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Molecular cloning of a cDNA encoding enolase from the filamentous fungus, *Aspergillus oryzae*

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Abstract A 1.6-kbp full-length cDNA for the *Aspergillus oryzae* enolase gene (*enoA*) was cloned. The sequenced insert contained a continuous open reading frame of 1314 bp encoding a protein of molecular weight 47 405. Among all enolases sequenced to-date, the deduced amino-acid sequence showed the highest homology (74.9%) with *Candida albicans* enolase (*ENO1*). Strong codon biases and multiple transcription start sites downstream from CT-blocks in the 5'-flanking region suggested strong expression. *enoA* mRNA was found to occupy approximately 3% (w/w) of total mRNA of *A. oryzae* by quantitative RT-PCR. This strong transcription was dependent on the carbon source in the medium and correlated with the growth rate of the mycelium.

Key words Enolase · *Aspergillus oryzae* · cDNA

Introduction

Aspergillus oryzae is an important filamentous fungus in the Japanese beverage and fermentation industries, and is used to produce industrially valuable hydrolytic enzymes. *A. oryzae* is considered a favorable host for heterologous protein production (Barbesgaard et al. 1992) owing to its ability to secrete large amounts of protein (Christensen et al. 1988) and its wide range of application in food indus-

tries. However, lack of basic knowledge on its gene structure and the regulation of its gene expression makes more extensive application difficult.

Enolase (2-phospho-D-glycerate hydrolase, EC 4.2.1.11) is a ubiquitous enzyme that catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate in glycolysis and gluconeogenesis. Enolases from a variety of sources, such as bacteria, yeast, *Drosophila*, amphibian, bird, plant and human, have been found to be highly conserved (Van Der Streten et al. 1991). In *Saccharomyces cerevisiae*, enolase is encoded by two genes (*ENO1* and *ENO2*) whose transcription is mediated by multifunctional transcriptional modulators which bind to short common DNA sequences in the 5' region, as observed in several house-keeping genes (Brindle et al. 1990; Shore and Nasmyth 1987). The enzyme encoded by *ENO1* increases during the stationary phase (Iida and Yahara 1985), whereas enolase encoded by *ENO2* increases when glucose is present in the growth medium (McAlister and Holland 1982). Complex regulatory mechanisms which govern the expression of the *ENO1* and *ENO2* genes involve *cis*-acting elements and *trans*-acting positive regulators (Machida et al. 1988, 1989; Brindle et al. 1990) including products of the *GCR1* (Brindle et al. 1990), *GCR2* (Uemura and Jigami 1992a) and *GCR3* (Uemura and Jigami 1992b) genes.

Enolase is a very highly expressed protein in many organisms including yeast, especially in cases in which the rate of glycolysis is high (Holland and Holland 1978). Based on the relatively close evolutionary relationship between *S. cerevisiae* and the filamentous fungus *A. oryzae*, *A. oryzae* enolase is likely to be an abundant protein whose expression is regulated by transcriptional modulators which control multiple genes, and the 5'-flanking regions of its gene may be useful for foreign gene expression in *A. oryzae*.

Recently, the genomic DNA which most likely encodes *A. oryzae* enolase (*enoA*) has been cloned and sequenced (Machida et al. 1996). The 5' non-coding sequence, including the surroundings of the translation initiation codon of *A. oryzae enoA*, shows characteristics of highly expressed

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genes including glycolytic genes in yeast (Machida et al. 1996). The *A. oryzae enoA* gene would thus appear to be highly expressed to the same extent as the enolase genes of other organisms.

This paper reports the cloning of cDNA corresponding to the *A. oryzae enoA* gene and demonstrates *enoA* mRNA to be a very abundant transcript, as expected from nucleotide sequencing data. Regulation of the *enoA* gene in various carbon sources is discussed.

Materials and methods

Strains, media, and reagents. The *A. oryzae* strain RIB40 was used for total RNA preparation. Dextrin-peptone (DP) medium, consisting of 2% dextrin, 1% polypeptone, 0.5% KH_2PO_4 and 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, served as the complete medium (Jimura et al. 1987). Czapek-Dox (CD) medium, consisting of 0.3% NaNO_3 , 0.2% KCl, 0.1% KH_2PO_4 , 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and 2% glucose, was the minimal medium (Jimura et al. 1987). Modified CD medium was supplemented with 1% polypeptone (Hata et al. 1992). *Escherichia coli* strain, XL1-Blue (Short et al. 1988) was grown in LB broth (10% tryptone, 5% yeast extract and 5% NaCl) or 2×YT (16% tryptone, 10% yeast extract and 5% NaCl). Oligonucleotide, AEPX576 (5'TTCTTCTTGGCGAGAGTCTTGAGC 3', designed by Oligo 5.0 software as a sequencing primer at very high stringency), labelled at 5'-termini with the fluorescent dye IRD41, was synthesized by Aloka, Japan.

Preparation of *A. oryzae* mRNA and the corresponding cDNA. Total RNA was isolated from approximately 4 g of wet-weight mycelium and grown in DP medium for 22 h at 30°C, according to the guanidine thiocyanate-method (Chirgwin et al. 1979). Finally, 4.6 mg of total RNA purified by ultracentrifugation in the presence of CsCl was dissolved in 500 µl of TE and stored at -80°C until use. Approximately 30 µg of polyadenylated [poly(A)] RNA was prepared from 1.4 mg of total RNA using oligo(dT)-cellulose column (Pharmacia) purification. First- and second-strand cDNA syntheses of poly(A) RNA were carried out using oligo(dT) as the primer.

