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Molecular Cloning and Characterization of the *Candida albicans* Enolase Gene

A. BRETT MASON,[1]† HELEN R. BUCKLEY,[2] AND JESSICA A. GORMAN[1]*

Department of Microbial Molecular Biology, Bristol-Myers Squibb Pharmaceutical Research Institute, P.O. Box 4000, Princeton, New Jersey 08543-4000,[1] and Microbiology and Immunology Department, Temple University Medical School, Philadelphia, Pennsylvania 19140[2]

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A DNA clone containing the putative *Candida albicans* enolase gene (*ENO1*) was isolated from a genomic DNA library. The sequenced insert contained a continuous open reading frame of 1,320 bp. The predicted 440-amino-acid protein is 78 and 76% identical, respectively, to *Saccharomyces cerevisiae* enolase proteins 1 and 2. Only one enolase gene could be detected in *C. albicans* genomic DNA by Southern analysis with a homologous probe. Northern (RNA) analysis detected a single, abundant *C. albicans ENO1* transcript of approximately 1,600 nucleotides. When cells were grown on glucose, levels of *ENO1* mRNA were markedly increased by comparison with *ENO1* mRNA levels in cells grown on ethanol, a gluconeogenic carbon source. In contrast to this glucose-mediated transcriptional induction, the carbon source had no dramatic effect on the levels of enolase protein or enzyme activity in the *C. albicans* strains tested. These results suggest that posttranscriptional mechanisms are responsible for modulating expression of the *C. albicans* enolase gene.

Enolase (EC 4.2.1.11) catalyzes the reversible dehydration of 2-phosphoglycerate to phosphoenolpyruvate. Genes that encode enolase proteins have been cloned from lower eukaryotic and vertebrate cell sources (11, 19, 30, 33), and in each organism, multiple nonidentical enolase genes have been identified. Enolase is biologically active only as a dimer, and the enzyme subunits may be products of different enolase genes. *Saccharomyces cerevisiae*, for example, possesses two enolase genes, *ENO1* and *ENO2*. Random pairing of enolase monomers generates three isoenzymes of the catalytically active dimer (21).

Both *S. cerevisiae* enolase genes are expressed during vegetative cell growth (21). Transcription of the *ENO2* gene is induced 20-fold when the cells are grown in glucose rather than a gluconeogenic carbon source, while transcription of the *S. cerevisiae ENO1* gene is maintained at a constant level independently of the carbon source (21). The complex regulatory mechanisms which govern expression of the *ENO1* and *ENO2* genes involve a number of *cis*-acting elements and a *trans*-acting, positive regulator identified as the *GCR1* gene product (3). In amphibian, avian, and mammalian tissues, the type of enolase isoenzyme synthesized is dependent upon the developmental stage and/or tissue type (33). Induction of enolase synthesis has also been variously reported as a consequence of the developmental stage (33), growth factor-stimulated cell growth (19), or cellular transformation (29).

Candida albicans enolase was first described as a cytoplasmic antigen (M_r, 48,000) that elicited a strong antibody response in a high percentage of patients with invasive candidiasis (38). Subsequent studies identified this protein as a monomer of the glycolytic enzyme enolase (8, 18). This report describes the cloning and sequencing of *C. albicans* enolase gene *ENO1*. Isolation of the *C. albicans ENO1* gene was used as the starting point for studying enolase expression in this organism.

* Corresponding author.
† Present address: Dental School, University of Otago, Dunedin, New Zealand.

MATERIALS AND METHODS

Strains, plasmids, and culture media. YEPD (1% yeast extract, 2% peptone, 2% glucose) was used as the standard growth medium. The yeast strains used included *C. albicans* B-792 (15), FC18 (42), and B311 (ATCC 32354) and *S. cerevisiae* F762 (MATa *trp1Δ1 ura3-52*). To observe the effect of the carbon source on *C. albicans* enolase expression, cultures were grown at 24.5°C in liquid synthetic medium for *C. albicans* (SMC; 17) containing either glucose (1.25%, wt/vol) or ethanol (3%, vol/vol) as the carbon source. Phagemid vector pBluescript I KS+ (Stratagene) was used for subcloning and DNA sequencing. Plasmid peno8 (11), containing the *S. cerevisiae ENO2* gene, was kindly provided by M. Holland. The *C. albicans* genomic library in low-copy yeast vector pYSK35 (7) was provided by Y. Koltin.

Southern analysis. Southern analyses were performed by standard procedures (31), with yeast genomic DNA prepared as described by Sherman et al. (35). After electrophoresis, DNA was transferred to a nylon membrane (Nytran; Schleicher & Schuell) or the gel was dried for direct hybridization (39). Prehybridizations were performed for 1 h at 65°C in a solution of 1× Denhardt's reagent–1% sodium dodecyl sulfate (SDS)–6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing salmon sperm DNA at a 0.5-mg/ml final concentration.

Double-stranded DNA probes were labelled with [α-^{32}P] dCTP by random oligonucleotide priming (6). Hybridizations were done at 65°C for 8 to 16 h in the same solution used for prehybridization, except that the final concentration of Denhardt's reagent was 10-fold higher. Dried gels or membranes were then washed four times at 65°C with 2× SSC–0.1% SDS for 10 min each time and then three times at 65°C with 0.5× SSC–0.1% SDS for 10 min each time.

Restriction mapping and DNA sequencing. A 3.8-kb *Stu*I-*Sma*I DNA fragment of the *C. albicans* DNA insert in a

positive library clone was subcloned into the *Eco*RV site of pBSI(KS+) to facilitate restriction mapping and DNA sequencing. This construct was designated pBMBS10. Two series of deletions were generated, starting from each end of the insert by digestion with exonuclease III for staggered time intervals, and then single-stranded DNA was eliminated with mung bean nuclease (31). Blunt-end ligation yielded an assortment of pBMBS10 derivatives. Size-selected plasmids were used for double-stranded DNA sequencing (14) with Sequenase version I (USB) by utilizing the T3 and T7 primer sites flanking the pBSI(KS+) multiple cloning site. Gaps were covered by sequencing with synthetic oligonucleotide primers with pBMBS10 as a template. Sequence analyses and data base searches employed the University of Wisconsin Genetics Computer Group programs (5).

