An n-Alkane-Responsive Promoter Element Found in the Gene Encoding the Peroxisomal Protein of Candida tropicalis Does Not Contain a C₆ Zinc Cluster DNA-Binding Motif

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When an asporogenic diploid yeast, Candida tropicalis, is cultivated on n-alkane, the expression of the genes encoding enzymes of the peroxisomal β-oxidation pathway is highly induced. An upstream activation sequence (UAS) which can induce transcription in response to n-alkane (UAS_ALK) was identified on the promoter region of the peroxisomal 3-ketoacyl coenzyme A (CoA) thiolase gene of C. tropicalis (CT-T3A). The 29-bp region (from −289 to −261) present upstream of the TATA sequence was sufficient to induce n-alkane-dependent expression of a reporter gene. Besides n-alkane, UAS_ALK-dependent gene expression also occurred in the cells grown on oleic acid. Several kinds of mutant UAS_ALK were constructed and tested for their UAS activity. It was clarified that the important nucleotides for UAS_ALK activity were located within 10-bp region from −273 to −264 (5′-TCTTCGCACAC-3′). This region did not contain a CGG triplet and therefore differed from the sequence of the oleate-response element (ORE), which is a UAS found on the promoter region of 3-ketoacyl-CoA thiolase gene of Saccharomyces cerevisiae. Similar sequences to UAS_ALK were also found on several peroxisomal enzyme-encoding genes of C. tropicalis.

Candida tropicalis (strain pK233) is an asporogenic diploid yeast, which can utilize n-alkanes as the sole carbon and energy source. During utilization of n-alkanes or fatty acids, a profound development of peroxisomes occurs in the cells, which is a major characteristic of this yeast (26). Enzymes localized in peroxisomes, such as the enzymes of the fatty acid β-oxidation pathway and of the glyoxylate pathway, are also induced along with the peroxisome proliferation (14, 37).

Thiolase is an enzyme which catalyzes the final step of the β-oxidation pathway. There are three thiolase isozymes in n-alkane-grown C. tropicalis: two acetoyctyl coenzyme A (CoA) thiolases (thiolase I), one of which is localized in cytosol (Cs-thiolase I) and one of which is localized in the peroxisome (Ps-thiolase I), and one peroxisomal 3-ketoacyl-CoA thiolase (thiolase III) (17–19). Only Cs-thiolase I is found in the cells grown on glucose. Cs-thiolase I and Ps-thiolase I are encoded (thiolase III) (17–19). Only Cs-thiolase I is found in the cells grown on glucose. Cs-thiolase I and Ps-thiolase I are encoded by the same pair of alleles (CT-T1A and CT-T1B) (9, 16), and expression of the genes is highly induced on n-alkane, whereas low but finite expression occurs in cells grown on glucose (10). Thiolase III is encoded by another pair of alleles (CT-T3A and CT-T3B) (10), and their expression is highly induced on n-alkane but completely repressed on glucose.

In Saccharomyces cerevisiae, induction of peroxisomal 3-ketoacyl-CoA thiolase (encoded by FOX3/POT1) is mediated via an upstream activation sequence (UAS) called the oleate activation sequence (ORE) (5), which enables us to introduce exogenous DNA into C. tropicalis with a form of episomal vector (6).

In this study, using the transformation procedure and the episomal vector system developed for C. tropicalis, we have identified a UAS, which can induce transcription in response to n-alkane (designated UAS_ALK), on the promoter region of CT-T3A. In comparing its sequence with that of ORE, the possibility was suggested that the molecular mechanism inducing peroxisomal 3-ketoacyl-CoA thiolase in C. tropicalis was essentially different from the ORE-mediated induction mechanism in S. cerevisiae.

MATERIALS AND METHODS

Strains and media. C. tropicalis SU-2 (ATCC 20913) (ura3/a/ura3b) (5), derived from C. tropicalis pK233 (ATCC 20336), was used as a host strain for transformation. Escherichia coli strain DH5α (29) was used for gene manipulation.

C. tropicalis was cultivated aerobically at 30°C in a medium containing glucose (16.5 g/liter), n-alkane mixture (C₁₀ to C₆: 10 ml/liter), oleic acid (5 ml/liter), glyceral (20 g/liter), sodium acetate (13.6 g/liter), sodium propionate (10 g/liter), or sodium butyrate (11 g/liter) as the sole carbon source (15, 39). The pH was adjusted to 5.2 for glucose, n-alkane, oleic acid, and glyceral media or to 6.0 for acetate, propionate and butyrate media. Tween 80 (0.5 ml/liter) was added to the n-alkane and oleic acid media. The basic composition of the medium was as follows: 5.0 g of NH₄H₂PO₄, 2.5 g of KH₂PO₄, 1.0 g of MgSO₄·7H₂O, 0.02 g of FeCl₃·6H₂O, and 1.0 ml of corn steep liquor per liter of tap water (39).