PCR amplification of *A. oryzae enoA* cDNA. To bring about the GST-fusion *A. oryzae enoA* cDNA was prepared by the polymerase chain reaction (PCR) from the cDNA pool with the *enoA*-specific primers, AOENOP1 (5'GTCGACATGCCTATCACCAAGATCCACG 3', 28-mer) and AOENOP2 (5'GTCGACGTCATATAATCATACGG TTCATTT 3', 30-mer), which contained mutations at nucleotide (nt) positions 4 (A to G) and 6 (A to C) for AOENOP1 and position 3 (A with to C) for AOENOP2 to introduce *SalI* sites at both ends of the amplified product. Amplified cDNA for the *A. oryzae enoA* gene was purified by agarose-gel electrophoresis and cloned into the pCRII vector (Invitrogen), creating pCR2-AOENOA. Rapid amplification of cDNA ends (RACE) and fusion of the RACE products to obtain full-length cDNA for the *A. oryzae enoA* gene were performed with a Marathon cDNA Amplification Kit (Clontech) in combination with long-distance PCR (Barnes 1994; Cheng et al. 1994) using an LA-PCR Kit (Takara Shuzo, Japan) according to the instructions of the manufacturer. The *A. oryzae enoA*-specific primers AOENOP1 and AOENOP2 were used for 3'- and 5'-RACE, respectively.

Sequencing. The purified 1.3-kbp *SalI* cDNA fragment was subcloned into pBluescript and sequenced by the cycle sequencing reaction with *Taq* DNA polymerase (Perkin-Elmer) and SequiTherm DNA polymerase (Epicentre Technologies) using Applied Biosystems 373A and LI-COR Model 4000L DNA sequencers, respectively. Sequence data were compiled by Sequencher software (Gene Codes, USA). Nucleotide misincorporation during PCR was followed by comparison with the sequence of the genomic *enoA* gene (Machida et al. 1996).

Northern-blot analysis. RNA was electrophoresed on 1.0% agarose (Agarose H, Nippon Gene, Japan) containing 6.7% formaldehyde (Sambrook et al. 1989) and blotted onto a nitrocellulose membrane (approximately 7 cm × 12 cm, Bio-Rad) for 3 h by vacuum transfer (Nikkyo, Japan). Digoxigenin (DIG)-labelled RNA-probe was prepared by transcribing the 2.9-kbp *BglII* fragment (Machida et al. 1996) containing an entire enolase coding region in the presence of DIG-dUTP (Boehringer). Pre-hybridization and hybridization were conducted at 68°C for 1 h in 10 ml of pre-hybridization solution [5×SSC, 50% formamide, 0.1% sarcosine, 0.02% SDS and 0.05% Blocking Reagent (Boehringer)] and overnight in a 1.5-ml pre-hybridization solution containing approximately 0.25 µg of DIG-labelled RNA probe. After washing twice at room temperature in 25 ml of 2×SSC containing 0.1% SDS and then twice at 68°C in 25 ml of 0.1×SSC containing 0.1% SDS, the membrane was rinsed in 40 ml of buffer 1 (100 mM maleic acid, 150 mM NaCl, adjusted to pH 7.5 with NaOH) and blocked with 40 ml of buffer 1 containing 0.5% blocking reagent at room temperature for 30 min. Finally the membrane was incubated with buffer 1 containing 3 U of Anti-DIG-Fab-alkaline phosphatase conjugate (Boehringer) at room temperature for 30 min. After washing twice with 40 ml of buffer 1 at room temperature for 15 min, hybridized signals were detected while incubating the membrane in buffered solution [100 mM Tris-HCl (pH 9.5), 0.1 M NaCl, 50 mM MgCl_2] containing 0.33 mg/ml of nitro-blue tetrazolium salt and 0.18 mg/ml of 5-bromo-4-chloro-3-indolyl phosphate. The coloring detection reaction was terminated by incubating the membrane for 10 min in 10 mM Tris-HCl (pH 7.5) containing 1 mM EDTA. De-ionized water, 20×SSC stock solution, buffer 1, and a blocking reagent stock solution (10% blocking reagent in buffer 1) were treated with diethyl pyrocarbonate and autoclaved. The molecular weight of the RNA hybridized to the *A. oryzae enoA* probe was determined from the standard curve, using DIG-labelled RNA molecular-weight standards (Boehringer).

Primer extension. DNA-free total RNA (9.0 µg) or poly(A) RNA (0.4 µg) and IRD41-labelled primer, AEPX576, (0.33 pmol) were dissolved in 5 µl of PE-buffer [50 mM Tris-HCl (pH 8.3), 50 mM KCl, 10 mM MgCl_2 , 10 mM dithiothreitol (DTT), 1 mM each dNTP and 1 mM spermidine] and hybridized by incubating first at 58°C for 20 min and then at room temperature for 10 min. Following the addition of 5 µl of PE-buffer containing 5.6 mM sodium pyrophosphate, eight units of human placenta RNase inhibitor (Boehringer) and one unit of AMV reverse transcriptase (Promega), the primer extension reaction was carried out at 43°C for 30 min. The synthesized cDNA was ethanol-precipitated, washed with 70% ethanol and dissolved in 2 µl of nuclease-free water. After adding 1.4 µl of 95% formamide containing 10 mM of ethylene-diamine tetra-acetic acid (EDTA), 0.1% xylene cyanol and 0.1% bromophenol blue, 1.5 µl of the reaction mixture were heat denatured at 95°C for 5 min and analyzed by a LI-COR Model 4000L DNA sequencer. Dideoxy chain-termination reactions were carried out using a SequiTherm Long-Read Cycle Sequencing Kit-LC (Epicentre Technologies) with fluorescent AEPX576 as the primer and the plasmid vector pCRII containing full-length cDNA as the template. The standard sequencing procedure was followed, with 7-deaza-dGTP in the termination mixture replaced by unmodified dGTP.

