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Genetic Organization and mRNA Expression of Enolase Genes of *Candida albicans*

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In previous work, we cloned a *Candida albicans* cDNA for the glycolytic enzyme enolase and found a single, abundant enolase transcript on Northern (RNA) blots and a single protein on immunoblots, using antiserum raised against a recombinant enolase fusion protein. Because *C. albicans* enolase is abundantly produced during infection and elicits strong host immune responses, the mechanisms regulating enolase production are important for understanding the growth of *C. albicans* in vivo. To obtain more information on enolase gene expression by *C. albicans*, we used the enolase cDNA clone to investigate the genetic organization of enolase genes and the steady-state levels of enolase mRNA under several growth conditions. Gene disruption techniques in combination with Southern blot analyses of genomic DNA showed the presence of two enolase gene loci that could be distinguished by the locations of *Cla*I and *Mnl*I sites in their 3′ flanking regions. Enolase steady-state mRNA levels were greatest during the middle phase of the logarithmic growth curve and were low during stationary phase. Minimal differences in enolase mRNA levels between yeast cells and hyphae were found. Propagation of *C. albicans* in glucose did not cause increased enolase mRNA levels compared with growth in a nonfermentable carbon source (pyruvate). It was concluded that two gene loci exist for *C. albicans* enolase and that enolase mRNA is constitutively produced at high levels during active metabolism.

Enolase, 2-phospho-D-glycerate hydrolyase (EC 4.2.1.11), is a cytoplasmic enzyme required for glycolysis. Enolase genes from a wide variety of eucaryotic cells exist as a gene family whose members encode isozymes that are developmentally regulated (28, 33, 48). Three isozymes that display tissue-specific expression are found in mammalian cells (28, 43, 44). In plants, enolase mRNA levels vary depending on the tissue source and on the stage of the fruit ripening process (33, 61). Enolase has been studied in two yeast species, *Saccharomyces cerevisiae* and *Candida albicans*, and in both it has been found that enolase and enolase mRNA are among the most abundant proteins and mRNAs in vegetative cells (18, 55, 56). In *S. cerevisiae*, enolase is encoded by two genes, *ENO1* and *ENO2*, that are differentially regulated depending on carbon source and growth phase (32) but are coordinately regulated with other glycolytic enzymes (29). Regulated expression of enolase genes in mammalian cells and in *S. cerevisiae* is mediated primarily through *cis*-acting promoters and *trans*-acting DNA-binding proteins that affect transcription (2, 5, 60). Although examples of plants and bacteria with single enolase genes exist (24, 66), the presence of multiple genes that are developmentally regulated is a characteristic feature of enolase gene expression throughout evolution in the majority of organisms that have been studied.

C. albicans is a diploid pathogen whose life cycle is characterized by facile interconversion between budding yeast and filamentous hyphal forms. Morphogenesis between budding and filamentous growth is correlated with pathogenesis in vivo and is regulated in vitro by changes in environmental factors such as temperature, pH, and carbon and nitrogen sources (7, 19, 41). The ecological niche of *C. albicans* is the gastrointestinal tract of warm-blooded animals, where the organism usually exists in harmony with other microbial flora and the host. However, severe infections occur in immunosuppressed hosts or those with other physiological, mechanical, or iatrogenic risk factors such as antibiotic treatment, presence of indwelling catheters, surgical trauma, or diabetes (39). Management of candidiasis is troublesome because of the small number of available nontoxic antifungal drugs and the difficulties inherent in recognizing internal infections (65).

C. albicans enolase is abundantly expressed both in vivo and in vitro (56, 64). The in vivo abundance is shown by its presence in the blood of patients with disseminated candidiasis and as such has been shown to be useful as a marker of internal infection (64). In addition, *C. albicans* enolase is an immunodominant antigen in that elevated antibody responses, compared with responses to other proteins in crude candidal extracts, are produced in infected patients who are not immunosuppressed (54). The presence of antibodies to enolase is associated with abnormal overgrowth and proliferation of *C. albicans* and thus can be useful in differentiating those who are infected from those who are merely colonized (16, 62). Recently, patients allergic to *C. albicans* have also been shown to have antibodies to *C. albicans* enolase (20).

The abundance of *C. albicans* enolase during infections and the presence of homologous host immune responses suggest that enolase is necessary for in vivo growth and contributes to host-parasite interactions. Despite its potential importance in infections, little is known about mechanisms regulating production of *C. albicans* enolase. To obtain more information on enolase gene expression by *C. albicans*, we used a recently isolated cDNA clone to investigate the genetic organization of enolase genes and the steady-state levels of enolase mRNA under several growth conditions. Gene disruption techniques in combination with Southern blot analyses of genomic DNA showed the presence of two enolase gene loci. Enolase steady-state mRNA levels were greatest during the middle phase of the logarithmic growth curve and were low during stationary phase. Minimal differences in enolase mRNA levels between

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TABLE 1. *C. albicans* strains used

Strain	Genotype	Parent strain	Reference
SC5314	Wild type		23
CAI-4	Δ*ura3*::*imm*434/Δ*ura3*::*imm*434	SC5314	12
UnoPP-1, 2, and 3	Δ*ura3*::*imm*434/Δ*ura3*::*imm*434 Δ*eno1*::*URA3*/*ENO1*	CAI-4	This work

TABLE 2. Plasmids and DNA probes

Plasmid	Probe	Fragment	Reference
p24eno	cENO	1.4-kb *Xba*I-*Xho*I	55
	cENO-mid	0.48-kb *Eco*RI (see Fig. 5)	55
	cENO-3′	0.62-kb *Eco*RI (see Fig. 5)	55
pMK22	*URA3*	1.3-kb *Rsa*I-*Xba*I	22
pCAct1	*ACT1*	2.0-kb *Sal*I	25
pLE219	*LEU2*	2.7-kb *Eco*RI	21
p24enura			This work

yeasts and hyphae were found. Growth of *C. albicans* in glucose did not cause increased enolase mRNA levels compared with growth in a nonfermentable carbon source (pyruvate). It was concluded that two gene loci exist for *C. albicans* enolase and that enolase mRNA is constitutively produced at high levels during active metabolism.

MATERIALS AND METHODS

Reagents. DNA polymerase and T4 DNA ligase were purchased from Gibco BRL Life Technologies, Gaithersburg, Md. Restriction enzymes, BAL 31 exonuclease, and T4 polynucleotide kinase were from Promega Corp., Madison, Wis. [α-^{32}P]dGTP, [γ-^{32}P]dATP, and [α-^{32}P]dATP were purchased from DuPont NEN, Wilmington, Del. Avian myeloblastosis virus reverse transcriptase, Sequenase, and sequencing reagents were purchased from U.S. Biochemical, Cleveland, Ohio. RNase A and Sephadex G-50 Quickspin columns were from Boehringer Mannheim Corp., Indianapolis, Ind. Random hexamers for random primer labeling were from Pharmacia Biotec Inc., Piscataway, N.J. Nitrocellulose was purchased from Schleicher & Schuell, Inc., Keene, N.H. Qiagen columns were from Qiagen, Inc., Chatsworth, Calif. Geneclean was from Research Products International Corp., Mount Prospect, Ill. DNA and RNA standards were from BRL. Amino acids, salts, β-glucuronidase, and other chemicals were from Sigma Chemical Company, St. Louis, Mo. Zymolyase was from ICN Biomedicals Inc., Costa Mesa, Calif. Yeast nitrogen base, yeast extract, and peptone were from Difco Laboratories, Detroit, Mich.

