

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

TAMOTSU KANAI, AKIHIRO HARA, NAOKI KANAYAMA, MITSUYOSHI UEDA,
AND ATSUO TANAKA*

Laboratory of Applied Biological Chemistry, Department of Synthetic Chemistry and Biological Chemistry,
Graduate School of Engineering, Kyoto University, Yoshida, Sakyo-ku, Kyoto 606-8501, Japan

Received 22 October 1999/Accepted 9 February 2000

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'-TCCTGCACAC-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 acetoacetyl 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 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 response element (ORE) (3, 12, 30, 31). ORE also exists on the upstream regions of genes encoding enzymes relating to the β -oxidation pathway (*FOX1* and *FOX2*) and fatty acid metabolism (*SPS19* and *ECII*) and proteins relating to peroxisomal biogenesis (*PEX1* and *PEX11*) (13). The transcriptional acti-

vation through ORE occurs by the binding of a heterodimeric protein complex consisting of Oaf1p and Oaf2p/Pip2p (12, 13, 22, 30, 31). Interestingly, *OAF2/PIP2* itself is also regulated by ORE whereas *OAF1* is not (12, 13, 31). However, the effect of this difference in the regulation mechanisms is not clear.

The molecular mechanism underlying the induction of peroxisomal enzymes or peroxisome itself is unclear for *C. tropicalis*, because no appropriate host-vector system has been available. Recently, using *ura3* derivatives of *C. tropicalis*, we have developed a transformation system for introducing exogenous DNA into the genomic DNA of *C. tropicalis* (9). We have also cloned an autonomously replicating sequence (ARS) from *C. tropicalis*, which enabled 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) (*ura3a/ura3b*) (5), derived from *C. tropicalis* pK233 (ATCC 20336), was used as a host strain for transformation. *Escherichia coli* strain DH5 α (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 C₁₃; 10 ml/liter), oleic acid (5 ml/liter), glycerol (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 glycerol 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).

* Corresponding author. Mailing address: Laboratory of Applied Biological Chemistry, Department of Synthetic Chemistry and Biological Chemistry, Graduate School of Engineering, Kyoto University, Yoshida, Sakyo-ku, Kyoto 606-8501, Japan. Phone: 81-75-753-5524. Fax: 81-75-753-5534. E-mail: atsuo@sbchem.kyoto-u.ac.jp.

TABLE 1. Oligonucleotides used in this study

Name	Sequence
PRT3AJ-1	5'-CATGCTCGAGGTGTTGAATATGTGC-3'
T3(-550S)	5'-TAGGTCGACCCGCGGTATCAACCATCGTCC-3'
T3(-473S)	5'-TCTGTCGACATTTGGTGGGTCTGCCCCC-3'
T3(-407S)	5'-CACGTCGACACATCCCGCTTAGTTGCGGG-3'
T3(-382S)	5'-CGGGTCGACGAACGTGTATTCCCGTAG-3'
T3(-343S)	5'-GTCGTCGACTAGTCACCCGCTTCTGCC-3'
T3(-310S)	5'-GGTGTGCTCAAAAGCTGGCATAAATG-3'
T3(-289S)	5'-ATAAGTCGACAAAAAAGCAGCATCCTGCACACAAC-3'
T3(-281S)	5'-CGAAGTCGACGCACAGCATCCTGCACACAAC-3'
T3(-270S)	5'-ACAGTCGACTGCACACAACCTGCTCAG-3'
T3(-230S)	5'-TGGGTCGACAGAAAACCTCGGCTTAAACC-3'
T3(-310X)	5'-CCAGCTCGAGAGGCAAAACCCAGATGCTGCTTTTTTTTG-3'
T3(-289X)	5'-CTTCTCGAGCGTCATTTATGCCAGCTTTGA-3'
T3(-260X)	5'-CTGCTCGAGGTTGTGTGCAGGATGCTGTGC-3'
T3UAS(M1-2)	5'-GGGCTCGAGGTTGTGTGCAGGATGCTATGCTTTTTTTTG-3'
T3UAS(M2-2)	5'-GGGCTCGAGGTTGTGTGCAGGATAATGTGCTTTTTTTTG-3'
T3UAS(M3-2)	5'-GGGCTCGAGGTTGTGTGCAAAATGCTGTGCTTTTTTTTG-3'
T3UAS(M4-2)	5'-GGGCTCGAGGTTGTGTAAAGGATGCTGTGCTTTTTTTTG-3'
T3UAS(M5-2)	5'-GGGCTCGAGGTTGTGTATGCAGGATGCTGTGCTTTTTTTTG-3'
T3UAS(M6-2)	5'-GGGCTCGAGGTTATGTGCAGGATGCTGTGCTTTTTTTTG-3'
T3UAS(M1-3)	5'-AAAAGTCGACAAAAAAGCATAGCATCCTGCACACAAC-3'
T3UAS(M2-3)	5'-AAAAGTCGACAAAAAAGCATTATCCTGCACACAAC-3'
T3UAS(M3-3)	5'-AAAAGTCGACAAAAAAGCAGCATTTTGCACACAAC-3'
T3UAS(M4-3)	5'-AAAAGTCGACAAAAAAGCAGCATCCTTTACACAAC-3'
T3UAS(M5-3)	5'-AAAAGTCGACAAAAAAGCAGCATCCTGCATACAAC-3'
T3UAS(M6-3)	5'-AAAAGTCGACAAAAAAGCAGCATCCTGCACATAAC-3'
T3UAS(WTB-1)	5'-AAAAGTCGACAAAAAAGCAGCGCTCCTGCACACGAC-3'
T3UAS(WTB-2)	5'-AGGGCTCGAGGTCGTGTGCAGGACGCCGTGTTTTTTTG-3'