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Plasmid construction. Lac4 encoding Kluyveromyces lactis β-galactosidase was amplified using primers 5'-AAGCTTCGAGGTTAACTATCTGATGGATTCTGAGC-3' and 5'-CTGCTTCGAGCTTAACTACTGATTGCTGATGCTG-3'. The genomic DNA of Kl. lactis IFO1267 (ATCC5558) was used as a template DNA. The amplified Lac4 fragment cut with SalI and XhoI was inserted into the SalI site of pUC-UR3, in which the 1.7-kbp C. tropicalis UR3 was inserted into pUC19 (11), and the subclone was named pUL4. The ARS of C. tropicalis 1098 was amplified using primers 5'-AAAAGTCGACATTTGGTGGGTCTGCCCCCC-3' and 5'-TCGGCTGACGTAGAAAGCATCGACCTCTGACAGAC-3', and one of the following primers: T3(270S), or T3(310S), and one of the following primers: T3(270S), or T3(310S), or T3(281S). The amplified fragment cut with T3(270S), T3(310S), or T3(281S) was inserted into the XhoI site of Bluescript-Xh (named Bluescript-ARS). Bluescript-ARS was cut with KpnI, treated with T4 DNA polymerase (blunting), digested with SalI, and a 1.4-kbp fragment containing ARS was eluted. This fragment was inserted into the XhoI site of Bluescript-Xh (named BlueScript-ARS). BlueScript-ARS was cut with KpnI, treated with T4 DNA polymerase (blunting), digested with SalI, and a 1.4-kbp fragment containing ARS was eluted. This fragment was ligated with the SalI-SmaI fragment of pUL4 containing C. tropicalis UR43 and Lac4, to make pUL4.

All deletion fragments were prepared either by PCR with pT7B9 (11) as a template or by annealing of two oligonucleotides. All the oligonucleotides used in this study are listed in Table 1. PCR was performed using primer PRT3A1 and one of the following primers: T3(550S), T3(473S), T3(382S), T3(343S), T3(310S), T3(289S), T3(270S), or T3(260S).

### Table 1. Oligonucleotides used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRT3A1-1</td>
<td>5'-CATGTCGAGGAGTGGTTTGGTAATGTTGC-3'</td>
</tr>
<tr>
<td>T3(550S)</td>
<td>5'-TGGTACCGCCCGGCTACCAATCTATCGTCCTCCA-3'</td>
</tr>
<tr>
<td>T3(473S)</td>
<td>5'-TCGTCGACTCTTGTTGAGGTTGGGTCGCC-3'</td>
</tr>
<tr>
<td>T3(407S)</td>
<td>5'-CAAGCTGCAATCGGCTTTGATGGGCG-3'</td>
</tr>
<tr>
<td>T3(382S)</td>
<td>5'-CGGTCGACACCAGTCGATTTCCATGAT-3'</td>
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<tr>
<td>T3(343S)</td>
<td>5'-GTCGCAGTTACTGCCGCCCTTGGC-3'</td>
</tr>
<tr>
<td>T3(310S)</td>
<td>5'-GGTCGACTCAAACTGGCATAAAAGT-3'</td>
</tr>
<tr>
<td>T3(281S)</td>
<td>5'-CAAGTCGACGACAGCAGACACGATGCTGACACAC-3'</td>
</tr>
<tr>
<td>T3(270S)</td>
<td>5'-ACAGTTCGACTGCAACACACCTGGTCTG-3'</td>
</tr>
<tr>
<td>T3(260S)</td>
<td>5'-TGGTTGCAGAAAGACCTCGGTATAA-3'</td>
</tr>
<tr>
<td>T3(230S)</td>
<td>5'-CCACGTCAGAGGGAACCCCAAGATGCG-3'</td>
</tr>
<tr>
<td>T3(289S)</td>
<td>5'-CTTCGTCAGGTTGTTGACGATGCTGTCG-3'</td>
</tr>
<tr>
<td>T3(270S)</td>
<td>5'-TGGTTGCAGAAAGACCTCGGTATAA-3'</td>
</tr>
<tr>
<td>T3(260S)</td>
<td>5'-CCACGTCAGAGGGAACCCCAAGATGCG-3'</td>
</tr>
<tr>
<td>T3(230S)</td>
<td>5'-CTTCGTCAGGTTGTTGACGATGCTGTCG-3'</td>
</tr>
</tbody>
</table>

Sequenced. The annealed fragments were filled in with the Klenow fragment, cut with SalI and XhoI, and introduced into the SalI site of pUTA230. The nucleotide sequences of all the deletion fragments were checked using ABI DNA sequencer model 373.