Production and purification of fusion protein. The 1.3-kbp *SalI* fragment containing the entire coding region of *A. oryzae enoA* was recovered from pCR2-AOENOA and subcloned into the *SalI* site of the pGEX-4T-3 plasmid vector (Smith and Johnson 1988), yielding pGEX-AOENOA. Plasmid DNA from ampicillin-resistant transformants of *E. coli* XL1-Blue was analyzed by restriction digestion to identify transformants containing inserts in the appropriate directions. Fusion-protein induction and purification were performed as described previously (Kaelin et al. 1992). The proteins were electrophoresed on a sodium dodecyl sulphate (SDS)-polyacrylamide gradient (4–20%) gel (Laemmli 1970) and stained with Coomassie blue.

Enolase enzyme assay. The enzymatic activity of enolase was determined by the coupled assay described by Maitra and Lobo (1971), with slight modification. The reaction mixture consisted of 50 mM

triethanolamine-HCl (pH 7.4), 10 mM $MgCl_2$, 0.16 mM NADH, 1.0 mM ADP, 1.0 mM glycerate-2-phosphate, 2.3 U lactate dehydrogenase (EC 1.1.1.27) (Boehringer) and 2.3 U pyruvate kinase (EC 2.7.1.11) (Boehringer) as the standard and monitored by a U-2000 spectrophotometer (Hitachi, Japan) at room temperature. One unit of enolase activity was defined as the conversion of 1 mmol NADH to NAD per min, based on an extinction coefficient at 340 nm for NADH of $6.22 \text{ mM}^{-1} \text{ cm}^{-1}$. The reaction mixture without the substrate showed no measurable changes in absorbance.

Results

DNA sequence

The 1.3-kbp cDNA fragment amplified by the primer pair AOENOP1 and AOENOP2 was cloned into the pCRII vector, yielding pCAO-ENOC1. Two independent clones were picked up and subcloned onto the pBluescript vector (Stratagene) to allow the sequencing of both strands (the GenBank, EMBL and DDBJ Nucleotide Sequence Database accession No. D64113). As expected from the genomic sequence, there was one long open reading frame (ORF) of 1314 bp, starting at a methionine codon (ATG, nt position 21) and ending at a stop codon (TAA, nt position 1335). Alignment of the deduced amino-acid (aa) sequence with that of enolase 2 from *S. cerevisiae* strongly suggested the methionine at nt position 21 to be the initial amino acid in

the protein. The ORF predicted a protein of molecular weight 47 405 consisting of 438 aa.

A 1.6-kbp DNA fragment from the 5'- and 3'-RACE was cloned into the pCRII vector, yielding pCAO-ENOCF1. The 5'-flanking regions of the resulting three independent clones each contained 14, 18 and 20 additional nucleotides from the initial methionine. The 3'-flanking regions contained a poly(A) tract of 4–11 nucleotides following the AATAAA sequence located at nt position 1530, suggesting this AATAAA sequence to function as a polyadenylation signal in *A. oryzae* as in other filamentous fungi (Ballance 1986).

A computer search of data bases showed the predicted aa sequence of *A. oryzae* enolase to be quite similar to the predicted enolase 2 (71.3% identical aa) encoded by the *ENO2* gene of *S. cerevisiae* (Holland et al. 1981) (Fig. 1). Of all the enolases in the data bases, *A. oryzae* enolase showed the greatest similarity to *Candida albicans* enolase (Sundstrom and Aliaga 1992) (74.9% identical), but less similarity to *Schizosaccharomyces pombe* (Jackson and Lopes 1995) (66.9%), *Drosophila* (Bishop and Corces 1990) (64.8%), human (Peshavaria et al. 1989) (63.9%), maize (Lal et al. 1991) (58.7%) and *Bacillus subtilis* (Leyva-Vazquez and Setlow 1994) (50.7%) enolases (partly shown in Fig. 1). All aa residues essential for catalytic activity (asterisks in Fig. 1) and those involved in subunit interactions (open circles in Fig. 1) (Sundstrom and Aliaga 1992) were conserved in *A. oryzae* enolase.

Fig. 1 Multiple alignment of amino-acid sequences of various enolases. Dots indicate residues identical to those of the predicted *A. oryzae* enolase.

Asterisks and open circles indicate residues important for catalytic activity and subunit interactions, respectively (Sundstrom and Aliaga 1992). Dashes indicate gaps introduced to fit the sequences. Homology (percent identity) of *A. oryzae* *enoA* to other enolases is given at the end of each sequence. *AoA* *A. oryzae* *enoA* (this study); *Ca1* *C. albicans* (Sundstrom and Aliaga 1992); *Sc1* *S. cerevisiae* *ENO1* (Holland et al. 1981); *Sc2* *S. cerevisiae* *ENO2* (Holland et al. 1981); *Sp1* *Schizosaccharomyces pombe* (Jackson and Lopes 1995); *con* conserved residues