Preparation of total RNA and mRNA from *C. albicans*. Total RNA was prepared from mid-log-phase (A_{540}, ~0.5) yeast cells grown in SMC with 1.25% glucose or 3% ethanol. Cells were disrupted by vortexing with glass beads (five 1-min pulses) in the presence of an RNase inhibitor mixture (vanadyl ribonucleoside complex; GIBCO BRL). Cells were kept on ice between pulses. Whole cells, cell debris, glass beads, and chromosomal DNA were spun out by centrifugation at 4°C for 20 min. The supernatant was transferred to a fresh tube and extracted five times with an equal volume of phenol-chloroform (1:1). The RNA was ethanol precipitated, washed with 70% ethanol, dried, dissolved in diethyl pyrocarbonate-treated water, and quantitated on the basis of its A_{260}. Polyadenylated mRNA was purified from total RNA by batch absorption with oligo(dT)-cellulose (Pharmacia LKB).

RNA analysis. RNA samples, including an RNA ladder (GIBCO BRL) for size comparison, were separated on formaldehyde–1% agarose gels (31). After electrophoresis, gels were washed for 45 min with several changes of diethyl pyrocarbonate-treated, deionized water and then dried under vacuum at 60°C for 2.5 h. Direct gel hybridization with oligolabelled DNA probes was performed without prehybridization. The hybridization solution was 0.5 M sodium phosphate (pH 7.2) containing 1 mM EDTA, 7% SDS, and 0.5 mg of salmon sperm DNA per ml. Posthybridization, gels were washed twice, for 10 min each time, with 0.3 M sodium phosphate (pH 7.2)–1 mM EDTA–5% SDS at 65°C and then five times, for 10 min each time, with 0.3 M sodium phosphate (pH 7.2)–1 mM EDTA–1% SDS at 65°C.

Primer extension analysis. An oligonucleotide complementary to nucleotides 5 to −30 of the *C. albicans* sequence shown in Fig. 2 was used to map the 5′ termini of *C. albicans* enolase mRNAs by primer extension. This oligonucleotide (5′-AGACATTGTTGTAATATTCCTGAATTATCAATT GAT-3′) was designated XT1. *C. albicans* mRNA (10 μg) was dissolved in 6 μl of TE (pH 7.5), containing 10^5 cpm of the end-labelled primer (31). The primer was annealed to the template RNA by heating the mixture to 80°C and then slowly cooling it. One microliter of 10× reverse transcriptase buffer (31), 1 μl of deoxynucleoside triphosphates (10 mM), 0.2 μl of actinomycin D (100 μg/ml), 0.2 μl of RNase Block II (GIBCO BRL), 0.6 μl of diethyl pyrocarbonate-treated water, and 10 U of Moloney murine leukemia virus reverse transcriptase (Stratagene) were added. The reaction mixture was incubated at 42°C for 60 min, and then the reaction was stopped by adding 1 μl of 0.5 M EDTA (pH 8.0). RNA in the sample was digested with 1 U of RNase (GIBCO BRL) for 30 min at 37°C. The sample was adjusted to 200 μl with TE (pH 7.5), phenol-chloroform extracted, and ethanol precipitated. The dried sample was suspended in 5 μl of DNA sequencing dye and run on a 7% sequencing gel

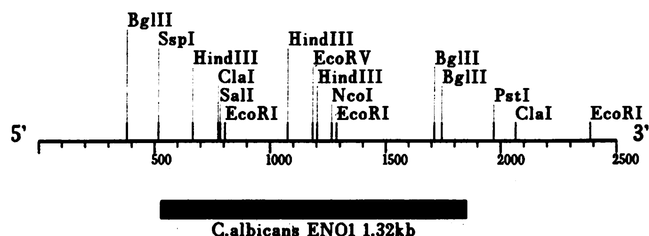

FIG. 1. Restriction map of the *C. albicans ENO1* gene. The 1.32-kb coding region of the *ENO1* gene is indicated by the shaded bar below the map. The open reading frame runs from left to right.

in parallel with a known DNA sequence ladder and a sequencing sample prepared by using the end-labelled primer and pBMBS10 template.

Enolase assay and Western blot (immunoblot) analysis. Cytoplasmic extracts for enzyme and protein analysis were prepared from the same *C. albicans* cultures as used for RNA preparation (see above). Cells were washed twice in ice-cold, sterile, deionized water, suspended in 0.1 M Tris (pH 7.0), and broken by vortexing with glass beads (five 1-min pulses). Protein concentrations in the lysates were estimated using BCA protein assay reagent (Pierce). Enolase activity was assayed as described by Westhead and McLean (41). A conjugate of goat anti-mouse immunoglobulins G, A, and M and alkaline phosphatase (GIBCO BRL) was used to develop Western blots.

Nucleotide sequence accession number. The nucleotide sequence of the *C. albicans ENO1* gene and flanking sequences has been submitted to GenBank and assigned accession no. L04943.

RESULTS

Cloning of the *C. albicans ENO1* gene. Preliminary Southern analysis showed that a 0.8-kb *Ava*II-*Bgl*II fragment of the *S. cerevisiae ENO2* gene (11) hybridized to homologous sequences in genomic *C. albicans* DNA (data not shown). This heterologous probe was used to screen a *C. albicans* genomic DNA library by colony hybridization. Four positive clones, containing identical inserts, were obtained. The insert was subcloned as a 3.8-kb *Stu*I-*Sma*I fragment into the *Eco*RV site of pBSI(KS+), yielding plasmid pBMBS10.