Strains. Fungal strains used in this study are described in Table 1 and were maintained at −70°C in media with 15% glycerol. Fungal strains from freezer stocks were cultured on YPD (42) medium (1% yeast extract, 2% peptone, 2% glucose, plus 1.5% agar for solid medium) at 25°C for 2 days and then stored at 4°C. New cultures were prepared from freezer stocks every 2 to 3 weeks. *Escherichia coli* HB101 (4) was used as a transformation host to amplify p24enura. Strain PS-1, a derivative of NM522 blue containing plasmid p24eno, was prepared as described previously (55). Bacterial strains were cultured on LB medium (46) (1% tryptone, 0.5% yeast extract, 1% sodium chloride, plus 1.5% agar for solid medium) at 37°C overnight. Strains containing plasmids were cultured on LB with 100 μg of ampicillin per ml.

Growth of organisms. Conditions for growing organisms in yeast or hyphal forms in modified Lee's medium (7), using cells in balanced growth as an inoculum, have been described previously (55, 58). To collect cells in various phases of logarithmic growth, *C. albicans* was grown overnight to stationary phase in modified Lee's medium (pH 4.5) at 30°C and then diluted into fresh medium at a cell density of 20 Klett units, using a 520- to 580-nm-pore-size filter. Organisms were harvested in early, middle, or late logarithmic phase or stationary phase (60, 150, 220, or >250 Klett units, respectively). To prepare glucose- or pyruvate-grown cells, *C. albicans* was grown overnight to stationary phase in yeast nitrogen base supplemented with either glucose or pyruvate (100 mM) as the carbon source at 30°C. Cultures were diluted to a density of 20 to 25 Klett units in fresh medium and incubated at 30°C with shaking (250 rpm) to mid-log phase (145 and 85 Klett units for cells grown in media with glucose and pyruvate, respectively). The generation times of cells grown in glucose and pyruvate were 1.75 and 3.54 h, respectively.

Plasmids, probes, and constructs. The plasmids used to prepare probes are described in Table 2. Isolation of plasmid p24eno containing the *C. albicans* enolase cDNA (cENO) has been previously described (55). Plasmid p24enura was constructed from p24eno by replacement of 445 bp within the enolase gene with the *C. albicans URA3* gene (21) (Fig. 1). The combined effect of the 445-bp deletion and the 1.3-kb insertion resulted in a net increase of 0.85 kb of the disrupted enolase cDNA relative to the wild-type cDNA.

Disruption of *C. albicans* enolase. Plasmid p24enura was digested to completion with *Xba*I and *Xho*I to release the 2.3-kb *URA3*-disrupted cENO from p24enura (Fig. 1). To ensure that DNA ends were within *C. albicans* DNA rather than polylinker sequences, the 2.3-kb *Xba*I-*Xho*I fragment was briefly digested with BAL 31 nuclease according to the directions of the manufacturer. Approximately 70 bp, consisting of approximately 30 bp of polylinker region and 40 bp of cENO, were removed from each end of the 2.3-kb construct. Transformation of the *C. albicans ura3* mutant CAI-4 (12) by using 10 μg of digested plasmid DNA was performed as described by Kurtz et al. (23), taking advantage of the fact that in *C. albicans*, linear DNA containing sequences homologous to those in the genome recombine at the corresponding locus in *C. albicans* genomic DNA (22). Three *ura*$^+$ *C. albicans* (UnoPP1 to -3) transformants were isolated on selective medium lacking uridine. The transformants were tested for the ability to produce germ tubes in TCM199 as previously described (59).

Southern blot analysis. Procedures for Southern blot analyses were similar to those previously described (58), with minor modifications. Genomic DNA was prepared by the method of Scherer and Stevens (47), and electrophoresis was performed by using 0.8% agarose gels (20 by 20 cm) according to standard procedures (46). Nitrocellulose blots were probed with 2×10^6 cpm of gel-purified DNA probes (Table 2) that had been labeled with [α-^{32}P]dGTP by the random primer method (10, 11) in a solution containing 50% deionized formamide, 5× Denhardt's solution (1× Denhardt's solution is 0.1% Ficoll, 0.1% bovine serum albumin, and 0.1% polyvinylpyrrolidone), 5× SSPE (20× SSPE is 3.6 M NaCl plus 200 mM NaH_2PO_4 plus 20 mM EDTA [pH 7.4]), and 100 μg of denatured salmon sperm DNA per ml. Filters were hybridized overnight at 37°C with gentle agitation. Filters were washed twice at room temperature for 15 min in a solution of 0.1% sodium dodecyl sulfate and 0.5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.0]). In experiments in which the hybridization signals of enolase (cENO) and actin (*ACT1*) nucleic acid probes were compared, probes were radiolabeled to similar specific activities (1.11×10^6 and 8.86×10^5 cpm/ng for cENO and *ACT1*, respectively), using ethidium bromide fluorescence to estimate the DNA concentration (46). Similar procedures have been used previously to compare levels of hybridization of different probes (27, 58).

Autoradiographic intensities of hybridization bands were measured following exposure times in the linear range of the film, using a scanning densitometer (model GS300; Hoefer Scientific Instruments, San Francisco, Calif.). Raw values were adjusted to account for differences in size and nucleotide composition (42.8 and 34.7% GC for cENO and *ACT1*, respectively) of the two probes. The ratio of the cENO to *ACT1* hybridization band intensities was determined.

Slot blot analysis. Total RNA from yeast or hyphal growth forms was prepared by the method of Schmitt et al. (49). Slot blot analysis was performed as previously described (55), with minor modifications. Briefly, total RNA samples were dissolved in a 50% formamide–7% formaldehyde–150 mM sodium chloride–15 mM sodium citrate solution and incubated at 68°C for 15 min. Samples were cooled on ice and applied to the nitrocellulose by vacuum filtration, using a slot

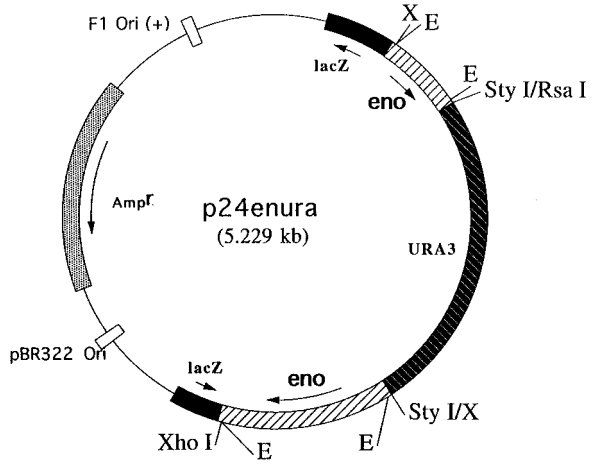


FIG. 1. Diagram of p24enura. The 445-bp *Sty*I fragment in the center of the enolase coding region was replaced with a 1.3-kb *Rsa*I-*Xba*I fragment containing the *C. albicans URA3* gene (22). E and X represent restriction enzymes *Eco*RI and *Xba*I, respectively.