AoA	MPITKI ^o HARSVYDSRG ^o NP ^o TV ^o EDV ^o VTETGLHRAIVPSGASTGQHEAHEL ^o RDGDKTHWGGKGLKAVEN ^o NKTIAPAVIEENLDVKDQSKV	
Ca1	MSYA.....Y.....FT.DK..F.S.....V...L.....SK.L.....A...DI...L.KAKI..V...A.I	
Sc1	AVS.VY.....ELT..K.VF.S.....V...L.M.....SK.M.....H..K...DV...FVKA.I....KA	
Sc2	AVS.VY.....ELT..K.VF.S.....V...L.M.....SK.M.....MN..N...NV...A.FVKA.....KA	
Sp1	A.Q.VF..QI.ALG.....LT.....I.....TW..L.M.....K.....NL.....VKA.....T..KAA	
con	K AR Y GNPTVEV G R IVP ^o SGASTG EA E RD DK W GRGV AV NVN IA A DV DQ	
AoA	DEFLKKLDGSANKSNL ^o GANA ^o ILGVSLAI ^o AKAGAAEKGVPLYAHISDL ^o AGTKK-PYVLVPFPQNVNLGGSHAGGR [*] LAFQEFMIVPSAAPS [*] F	
Ca1	LS...TP...K.....A.N.A.AQ.L...K..ANISNA..GKF.....A.....A.TGVST.	
Sc1	.D..IS...T...K.....ASR.A...N...K.LA..SKS.TS.....L.....A...A.TG.KT.	
Sc2	.D..LS...T...K.....M.A.R.A...N...Q.LA..SKS.TS.....L.....A...A.TG.KT.	
Sp1	...L...TE...K.....M.ICR...Q.KL..WKY.AENF...G.....SF.....D.....L.TG...	
con	D FL LDG NKS LGANA ^o ILGVS A A AA PL K VLPVP NVLNGGSHAGG LA QEFMI P F	
AoA	SEALRQGAEEVYQRLKTLAKKKYGG [*] SAGNVGDEGGVAPD [*] IQTAEALDLITEAQAGYTGKMKIAMDVASSEFYKADVKKYDLDFKNPDS	
Ca1	I.S...HN..S.T.....K.PK.....MD..DK...K..VG.....G.....E.	
Sc1	A...I.S...HN..S.T..R..A.....N.....VD..KA..HD..V..GL.C....F..G.....N.	
Sc2	A..M.I.S...HN..S.T..R..A.....N.....VD..KA..HD..V..GL.C....F..G.....E.	
Sp1	...M.W...T..T..S.L...R..S.....I...L..PQ.....V...NK...E..L..GL..T....V..G.....LAAPK	
con	EA R G E Y LK KK YG SAGNVGDEGG AP T EALDLI AI AG GK I D SSEF D KYDLD K	
AoA	DSSKWLTYEQLADLYKTLASKPYIVSTEDPFAEDDWEAVSYFY-KTSD-FQIVGDDLTVTNPLRIKEMETKWNALLIKVNQIGTLTES	
Ca1	.P....SGP.....EQ.I.E.....D..VII.FERVG.KI.....T...T...K..A.....	
Sc1	.K....GP.....HS.MKR.....H...FRTAGI...A.....K..AT...K..AD.....S...	
Sc2	.K....GVE...M.HS.MKR.....H...FRTAGI...A.....K..AT...K..AD.....S...	
Sp1	KPENK...Q..T...VE.SK.....DQ...S...TH--M.AETD.....VK.IRT...DK.CA.....SVT...	
con	L L D V L YPIVSTEDPF DDW AW QIV DDLTVTN R AI K ALLIKVNQIG ES	
AoA	IQA [*] KDSYADN [*] GWGMVSHRSGETEDAT [*] IADTAVGLRSGQIKTGAPARSERLAKINQHRIEEELGNNAIYAGEKFRITSNL	
Ca1	N...AG.....TF...LS.....SE.....KD.QKASQ. 74.9%	
Sc1	.K..Q..E.AG.....TF...LV...T.....D..VF...N..HHGDK. 72.2%	
Sc2	.K..Q..E.AN.....TF...LV...T.....D..VF...N..HHGDK. 71.3%	
Sp1	LNVRMLHAGL.....G.A.TE.SHLT..EALVL.S...C.....YEL.....SRG...AHAGKYTKAAKF 66.9%	
con	S AGVMVSHRSG T D I VG GQ K GAP RSERLAK N LRIIEELG AG	

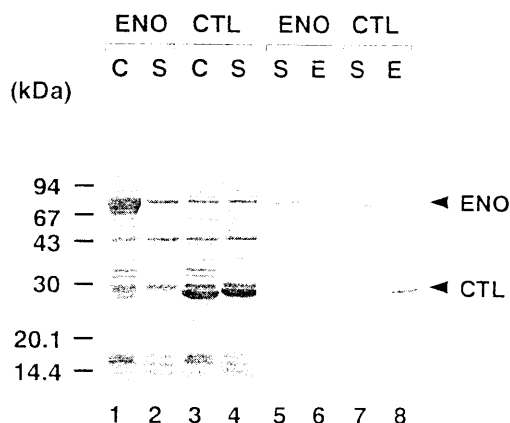


Fig. 2 Detection of *A. oryzae* enolase fused to glutathione S-transferase. Crude extract was prepared from the XL1-Blue transformant harboring the pGEX plasmid vector containing *A. oryzae* enolase cDNA fused to GST in-frame (lanes 1, 2, 5 and 6) and out of frame (lanes 3, 4, 7 and 8) as described in Materials and methods. Fusion proteins were induced by adding 1 mM (lanes 1 to 4) or 0.01 mM (lanes 5 to 8) of IPTG. Proteins in the sonic cell extract (C) (lanes 1 and 3), in the soluble fraction (S) (lanes 2, 4, 5 and 7) and the eluate from the Glutathione Sepharose 4B chromatography (E) (lanes 6 and 8) were electrophoresed on SDS-polyacrylamide gel (5–20% gradient). Proteins amounts were approximately 10 µg, except for 0.46 µg and 1.4 µg in lanes 6 and 8, respectively

Codon usage of the *A. oryzae* *enoA* gene was highly biased and virtually identical to the codon bias reported so far in *Aspergillus* genes (Ballance 1986). The preference of GGU for Gly (25 GGU in 35 Gly codons) and the rare usage of AGN (6 AGC for Ser in 438 codons) observed in highly expressed genes of *Neurospora crassa* (Ballance 1986) were also noted in the *A. oryzae* *enoA* gene. Rare usage of codons ending with A (only 7 codons ending with A among 438 codons) may possibly be a unique feature in highly expressed genes. Several codons (AGA, CAA, CCA, UGU and UUG, encoding Arg, Gln, Pro, Cys and Leu, respectively) preferred in filamentous fungi but not yeast (Ballance 1986), are also preferred in the *A. oryzae* *enoA* gene.

