Structure and Regulatory Expression of A Single Copy Alternative Oxidase Gene from the Yeast *Pichia anomala*

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To investigate the regulatory mechanism of alternative oxidase gene expression, genomic DNA was cloned from the yeast *Pichia anomala*. Genomic Southern blot analysis suggested that a single copy nuclear gene encoded an alternative oxidase in the yeast. The nucleotide sequence showed an uninterrupted coding region for the alternative oxidase protein. In the upstream region from the transcription initiation site found by primer extension analysis, CCAAT, TATAA, and UAS2-like elements were detected. The UAS2 is the element involved in transcriptional regulation by carbon source and the target site for the factor, HAP2/3/4/5 protein complex, in *Saccharomyces cerevisiae*. By a gel mobility shift assay, a specific retardation band was detected when a protein extract from cells grown on an inducing carbon source was incubated with a UAS2-containing probe. These results suggest that carbon source regulation of alternative oxidase gene expression is mediated by the UAS2-like element and a HAP-like factor in *P. anomala*.

Key words: alternative oxidase; cyanide-resistant respiration; fungi; mitochondria; nuclear gene

Alternative oxidase is a cyanide-resistant terminal oxidase and constitutes a “cyanide-resistant,” “cyanide-insensitive,” or “alternative” respiratory pathway. In many higher plants and eucaryotic microorganisms, including yeast, filamentous fungi, green algae, amoebae, and trypanosomes, an alternative oxidase has been found and identified. This second terminal oxidase transfers electrons from ubiquinone to oxygen to produce water in a cyanide-resistant and salicylhydroxamate (SHAM)-sensitive way. Since the enzyme is not coupled to oxidative phosphorylation, the alternative pathway is thought to generate heat.

Alternative oxidase is a highly regulated enzyme. At the enzyme level, pyruvate and diithiothreitol (DTT) activate alternative oxidase in higher plants. The enzyme is also activated by purine nucleoside 5’-monophosphate in yeast and amoeba. Expression of the alternative oxidase gene is also highly regulated. Namely, transcription of this gene changes dramatically in response to various environmental conditions. Inhibition of mitochondrial cyanide-sensitive respiratory pathway by antimycin A induces alternative oxidase gene expression in yeast, tobacco, and *Arabidopsis thaliana*. Inhibition of mitochondrial protein synthesis by chloramphenicol induces alternative oxidase mRNA transcription in *Neurospora crassa*. These phenomena are very interesting because the function or state of mitochondria affects nuclear gene expression. Under physiological conditions, expression of yeast alternative oxidase gene is regulated by osmolarity, sulfur-containing compounds, and carbon source. In higher plants, alternative oxidase gene transcription is induced by cold temperature or some natural chemicals such as hydrogen peroxide, salicylate, citrate, and cysteine. To study regulatory mechanism of alternative oxidase gene expression, structure and expression of alternative oxidase gene were investigated in the yeast *Pichia anomala*.

Materials and Methods

Construction and screening of genomic DNA Library. *Pichia anomala*, former *Hansenula anomala*, strain LKBY-1 was grown aerobically and spheroplasts were prepared as described previously. High-molecular-weight DNA was purified from the spheroplasts by the method described by Murray and Thompson. After partial digestion of DNA with *Sal*I and size fractionation by sucrose density gradient centrifugation, the resulting 15-kbp DNA fragments were inserted into BamHI arms of the lambda GEM12 vector (Promega, USA). The library was screened by plaque hybridization with the 32P-labeled 0.6-kbp BamHI--SacI fragment of *P. anomala* alternative oxidase cDNA.

Genomic Southern blot hybridization. *P. anomala* genomic DNA was completely digested with *Bam*HI, *Eco*RI, *Sal*I, *Kpn*I, or *Xho*I. Resulting restriction fragments from 10 μg of DNA were separated by 0.6% agarose gel electrophoresis and transferred to a nylon membrane. Hybridization and detection were done with the Gene Images labeling and detection system (Amerham, UK). The 1.2-kbp *Bam*HI--*Sac*I fragment of pALXHAn6 covering most of the protein coding region was used as the hybridization probe.