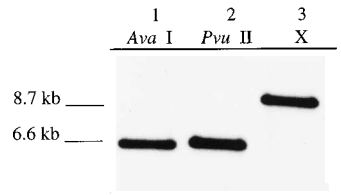
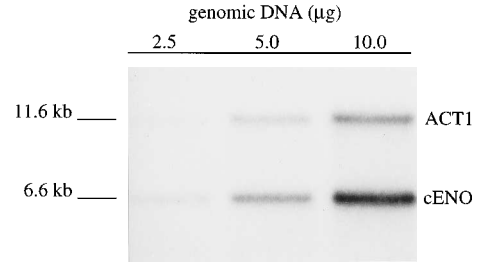


FIG. 2. Southern blot analysis of *C. albicans* SC5314 genomic DNA digested with *Ava*I, *Pvu*II, and *Xba*I (X) and probed with cENO.

blot apparatus (model PR648; Hoefer Scientific Instruments). Slot blot analyses were performed on three different RNA samples for each condition. Hybridization of slot blots was performed as described for Southern blot analysis.

Primer extension. A DNA oligonucleotide probe (ppenol) complementary to nucleotides 25 through 45 downstream of the AUG initiation codon was prepared with an automated Milligen Cyclone Plus Synthesizer using phosphoramidite precursors. Primer extension was performed according to standard techniques (46). The length of the primer extension product was determined by electrophoresis adjacent to a sequencing ladder that was generated by using the synthesized oligonucleotide, ppenol, as a primer and cENO as the template. Samples were separated on a sequencing gel containing 6% polyacrylamide and 7.5 M urea.



Hybridization intensities of cENO and *ACT1* probes to C. albicans genomic DNA

	DNA (μg)		
	2.5	5.0	10.0
ACT1	271	1,280	3,730
cENO	507	2,505	7,241
cENO:*ACT1*	1.87	1.96	1.94

FIG. 3. Southern blot analysis of increasing amounts of SC5314 genomic DNA digested with *Ava*I and probed simultaneously with cENO and *ACT1*. The intensities of hybridization bands were quantitated by densitometry.

RESULTS

Southern blot analysis. A *C. albicans* cDNA clone encoding enolase (55) was used to investigate the genetic organization of enolase DNA. Southern transfers of *C. albicans* SC5314 genomic DNA digested with the restriction endonucleases *Ava*I, *Pvu*II, and *Xba*I, which do not cut within the enolase coding region, showed that a single DNA fragment hybridized to the labeled 1.4-kb cENO probe (Fig. 2). The presence of single hybridization fragments for each enzyme suggested the presence of a single gene locus. However, this result would also be consistent with the presence of multiple closely related loci. Additional experiments were designed to investigate the number of enolase gene loci in *C. albicans*.

First, experiments were performed to compare the hybridization signals of cENO and probes for known single-locus genes. Since the *C. albicans* actin gene has been shown by Lasker and Riggsby (25) to be present in single copy, a nitrocellulose membrane containing three different concentrations of *Ava*I-digested genomic DNA was simultaneously probed with *ACT1* and cENO as described in Materials and Methods. *ACT1* and cENO hybridized to *Ava*I fragments of 11.6 and 6.6 kb, respectively (Fig. 3). For each concentration, the hybridization signal intensity for the cENO probe was approximately twofold greater than that generated by *ACT1*. Equivalent results were obtained in four separate experiments performed as described above. Similar results were obtained when a probe for *LEU2*, which is also a single-locus gene (21), was used instead of actin (not shown). It is unlikely that the differing hybridization intensities seen with the *ACT1* and cENO probes were a result of differences in transfer efficiency because hybridization intensities of larger fragments were not less than those of smaller ones when cENO was used as the probe (Fig. 4A; compare lane 4 with lanes 2 and 6). These results led to skepticism regarding the presence of a single gene locus encoding enolase in *C. albicans* and suggested that multiple loci might be present.

Since closely related genes can sometimes be distinguished by sequence differences in their 5′ and 3′ nontranslated regions, additional Southern blots were performed with enzymes with unique sites in cENO that would release genomic DNA fragments with one end in the enolase coding region and the other in either the 5′ or 3′ flanking region. An internal *Eco*RI fragment, representing the central 478 bp of cENO, that did not contain restriction sites for the selected enzymes was used as a probe. The locations of the restriction sites and the probe (cENO-mid) are shown in Fig. 5. If two similar enolase loci with differences in flanking regions were present, DNA digested with one or more of the restriction enzymes would result in two rather than one hybridization band in Southern blot analyses.

Although digestion of genomic DNA with several restriction endonucleases resulted in a single fragment that hybridized to the internal probe, two fragments were released following digestion with *Cla*I (Fig. 4A, lane 5). The *Cla*I data suggested that there were two enolase gene loci differing in their 3′ untranslated regions, one having a proximal and the other having a distal *Cla*I site relative to the coding region. Identical results were found with three other wild-type strains of *C. albicans* (441B, B311, and ATCC 38696) (not shown). Both of the *Cla*I fragments most likely represent enolase DNA and not cross-hybridization of the intragenic probe with other glycolytic enzyme genes because of the stringency of the hybridization conditions used and because of the lack of nucleotide sequence conservation between glycolytic enzymes, despite striking secondary structure conservation (13). Digestion with *Mnl*I, which also digests cENO at a unique site upstream of the probe, similarly produced two hybridization fragments (Fig. 4B). The relative positions of the *Cla*I and *Mnl*I sites on the two putative gene loci are not known. The fact that digestion with *Sal*I produced a single fragment of 6.9 kb suggested that the differences in 3′ flanking regions were limited. No variability was found in the 5′ flanking regions of the two putative loci, as shown by the presence of a single hybridization band when restriction enzymes *Ava*II, *Bgl*II, and *Bsp* 1286 were used. Other experiments showed that the *Eco*RI sites in the flanking regions did not show variability (not shown). A diagram of the restriction sites in the 5′ and 3′ flanking regions of the enolase loci derived from Southern blot analysis of *C. albicans* genomic DNA, including the variable positions of *Cla*I and *Mnl*I sites, is shown in Fig. 5.

Disruption of one of the *C. albicans* enolase genes by *URA3*. Although the previous results strongly implicated the presence of two gene loci for enolase in *C. albicans*, the possibility remained that the two fragments that hybridized to cENO

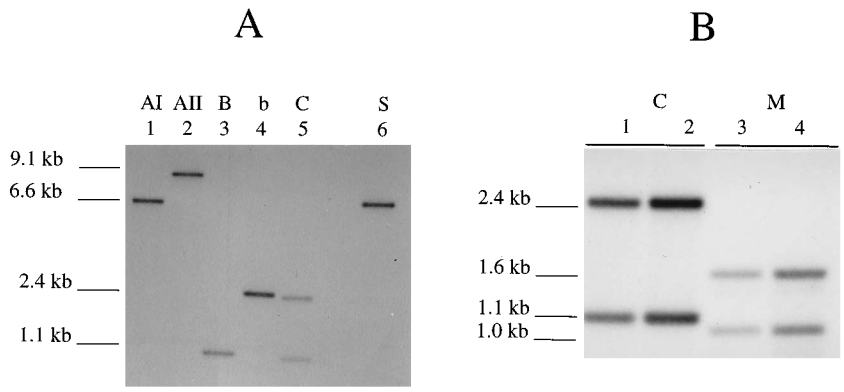


FIG. 4. Southern blot analysis of *C. albicans* SC5314 genomic DNA digested with restriction endonucleases that have unique sites in the 5′ or 3′ coding region of cENO. (A) Genomic DNA was digested with *Ava*I (AI), *Ava*II (AII), *Bgl*II (B), *Bsp* 1286 (b), *Cla*I (C), and *Sal*I (S) (lanes 1 to 6) and probed with the internal 478 bp of cENO (see Fig. 5). (B) Genomic DNA (5 μg [lanes 1 and 3] and 10 μg [lanes 2 and 4]) was digested with *Cla*I (C) and *Mnl*I (M) and probed with cENO-3′ (see Fig. 5).

