Note

Molecular Cloning of a Genomic DNA for Enolase from *Aspergillus oryzae*

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We have isolated an enolase gene (enol) from *Aspergillus oryzae* by heterologous hybridization using the corresponding *Saccharomyces cerevisiae* ENO2 gene as a probe. A 2.9-kb BglII fragment contained the entire structural gene enol including 5′- and 3′-flanking regions. The homology between *A. oryzae* enol and *S. cerevisiae* ENO2 genes is 66.9% when introns are removed. Genomic Southern analysis indicated that there is only one enolase gene in *A. oryzae*.

**Key words:** enolase; *Aspergillus oryzae*; genomic DNA

*Aspergillus oryzae* is an important filamentous fungus used in the Japanese beverage and fermentation industries, as well as to produce industrially valuable hydrolytic enzymes. *A. oryzae* is considered to be a non-pathogenic and favorable host for heterologous protein production13 because of its ability to secrete a large amount of proteins.14 However, the lack of basic knowledge about its gene structure and regulation of gene expression makes it difficult to put *A. oryzae* to more extensive industrial use.

Enolase has been reported to be one of the most highly expressed proteins in some organisms including yeast, especially those in which there is a high rate of glycolysis.15 It is, therefore, expected that the *A. oryzae* enolase gene may be also highly expressed depending on its strong promoter activity, and that the 5-flanking region of its gene may be useful for gene expression and transformation studies with *A. oryzae*.

The 1.2-kb 4rcl-fragment of pACYC177-ENO138, which contains the entire *S. cerevisiae* enolase 2 structural gene, ENO2,16 was recovered and subcloned onto pBluescript. A DIG-labeled RNA probe, which had a more sensitive signal than the DIG-labeled DNA probe in our experiments, was transcribed by T7 RNA polymerase after restriction digestion to exclude contamination by the vector sequence in the RNA probe. A 4.5-kb EcoRI fragment was detected on the Southern hybridization of complete *EcoR*1 digests of genomic DNA from *A. oryzae* strain R1B40.17 Approximately 30,000 plaques of the 5′10 genomic library containing 3.5-6.0 kb EcoRI-fragments of *A. oryzae* DNA were screened and three positive clones were isolated. A restriction map of the insert showed that the three positive clones had a common 4.5-kb EcoRI fragment. However, since the fragment contained only the downstream half of the enolase gene, 5000 plaques from the genomic gene library inserted into JM36L3 were re-screened by hybridization with the probe prepared from the downstream half of the *A. oryzae* enolase gene. Three positive clones were selected and the 2.9-kb BglII-fragment common in all the three clones was subcloned into pBluescript and sequenced (Fig. 1). Because the three JM36L3 clones seemed to be derived from a single clone of an original library as judged from the restriction digestion, the presence of the upstream half of the BglII-EcoRI fragment was confirmed by cloning the upstream 12-kb EcoRI fragment by screening the 5′10 library containing the EcoRI fragment of *A. oryzae* DNA.

Comparison of the nucleotide (nt) sequence of the 2.9-kb BglII fragment and the *S. cerevisiae* ENO2 gene showed that the coding region of the *A. oryzae* enolase gene (enol) consisted of five exons interrupted by four introns and encoded 438 amino acids (aa) residues. When these four introns were removed from the nt sequence, the homology between both genes is 66.9%, and the deduced aa sequence had a high degree of homology (71.3%, identical) with that of *S. cerevisiae* enolase 2 protein.

All four introns start with a GT and end with an AG sequence, which is known to be a general feature of introns.18–20 Sequences homologous to the internal consensus sequence, PuCTPuAC, found in the 3′ terminus of the fungal subsequences20 were also observed in all the four intervening sequences.

The sequence around the AUG codon (underlined in 5′-CAAUAUGGC) which is thought to be important in the efficiency of translation initiation in eukaryotes20 closely resembles the consensus sequence for filamentous fungi (5′-CACAACUGCCG28) and the consensus sequence 5′-AAAUGG in glycolytic genes of *S. cerevisiae*.11

The 5′-flanking region contained a putative TATA box at nt −94 from the start codon, but did not contain a putative CATT sequence at the expected region. The C + T-rich motif between TATA-box and start codon ATG, which is found especially in highly expressed genes, such as the *Aspergillus niger* pgk gene,21 was found in the enolase promoter. These results together with the several reports about the expression of enolase, strongly suggest that *A. oryzae* enolase is highly expressed under transcriptional control.

The RAPI-binding sequence13 (5′-RMACCCANNCCYY, where R is A or G, M is A or C, and Y is T or C) which is observed in the transcription regulatory region of *S. cerevisiae* ENO1 and ENO2 genes, was not observed in the 5′-flanking region of *A. oryzae* enol gene. This suggests that the recognition sequence of the factor may be different from that of *S. cerevisiae* or that the general transcription factor, such as Rap1p,13 may not be present in *A. oryzae*.

The consensus sequence 5′-AATTTA thought to be a polyadenylation signal of higher eukaryotes13 was found at the position +195 in the 3′-flanking region of the enolase gene. The unique inverted repeats related to the function of the transcription termination13 were not found in the 3′-flanking region.

Genomic Southern analysis using *A. oryzae* enol4 gene as a probe showed a single band on any restriction digests of *A. oryzae* DNA (Fig. 2). This indicates that there is only one enolase gene in *A. oryzae* while there are the two enolase structural genes, ENO1 and ENO2, per haploid genome of *S. cerevisiae*. Since the yeast genes are regulated in different manners, it is also interesting to know how the *A. oryzae* gene is regulated.
Fig. 1. Restriction Map and nt Sequence of A. oryzae eno A gene.

(A) Restriction map of the isolated 2.9-kb BglII fragment containing the A. oryzae eno A gene. The protein-coding regions (exons) are indicated by solid boxes. The direction of transcription is shown by an arrow. B: BglII; Sc, SacI; N, NcoI; S, SalI; H, HindIII; Sm, Smal; P, PstI; E, EcoRI. The 2.4-kb EcoRI fragment of the insert in the isolated phase clone was subcloned into pBluescript. (B) The nt sequence of the enolase gene and the deduced aa sequence. A set of unidirectional deletions of the BglII fragment was constructed in both directions using the restriction sites. Nucleotide sequencing was done by the dideoxynucleotide chain termination method using automated DNA sequencers (Applied Biosystems Model 373A and L1 COR Model 4000L). Localization of the coding region and positions of the introns were deduced from the sequence homology with the E. no2 gene of S. cerevisiae. Putative introns and 5'- and 3'- untranslated regions are written in lower-case. Putative TATA in the 5'-flanking region and a polyadenylation signal in the 3'-flanking region are doubly underlined. The sequences homologous to the internal consensus sequence, PuTPuAC, in the intervening sequences are singly underlined. The nt sequence data reported will appear in the GenBank EMBL and DDBJ Nucleotide Sequence Database under the accession No. D00941.
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**References**