Assay of fusion protein for enolase enzymatic activity

The XL1-Blue transformant harboring pGEX-AOENOA, which encodes *A. oryzae* enolase fused to the C-terminus of GST, was cultured in 2×YT medium containing 2% glucose and 100 µg/ml of ampicillin at 37°C for 3 h. The expression of the GST-enolase fusion protein was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG). The transformant harboring the pGEX vector containing the same *enoA* gene in a different reading frame with an additional 15 aa at the C-terminus of GST, served as a control. The cells were disrupted by sonication and cell debris was precipitated by centrifugation. Induction of the fusion protein by 1 mM IPTG showed a strong band at a molecular weight of 74 kDa, corresponding to that of the GST-eno-

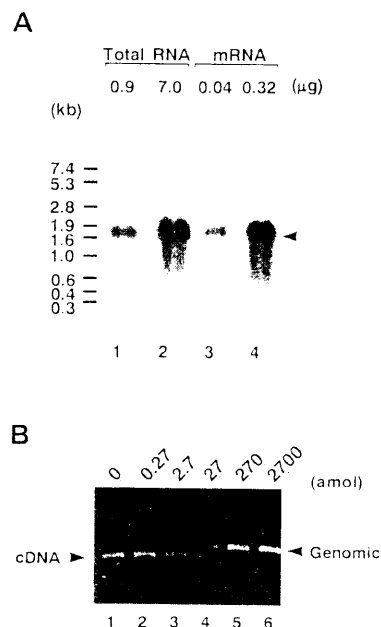


Fig. 3A, B Detection and quantitation of *A. oryzae* *enoA* mRNA. **A** Northern-blot analysis of *A. oryzae* enolase mRNA. RNA was prepared from an *A. oryzae* mycelium cultured in DP medium as described in Materials and methods. DIG-labelled RNA probe transcribed from the genomic *enoA* gene was hybridized to the blot of 0.9 µg (lane 1) or 7.0 µg (lane 2) of total RNA and 0.04 µg (lane 3) or 0.32 µg (lane 4) of poly(A) RNA. The position of enolase mRNA is indicated by an arrow at 1.7 kb. **B** competitive PCR was performed with AOENOP1 and AOENOP2 as primers and 0.4 ng of *A. oryzae* cDNA as a template. The indicated amounts of competitor DNA containing genomic *A. oryzae* *enoA* were added to the PCR reaction

lase fusion in the sonicated extract (Fig. 2, lane 1). Most of the band disappeared after centrifugation to remove bacterial debris (Fig. 2, lane 2), suggesting the formation of an inclusion body in the fusion protein. The yield of purified fusion protein through Glutathione Sepharose 4B was not significantly improved by increasing the IPTG concentration from 0.01 mM to 1 mM (data not shown). The formation of an inclusion body may thus be augmented depending on the expression of the fusion protein. The presence of an incorrectly folded fusion protein may, by copurification, reduce the enolase-specific activity of the purified fusion protein. Consequently, 0.01 mM IPTG was used for the induction whereby the formation of an inclusion body would be minimal and without significant loss of the purified fusion protein. The specific enolase activity of purified fusion protein (Fig. 2, lane 6) and for standard rat muscle enolase was 2.34 and 40 U/mg, respectively. The observed activity for the enolase fusion protein was at least 100-times the detection limit under the present experimental conditions. No enzymatic activity was observed when control fusion protein was used or when glycerate-2-phosphate or ADP was omitted from the reaction mixture.

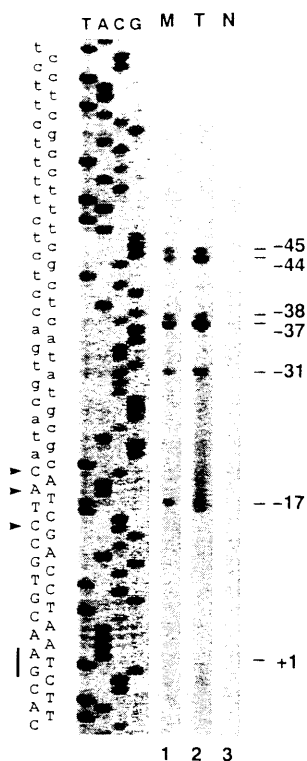


Fig. 4 Mapping of transcription initiation sites. The reverse transcription reaction was carried out by AMV reverse transcriptase using the fluorescent primer AEPX576 in the presence of poly(A) RNA (*lane 1*) or total RNA (*lane 2*) from *A. oryzae*, or without RNA (*lane 3*). The sequencing ladder was prepared by the sequencing reaction of pCAO-ENOCF1, containing a 1.6-kbp *enoA* cDNA fragment, using the same fluorescent primer as described in Materials and methods. The nucleotide sequence of the 5' flanking region of *enoA* is assigned to the left. *Upper and lower case letters* indicate regions where the corresponding ladder is derived from the 5' terminal region of *enoA* cDNA and the pCRII vector, respectively. *Solid horizontal bars* on the right and *arrow heads* on the left indicate the positions of fluorescent products by the primer extension reaction and the 5'-ends of the RACE products, respectively. *Numbers* indicate nucleotide positions when the first A of the translation initiation site is designated +1. The *vertical bar* on the left indicates the putative translation initiation site