Plasmid construction. *Lac4* encoding *Kluyveromyces lactis* β -galactosidase was amplified using primers 5'-AACTGTCGACTATGTCTTGCCTTATTCCTGA G-3' and 5'-CTGTCTCGAGCTTAACGGTCTAATCGTTAATCAG-3'. The genomic DNA of *K. lactis* IFO1267 (ATCC8585) was used as a template DNA. The amplified *Lac4* fragment cut with *SalI* and *XhoI* was inserted into the *SalI* site of pUC-URA3, in which the 1.7-kbp *C. tropicalis* *URA3* was inserted into pUC19 (11), and the subclone was named pUL4. The ARS of *C. tropicalis* 1098 was amplified using primers 5'-AAAAGTCGACACATTTCCTCCGAAAAGT GCCACC-3' and 5'-AAAAGTCGACGGTTAATGTGTCATGATAATAATGGT TTC-3', with pUCNUA1 (6) as a template DNA. Bluescript II(SK+) cut with *SspI* was filled in using T4 DNA polymerase and joined with an *XhoI* linker (named Bluescript-Xh), and the amplified ARS fragment cut by *SalI* 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* *URA3* and *LAC4*, to make pUAL4.

All deletion fragments were prepared either by PCR using pT37Bg (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 PRT3AJ-1 and one of the following primers: T3(-550S), T3(-473S), T3(-407S), T3(-382S), T3(-343S), T3(-310S), T3(-289S), T3(-270S), or T3(-230S). Each amplified fragment was cut with *SalI* and *XhoI* and inserted into the *SalI* site of pUAL4 to construct plasmid pUTA550, pUTA473, pUTA407, pUTA382, pUTA343, pUTA310, pUTA289, pUTA270, and pUTA230, respectively. To construct plasmids pUTA311R, pUTA290R, and pUTA261R, PCR was performed with primer T3(-550S) and plus primer T3(-310X), T3(-289X), and T3(-260X), respectively. The amplified fragment cut with *SalI* and *XhoI* was inserted into the *SalI* site of pUTA230. Plasmids pUTA03F, pUTA03R, pUTA04F, and pUTA05F were constructed by the same method as above using the following set of primers: T3(-310S) and T3(-260X) for pUTA03F and pUTA03R, T3(-310S) and T3(-289X) for pUTA04F, and T3(-289S) and T3(-260X) for pUTA05F.

To construct pUTA11, pUTA12, pUTA13, pUTA14, pUTA15, pUTA16, pUTA17, and pUTA18, two complementary oligonucleotides [T3UAS(M1-2) and T3UAS(M1-3) for pUTA11, T3UAS(M2-2) and T3UAS(M2-3) for pUTA12, T3UAS(M3-2) and T3UAS(M3-3) for pUTA13, T3UAS(M4-2) and T3UAS(M4-3) for pUTA14, T3UAS(M5-2) and T3UAS(M5-3) for pUTA15, T3UAS(M6-2) and T3UAS(M6-3) for pUTA16, T3(-281S) and T3(-260X) for pUTA17, and T3UAS(WTB-1) and T3UAS(WTB-2) for pUTA18] were an-

nealed. 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 μ l) in Z buffer (940 μ l) (24) was incubated at 30°C for 1 min, 10 mM MUG solution (10 μ l) was added, and the increase in fluorescence was measured with a Hitachi fluorophotometer model 650-10S (excitation, 360 nm; emission, 449 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 μ F, and 201 Ω) (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 promoter region of *CT-3A* and *CT-3B* will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers AB025647 and AB025648, respectively.