***C. albicans ENO1* DNA sequence and predicted amino acid sequence.** A partial restriction map of the *C. albicans* insert in plasmid pBMBS10 is shown in Fig. 1. The nucleotide sequence of the putative enolase gene, with its 5′ and 3′ flanking regions, is shown in Fig. 2. A single continuous open reading frame of 1,320 bp is present. The deduced amino acid sequence is included beneath the nucleotide sequence. The DNA sequence was identified as encoding an enolase on the basis of its high homology to the *S. cerevisiae* enolase genes. Comparison of the *C. albicans ENO1* DNA sequence with those of *S. cerevisiae ENO1* and *ENO2* revealed homologies of 80 and 79%, respectively.

In the 5′ flanking region of *C. albicans ENO1*, there is a putative TATA box (nucleotides −127 to −123) followed by a pyrimidine-rich stretch of nucleotides. In the region from −111 to −47, only 5 of 65 nucleotides are purines (46 of 65 are T). At the 3′ end of the *C. albicans ENO1* DNA sequence, the region immediately downstream of the UAA stop codon (Fig. 2) contains 30 nucleotides of dyad symmetry within a 38-nucleotide range (nucleotides 1334 to 1371). The secondary structure produced by this sequence may

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-528  GGGATCAAGATTTGTTTACAGGTGATGCCTTTTTTTAGAACTTATGCGTTGCAAAAGTTG  -469

-468  ACTAACGATTTCTATAAGGTGATCCACACTAATTATACAAACGTACAAACAGACATACTT  -409

-408  TTCCTGCGTTCACCTGATGTTGGCCAGATTTCTCTCTTCATTGCATAGAACATAACCACA  -349

-348  CTAGGGCAACAGAAAAAAAAAAAAAAAAGTGAATCCGGGAAGTTGTGTTCCATTCATTATA  -289

-288  TGTCTACTACTGCATATGAGTAGCCCACCCACCACCACCATAGTAAGTTTTTGTGTATGC  -229

-228  GCGCCGTCAGTTATTTCATTTCTGAATTTTTCAACCACCTTACTCCCTTTATTGTTGATT  -169

-168  GACAATTTTGCTCACAGTAAGATCTTTTAGACTCCAATTAATATAAAATAAGTCTGATTT  -109
                                                ######

-108  TCCAATTCCTGTTTTTTCTTTTTTTTTCTGTTTCTATTTCTTTCCTTTTCTCCCTTTTTT  -49

-48   TTAATTCTTCATTCAATCATCAATTGATAATTCAGGAATATTACAACAATGTCTTACGCC  11
      _++      ++                                     M  S  Y  A      4
                   <------------------XT1-------------|*

12    ACTAAAATCCACGCCAGATACGTCTACGACTCCAGAGGTAACCCAACCGTTGAAGTTGAT  71
      T  K  I  H  A  R  Y  V  Y  D  S  R  G  N  P  T  V  E  V  D    24

72    TTCACCACCGACAAAGGTTTATTCAGATCAATTGTCCCATCTGGTGCCTCTACTGGTGTC  131
      F  T  T  D  K  G  L  F  R  S  I  V  P  S  G  A  S  T  G  V    44

132   CACGAAGCTTTGGAATTGAGAGATGGTGACAAATCCAAATGGTTAGGTAAAGGTGTTTTG  191
      H  E  A  L  E  L  R  D  G  D  K  S  K  W  L  G  K  G  V  L    64

192   AAAGCCGTTGCCAATGTTAATGACATCATTGCTCCAGCTTTAATCAAAGCCAAGATCGAT  251
      K  A  V  A  N  V  N  D  I  I  A  P  A  L  I  K  A  K  I  D    84

252   GTTGTCGACCAAGCTAAGATTGATGAATTCTTGTTGTCCTTGGACGGTACTCCAAACAAA  311
      V  V  D  Q  A  K  I  D  E  F  L  L  S  L  D  G  T  P  N  K    104

312   TCCAAATTGGGTGCCAATGCTATCTTGGGTGTTTCTTTGGCTGCTGCCAATGCTGCCGCT  371
      S  K  L  G  A  N  A  I  L  G  V  S  L  A  A  A  N  A  A  A    124

372   GCTGCTCAAGGCATTCCATTGTACAAACACATTGCCAACATTTCCAATGCCAAGAAGGGT  431
      A  A  Q  G  I  P  L  Y  K  H  I  A  N  I  S  N  A  K  K  G    144

432   AAATTCGTTTTGCCAGTTCCATTTCAAAACGTTTTGAACGGTGGTTCCCATGCTGGTGGT  491
      K  F  V  L  P  V  P  F  Q  N  V  L  N  G  G  S  H  A  G  G    164

492   GCTTTAGCTTTCCAAGAATTTATGATTGCCCCAACTGGTGTCTCCACTTTCTCTGAAGCT  551
      A  L  A  F  Q  E  F  M  I  A  P  T  G  V  S  T  F  S  E  A    184

552   TTGAGAATTGGTTCAGAAGTTTACCACAACTTGAAATCTTTGACCAAGAAGAAATACGGT  611
      L  R  I  G  S  E  V  Y  H  N  L  K  S  L  T  K  K  K  Y  G    204

612   CAATCCGCTGGTAACGTCGGTGATGAAGGTGGTGTTGCTCCAGATATCAAAACTCCAAAG  671
      Q  S  A  G  N  V  G  D  E  G  G  V  A  P  D  I  K  T  P  K    224

672   GAAGCTTTGGACTTGATCATGGATGCCATTGACAAAGCCGGTTACAAAGGTAAGGTTGGT  731
      E  A  L  D  L  I  M  D  A  I  D  K  A  G  Y  K  G  K  V  G    244

732   ATTGCCATGGATGTTGCTTCATCTGAATTCTACAAGGACGGTAAATACGACTTGGACTTT  791
      I  A  M  D  V  A  S  S  E  F  Y  K  D  G  K  Y  D  L  D  F    264
                  *

792   AAAAACCCAGAATCCGACCCATCTAAATGGTTGTCTGGTCCACAATTGGCTGACTTATAT  851
      K  N  P  E  S  D  P  S  K  W  L  S  G  P  Q  L  A  D  L  Y    284

852   GAACAATTGATTTCCGAATACCCAATTGTTTCTATTGAAGATCCATTCGCTGAAGATGAC  911
      E  Q  L  I  S  E  Y  P  I  V  S  I  E  D  P  F  A  E  D  D    304