from 0.4 ng cDNA, as shown in Fig. 3B. The pBluescript plasmid vector containing the 2.9-kbp *Bgl*/II fragment (Machida et al. 1996) of an *A. oryzae enoA* genomic fragment was linearized at the unique *Xho*I site and used as the competitor. The plasmid containing sequences identical to the annealing sites of the two primers gave an amplified fragment longer (1.6 kbp) than that from cDNA (1.3 kbp) due to the presence of four introns in the enolase-coding region. The competitor plasmid (0–2700 amol) was added in different amounts to the PCR reaction and the cDNA calculated according to Price et al. (1992). The ratio of cDNA to genomic DNA bands in lanes of 2.7 amol and 27 amol indicated the amount of cDNA corresponding to the enolase to be 12 amol. Competitive PCR using primers for the Taka-amylase gene (*amyB*) (Tada et al. 1989) showed an expression similar to that of *amyB* mRNA (16 amol, data not shown). Taka-amylase is a very strongly expressed gene in *A. oryzae* under inducible culture conditions (Tada et al. 1991) and thus *A. oryzae enoA* should also be very strongly expressed.

Mapping of the *A. oryzae enoA* 5' transcription initiation site

The start sites of *A. oryzae* enolase mRNAs were determined by primer extension analysis. The reverse transcriptase reaction was initiated from the single-stranded fluorescent primer AEPX576 hybridized to total or poly(A) RNAs. The molecular-weight standard was prepared by the sequencing reaction of pCAO-ENOCF1 containing *enoA* cDNA using the same fluorescent primer as described in Materials and methods. Transcription start sites were mapped at positions -45/-44, -38/-37, -31 and -17 relative to the putative initiation codon (Fig. 4). The results were completely identical whether total or poly(A) RNA was used, though the background level was slightly higher in total RNA than in poly(A) RNA.

Effects of the carbon source on *enoA* gene transcription

A. oryzae was grown for 22 h at 30°C in modified CD medium containing 2% glucose or 2% pyruvate. Alternatively, *A. oryzae* mycelia were pre-cultivated for 20 h at 30°C in modified CD medium containing 2% glucose and, after being washed, the mycelia were inoculated in CD medium along with various carbon sources each at a final concentration of 3%. As shown in Table 1, the mycelia were able to grow in medium containing glucose or pyruvate when polypeptone had been added, although growth on glucose was more rapid. When synthetic medium was used, mycelia grew more rapidly on dextrin or glucose than on glycerol, but did not grow on pyruvate or in the absence of a carbon source.

Transcription of the *enoA* gene was monitored by Northern hybridization using the same *enoA* probe as in Fig. 3A. Ethidium bromide staining showed the amount of RNA to be essentially the same in each sample (Fig. 5, upper

Characterization of *A. oryzae* enolase mRNA

Northern analysis of total RNA and mRNA samples from *A. oryzae* RIB40 indicated a single *enoA* transcript of 1.7 kb (Fig. 3A), this size being consistent with the 1535 bp of the 5'- and 3'-RACE PCR product plus a poly(A) tract, assuming the nt length of the poly(A) tract to be approximately 200 bp. Only several-minutes incubation for the coloring reaction led to clear and dense bands in total RNA and mRNA, depending on the amounts of RNAs, suggesting strong expression of the *enoA* gene (Fig. 3A).

To confirm the above expression, *A. oryzae enoA* mRNA was determined by competitive PCR (Gilliland et al. 1990) using the cDNA pool prepared from poly(A) RNA of *A. oryzae* (Fig. 3B). A 1.3-kbp fragment was amplified after 24 cycles using AOENOP1 and AOENOP2 primers

Table 1 Effects of the carbon source on the growth of *A. oryzae* and recovery of poly(A) RNA by oligo(dT)-cellulose column purification; nd, not determined

Medium	Carbon source	Wet weight of mycelium (g)				Recovery of poly(A) RNA (%) ^c	
		0 h	8 h	22 h	48 h	1st	2nd
Modified CD ^a	Glucose	–	nd	6.1	23	6.9	4.0
	Pyruvate	–	nd	4.0	7.1	5.0	3.5
CD ^b	Dextrin	2.0	3.1	nd	6.5	7.3	5.1
	Glucose	2.0	3.2	nd	5.8	5.4	4.0
	Glycerol	2.0	2.6	nd	4.3	4.9	3.8
	Pyruvate	2.0	1.9	nd	1.4	4.4	3.5
	None	2.0	2.2	nd	1.4	5.3	3.6

^a Approximately 3×10^8 conidia were inoculated in 500 ml of modified CD medium and cultivated at 30°C in the presence of a 2% carbon source. Mycelia after 22 h cultivation were used for total RNA extraction

^b Two grams of mycelium pre-cultivated in modified CD medium containing 2% glucose for 20 h at 30°C was washed and inoculated into 500 ml of CD medium containing a 3% carbon source. Mycelia after 8 h cultivation were used for total RNA extraction

^c Poly(A) RNA was recovered from 400 µg of total RNA by a sequential two-step purification using an oligo(dT)-cellulose column. The amounts of RNA after each purification step are indicated, with total RNA set at 100%