following digestion with *Cla*I and *Mnl*I could also have resulted from chromosomal restriction fragment length polymorphisms since *C. albicans* is diploid. Models of *C. albicans* genomic DNA representing one or two gene loci for enolase are diagrammed in Fig. 6. A useful method for distinguishing between the two models is gene disruption. Replacement of one of the genes with recombined enolase DNA containing a selectable marker would result in transformants whose genomic DNA would or would not contain both *Cla*I fragments identical in size to those of the parental strain, depending on whether one or two enolase loci were present. The expected Southern blot results for disruption of one of the genes having a proximal *Cla*I site are shown beneath each model. Regardless of the gene copy number, a new hybridization band that would reflect the size increase of the disrupted enolase DNA relative to wild-type DNA was predicted. The new hybridization band should be detectable with individual probes for the selectable marker as well as for enolase DNA. Importantly, in the dual-locus model, both parental *Cla*I cENO hybridization bands would still be visible in the disrupted strains because simultaneous disruption of enolase DNA on both homologs is unlikely. Alternatively, if a single locus with a restriction fragment length polymorphism between homologs were present, only one of the parental *Cla*I bands would remain in DNA from disrupted strains.

To disrupt one of the enolase genes of *C. albicans*, we constructed p24enura, containing cENO with a central portion of the coding region replaced by the selectable *URA3* marker (Fig. 1). Prior to transformation, the construct was digested to completion with restriction enzymes to release the recombined enolase DNA from the plasmid (see Materials and Methods). To isolate *C. albicans* strains with wild-type enolase DNA replaced by the recombined DNA, the digested DNA was transformed into a *C. albicans* SC5314 isogenic *ura* mutant (CAI-4) (12). Three independent transformants, UnoPP-1, -2, and -3, were isolated on selective medium without uridine. Southern blot analysis of *Ava*I-digested genomic DNA from the wild-type, parental, and transformed *C. albicans* strains, using the *URA3* gene and a fragment containing the 3′ portion of the enolase gene (cENO-3′ shown in Fig. 5) as probes, confirmed that the construct had replaced one of the enolase genes. In addition to the parental cENO hybridization band, an extra fragment was present in the three transformants (Fig. 7). Southern blot analysis using genomic DNA digested with *Cla*I showed that a gene with a proximal *Cla*I site (1.1 kb) had been disrupted in each transformant. The size of the new fragment was 2.1 kb, consistent with the increased size of the construct relative to enolase cDNA (see Materials and Methods). To verify that the transformants were not derived from incompletely digested plasmid DNA, *Cla*I-digested genomic DNA from the transformants, wild-type, and parental strains were probed with the plasmid vector pIBI24. No hybridization was

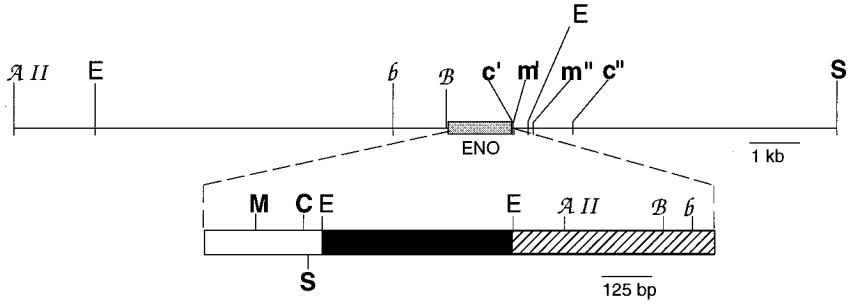


FIG. 5. Genetic organization of enolase genes derived from Southern blot analysis of genomic DNA. The coding region of the genomic DNA is indicated by the shaded box and the expanded region in the genetic organization diagram. Restriction sites in genomic DNA were determined by using the indicated enzymes and the cENO-mid probe (solid area in the expanded region). Italicized and boldfaced text denote enzymes used to map DNA in the 5′ and 3′ enolase coding regions, respectively. Sites for restriction enzymes are abbreviated as follows: A II (*Ava*II), E (*Eco*RI), B (*Bgl*II), b (*Bsp* 1286), S (*Sal*I), C (*Cla*I), and M (*Mnl*I). The proximal and distal sites for *Cla*I are written as c′ and c″. The proximal and distal sites for *Mnl*I are written as m′ and m″. The hatched area shows the location of the cENO-3′ probe that is produced by cutting at *Eco*RI sites in the coding region of cENO (the 3′ site) and in the cloning vector (55).

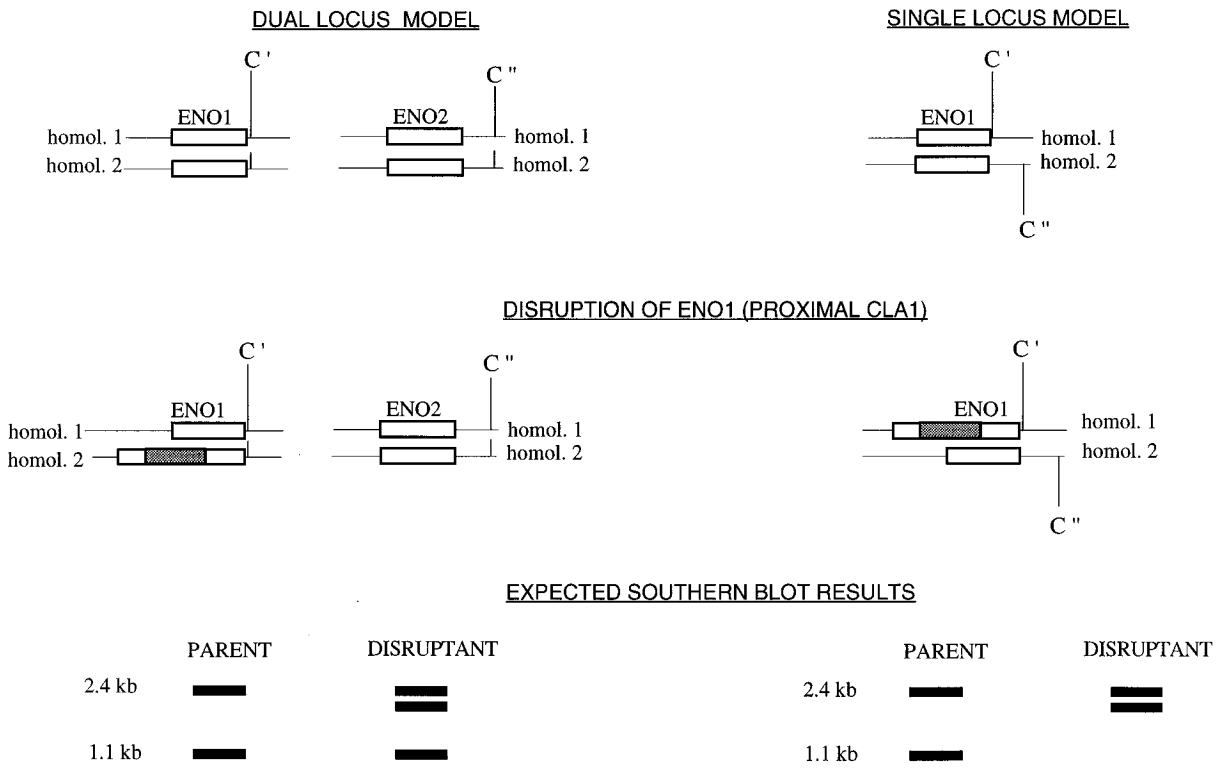


FIG. 6. Predicted arrangement of enolase genes in the genome of *C. albicans* DNA based on the presence of one or two enolase gene loci. For simplicity, the expected results are drawn only for disruption of an enolase gene with a proximal *Cla*I site. The *ENO1* proximal and distal *Cla*I sites are written c′ and c″, respectively. The expected Southern blot results are shown below each model.