912   TGGGATGCTTGGGTCCACTTCTTTGAAAGAGTTGGTGACAAGATCCAAATTGTCGGTGAT  971
      W  D  A  W  V  H  F  F  E  R  V  G  D  K  I  Q  I  V  G  D    324
                                          *  *

972   GATTTGACTGTCACTAACCCTACCAGAATCAAGACTGCCATTGAAAAGAAAGCCGCTAAT  1031
      D  L  T  V  T  N  P  T  R  I  K  T  A  I  E  K  K  A  A  N    344

1032  GCTTTGTTGTTGAAGGTTAACCAAATTGGTACTTTGACTGAATCTATACAAGCTGCTAAC  1091
      A  L  L  L  K  V  N  Q  I  G  T  L  T  E  S  I  Q  A  A  N    364

1092  GATTCTTACGCTGCTGGTTGGGGTGTCATGGTTTCCCACAGATCCGGTGAAACCGAAGAT  1151
      D  S  Y  A  A  G  W  G  V  M  V  S  H  R  S  G  E  T  E  D    384

1152  ACTTTCATTGCTGACTTGTCAGTTGGTTTAAGATCTGGTCAAATCAAGACTGGTGCTCCA  1211
      T  F  I  A  D  L  S  V  G  L  R  S  G  Q  I  K  T  G  A  P    404

1212  GCCAGATCTGAAAGATTGGCCAAATTGAACCAAATCTTGAGAATCGAAGAAGAATTAGGT  1271
      A  R  S  E  R  L  A  K  L  N  Q  I  L  R  I  E  E  E  L  G    424

1272  TCTGAAGCTATCTACGCTGGTAAAGATTTCCAAAAGGCTTCTCAATTGTAAGTTTGCCTG  1331
      S  E  A  I  Y  A  G  K  D  F  Q  K  A  S  Q  L                 440

1332  TGATTAAATAAAAAAAAGCTGGTGCTTTTTTTTCTTTTATAGGAACATCTTGAATATATG  1391

1392  AACTAATTAAATGATAATTTTTTACCCATCTTTACTCTTAATCACTGAGCTGCAGTCAAA  1451

1452  GAAAAAGGGATACAGCACCTGGTGAAGAGATGAACGGAGACTAACTTAGACGCGTTGATT  1511

1512  CTTTTTAATTGCACATTTTATTAATCGATGCTAACGTCTATTTACATATATTCTTTAGAG  1571

1572  ATATTATCTAGGGGCTTCAAATAATCTCTGGACAGCAATAAAAGTCTCTTCAAAGCTATT  1631

1632  GTATACGGCAATGGGGCTAATCTGATTACATCTGTCTTCTTTCGTCACAGATTATAGCAT  1691

1692  GATCATGCAAGTACGCATTAACTCGTCATGAGCTCTGTCTTTCATCGAAATGCGGTTGAA  1751

1752  ACATAATGGACAATTGACATCCTCTTTCAGCTGGATTCAAAGGAGTTAAAATTTTAAACC  1811

1812  CAAATTTGGAGTTTGATGTACTGGATTGTGGTATGTAATACTTGGAATTCGTCAATAGAT  1871

1872  CCTGTAAAAATTGAGTCAAAGCAACACTTTTTTCACGAAGTTTAGATACTCCACCCACTT  1931

1932  TAGCATACACTTCCAATGACGACTTCACAGCAACAACATCAAGAACAGAAGGATTTGACT  1991

1992  GTCTGTAAGAAAGAGCCGAGTTTATTGGATCAAACTCTTCTAACATTTTGAATCGTTCCT  2051

2052  GGGAGTTATTGCCCCACCAACCAGCTAGTCTAGGAACGAAACTGCTCTTCTTCGTCTCTA  2111

2112  TAGTGTA  2118
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FIG. 2. Nucleotide sequence and predicted amino acid sequence of the *C. albicans ENO1* gene. The dashed line indicates the 36-mer oligonucleotide primer (XT1) used to map the *ENO1* transcription initiation sites (+). Other sequence features in the noncoding regions include the putative TATA box (#), a pyrimidine-rich sequence of 65 nucleotides in the 5′ flanking region (underlined), and a region of dyad symmetry just 3′ of the UAA stop codon (underlined). Amino acids of particular interest are also indicated (*).

contribute to termination of transcription. At the 3′ end of this putative termination-promoting sequence is a heptanucleotide sequence, TTTTATA, which is almost identical to the yeast transcription termination signal, TTTTTATA (9). In addition, there is a termination signal typical of higher eukaryotic genes at the 5′ end of the region of dyad symmetry (i.e., AATAAA) (1) which is repeated 254 nucleotides downstream. These findings suggest that termination and polyadenylation of the *C. albicans* enolase transcript probably occur within, or just downstream from, the dyad symmetrical sequence.

C. albicans enolase is 440 amino acids long, 3 amino acids longer than either of the *S. cerevisiae* enolase proteins. The deduced amino acid sequence shows a high degree of homology with the seven eukaryotic enolases listed in Fig. 3, the highest being shared with *S. cerevisiae ENO1* and *ENO2* (78 and 76%, respectively). In common with the *S. cerevisiae* enolase genes and highly expressed *C. albicans* genes (Table 1), *C. albicans ENO1* shows a biased codon usage pattern. The protein begins at a typical eukaryotic AUG start codon and terminates at a UAA stop codon.

Mapping of the *C. albicans ENO1* 5′ transcription initiation site. The start sites of *C. albicans* enolase mRNAs were determined by primer extension analysis. mRNA from yeast phase cells of *C. albicans* FC18 was used as a template for primer extension analysis with single-stranded synthetic oligonucleotide primer XT1. The cDNAs generated mapped to two major sites, positions (i) −37 and −36 and (ii) −46 and −45 relative to the putative initiation codon (Fig. 4). There may also be a low frequency of transcription initiation at nucleotide positions −72 and −71. Multiple transcription initiation sites are commonplace in *S. cerevisiae* (34), so it is perhaps not surprising to find multiple transcription start sites upstream of the *C. albicans ENO1* gene.