RESULTS

To evaluate the activity of promoter elements to induce transcription in *C. tropicalis*, pUAL4, a shuttle vector which can replicate in both *E. coli* and *C. tropicalis*, was first constructed by the method described in Materials and Methods. pUAL4 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 multicloning site was introduced before the translation initiation codon of

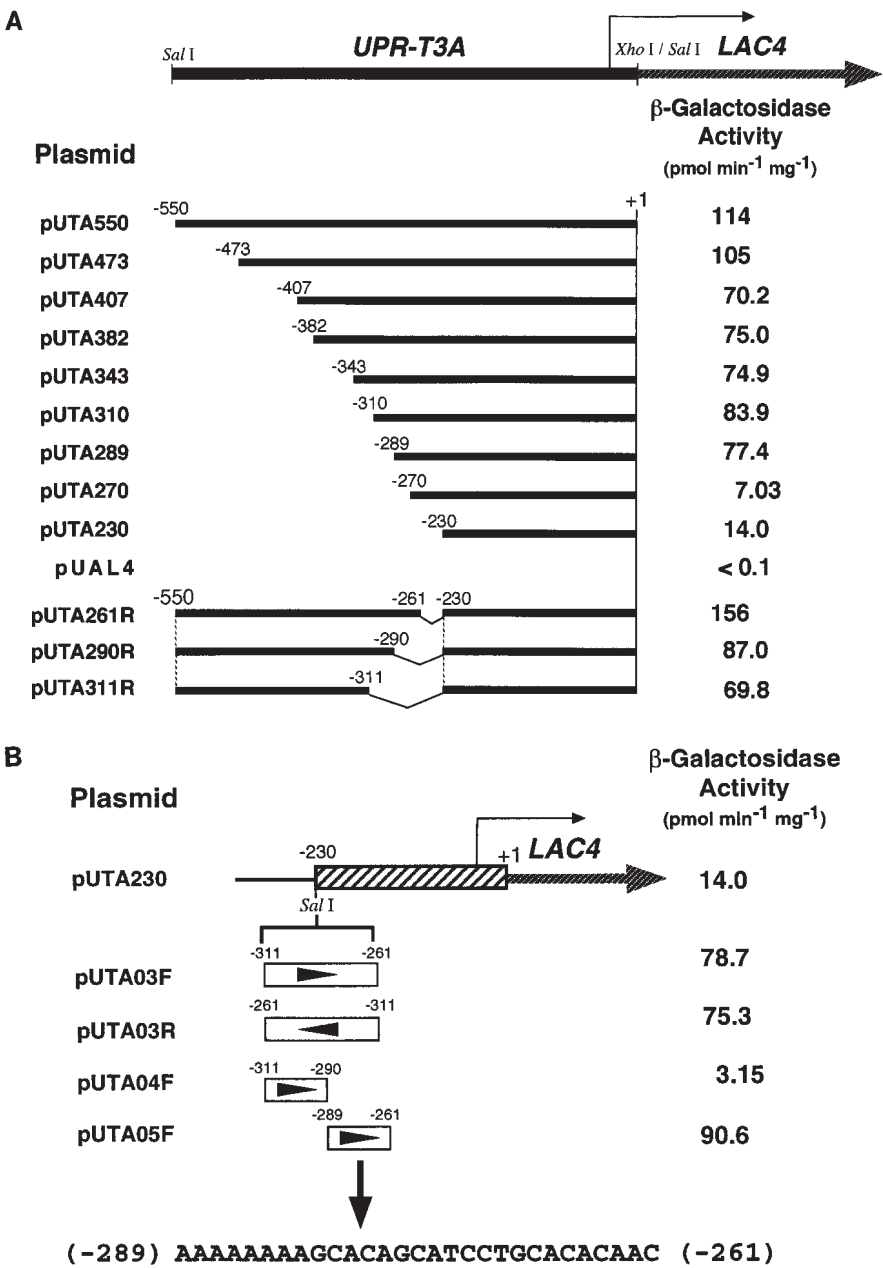


FIG. 1. Deletion fragments of the *CT-T3A* promoter and β -galactosidase activity in cells grown on *n*-alkane. The β -galactosidase activity after 24 h on *n*-alkane is shown (initial optical density at 570 nm = 0.2). Arrowheads indicate the direction of inserted fragments in panel B.