found (not shown). These results strongly supported the presence of two enolase gene loci in *C. albicans* as diagrammed in Fig. 6. As expected, the new *Cla*I and *Ava*I fragments specific to the transformants hybridized to *URA3* as well as to cENO-3′. To provide evidence that changes in the genetic organization of genes unrelated to enolase had not occurred, Southern transfers were stripped and probed with *C. albicans ACT1* and *LEU2* (not shown). No differences between transformants, parental, and wild-type strains were seen in the sizes of *LEU2* and *ACT1* hybridization bands. The data support the presence of two enolase loci, each locus having two genes, since *C. albicans* is diploid. Microscopic analysis of log-phase cells of *C. albicans* SC5314 and UnoPP-1, 2, and 3 revealed no differences among the three strains. All were able to undergo the yeast-to-hypha conversion normally, and growth rates were indistinguishable (not shown).

Mapping the translational start site(s) of enolase mRNA. Primer extension analysis was used to map the translational start site(s) of the enolase mRNA. The results were similar to those found by others (31). We detected two primer extension products whose start sites were found to be 36 and 44 nucleotides upstream from the AUG initiation codon.

Steady-state enolase mRNA levels in *C. albicans* under various growth conditions. Slot blot analysis using cENO as the probe was used to determine relative enolase mRNA levels under various conditions. However, Northern (RNA) blot analysis was performed prior to slot blot analyses to ensure that RNA samples were not degraded. In all experiments, a single, focused mRNA species of 1.5 kb, as described previously (55), was found (not shown).

(i) Effect of morphology. RNA from hyphae (grown in Lee's medium pH 6.5 at 37°C) was compared with RNA from yeasts grown in Lee's media pH 4.5 and 6.5 at 25°C and pH 4.5 at 37°C. A representative slot blot is shown in Fig. 8A. The relative amounts of enolase under the four conditions as determined by densitometry were yeast (25°C, pH 6.5) > hyphae (37°C, pH 6.5) > yeast (25°C, pH 4.5) > yeast (37°C, pH 4.5). The differences in enolase mRNA levels between yeast forms grown at 25°C and hyphal forms were minimal. The ratios of enolase mRNA levels from hyphal forms (pH 6.5, 37°C) to yeast forms grown at room temperature were close to 1 (1.3 and 0.7 for cells grown at pH 4.5 and 6.5, respectively). Thus, subtle variations in enolase mRNA levels were seen but were not correlated with cellular morphology.

(ii) Effect of growth phase. Slot blot analysis of total RNA isolated from *C. albicans* SC5314 during early, middle, and late logarithmic phases and stationary phase and probed with labeled cENO indicated that levels of enolase increase during logarithmic growth but are lower in stationary phase (Fig. 8B). Densitometric scans of the corresponding autoradiograms indicated that levels of enolase mRNA doubled in mid-log phase relative to early log phase. Levels in late log phase decreased by 25% relative to mid-log phase. Stationary phase cells contained very little enolase mRNA.

(iii) Effect of carbon source. *C. albicans* SC5314 was grown to mid-log phase in medium containing either 100 mM glucose or pyruvate as the carbon source as described in Materials and Methods. Relative to total RNA, enolase mRNA levels were similar in the two media (Fig. 8C) despite the twofold-lower growth rate of cells grown in pyruvate compared with glucose-grown cells (see Materials and Methods).

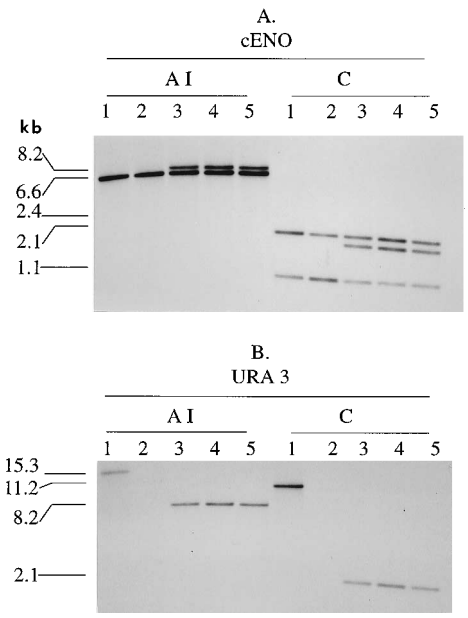


FIG. 7. Southern blot analysis of the *ura3*$^+$ transformants of *C. albicans* CAI-4. Genomic DNA was digested with *Ava*I (AI) or *Cla*I (C) and probed with 3′-terminal *Eco*RI fragment (hatched region in Fig. 5) of cENO (A) or *URA3* (B) from *C. albicans* SC5314 (lane 1), CAI-4 (lane 2), and UnoPP-1, 2, and 3 (lanes 3 to 5).

DISCUSSION

Results from our previous studies and those of other investigators suggested that a single gene locus encodes enolase in *C. albicans* (31, 54, 55, 56). A single protein was detected in immunoblotting and immunoprecipitation experiments (55, 56), arguing against the presence of multiple isozymes of *C. albicans* enolase as is found in other organisms, including *S. cerevisiae* (15, 32, 50). In addition, a single mRNA species was detected in Northern blotting experiments using an enolase cDNA probe (31, 55, 56). Similarly, results of our initial Southern blotting experiments agreed with those of others (31) and also suggested the presence of a single locus. However, the frequent presence of multiple genes for enolase in other organisms prompted us to investigate the enolase gene copy number in *C. albicans* more thoroughly. Further investigation using three experimental approaches pointed to the existence of two enolase gene loci in the genome of *C. albicans*. First, comparison of hybridization intensities with single-locus genes suggested multiple loci. Second, restriction enzyme analysis of genomic DNA suggested the presence of sequence variation in the 3′ flanking regions of two putative loci. The strongest evidence was provided by a gene disruption experiment, in which DNAs representative of both parental enolase loci were present in three independent transformants. This result could not have occurred if only a single locus were present.

Although the experiments described in this report did not provide direct evidence that both loci produce functional genes, the presence of nonfunctional enolase genes is unlikely. Duplicated genes are expected to be maintained only if the extra copies are beneficial to the organism (1). The fact that no differences in growth rate were found between the disruptants and wild-type *C. albicans* does not necessarily mean that the disrupted gene was not functional, since a loss of one of four copies might not have led to a measurable decrease in growth rate. Finally, although the incidence of nonfunctional genes in *C. albicans* is not known, nonfunctional genes in an organism of similar genome size, *S. cerevisiae*, is low (40).