***C. albicans* enolase is encoded by a single gene.** Since multiple enolase genes have been demonstrated in several

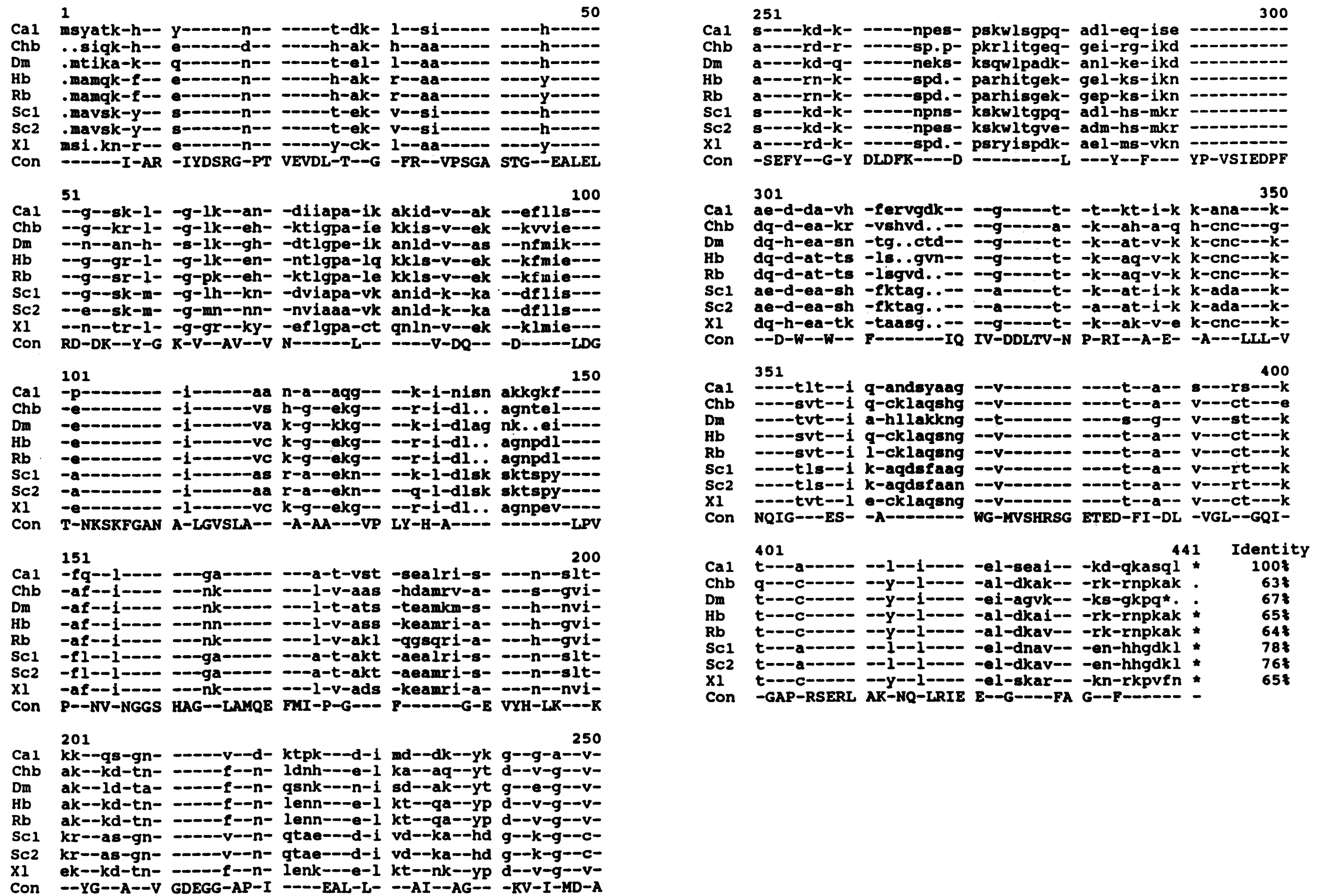

FIG. 3. Comparison of the predicted amino acid sequence of *C. albicans* enolase with those of seven other enolases. All sequences were gap fitted (5) to the *C. albicans* sequence (Cal). The other enolases are as follows: Chb, chicken β (30); Dm, *Drosophila melanogaster* (2); Hb, human β (28); Rb, rat β (27); Sc1, *S. cerevisiae ENO1* (11); Sc2, *S. cerevisiae ENO2* (11); Xl, *Xenopus laevis ENO1* (33); Con, consensus sequence showing amino acids conserved in all eight sequences. Homology (percent identity) of *C. albicans* ENO1p to other enolases is given at the end of each sequence.

eukaryotic species, evidence of additional *C. albicans* enolase genes was sought by Southern analysis. To look for any other gene(s) with homology to *ENO1*, an *Nco*I-*Pst*I fragment of the *C. albicans ENO1* gene (Fig. 1), which includes 586 nucleotides of the coding sequence plus 123 nucleotides of the 3′ flanking sequence, was hybridized against *C. albicans* and *S. cerevisiae* genomic DNAs digested with *Bam*HI or *Xba*I. As neither of these restriction enzymes has a cleavage site within the *S. cerevisiae ENO1* or *ENO2* or *C. albicans ENO1* gene, each hybridization band should represent a unique enolase gene. As expected, two *S. cerevisiae* DNA fragments were recognized by the probe. Only one band was evident in the *C. albicans* genomic DNA (Fig. 5). This strongly suggests that there is only a single enolase gene per haploid *C. albicans* genome.

TABLE 1. Codon bias observed for yeast enolase proteins and other *C. albicans* proteins with high-level expression

Protein	Codon bias[a]	Reference
C. albicans enolase	35/61	This report
S. cerevisiae enolase 1	32/61	11
S. cerevisiae enolase 2	31/61	11
C. albicans H^+-ATPase	42/61	24
C. albicans β-tubulin	42/61	36

[a] Total number of codons used/total number possible.