Primer extension analysis. The synthetic primer, TTTTCAAGGATCTGACCTACAAGCAGAG, was radiolabeled at 5’-terminus by T4 polynucleotide kinase and [γ-32P]ATP. Total RNA (30 μg) prepared from antimycin A-treated yeast was hybridized with 2 × 107 cpm

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Abbreviations: DTT, diithiothreitol; PIPES, piperazine-1,4-bis(2-ethanesulfonic acid); SHAM, salicylhydroxamic acid
of primer in 25 μl of 0.4 M LiCl, 1 mM EDTA, and 40 mM piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES)-NaOH buffer (pH 6.4) at 42°C for 16 h. After RNA-primer hybrid was recovered by ethanol precipitation, extension reaction was done in a 6-μl reaction mixture composed of 60 mM KCl, 10 mM MgCl₂, 5 mM DTT, 1 mM dNTPs, 33 μg/ml actinomycin D, 0.25 U/μl RNASin, 3.3 U/μl AMV reverse transcriptase-XL, and 20 mM Tris-HCl buffer (pH 8.3) at 43°C for 90 min. To produce the sequencing ladder, the dideoxynucleotide chain termination reaction was done after the same amounts of the end-labeled primer were annealed with 0.5 μg of pALXHan6, which carried the 3.7-kbp EcoRI-EcoRV fragment on pBluescript SK(+). Primer extension and dideoxy sequencing products were analyzed by denaturing polyacrylamide gel electrophoresis and autoradiography.

Gel retardation assay. P. anomala was grown in medium composed of 2% Bacto-peatone, 1% Bacto-yeast extract, and 3% carbon source. Exponentially growing cells were harvested by centrifugation. Protein extracts were prepared after disruption of cells with glass beads as described by Zhang and Guarente except that protease inhibitors were omitted. Probe DNA was prepared by labeling 5'-termini of synthetic double stranded DNA of the sequence CGTTTTGAACCAATTCAATACAAATA with ³²P by T4 polynucleotide kinase reaction. This probe DNA contained core UAS₂, namely the sequence TGAATTGGT. Binding reactions were done in 5 μl of reaction mixture composed of 50 mM NaCl, 4 mM MgCl₂, 0.5 mM DTT, 5% glycerol, 0.01% bromophenol blue, 50 ng/μl sonicated salmon sperm DNA, 5 mM HEPES-KOH buffer (pH 7.9), 0.1 ng/μl probe DNA, and 10 μg/μl protein extract. After 1 h of incubation at 25°C, products were electrophoresed on 5% polyacrylamide gel and autoradiographed as described.

Other methods. Protein was measured by the method of Lowry et al. using bovine serum albumin as the standard. DNA handling was done by established methods.

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**Results**

Among 5 × 10⁴ plaques of genomic library screened, nine clones hybridized with the probe for *P. anomala* alternative oxidase. Three overlapping clones were selected to make a restriction enzyme map of the alternative oxidase gene and its flanking region (Fig. 1). *P. anomala* genomic DNA was analyzed by genomic Southern blotting after digestion with *BamHI*, EcoRI, *SalI*, *KpnI*, or *XhoI*. A single DNA fragment was detected for each enzyme (Fig. 2). Further, the size of these hybridized fragments was identical with that deduced from the restriction enzyme maps shown in Fig. 1 and reported previously. These results suggested that the *P. anomala* alternative oxidase was encoded by a single copy nuclear gene.

Since the 3.7-kbp EcoRI-EcoRV fragment seemed to contain an entire alternative oxidase gene (Fig. 1), the complete nucleotide sequence of this fragment from the clone *ALXHan6* was analyzed. As shown in Fig. 3, this fragment contained an uninterrupted coding region for the alternative oxidase precursor protein. The transcription start site for this gene was analyzed by the primer extension method (Fig. 4). The longest product seemed to represent the transcription initiation site. It was in the position at -48 from the first nucleotide of the protein.

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**Fig. 1.** Restriction Enzyme Maps of Genomic Clones for *Pichia anomala* Alternative Oxidase.

The arrow head shows the position and orientation of alternative oxidase coding region.

**Fig. 2.** Genomic Southern Blot Analysis of the Yeast Alternative Oxidase Gene.

Total yeast DNA (10 μg) was digested completely with the indicated restriction enzyme and Southern blotted as described under "Materials and Methods".
Fig. 3. Nucleotide Sequence of Alternate Oxidase Gene and Its Flanking Region.

Nucleotides indicated by italics correspond to previously reported cDNA sequence (DDBJ/EMBL/GenBank databases accession number D00741). TATAA, CCAAT, and UAS2-like elements are shown by underline, double underline, and outline, respectively. The downward triangle indicates the transcription initiation site identified by primer extension analysis. Sequence element for yeast polyadenylation signal is shown by dotted nucleotides. Although upstream sequence from the position at −811 is excluded here, complete nucleotide sequence of the 3.7-kbp EcoRI-EcoRV fragment of pALXHan6 will appear in the alternate accession number AB026726.
coding region (Fig. 3). Several shorter extension products might be due to immature termination of the extension reaction or termination at the degraded 5'-end.