LAC4 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

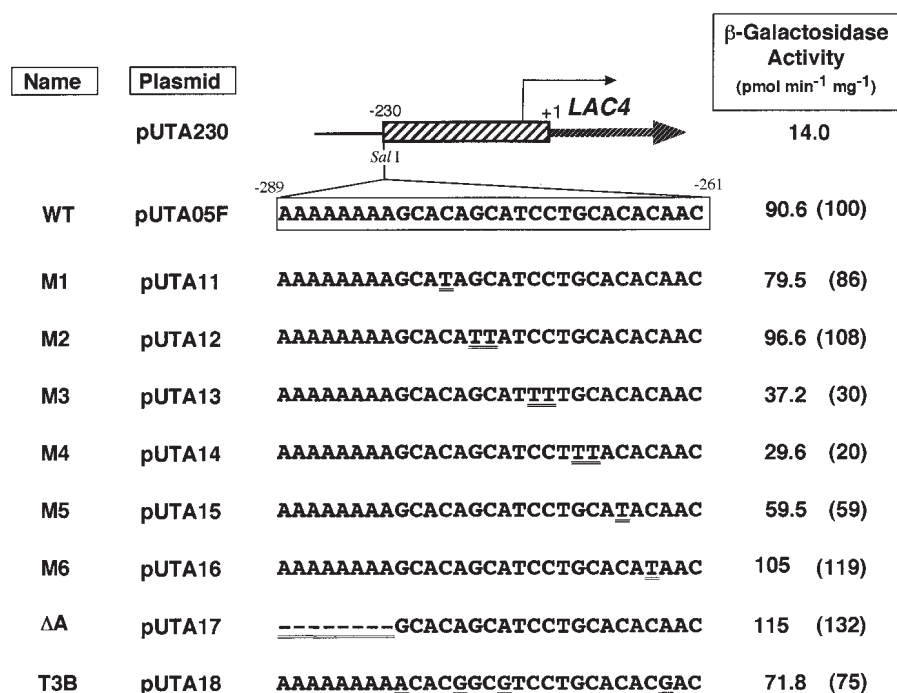


FIG. 2. UAS activity of UAS_{ALK} and its mutants on *n*-alkane. The nucleotides different from those of wild-type (WT) UAS_{ALK} are double underlined. The nucleotides in pUTA18 different from those of wild-type UAS_{ALK} are underlined. The number in parentheses indicates the UAS-dependent transcription inducing activity, where the activity of wild-type UAS_{ALK} was set as 100.

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'-CCTGCTCAGTGTGACAGGTGGTGGTGAAT-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_{ALK}) 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_{ALK} (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 compa-

table (over 80%) UAS activity to the wild-type UAS_{ALK}. 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_{ALK} 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_{ALK} activity. Furthermore, the upstream sequence of *CT-T3B* corresponding to the region of UAS_{ALK} 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_{ALK} 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_{ALK}-independent transcriptional activation. When the cells were cultivated on glucose, glycerol, or acetate, no UAS_{ALK}-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_{ALK}-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_{ALK}.

In *S. cerevisiae*, the transcription of 3-ketoacyl-CoA thiolase encoded by *FOX3/POT1* is induced by oleic acid (4, 8). Accordingly, UAS_{ALK} 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_{ALK}) or pUTA17 (with ΔA derivative of UAS_{ALK}) (activ-

TABLE 2. Effect of different carbon sources on UAS_{ALK}-mediated transcriptional activation

Carbon source	β-Galactosidase activity (pmol min ⁻¹ mg ⁻¹) ^a		Activation (fold)
	Control	+UAS _{ALK}	
Glucose	<1.0	<1.0	
Glycerol	18.2	15.8	0.87
<i>n</i> -Alkane	3.5	71.7	20.5
Acetate	11.1	10.9	0.98
Propionate	24.7	127	5.15
Butyrate	381	1,260	3.30

^a Control indicates pUTA230-transformed cells, and +UAS_{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 0.4 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.

ity of 22.4 and 26.0 pmol/min/mg, respectively) compared with the activity in those harboring pUTA230 (without UAS_{ALK}) (2.51 pmol/min/mg), indicating that UAS_{ALK} is also active in the oleic acid-grown cells. However, the cells harboring pUTA550 (with total *UPR-T34*) had twice the β-galactosidase activity (45.0 pmol/min/mg) as that of the pUTA05F-harboring cells, demonstrating the possible existence of another UAS(s) in addition to the UAS_{ALK} in response to oleic acid on *UPR-T34*.