Characterization of *C. albicans* enolase mRNA. Northern (RNA) analysis of total RNA samples from two strains of *C. albicans* revealed a single *ENO1* mRNA of 1.6 kb (Fig. 6A). This size is consistent with the short mRNA leader sequence suggested by primer extension, an open reading frame of 1,320 bp and a 3′ tail plus a poly(A) tract with a combined length of approximately 240 nucleotides.

Effect of carbon source on expression of *C. albicans* enolase. The influences of different carbon sources on expression of the *C. albicans ENO1* gene were investigated by using two *C. albicans* strains grown in medium containing either ethanol or glucose. The growth rate of both strains was three to four times faster in glucose-containing than in ethanol-containing medium. Levels of the 1.6-kb *ENO1* transcript in *C. albicans* B311 and FC18 grown on glucose were 6- and 13-fold higher, respectively, than the levels of *ENO1* mRNA in the same strains grown on ethanol (Fig. 6A). As an internal control for mRNA levels, the gel shown in Fig. 6A was rehybridized with a *C. albicans* actin probe. Actin was chosen because *S. cerevisiae* actin mRNA levels are similar under glycolytic and gluconeogenic conditions (20). Unex-

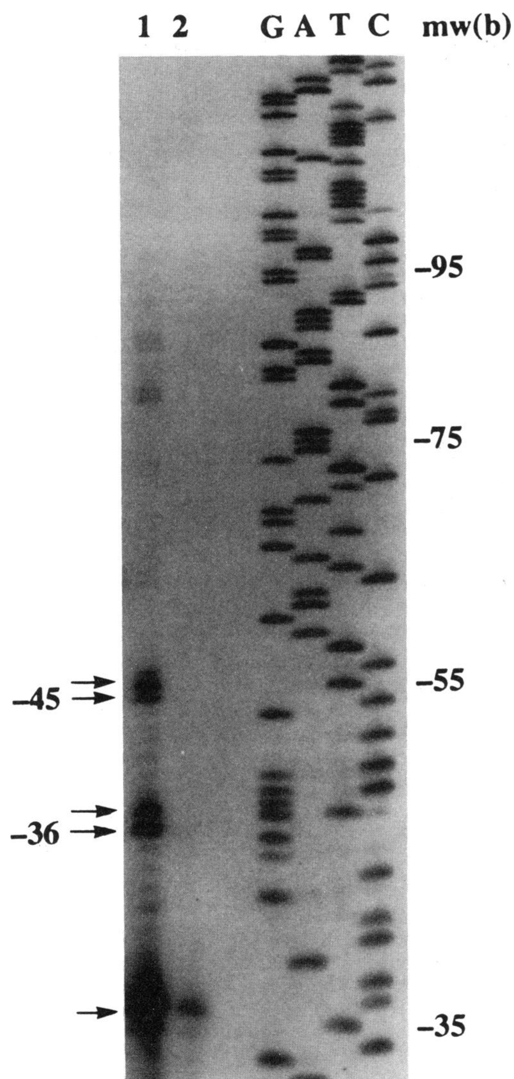

FIG. 4. Primer extension analysis to map the mRNA start sites of *C. albicans ENO1*. Lanes: 1, 3 μl of RNase-treated primer extension mixture (double arrows indicate major transcription initiation sites); 2, nonextended, end-labelled XT1 primer only (single arrow). The sequencing ladder represents an unrelated DNA with a known sequence, included for molecular size estimation. Start site positions were confirmed by sequencing pBMBS10 by using the end-labelled XT1 primer (data not shown).

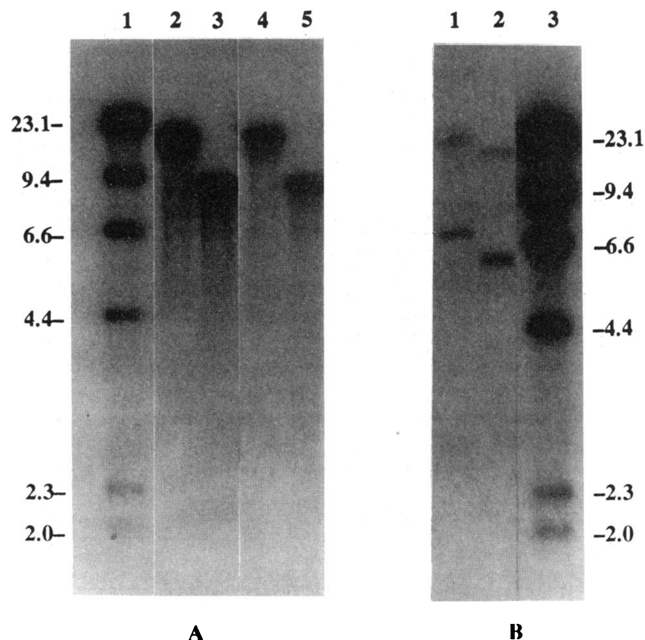

FIG. 5. Southern hybridization of *C. albicans ENO1* to restricted genomic DNAs of *C. albicans* and *S. cerevisiae*. The probe was an *Nco*I-*Pst*I fragment of *C. albicans ENO1*. (A) Lanes: 1, λ *Hin*dIII markers; 2 and 3, *C. albicans* B311 genomic DNA digested with *Bam*HI and *Xba*I, respectively; 4 and 5, *C. albicans* B792 genomic DNA digested with *Bam*HI and *Xba*I, respectively. (B) Lanes: 1 and 2, *S. cerevisiae* F762 genomic DNA digested with *Bam*HI and *Xba*I, respectively; 3, λ *Hin*dIII markers. The numbers to the sides are sizes of the marker DNA fragments in kilobases.

pectedly, much the same level of induction was seen for actin mRNA as was seen for enolase (data not shown). This suggests that in *C. albicans*, transcription of the actin gene is also elevated in glucose-grown cells.