Comparative studies of gene expression between yeast and hyphal forms of *C. albicans* are important for understanding differences between the two forms that could contribute to infections. Genes whose mRNA is present only during elongated hyphal growth (3) or only during yeast growth (53) have recently been described, establishing the existence of differential gene expression during morphogenesis. In contrast to these genes, *C. albicans* enolase mRNA levels were not related to morphology. Similar results have been reported for EF-1α, EF-3, actin, exoglucanase, and calmodulin genes (8, 26, 37, 57, 58). These findings are consistent with early studies comparing protein profiles of yeast and hyphae: it was concluded that most genes are not differentially expressed (6, 7, 30).

Several studies have shown that enolase and other glycolytic enzymes of *S. cerevisiae* are regulated by controlling the

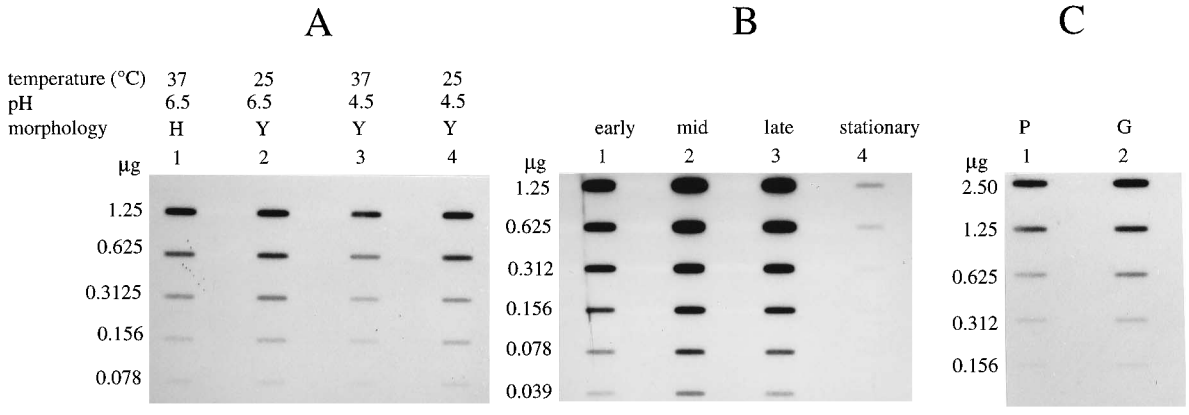


FIG. 8. Expression of enolase mRNA under various growth conditions. (A) Comparison of enolase mRNA levels in yeast and hyphal growth forms of *C. albicans*. Twofold dilutions of total RNA from yeast forms (Y) grown at 25°C (room temperature) at pH 4.5 and 6.5 (lanes 2 and 4, respectively) and at 37°C at pH 4.5 (lane 3) and from hyphal forms (H) grown at 37°C, pH 6.5 (lane 1), were applied to a nitrocellulose membrane and probed with cENO. (B) Relative enolase mRNA levels in *C. albicans* cells in early, middle, and late logarithmic and stationary phases of growth. Two-fold dilutions of total RNA from yeast forms grown at 30°C to early, middle, and late logarithmic and stationary growth were applied to a nitrocellulose membrane and probed with cENO. (C) Slot blot analysis to compare enolase mRNA levels in yeast forms of *C. albicans* grown in fermentable and nonfermentable carbon sources. Twofold dilutions of total RNA from yeasts grown with pyruvate (P) or glucose (G) as the carbon source were applied to a nitrocellulose membrane and probed with cENO.

amount of mRNA. Cells grown in glucose have higher glycolytic enzyme mRNA levels relative those grown in nonfermentable carbon sources such as ethanol, lactate, or a combination of glycerol and lactate (32, 36). The possibility existed that *C. albicans* enolase mRNA levels were also modulated by the carbon source used to propagate the cells. In attempts to define growth conditions suitable for investigating the effect of carbon source on *C. albicans* enolase mRNA levels, we found that growth of *C. albicans* was minimal on ethanol and that no growth was found when glycerol, lactate, or acetate was used (not shown). Poor growth on ethanol has previously been reported for *C. albicans* (9, 67). However, the twofold-higher growth rate of *C. albicans* on pyruvate relative to glucose was similar to the growth rates of *S. cerevisiae* on nonfermentable carbon sources relative to glucose (32, 36). Therefore, pyruvate was considered to be a suitable nonfermentable carbon source for similar studies of *C. albicans*. The finding that no differences in enolase mRNA levels (relative to total RNA) were found between *C. albicans* grown on glucose and pyruvate underscored early observations that glycolytic enzyme activity is less affected by glucose in *C. albicans* cells than in *S. cerevisiae* cells (29). In general, *C. albicans* responses to glucose appear different from those of *S. cerevisiae*. For example, in contrast to *S. cerevisiae*, *C. albicans* respiration is glucose insensitive (38) and the kinetics of the plasma membrane H^+-ATPase are less affected by glucose (35).

The most dramatic changes in enolase mRNA levels were observed during the growth cycle, with the highest levels occurring in mid-log phase followed by minimal levels in stationary phase. These results suggest that *C. albicans* is adapted to production of high levels of enolase mRNA during periods of high metabolic activity, a finding that is consistent with enolase production in other organisms (51, 61). Overall, the results are consistent with unregulated constitutive expression of *C. albicans* enolase during most environmental conditions.

Although reasons for the presence of two enolase genes are unknown, it is possible that gene-specific regulation is present and functions in adaptation to changing environmental conditions. Such regulation would not have been detected in this study since the mRNA levels reflected the combined expression of multiple genes. Alternatively, multiple genes might contribute to the large amount of enolase in *C. albicans*. It has been estimated that enolase constitutes 0.7 and 2% of the total protein of yeast and hyphal forms of *C. albicans*, respectively (56). Other abundant fungal proteins encoded by gene families include glyceraldehyde-3-phosphate dehydrogenase (17), EF-1α (57), ribosomal proteins (14, 34), and histones (52).

The production of abundant enolase in patients with disseminated candidiasis reflects the use of the glycolytic pathway by *C. albicans* in vivo and suggests that enolase and possibly other glycolytic enzymes might be useful antifungal targets. Further support for the hypothesis that interference with fungal glycolysis could modulate candidiasis is given by experiments showing that mice that were fed glucose-supplemented diets contracted more severe mucosal infections than control animals did (63). The enhanced susceptibility of individuals with diabetes (39) and the correlation of high-carbohydrate diets with candidiasis (45) also support the importance of glucose in promoting growth of *C. albicans* in vivo. Further studies on the mechanism of expression of genes that are expressed in response to glucose-rich environments are essential for understanding the proliferation of *C. albicans* in vivo.