β-Galactosidase assay. β-Galactosidase activity was determined by measuring the hydrolysis of 4-methylumbelliferyl-β-D-galactopyranoside (MUG; Molecular Probes) (2, 42). Enzyme solution (50 μl) in Z buffer (940 μl) (24) was incubated at 30°C for 1 min, 10 mM MUG solution (10 μl) was added, and the increase in fluorescence was measured with a Hitachi Fluorophotometer model 650-10S (excitation, 360 nm; emission, 495 nm). 7-Hydroxy-4-methylcoumarin (Molecular Probes) dissolved in 100 mM sodium phosphate buffer (pH 7.0) was used as the reference standard. All activities are the mean values of at least two experiments.

Other methods. Transformation of C. tropicalis was carried out by electroporation (1,000 V, 25 μF, and 300 μF) (11). The protein concentration was assayed by the Bradford method using bovine serum albumin as the standard (1).

Nucleotide sequence accession number. Nucleotide sequence data of the region of CT-3A and CT-3B will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers AB025667 and AB025668, respectively.

RESULTS

To evaluate the activity of promoter elements to induce transcription in C. tropicalis, pUL4, a shuttle vector, which can replicate in both E. coli and C. tropicalis, was first constructed by the method described in Materials and Methods. pUL4 contains an ARS from C. tropicalis (6), URA3 of C. tropicalis (11), and Lac4 encoding β-galactosidase of K. lactis (27). In Candida yeasts, Lac4 instead of LacZ has usually been used as the source of the β-galactosidase gene (20, 21, 23), because several Candida yeasts translate the CUG codon as Ser instead of Leu, and Lac4 contains fewer CUG codons than LacZ does (3 for Lac4 and 53 for LacZ). A multiloning site was introduced before the translation initiation codon of

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so that the promoter sequence to be tested could be inserted.

The nucleotide sequences of the upstream regions of CT-T3A and CT-T3B (about 1.5 kbp) were determined. A 550-bp upstream region of CT-T3A (from −1 to −550; UPR-T3A) was introduced into pUAL4, and the resulting plasmid (pUTA550) was transformed into C. tropicalis SU-2. The transformant was then grown on either glucose or n-alkane as the sole carbon source, and the intracellular β-galactosidase activity was measured. β-Galactosidase activity in the glucose-grown cells was almost negligible (less than 0.1 pmol min⁻¹ mg⁻¹), while over 1,000 times more β-galactosidase activity was detected for the n-alkane-grown cells (Fig. 1A). This result indicated that a 550-bp UPR-T3A contained a sufficient region(s) to induce transcription in response to n-alkane.

A series of deletion fragments of UPR-T3A were constructed (pUTA550 to pUTA230), and their abilities to induce transcription by n-alkane were compared (Fig. 1A). When grown on glucose, all deletion mutants showed no detectable β-galactosidase activity (less than 0.1 pmol min⁻¹ mg⁻¹). In the cells grown on n-alkane, significant levels of β-galactosidase activity were detected from pUTA550 to pUTA289. The β-Galactosidase activity dropped sharply between pUTA289 and pUTA270, suggesting the existence of a UAS around −270 to −289. Three internal deletion mutants (pUTA261R, pUTA290R, and pUTA311R) were also constructed in which

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**FIG. 1.** Deletion fragments of the CT-T3A promoter and β-galactosidase activity in cells grown on n-alkane. The β-galactosidase activity after 24 h on n-alkane is shown (initial optical density at 570 nm = 0.2). Arrowheads indicate the direction of inserted fragments in panel B.
the region between −231 to −260, between −231 to −289, or between −231 to −310 was deleted, respectively (Fig. 1A). The relatively higher activities detected for pUTA261R than for pUTA290R and pUTA311R might be explained by the existence of upstream repression sequence (URS) in the region between −231 and −260. The putative URS between −231 and −260 and the UAS between −270 and −289 would be present. A noticeable sequence for URS could not be detected in the sequence between −231 and −260; 5′-CCTGCTCAGT GTGACAGGTGTTGTTGAAT-3′.