Measurement of the level of enolase activity failed to demonstrate a dramatic difference between glucose- and ethanol-grown cells (Table 2). In strain B311, it was 50% higher in glucose-grown cells than in ethanol-grown cells, while in strain FC18, it was elevated only 20% by growth on glucose. Measurement of total enolase protein by immunoblotting (Fig. 6B) also indicated that use of a different carbon source had little or no effect on the total amount of the enzyme present in the cells during log-phase growth.

DISCUSSION

***C. albicans* enolase.** Comparison of the predicted amino acid sequence of *C. albicans* enolase with enolase sequences from other eukaryotes shows a high degree of evolutionary conservation (Fig. 3). Homology has been retained throughout the protein, and there are numerous regions of identity among the eight sequences analyzed. The consensus sequence comprises 236 invariant amino acids arranged, for the most part, in short tracts of highly conserved sequences. Predictably, the enolase sequences most similar to *C. albicans* enolase are those from *S. cerevisiae*. On the basis of the proposed crystal structure of *S. cerevisiae* apo-enolase A, tracts of highly conserved sequences correspond to β strands which form the central barrel that makes up the active site of the enzyme (37).

The three additional amino acids in *C. albicans* enolase relative to the two *S. cerevisiae* enolases (Tyr-3, Asp-317, and Lys-318) do not alter the overall charge of the protein and are not predicted to have a significant impact on protein folding or active-site conformation. Amino acids identified in *S. cerevisiae* enolase as being part of the active site or involved in metal ion (Mg^{2+}) binding (16, 37) are conserved in the *C. albicans* enolase protein, with one exception. At position 249 (Fig. 3), the single cysteine in *S. cerevisiae* enolase has been replaced by valine in *C. albicans* enolase. Placement of the sulfhydryl group of the cysteine at the active site of *S. cerevisiae* enolase was suggested by Weiss and colleagues (40) and confirmed by the enolase crystal structure (37). Therefore, it is of interest that this residue is not present in the *C. albicans* enzyme. While it is possible that the deduced amino acid sequence of ENO1p has been

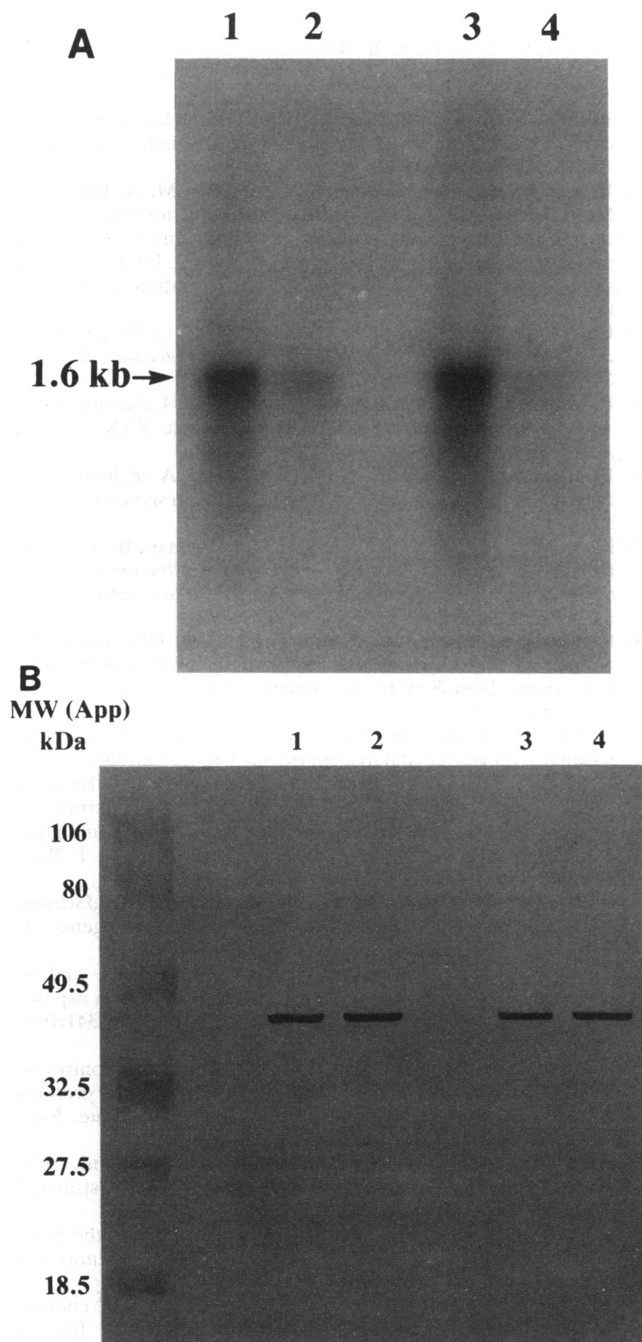

FIG. 6. Analysis of *C. albicans ENO1* expression after growth on glucose or ethanol. RNA and protein preparations were made from cells in the early log phase of growth (A_{540}, ~0.5). (A) Identification of the transcript encoded by the *ENO1* gene in total cellular RNA (1 μg per lane) from *C. albicans* B311 grown on glucose (lane 1) or ethanol (lane 2). Lanes 3 and 4 contained total cellular RNA (1 μg per lane) from *C. albicans* FC18 grown on glucose and ethanol, respectively. The DNA probe was an *NcoI-PstI* fragment of *C. albicans ENO1*. (B) Equivalent amounts of protein (estimated by BCA assay and confirmed by silver-stained SDS-polyacrylamide gel electrophoresis) were separated by SDS-10% polyacrylamide gel electrophoresis and then Western blotted by using a murine monoclonal antibody to *C. albicans* enolase. Ten micrograms of total cell protein from *C. albicans* B311 grown on glucose (lane 1) or ethanol (lane 2) or *C. albicans* FC18 grown on glucose (lane 3) or ethanol (lane 4) was used. MW (App), apparent molecular mass.

TABLE 2. Effect of carbon source on enolase enzyme activity in *C. albicans*[a]

C. albicans strain	Carbon source	Enolase activity[b] (U/mg of protein)
B311	Glucose	0.82
B311	Ethanol	0.44
FC18	Glucose	0.55
FC18	Ethanol	0.44

[a] Cells were grown to an A_{540} of ~0.5 in either SMC-glucose or SMC-ethanol at 24.5°C.

