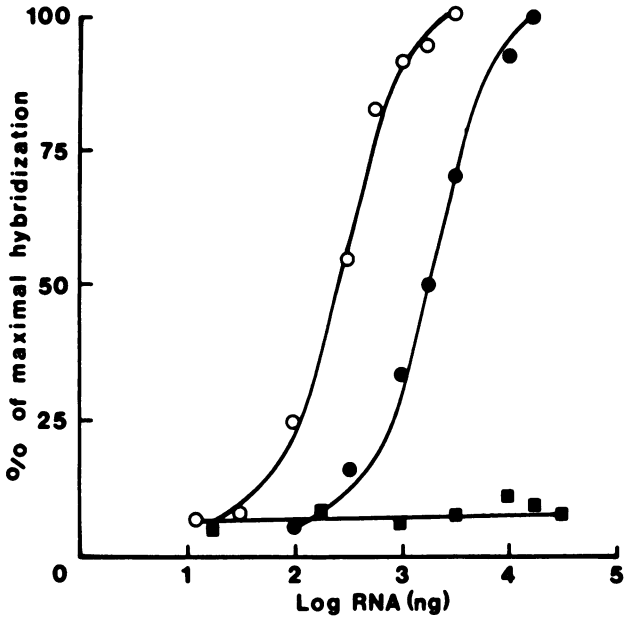

FIG. 5. Quantitation of PAL mRNA in *R. toruloides*. Total RNA was hybridized to ^{32}P-labeled cDNA to PAL mRNA as described in the text. The percentage of maximal hybridization was plotted against the log RNA added to the reaction. RNA was extracted from cells grown on phenylalanine minimal medium (○), glucose-phenylalanine minimal medium (●), and glucose-ammonia minimal medium (■). Comparative levels of PAL mRNA in total RNA preparations were determined from the amount of RNA required to obtain 50% of the maximal hybridization.

tions. Representative hybridization curves are shown in Fig. 5. Between 80 and 90% of the input cDNA was protected from S1 nuclease digestion when hybridization was maximal. The S1 nuclease-resistant background was ca. 7% of the input probe. The relative concentration of PAL mRNA was determined from the quantity of RNA required to produce 50% of the maximal hybridization. The results (Table 2) show that PAL mRNA was ca. seven times higher in cells grown on phenylalanine medium than in glucose-phenylalanine-grown cells. No PAL mRNA was detected in yeast cells grown on glucose-ammonia minimal medium.

The actual quantity of PAL mRNA in fully induced yeast cells was determined by comparison of the hybridization of total RNA and HS2 (which contains the 0.6-kb DNA fragment coding for part of PAL mRNA) with that of ^{32}P-labeled PAL cDNA. Hybridization conditions were 48 h at 65°C in 4× SSC (SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–5× Denhardt solution–0.1% SDS. Under these conditions DNA-DNA and DNA-RNA hybridizations are equivalent (9). Results showed that 5 pg of DNA and 260 ng of RNA were required to produce 50% of the maximal hybridization. Therefore 0.0072% of the total RNA is PAL mRNA. Assuming the mRNA is 0.5% of the total RNA (this is the percentage of *R. toruloides* RNA which is polyadenylated), the PAL mRNA represents 1.54% of the total mRNA.

PAL mRNA synthesis and degradation. PAL mRNA induction was determined by measuring levels of PAL mRNA in *R. toruloides* transferred from noninducing (glucose-ammonia) to inducing (phenylalanine) growth conditions. Results showed (Fig. 6A) a rapid induction of PAL mRNA when yeast cells were transferred to phenylalanine medium. Maximum levels of PAL mRNA were observed within 60 min of transfer to inducing conditions.

The degradation rate of PAL mRNA was determined by pulse-labeling RNA with [8-^{3}H]adenine when cells were grown in different physiological conditions. Results presented in Fig. 6B and Table 2 show that PAL mRNA decayed exponentially with time in all growth conditions tested. The half-life of PAL mRNA was ca. 8 min in inducing (phenylalanine), noninducing (glucose-ammonia), and glucose-phenylalanine media. This is similar to the half-life of functional PAL mRNA (13). These data show that PAL mRNA concentrations are not regulated by phenylalanine, glucose, or ammonia at the level of mRNA degradation.

DISCUSSION

The PAL structural gene has been cloned in *E. coli* and localized to a 2.7-kb *Xho*I-*Sal*I DNA restriction fragment. The size of PAL mRNA was shown to be 2.5 kb. From the known subunit molecular weight (75,000 [12]) and amino acid composition (G. Jack, unpublished results) of the enzyme, the size of the protein coding region of the gene is 2.362 kb. The 138 bases which do not code for the PAL protein are too small to encode a second protein, particularly when one considers that at least 50 of these bases consist of the 3′ polyadenylated tail found in *R. toruloides* mRNA (13). This implies that PAL mRNA, in common with nearly all eucaryotic mRNAs, is monocistronic.

Results presented in this study describe the construction and use of a DNA hybridization probe to quantitate PAL mRNA levels. Results showed that changes in total PAL mRNA paralleled changes in both functional PAL mRNA and PAL antigen when the yeast cells were grown in different physiological conditions. The half-life of PAL mRNA was ca. 8 min in all growth conditions. This is similar to the degradation rate of mRNAs studied in *Saccharomyces cerevisiae* (4, 35). These data show that glucose, phenylalanine, and ammonia control PAL synthesis at the level of transcription, as is the case for other inducible yeast enzymes, such as orotidine-5′-phosphate decarboxylase (4) and isopropylmalate synthetase (19).