To determine the region functioning as the UAS, the following plasmids were constructed in which the sequence between −311 and −261 was inserted into the SalI site of pUTA230 (pUTA03F) (Fig. 1B). pUTA230, which contained the TATA sequence of UPR-T3A, did not have UAS activity by itself (Fig. 1A). β-Galactosidase activity in pUTA03F-transformed cells grown on n-alkane was significantly higher than that in pUTA230-transformed cells (Fig. 1B). Moreover, pUTA03R, in which the same sequence was inserted in the opposite direction to pUTA03F, also showed a significant increase in β-galactosidase activity. On the other hand, when grown on glucose, neither pUTA03F-transformed nor pUTA03R-transformed cells, together with pUTA230-transformed cells, showed β-galactosidase activity (data not shown). These results demonstrated the presence of an n-alkane-responsive UAS (designated UAS_{n-alkane}) in the region between −311 and −261. Further deletion analysis indicated that the 29-bp region between −289 and −261 contained sufficient sequences for UAS_{n-alkane} (pUTA05F in Fig. 1B).

To find the important nucleotide sequences inside this 29-bp region, a series of point mutations were introduced. First, six kinds of mutants (M1 to M6) were made in which one or two adjacent guanine and/or cytosine nucleotides were changed into thymine nucleotides, and their UAS activities were compared (Fig. 2). Mutants M1, M2, and M6 had almost comparable (over 80%) UAS activity to the wild-type UAS_{n-alkane}. On the other hand, mutants M3, M4, and M5 had lower UAS activity, showing 30, 20, and 59% of the wild-type activity, respectively. Moreover, a mutant, in which the adenine stretch located between −289 and −282 was deleted (ΔA) had a UAS_{n-alkane} activity comparable to the wild-type activity. These results indicate that nucleotide positions changed in mutant M4 (positions −268 and −269) are particularly important for UAS_{n-alkane} activity. Furthermore, the upstream sequence of CT-T3B corresponding to the region of UAS_{n-alkane} was tested for its UAS activity, and the result indicated that this region also had sufficient UAS activity (75% of that of CT-T3A).

Expression of thiolase III is induced not only by n-alkane but also by other carbon sources, such as butyrate (10, 17). Therefore, it is of interest to examine whether UAS_{n-alkane} induces gene expression by other carbon sources. Cells transformed with pUTA05F were cultivated on glucose, glycerol, n-alkane, acetate, propionate, or butyrate as the sole carbon source, and intracellular β-galactosidase activities were compared (Table 2). Cells transformed with pUTA230 were used as a control for estimating UAS_{n-alkane}-independent transcriptional activation. When the cells were cultivated on glucose, glycerol, or acetate, no UAS_{n-alkane}-dependent increase of β-galactosidase activity was observed. On the other hand, in cells grown on propionate or butyrate as well as on n-alkane, a UAS_{n-alkane}-dependent increase of β-galactosidase activity was observed. These results demonstrate that induction of the expression of the thiolase III gene in the propionate- or butyrate-grown cells occurs, at least in part, by a common mechanism that acts through UAS_{n-alkane}.

In S. cerevisiae, the transcription of 3-ketoacyl-CoA thiolase encoded by FOX3/POT1 is induced by oleic acid (4, 8). Accordingly, UAS_{n-alkane} was tested to find whether it can induce transcription by oleic acid. β-Galactosidase activity was increased in the oleic acid-grown cells harboring pUTA05F (with UAS_{n-alkane}) or pUTA17 (with ΔA derivative of UAS_{n-alkane}) (activ-
The 12-bp sequence including these positions was selected, and similar motifs were searched for promoters of genes encoding several *C. tropicalis* peroxisomal enzymes (Fig. 3). In this 12-bp sequence, the marginal positions were not crucial for the UAS\textsubscript{ALK} activity, because, as for CT-T3B, the marginal positions of the corresponding 12-bp sequence were different from those of CT-T3A but the region still had the UAS\textsubscript{ALK} activity (Fig. 2). In POX18 and KAT, regions were found in which internal 10 bp of UAS\textsubscript{ALK} (5'-TCCTGGCACAC-3') was completely conserved. KAT encodes catalase, a marker enzyme of peroxisome, which is highly induced by n-alkane (28, 34, 40, 41). Therefore, it is reasonable to consider that this region functions as a UAS\textsubscript{ALK}. POX18 of *C. tropicalis* (POX18) encodes a nonspecific lipid transfer protein which is induced by oleic acid (35, 36). Although it is not clear at present whether the expression of *C. tropicalis* POX18 is induced by n-alkane, the expression of *Candida maltosa* POX18 is inducible by n-alkane (7). The UAS\textsubscript{ALK} can also induce transcription by oleic acid; therefore, it seems probable that the expression of *C. tropicalis* POX18 is induced by n-alkane by the common mechanism through UAS\textsubscript{ALK} as in the oleic acid-grown cells. However, whether the sequences shown in Fig. 3 actually have the UAS\textsubscript{ALK} activity should be determined by experiments.