[b] Units of enolase activity are defined as Δ_{230nm} per minute per milliliter. Averages of three measurements are shown.

compromised by nonstandard codon usage, such as that reported for some *Candida* proteins (12, 32), this appears unlikely to account for the lack of a Cys residue at position 249. No evidence of a cysteine residue was found by amino acid analysis of purified *C. albicans* enolase (18a). The presence of Val rather than Cys at residue 249 is also consistent with the amino acid sequences of eukaryotic enolases other than the two *S. cerevisiae* proteins (Fig. 3).

Similarities between the 5′ noncoding regions of fungal enolase genes. The locations of the major transcription initiation sites in *C. albicans ENO1* ([i] −37 and −36 and [ii] −46 and −45) are similar to those for the *S. cerevisiae ENO1* and *ENO2* genes, where the 5′ ends of the mRNAs have been mapped to positions (i) −41 and −40 and (ii) −36, respectively (10). The pyrimidine-rich sequence upstream of the *C. albicans ENO1* gene (−111 to −47) is analogous to 5′ noncoding sequences in the *S. cerevisiae ENO1* (−110 to −46) and *ENO2* (−113 to −39) genes (11). In all three genes, this sequence is located between the TATAA box and the AUG codon. McNeil (23) presented evidence that such a stretch of pyrimidines serves to reduce the level of aberrant transcriptional initiation in highly expressed *S. cerevisiae* genes, thereby improving the yield of functional mRNAs and limiting nucleoside triphosphate consumption. In keeping with this theory, transcription initiates at nucleotides immediately downstream from the proposed CT blocks in *C. albicans ENO1* (Fig. 2) and in both *S. cerevisiae ENO1* and *ENO2* (10). A similarly located pyrimidine-rich sequence is found upstream of the highly expressed *C. albicans* H^+-ATPase gene *PMA1* (24).

***C. albicans* has only one enolase gene.** *C. albicans* possesses a single enolase gene per haploid genome. This conclusion, which was initially suggested by biochemical studies (18), was confirmed by Southern blot analysis. The catalytically active enzyme must, therefore, exist as a homodimer of the *ENO1* gene product. Why should this be the case, when other eukaryotes, including *S. cerevisiae*, possess multiple enolase genes? Possibly it is related to the cellular conditions under which the enzyme is normally required. In higher eukaryotes, expression of a particular enolase isoenzyme is usually specific for a cell type and/or developmental stage of the organism (33). *S. cerevisiae* occupies a dynamic environmental niche and may benefit from having more than one form of enolase in much the same way that higher organisms have enolase isoenzymes devoted to specific functions. For example, *S. cerevisiae* ENO1p could act as a failsafe to ensure that glycolysis continues when the cellular or environmental conditions do not favor expression of ENO2p. *C. albicans*, on the other hand, may need only a single type of enolase gene because, as a

commensal organism, it has adapted to an environment where the temperature and carbon source are relatively constant.

Expression of the *C. albicans ENO1* gene. *C. albicans* enolase mRNA was detected as an abundant transcript in cells grown on either a glycolytic or a gluconeogenic carbon source, although the amount of the *ENO1* transcript in glucose-grown cells was several times greater than that detected in cells grown on ethanol. This induction is reminiscent of the 20-fold glucose-mediated induction of the *S. cerevisiae ENO2* gene (21). It should be noted that Northern analyses (Fig. 6A) compared samples of total cellular RNA and that actin, unexpectedly, did not serve as an appropriate control for normalization of mRNA levels. Therefore, qualification of enolase mRNA levels may have been skewed by glucose-induced changes in the synthesis of *C. albicans* non-mRNA (primarily ribosomal RNA). This effect might be substantial. *S. cerevisiae* growing on glucose synthesizes rRNA at a rate seven times higher than when utilizing ethanol (13). If this differential synthesis also occurs in *C. albicans*, the values for glucose-mediated transcriptional induction for *C. albicans* B311 (6-fold) and FC18 (13-fold) represent considerable underestimates. The apparent difference in enolase mRNA levels between the two *C. albicans* strains may actually reflect differences in the amount of rRNA synthesized in relation to total cellular RNA. We are attempting to identify a *C. albicans* mRNA whose synthesis is not influenced by glucose.

The elevated transcription of *C. albicans ENO1* in glucose-grown cells was not accompanied by significant elevations in the levels of ENO1p (Fig. 6B) or enolase activity (Table 2). This suggests that the cytoplasmic levels of enolase are regulated posttranscriptionally. This could be achieved through more rapid degradation of *ENO1* mRNA when cells are grown on glucose. Decreased mRNA stability would effectively reduce the amount of protein synthesized from each *ENO1* mRNA, resulting in levels of protein synthesis that do not rise in proportion to increased transcription of the gene. This explanation, however, conflicts with the demonstrated stability of glycolytic mRNAs in *S. cerevisiae* (26).

S. cerevisiae provides examples of other posttranscriptional mechanisms that might regulate enolase expression despite elevated mRNA levels. For example, overexpression of *PYK1* mRNA does not result in increased pyruvate kinase activity (25). As *PYK1* mRNA accumulates, translation of this message decreases, apparently because of reduced ribosomal loading on *PYK1* mRNAs (25). Alternatively, regulation can be imposed posttranslationally through an increased rate of protein turnover. This mechanism is used to avoid accumulation of excess *S. cerevisiae* ribosomal proteins when ribosomal protein mRNAs are overexpressed and translated at wild-type rates (43).

The posttranscriptional mechanism(s) by which *C. albicans* regulates its cytoplasmic enolase levels is unknown. In *S. cerevisiae*, the levels of most gluconeogenic enzymes are unaffected by carbon source changes (4, 22). Our results suggest that this is also true for *C. albicans* enolase, reflecting the enzyme's key role in both the glycolytic and gluconeogenic pathways.

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