PAL mRNA was shown to be ca. 1.5% of the total mRNA in fully induced cells. However, this calculation was made on the assumption that all of the *Sal*I-*Sst*I DNA restriction fragment codes for part of the PAL gene. This would not be the case if an intron of the PAL gene was present in the *Xba*I-*Sst*I sequence. In *S. cerevisiae* only ribosomal, tRNA, and actin genes contain introns. However, introns are not necessarily so rare in the basidiomycete yeast *R. toruloides*. As 2.7 kb of the yeast genome encodes the 2.5-kb PAL mRNA, any introns that are present would be small in comparison with those found in higher eucaryotes.

TABLE 2. PAL antigen and PAL mRNA levels in *R. toruloides*[a]

Growth conditions	PAL antigen (μg of PAL/mg of protein)	PAL mRNA[b] (%)	Half-life of PAL mRNA (min)
Phenylalanine	20	100	8.3
Phenylalanine-ammonia	21	95	7.4
Glucose-phenylalanine	3	15	8.7
Glucose-ammonia	0	0	7.2

[a] Isolation of RNA and protein and determination of PAL antigen, PAL mRNA, and the half-life of PAL mRNA were as described in the text.

[b] PAL mRNA levels in the various growth conditions were expressed as a percentage of PAL mRNA in phenylalanine minimal medium.

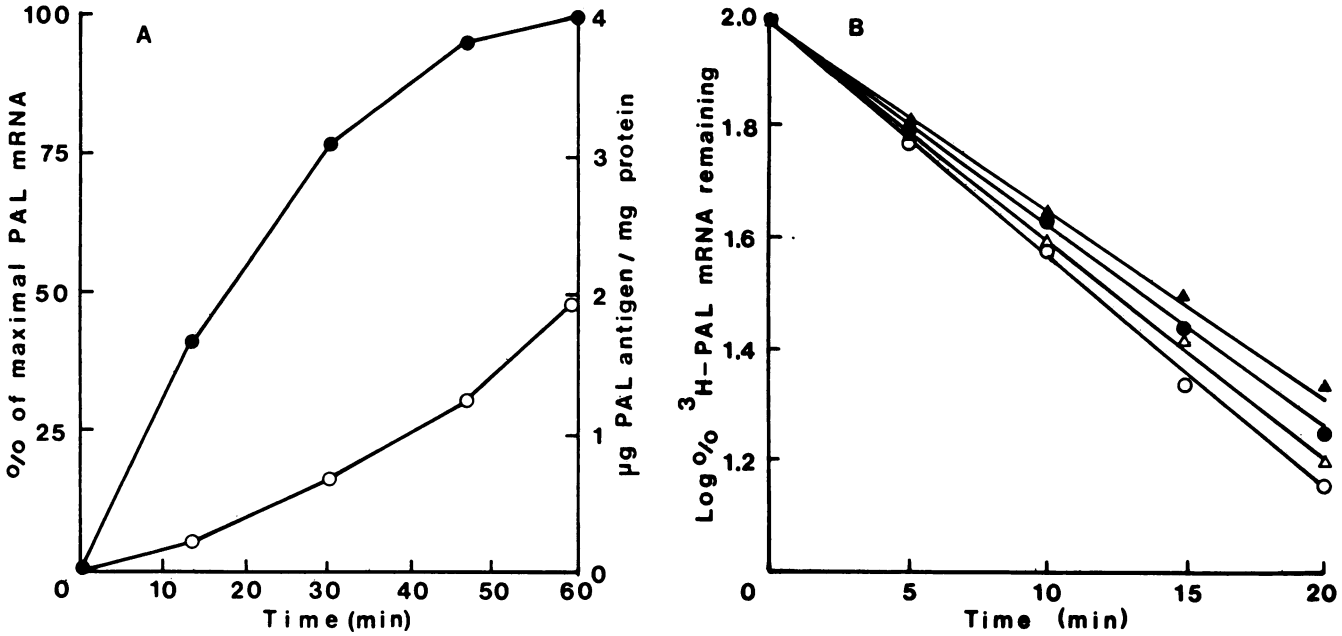

FIG. 6. Kinetics of PAL mRNA synthesis and degradation. (A) The kinetics of PAL antigen and PAL mRNA synthesis were determined as follows. *R. toruloides* cells grown on glucose-ammonia medium were harvested, washed in 0.15 M NaCl, and suspended in phenylalanine minimal medium. At regular intervals cells were removed and PAL antigen (○) and PAL mRNA (●) levels were determined as described in the text. Approximately 2,000 cpm of the probe hybridized to 1 μg of RNA at 100% induction. (B) The rate of PAL mRNA degradation was determined as follows. *R. toruloides* cells were grown in phenylalanine minimal medium or glucose-phenylalanine medium, labeled with [8-^{3}H]adenine, harvested, and washed as described in the text. The cells grown on the former medium were transferred to phenylalanine minimal medium (●), phenylalanine-ammonia (△), or glucose-ammonia medium ( ); *R. toruloides* cells grown on the latter medium were transferred to glucose-phenylalanine medium (▲). These media were supplemented with 5 mM nonradioactive adenine. At regular intervals after the net uptake of [^{3}H]adenine into RNA had stopped (ca. 10 min in nonradioactive medium), cells were removed, the RNA was extracted, and ^{3}H-PAL mRNA was determined by hybridization to PAL cDNA as described in the text. Time zero is when incorporation of [^{3}H]adenine into RNA ceased. At this time the specific activity of ^{3}H-PAL mRNA was 3,000 cpm/ng of PAL mRNA.

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