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REFERENCES

1. **Alberts, B., D. Bray, J. Lewis, M. Raff, K. Roberts, and J. Watson.** 1989. The organization and evolution of the nuclear genome, p. 599–612. *In* M. Robertson (ed.), Molecular biology of the cell, 2nd ed. Garland, New York.
2. **Andersen, J. K., D. A. Garber, C. A. Meaney, and X. O. Breakfield.** 1992. Gene transfer into mammalian central nervous system using herpes virus vectors: extended expression of bacterial *lacZ* in neurons using the neuron-specific enolase promoter. Hum. Gene Ther. **3:**487–499.
3. **Birse, C. E., M. Y. Irwin, W. A. Fonzi, and P. S. Sypherd.** 1993. Cloning and characterization of *ECE1*, a gene expressed in association with cell elongation of the dimorphic pathogen *Candida albicans*. Infect. Immun. **61:**3648–3655.
4. **Boyer, H. W., and D. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. **41:**459–472.
5. **Brindle, P. K., J. P. Holland, C. E. Willett, M. A. Innis, and M. J. Holland.** 1990. Multiple factors bind the upstream activation sites of the yeast enolase genes *ENO1* and *ENO2*: ABF1 protein, like repressor activator protein RAP1, binds *cis*-acting sequences which modulate repression or activation of transcription. Mol. Cell. Biol. **10:**4872–4885.
6. **Brown, L. A., and W. L. Chaffin.** 1981. Differential expression of cytoplasmic proteins during yeast bud and germ tube formation in *Candida albicans*. Can. J. Microbiol. **27:**580–585.
7. **Brummel, M., and D. R. Soll.** 1982. The temporal regulation of protein synthesis during synchronous bud or mycelial formation in the dimorphic yeast *Candida albicans*. Dev. Biol. **89:**211–224.
8. **Chambers, R. S., and P. A. Sullivan.** 1993. Expression of the exoglucanase gene in yeast and hyphal forms of *Candida albicans*. FEMS Microbiol. Lett. **111:**63–68.
9. **Encarnacion, M. R., J. Jimenez, and T. Benitez.** 1989. Ethanol inhibition of *Saccharomyces* and *Candida* enzymes. Curr. Genet. **15:**7–16.
10. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132:**6–13.
11. **Feinberg, A. P., and B. Vogelstein.** 1984. Addendum: A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **37:**266–267.
12. **Fonzi, W. A., and M. Y. Irwin.** 1993. Isogenic strain construction and gene mapping in *Candida albicans*. Genetics **134:**717–728.
13. **Fothergill-Gilmore, L. A.** 1986. The evolution of the glycolytic pathway. Trends Biochem. Sci. **11:**47–51.
14. **Gatermann, K. B., C. Teletski, T. Gross, and N. F. Kaufer.** 1989. A ribosomal protein gene family from *Schizosaccharomyces pombe* consisting of three active members. Curr. Genet. **16:**361–367.
15. **Giallongo, A., S. Feo, R. Moore, C. M. Croce, and L. C. Showe.** 1986. Molecular cloning and nucleotide sequence of a full-length cDNA for human alpha enolase. Proc. Natl. Acad. Sci. USA **83:**6741–6745.
16. **Gutierrez, J., C. Maroto, G. Piedrola, E. Martin, and J. A. Perez.** 1993. Circulating candida antigens and antibodies: useful markers of candidemia. J. Clin. Microbiol. **31:**2550–2552.
17. **Holland, J. P., L. Labieniec, C. Swimmer, and M. J. Holland.** 1983. Homologous nucleotide sequences at the 5′ termini of messenger RNAs synthesized from the yeast enolase and glyceraldehyde-3-phosphate dehydrogenase gene families. J. Biol. Chem. **258:**5291–5299.
18. **Holland, M. J., and J. P. Holland.** 1978. Isolation and identification of yeast messenger ribonucleic acids coding for enolase, glyceraldehyde-3-phosphate dehydrogenase, and phosphoglycerate kinase. Biochemistry **17:**4900–4907.
19. **Holmes, A. R., and M. G. Shepherd.** 1988. Nutritional factors determine germ tube formation in *Candida albicans*. J. Med. Vet. Mycol. **26:**127–131.
20. **Ishiguro, A., M. Homma, S. Torii, and K. Tanaka.** 1992. Identification of *Candida albicans* antigens reactive with immunoglobulin E antibody of human sera. Infect. Immun. **60:**1550–1557.
21. **Kelly, R. S., M. Miller, and M. B. Kurtz.** 1988. One-step gene disruption by cotransformation to isolate double auxotrophs in *Candida albicans*. Mol. Gen. Genet. **214:**24–31.
22. **Kelly, R. S., M. Miller, M. B. Kurtz, and D. R. Kirsch.** 1987. Directed mutagenesis in *Candida albicans*: one-step gene disruption to isolate *ura3* mutants. Mol. Cell. Biol. **7:**199–207.
23. **Kurtz, M. B., M. W. Cortelyou, and D. R. Kirsch.** 1986. Integrative transformation of *Candida albicans*, using a cloned *Candida ADE2* gene. Mol. Cell. Biol. **6:**142–149.
24. **Lal, S. K., S. Johnson, T. Conway, and P. M. Kelley.** 1991. Characterization of a maize cDNA that complements an enolase-deficient mutant of *Escherichia coli*. Plant Mol. Biol. **16:**787–795.
25. **Lasker, B. A., and W. S. Riggsby.** 1992. Transcription of the single actin gene of *Candida albicans* during the yeast-to-mycelium conversion. Exp. Mycol. **16:**155–162.
26. **Leuker, C. E., A. M. Hahn, and J. F. Ernst.** 1992. β-Galactosidase of *Kluyveromyces lactis* (Lac4p) as reporter of gene expression in *Candida*