In *C. maltosa*, NADPH-cytochrome P-450 reductase which is localized in the endoplasmic reticulum, is highly induced by n-alkane. By a reporter gene assay, the 0.47-kbp 5'-noncoding region of the gene was shown to be sufficient for the induction on n-tetradecane (25). We compared this region with UAS\textsubscript{ALK}. Although no region closely homologous to UAS\textsubscript{ALK} was detected, there were two CACAT motifs, the pentanucleotide sequence that responds to n-alkane (UAS\textsubscript{ALK}) in the n-alkane-assimilating yeast *C. tropicalis*. Deletion analysis delimitated the sequence of UAS\textsubscript{ALK} within 29 bp (from positions -289 to -261 of UPR-T3A). Further mutation analysis showed that the nucleotides that were changed in the M4 mutant (positions -268 and -269) were the most critical for the UAS\textsubscript{ALK} activity. The 12-bp sequence was observed, which supports our notion that these two motifs are necessary for the expression of the UAS\textsubscript{ALK}. We refer to these regions as UAS\textsubscript{ALK}.

**DISCUSSION**

We have identified the UAS sequence that responds to n-alkane (UAS\textsubscript{ALK}) in the n-alkane-assimilating yeast *C. tropicalis*. Deletion analysis delimitated the sequence of UAS\textsubscript{ALK} within 29 bp (from positions -289 to -261 of UPR-T3A). Further mutation analysis showed that the nucleotides that were changed in the M4 mutant (positions -268 and -269) were the most critical for the UAS\textsubscript{ALK} activity. The 12-bp sequence was observed, which supports our notion that these two motifs are necessary for the expression of the UAS\textsubscript{ALK}. We refer to these regions as UAS\textsubscript{ALK}.

![FIG. 3. Nucleotide sequences similar to UAS\textsubscript{ALK} found on promoters of *C. tropicalis* peroxisomal enzyme genes. POX2 and POX4 encode acyl-CoA oxidase (accession numbers for POX2 and POX4 are M18259 and M12160, respectively); BFE encodes the bifunctional enzyme (X57855); CAT encodes carnitine acyltransferase (D04549) (unpublished data); KAT encodes catalase (X13978, E01922) (unpublished data); POX18 encodes nonspecific lipid transfer protein (X53633 and M24440). The score indicates the number of the nucleotides that are the same as those of CT-T3A. The positions of changed nucleotides in the UAS\textsubscript{ALK} mutants (M3, M4, M5, and M6) are indicated above the sequences. Numbers on both sides of the sequences indicate the distance relative to the translational start codon.](http://jb.asm.org/)

**TABLE 2. Effect of different carbon sources on UAS\textsubscript{ALK}-mediated transcriptionsal activation**

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>β-Galactosidase activity (pmol min(^{-1}) mg(^{-1}))</th>
<th>Activation (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.0</td>
<td>1.0</td>
</tr>
<tr>
<td>+UAS\textsubscript{ALK}</td>
<td>25.0</td>
<td>5.0</td>
</tr>
</tbody>
</table>

* Control indicates pUTA230-transformed cells, and +UAS\textsubscript{ALK} indicates pUTA05F-transformed cells. The initial cell density (optical density at 570 nm) for each carbon source was as follows: 0.05 for glucose, 0.1 for glycerol, 0.2 for n-alkane and acetate, and 0.04 for propionate and butyrate. The cultivation time for each condition was as follows: 24 h for glucose- and glycerol-grown cells, 36 h for n-alkane- and acetate-grown cells, and 88 h for propionate- and butyrate-grown cells.
yeasts have differences in the regulation mechanism for the induction of peroxisomal enzymes. Further investigation involving the isolation of the factor(s) binding to UASALK will help to clarify the activation mechanism of the peroxisomal enzyme genes in C. tropicalis.

REFERENCES