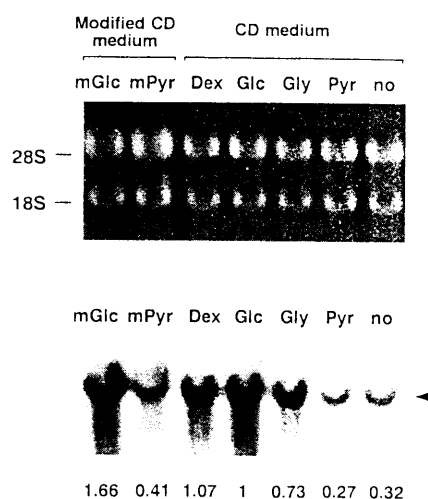


Fig. 5 Transcriptional regulation of the *enoA* gene. Total RNA was extracted from mycelia grown in modified CD medium containing 2% glucose (*mGlc*) or 2% pyruvate (*mPyr*) for 22 h at 30°C. Alternatively, mycelia incubated for 8 h in CD medium containing 3% dextrin (*Dex*), 3% glucose (*Glc*), 3% glycerol (*Gly*), 3% pyruvate (*Pyr*), or without carbon source (*no*) were used for total RNA extraction. Approximately 5 µg of total RNA were electrophoresed, stained with ethidium bromide (upper panel), and hybridized with the *enoA* probe after blotting (lower panel). The arrow indicates the position of the signal corresponding to the *enoA* mRNA. The intensity of ribosomal RNA and the hybridized bands was measured by an ARGUS image analyzing system equipped with a UV transilluminator. The amount of transcript, normalized by that of ribosomal RNA, is shown at the bottom of the lower panel below the Northern hybridization when the *enoA* mRNA level of cells from CD medium containing glucose was 1.0

panel). The 1.7-kb signal corresponding to the *enoA* transcript in the presence of glucose was approximately four-times stronger than in the presence of pyruvate when modified CD medium was used (relative ratios of 1.66 and 0.41 in Fig. 5, lower panel), suggesting transcription to be possibly induced by glucose. When mycelia were cultivated

in synthetic CD medium, band intensity in the presence of glucose was slightly less than that in modified CD medium containing glucose. As observed in modified CD medium, the transcription of pyruvate-grown cells was approximately one-fourth that of glucose-grown cells (relative ratio of 0.27 and 1) and nearly the same as that without a carbon source in synthetic CD medium (0.32). The transcription of glycerol-grown cells showed an expression intermediate between glucose-grown cells and pyruvate-grown cells.

The amounts of poly(A) RNA in the various media described above were determined by two-step purification using an oligo(dT)-cellulose column. Total RNA (400 µg) three-times less the maximum capacity of the column (approximately 1.25 mg total RNA) was used for the 1st purification. The recovery of poly(A) RNA against total RNA after the 1st and 2nd purifications was 4.4–7.3% and 3.5–5.1% (57–80% recovery in the 2nd purification), respectively, as shown in Table 1, indicating poly(A) RNA to be quantitatively recovered and highly purified by the two-step purification. The poly(A) RNA ratio of the highest recovery (5.1%, dextrin in CD medium) to the lowest recovery (3.5%, pyruvate in CD and modified CD media) for all seven samples was 1.5 and that of glucose- to pyruvate-grown cells in CD and modified-CD media, 1.1 (relative ratios of 4.0 and 3.5). Changes in the expression of the *enoA* gene under various culture conditions (about a 4-fold difference) are thus not due only to those in overall transcriptional levels (less than a 1.5-fold difference). *enoA* transcription was thus concluded to be specifically regulated by the carbon source employed.

Discussion

The DNA sequences of enolase from many organisms have been shown to be remarkably conserved from yeast to

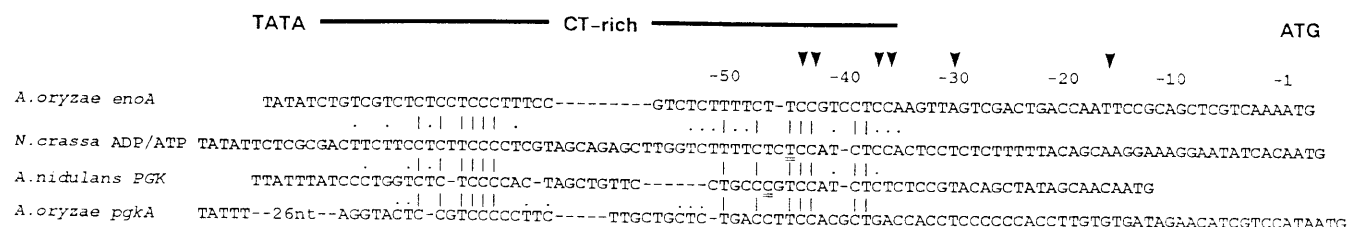


Fig. 6 Summary of transcription initiation sites and the 5' pyrimidine-rich regions. Nucleotides conserved in the pyrimidine-rich regions in all four genes, *A. oryzae enoA*, *N. crassa ADP/ATP* carrier protein gene, *A. nidulans PGK* gene and the *A. oryzae pgkA* gene, are indicated by vertical lines. Nucleotides identical to the *A. oryzae enoA* gene but not conserved in the four genes are indicated by dots. Arrow heads and double underlines represent the transcription initiation sites of *A. oryzae enoA* and those of the other genes, respectively.

mammals (Verma and Dutta 1994). A comparison of the predicted aa sequence of *A. oryzae* enolase with those of other eukaryotic enolases indicated a high degree of conservation (66.9–74.9%) as shown in Fig. 1. Homology was retained throughout the protein portion and numerous regions among the five sequences analyzed were identical. The predicted protein size (438 aa) was within the range reported for other enolases [from 433 aa for *Drosophila* (Bishop and Corces 1990) to 458 aa for human lung (Verma and Kurl 1993)] and most resembles that of *S. pombe* (439 aa; Jackson and Lopes 1995).