In the upstream part from the alternative oxidase gene (named ALX1), another reading frame was found (Fig. 3). A database search showed that the upstream partial reading frame encoded a protein similar in sequence to ubiquitin carboxyl-terminal hydrolases. These two reading frames were separated by 745 bp of spacer region. In the spacer region, there were some interesting sequence elements. Namely, TATAA and CCAAT boxes were found at −71 and −227, respectively (Fig. 3). Besides these basic promoter elements, a sequence matching with UAS2 consensus, TNRTTGTT or complementary ACCAAYNA, was discovered at position −161 (Fig. 3). This sequence TGATTGGTT was identical with the sequence of UAS2UP1, the most typical USA2 in Saccharomyces cerevisiae. The UAS2 was originally identified in the upstream region of the S. cerevisiae CYC1 gene as an element responsible for carbon source regulation of CYC1 transcription. In deed, expression of P. anomala alternative oxidase gene was regulated by the carbon source as reported previously. Namely, alternative oxidase gene expression was repressed by glucose but induced by glycerol, lactate, or raffinose. Two classes of polyadenylation sites were identified in S. cerevisiae. One class is the consensus sequence TTTTAT. In the 3'-untranslated region from the alternative oxidase gene, another class of the yeast polyadenylation signals, namely the sequence TAG–TAG–TAT, was found (Fig. 3).

To detect a transcription factor involved in carbon source control, gel mobility shift assays were done (Fig. 5). Protein extracts prepared from cells grown in medi-
um containing glucose, glycerol, or raffinose were incubated with the UAS2-containing probe DNA and then analyzed by polyacrylamide gel electrophoresis. Although most of the retardation bands were common among the three carbon sources, one band appeared to respond to inducing carbon sources (Fig. 5). Since this band disappeared when unlabeled probe DNA was added to the binding assay (Fig. 5, lanes 5 and 6), the interaction between the probe and factor should be specific to the sequence. These results suggested that the UAS2-like sequence mediated regulatory expression of the *P. anoma la* alternative oxidase gene by carbon source. The faster migrating band seemed to be due to nonspecific binding because it did not compete with the competitor. Another major retardation band did not change by carbon source but competed with the competitor RNA. In *S. cerevisiae*, an immature UAS2 binding protein complex lacking the transcriptional activation subunit usually formed a faster migrating retardation band. It might be possible that the third retardation band contained an immature protein complex.

**Discussion**

In higher plants, a small gene family encodes alternative oxidases. Typically, expression of each gene is regulated differentially. There are at least three alternative oxidase genes which are differentially expressed during development in soybean. In *A. thaliana*, only one gene is induced by antimycin A- or myxothiazol-treatment, though there are four alternative oxidase genes. In rice plants, two alternative oxidase genes have been identified. It is reported that expression of both genes is induced by low temperature. In other than higher plants, two alternative oxidase genes are reported in *Chlamydomonas reinhardtii* recently. This study suggests that a single copy nuclear gene encodes the *P. anoma la* alternative oxidase. In contrast to higher plants, the alternative oxidase gene is also reported as a single copy gene in filamentous fungi. Expression of the fungal alternative oxidase gene is highly regulated in respond to various environmental conditions. It is, therefore, interesting to note that only one alternative oxidase gene responds to all these conditions in fungi.

In this study, the first example of a possible controlling element involved in alternative oxidase gene expression is presented. Namely, the UAS2-like sequence is found in the upstream region of the *P. anoma la* alternative oxidase gene and suggested to be involved in the carbon source response of this gene. In *S. cerevisiae*, UAS2-like elements are identified upstream from several nuclear genes encoding mitochondrial protein. The UAS2 is also known as the target site for the transcription factor called HAP2/3/4/5 protein complex. HAP2 and HAP3 subunits are shown to retain DNA binding activity. HAP4 functions as a transcriptional activation subunit. When the carbon source is changed from glucose to lactate, the protein complex binds to UAS2 and activates transcription of these genes. Behavior of *P. anoma la* UAS2-binding factor in the gel mobility shift assay was similar with that of HAP2/3/4/5 complex. It seems likely, therefore, that the HAP2/3/4/5 homologue and the UAS2-like element mediate the carbon source regulation of alternative oxidase gene expression in *P. anoma la*. A transcription factor similar to the HAP complex is reported in the yeast *Kluyveromyces lactis* and the filamentous fungus *Aspergillus nidulans*. Transcriptional controlling systems mediated by HAP complex may be common among fungi.

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**References**


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