albicans and *C. tropicalis*. Mol. Gen. Genet. **235:**235–241.
27. **Linz, J. E., and P. S. Sypherd.** 1987. Expression of three genes for elongation factor 1α during morphogenesis of *Mucor racemosus*. Mol. Cell. Biol. **7:**1925–1932.
28. **Lucas, M., C. Goblet, A. Keller, N. Lamande, F. Gros, R. G. Whalen, and M. Lazar.** 1992. Modulation of embryonic and muscle-specific enolase gene products in the developing mouse hindlimb. Differentiation **51:**1–7.
29. **Maitra, P. K., and Z. Lobo.** 1971. A kinetic study of glycolytic enzyme synthesis in yeast. J. Biol. Chem. **246:**475–488.
30. **Manning, M., and T. G. Mitchell.** 1980. Analysis of cytoplasmic antigens of the yeast and mycelial phases of *Candida albicans* by two-dimensional electrophoresis. Infect. Immun. **30:**484–495.
31. **Mason, A. B., H. R. Buckley, and J. A. Gorman.** 1993. Molecular cloning and characterization of the *Candida albicans* enolase gene. J. Bacteriol. **175:** 2632–2639.
32. **McAlister, L., and M. J. Holland.** 1982. Targeted deletion of a yeast enolase structural gene: identification and isolation of yeast enolase isozymes. J. Biol. Chem. **257:**7181–7188.
33. **Miernyk, J. A., and D. T. Dennis.** 1992. A developmental analysis of the enolase isozymes from *Ricinus communis*. Plant Physiol. **99:**748–750.
34. **Molenaar, C. M., L. P. Woudt, A. E. Jansen, W. H. Mager, R. J. Planta, D. M. Donovan, and N. J. Pearson.** 1984. Structure and organization of two linked ribosomal protein genes in yeast. Nucleic Acids Res. **12:**7345–7358.
35. **Monk, B. C., M. B. Kurtz, J. A. Marrinan, and D. S. Perlin.** 1991. Cloning and characterization of the plasma membrane H^+-ATPase from *Candida albicans*. J. Bacteriol. **173:**6826–6836.
36. **Moore, P. A., F. A. Sagliocco, R. M. C. Wood, and A. P. Brown.** 1991. Yeast glycolytic mRNAs are differentially regulated. Mol. Cell. Biol. **11:**5330–5337.
37. **Myers, K. K., W. A. Fonzi, and P. S. Sypherd.** 1992. Isolation and sequence analysis of the gene for translation elongation factor-3 from *Candida albicans*. Nucleic Acids Res. **20:**1705–1710.
38. **Niimi, M., A. Kamiyama, and M. Tokunaga.** 1988. Respiration of medically important *Candida* species and *Saccharomyces cerevisiae* in relation to glucose effect. J. Med. Vet. Mycol. **26:**195–198.
39. **Odds, F. C.** 1988. Candida and candidosis, p. 93–114. Balliere Tindall, Philadelphia.
40. **Olson, M. V.** 1991. Genome structure and organization in *Saccharomyces cerevisiae*, p. 1–39. *In* J. R. Broach, J. R. Pringle, and E. W. Jones (ed.), The molecular biology of the yeast *Saccharomyces cerevisiae*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
41. **Pollack, J. H., and T. Hashimoto.** 1987. The role of glucose in the pH regulation of germ-tube formation in *Candida albicans*. J. Gen. Microbiol. **133:**415–424.
42. **Rose, M. D., F. Winston, and P. Hieter.** 1990. Methods in yeast genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
43. **Sakimura, K., E. Kushiya, M. Obinata, S. Odani, and Y. Takahashi.** 1985. Molecular cloning and the nucleotide sequence of cDNA for neuron-specific enolase messenger RNA of rat brain. Proc. Natl. Acad. Sci. USA **82:**7453–7457.
44. **Sakimura, K., E. Kushiya, M. Obinata, and Y. Takahashi.** 1985. Molecular cloning and the nucleotide sequence of cDNA to mRNA for non-neuronal enolase (αα enolase) of rat brain and liver. Nucleic Acids Res. **13:**4365–4378.
45. **Samaranayake, L. P.** 1986. Nutritional factors and oral candidosis. J. Oral Pathol. **15:**61–65.
46. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
47. **Scherer, S., and D. A. Stevens.** 1987. Application of DNA typing methods to epidemiology and taxonomy of *Candida* species. J. Clin. Microbiol. **25:**675–679.
48. **Schmechel, D. E., M. W. Brightman, and P. J. Marangows.** 1980. Neurons switch from non-neuronal enolase to neuron-specific enolase during differentiation. Brain Res. **190:**195–214.
49. **Schmitt, M. E., T. A. Brown, and B. L. Trumpower.** 1990. A rapid and simple method for preparation of RNA from *Saccharomyces cerevisiae*. Nucleic Acids Res. **18:**3091.
50. **Segil, N., A. Shrutkowski, M. B. Dworkin, and E. Dworkin-Rastl.** 1988. Enolase isoenzymes in adult and developing *Xenopus laevis* and characterization of a cloned enolase sequence. Biochem. J. **251:**31–39.
51. **Silverman, W. F.** 1992. Temporal and compartmental restriction of neuron-specific enolase expression in the rat mesostriatal system. Brain Res. **69:**31–39.
52. **Smith, M. M., and O. S. Anddresson.** 1983. DNA sequences of yeast H3 and H4 histone genes from two non-allelic gene sets encode identical H3 and H4 proteins. J. Mol. Biol. **169:**663–690.
53. **Srikantha, T., and D. R. Soll.** 1993. A white-specific gene in the white-opaque switching system of *Candida albicans*. Gene **131:**53–60.
54. **Strockbine, N. A., M. T. Largen, S. M. Zweibel, and H. R. Buckley.** 1984. Identification and molecular weight characterization of antigens from *Candida albicans* that are recognized by human sera. Infect. Immun. **43:**715–721.
55. **Sundstrom, P., and G. R. Aliaga.** 1992. Molecular cloning of cDNA and analysis of protein secondary structure of *Candida albicans* enolase, an abundant, immunodominant glycolytic enzyme. J. Bacteriol. **174:**6789–6799.
56. **Sundstrom, P., and G. R. Aliaga.** 1994. A subset of proteins found in culture supernatants of *Candida albicans* includes the abundant, immunodominant, glycolytic enzyme enolase. J. Infect. Dis. **169:**452–456.
57. **Sundstrom, P., M. Irwin, D. Smith, and P. S. Sypherd.** 1991. Both genes for Ef-1α in *Candida albicans* are translated. Mol. Microbiol. **5:**1703–1706.
58. **Sundstrom, P., D. Smith, and P. S. Sypherd.** 1990. Sequence analysis and expression of the two genes for elongation factor 1α from the dimorphic yeast *Candida albicans*. J. Bacteriol. **172:**2036–2045.
59. **Sundstrom, P. M., and G. E. Kenny.** 1984. Characterization of antigens specific to the surface of germ tubes of *Candida albicans* by immunofluorescence. Infect. Immun. **43:**850–855.
60. **Tornow, J., X. Zeng, W. Gao, and G. M. Santangelo.** 1993. GCR1, a transcriptional activator in *Saccharomyces cerevisiae*, complexes with RAP1 and can function without its DNA binding domain. EMBO **12:**2431–2437.
61. **Van Der Straeten, D., R. A. Rodrigues-Pousada, H. M. Goodman, and M. V. Montagu.** 1991. Plant enolase: gene structure, expression, and evolution. Plant Cell **3:**719–735.
62. **Van Deventer, A. J. M., H. J. A. van Vliet, L. Voogd, W. C. J. Hop, and W. H. F. Goessens.** 1993. Increased specificity of antibody detection in surgical patients with invasive candidiasis with cytoplasmic antigens depleted of mannan residues. J. Clin. Microbiol. **31:**994–997.
63. **Vargas, S. L., C. C. Patrick, G. D. Ayers, and W. T. Hughes.** 1993. Modulating effect of dietary carbohydrate supplementation on *Candida albicans* colonization and invasion in a neutropenic mouse model. Infect. Immun. **61:**619–626.
64. **Walsh, T. J., J. W. Hathorn, J. D. Sobel, W. G. Merz, V. Sanchez, S. M. Maret, H. R. Buckley, M. A. Pfaller, R. Schaufele, C. Silva, E. Navarro, J. Lecciones, P. Chandrasekar, J. Lee, and P. A. Pizzo.** 1991. Detection of circulating *Candida* enolase by immunoassay in patients with cancer and invasive candidiasis. N. Engl. J. Med. **324:**1026–1031.
65. **Walsh, T. J., and P. A. Pizzo.** 1993. Laboratory diagnosis of candidiasis, p. 109–135. *In* G. P. Bodey (ed.), Candidiasis: pathogenesis, diagnosis, and treatment, 2nd ed. Raven Press, New York.
66. **Weng, M., C. A. Makaroff, and H. Zalkin.** 1986. Nucleotide sequence of *Escherichia coli* pyr-g encoding ctp synthetase. J. Biol. Chem. **261:**5568–5574.
67. **Zeuthen, M. L., N. Dabrowa, C. M. Aniebo, and D. H. Howard.** 1988. Ethanol tolerance and the induction of stress proteins by ethanol in *Candida albicans*. J. Gen. Microbiol. **134:**1375–1384.