A comparison of the cDNA with the genomic DNA of the *A. oryzae enoA* gene (Machida et al. 1996) confirmed the presence of four introns in the protein-coding region predicted from the genomic sequence. The cDNA sequence has been shown to be completely identical to the genomic sequence of *A. oryzae* enolase following the removal of the four introns (Machida et al. 1996). Splicing sites in all introns obey the GT-AG rule of class-II introns and as shown by Ballance (1986) involved conserved intron sequences in filamentous fungi: [(1) 5'GTTAGT–45nt–GCTGAC–14nt–TAG 3', (2) 5'GTATGT–45nt–GCTGAT–19nt–TAG 3', (3) 5'GTAAGT–48nt–ACTAAC–12nt–TAG 3' and (4) 5'GTATGT–36nt–ACTGAA–20nt–CAG 3']. The lengths of the introns, (1) 74 nt, (2) 79 nt, (3) 75 nt and (4) 71 nt, are essentially the same as those of other filamentous fungi (Orbach et al. 1986).

The recombinant fusion protein produced from a clone harboring 1.3-kbp cDNA (pCR2-AOENOA) showed enolase enzymatic activity. This fusion protein was 1.6-times larger than that of the native enolase and thus the specific activity of GST-enolase fusion (3.62 U/mg) was 11-times less than that of rat muscle enolase (40 U/mg). Sundstrom and Aliaga (1992) reported the *C. albicans* GST-enolase fusion to have a specific activity of 49.7 U/mg, which is comparable to that of *S. cerevisiae* enolase. *C. albicans* fusion protein contains a space of more than 10 aa between the GST C-terminus and N-terminus of *C. albicans* enolase (Sundstrom and Aliaga 1992). Since *A. oryzae* enolase was prepared by PCR so as to make a *SalI* site immediately in front of the translation initiation codon, the GST-

enolase fusion of *A. oryzae* contained only two aa between GST and enolase. Hardly any of the GST-enolase fusion of *A. oryzae* could be recovered from a soluble fraction by induction with 1 mM of IPTG, whereas the GST-enolase fusion of *C. albicans* grown under normal induction conditions could be recovered. The insertion of a short aa space in the *A. oryzae* fusion protein may de-stabilize the GST-enolase fusion and so decrease the enolase-specific activity.

Competitive PCR showed the expression of the *enoA* gene to be comparable to that of the Taka-amylase gene (*amyB*), which is very strongly expressed in *A. oryzae* (Tada et al. 1991). *A. oryzae* enolase mRNA constituted approximately 3% (w/w) of the total mRNA. Preliminary screening of a λ gt10 library of *A. oryzae* cDNA with the DIG-labelled *enoA* RNA probe showed approximately 500 clones to be positive among 19 000 (2.6% positive). Thus, it may be concluded that *enoA* is a very strongly expressed gene in *A. oryzae*.

cDNAs generated by primer extension were mapped at six major sites, at positions –45/–44, –38/–37, –31 and –17 relative to the putative initiation codon (Fig. 4). The 5'-flanking region of the three independent clones of the RACE product terminated at –20, –18 and –14 (Fig. 4). Short cDNAs are preferentially amplified in a PCR mixture (Schaefer 1995) and the adaptor-ligation method fails to correctly produce full-length cDNAs (Edwards et al. 1991). The lengths of RACE products, whose 5' termini are distributed about the shortest products by primer extension, are thus considered reasonable.

Multiple transcription initiation has been noted in several other genes from filamentous fungi which possess a CT-rich region (Kinnaird and Fincham 1983; Boel et al. 1984; Mullaney et al. 1985), and is a common feature in yeast genes (Sentenac and Hall 1982) including enolase (Holland et al. 1983; Mason et al. 1993). The strongly expressed *A. oryzae enoA* gene should thus contain multiple transcription start sites.

Transcription initiation sites were mapped between the TATA box and AUG codon immediately downstream from the CT-blocks in the *enoA* gene (Fig. 6) as is the case with other fungal genes (Kinnaird and Fincham 1983; Arends and Sebald 1984; Clements and Roberts 1986). A stretch of pyrimidine-rich residues may improve the yield of functional mRNAs and limit nucleotide triphosphate consumption by lessening the aberrant transcriptional initiation of highly expressed genes in yeast (McNeil 1988). The results of primer extension in the present study suggest the same mechanism to effectively produce functional mRNAs of highly expressed genes in *A. oryzae*. Sequences in the

CT-rich region of *A. oryzae enoA* are highly homologous to those of other highly expressed genes of filamentous fungi such as the *N. crassa* ADP/ATP carrier protein gene (Arends and Sebald 1984) and the *Aspergillus nidulans* *PGK* gene (Clements and Roberts 1986), and is also homologous to the recently sequenced *A. oryzae pgkA* gene (Ogawa et al. 1994, Accession No. D28484) (Fig. 6). Thus, possibly, the sequence of the pyrimidine-rich region may be essential for relatively high gene expression in filamentous fungi.

enoA gene transcription appears inducible by glucose in contrast to cases with other glyconeogenic carbon sources such as glycerol and pyruvate, as has been observed for the *ENO2* gene of *S. cerevisiae*. In the present study, growth rate was affected by culture conditions and closely related to the transcription of *enoA*. However, since poly(A) RNA amounts were almost the same in all cells grown with different carbon sources and under varied culture conditions (Table 1), expression of the *enoA* gene was shown to be specifically regulated by the carbon source and to be growth-rate dependent. The correlation between *enoA* transcription and growth rate is reasonable since enolase is a house-keeping gene and *A. oryzae* most likely possesses only a single copy of it (Machida et al. 1996).

This is the first report to present results of the analysis of the glycolytic gene from *A. oryzae* and the cloning of enolase cDNA in filamentous fungi. The *A. oryzae* enolase gene was shown to be strongly expressed, and expression was regulated by the carbon source in the medium. Further analysis of the regulatory mechanism of the 5'-flanking region of the *enoA* gene will be important for the strong expression of foreign genes in *A. oryzae* and for the regulation of house-keeping genes in fungi.

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