DISCUSSION

We have identified the UAS sequence that responds to *n*-alkane (UAS_{ALK}) in the *n*-alkane-assimilating yeast *C. tropicalis*. Deletion analysis delimited the sequence of UAS_{ALK} within 29 bp (from positions -289 to -261 of *UPR-T34*). 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_{ALK} activity. 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_{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_{ALK} activity (Fig. 2). In *POX18* and *KAT*, regions were found in which internal 10 bp of UAS_{ALK} (5'-TCCTGCACAC-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_{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_{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_{ALK} as in the oleic acid-grown cells. However, whether the sequences shown in Fig. 3 actually have the UAS_{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_{ALK}. Although no region closely homologous to UAS_{ALK} was detected, there were two CACAT motifs, the pentanucleotide often found in the 5'-noncoding regions of P-450alk genes, encoding cytochrome P-450, of *C. maltosa* (25). UAS_{ALK} of *C. tropicalis* contains a CACACA sequence at its 3' end. Physiological and genetic evidence suggests that *C. tropicalis* and *C. maltosa* are closely related strains. Therefore, it is likely that the similar activation mechanisms are present in these yeasts, in which the CACA motif sequence might play an important role.

Besides *n*-alkane and oleic acid, UAS_{ALK}-dependent transcription also occurred with butyrate and propionate. These carbon sources can also induce the proliferation of peroxisomes in *C. tropicalis* (17, 39). *n*-Alkane or long-chain fatty acids incorporated in *C. tropicalis* are degraded through the fatty acid β-oxidation system localized in peroxisomes and are ultimately converted into butyryl- or propionyl-CoA. Therefore, the results of this study show that these short-chain fatty acids and/or their derivatives might be a true inducer(s) that causes UAS_{ALK}-dependent transcriptional activation.

In *S. cerevisiae*, the consensus sequence of ORE is suggested as inverted repeats of the CGG triplet with a spacing of 15 to 18 nucleotides (CGGN₁₅₋₁₈CCG) (13, 30). The CGG triplet repeat is the common consensus sequence for the C₆ zinc cluster family of fungal transcriptional regulators, such as Gal4p (32, 38). In fact, a heterodimeric protein complex consisting of Oaf1p and Oaf2p/Pip2p, both of which have a C₆ zinc cluster motif, was identified as the factor that binds to ORE (12, 22, 30, 31). On the other hand, UAS_{ALK} does not contain the CGG triplet repeat. This fact strongly suggests that the ORE-like regulation mechanism does not exist in *C. tropicalis*. Sloots et al. (33) investigated the regulation mechanism of the gene (*HDE*) encoding the peroxisomal bifunctional enzyme of *C. tropicalis* by introducing its upstream region into *S. cerevisiae*. By deletion analysis, they identified an oleic acid-responsive region located between positions -393 and -341. When this region was compared with UAS_{ALK}, no homologous sequence was observed, which supports our notion that these two

		M3	M4	M5	M6	Score
CT-T3A	[-274]	ATCCTGCACACA				[263] (12/12)
POX18	[-297]	GTCTGCACACA				[308] (11/12)
CT-T3B	[-268]	GTCTGCACACG				[257] (10/12)
KAT	[-998]	CTCTGCACACT				[-1009] (10/12)
POX4	[-264]	TTTTCACACA				[-253] (09/12)
BFE	[-226]	ACACTGCAGACA				[-237] (09/12)
CAT	[-272]	GTGGTGCACACA				[-261] (09/12)
POX2	[-432]	GTCTGCTCTCC				[-443] (08/12)
BFE	[-253]	CACATGCACACT				[-242] (08/12)
KAT	[-378]	GCCCCACACAC				[-367] (07/12)
POX2	[-251]	GGCATGCACGCT				[-240] (07/12)

FIG. 3. Nucleotide sequences similar to UAS_{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 (X57854); *CAT* encodes carnitine acetyltransferase (D84549) (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_{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.

yeasts have differences in the regulation mechanism for the induction of peroxisomal enzymes. Further investigation involving the isolation of the factor(s) binding to UAS_{ALK} and its characterization by comparison with Pip2p will help to clarify the activation mechanism of the peroxisomal enzyme genes in *C. tropicalis*.

REFERENCES

- Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254.
- Dangelmaier, C. A., and H. Holmsen. 1980. Determination of acid hydrolases in human platelets. *Anal. Biochem.* **104**:182–191.
- Einerhand, A. W., W. T. Kos, B. Distel, and H. F. Tabak. 1993. Characterization of a transcriptional control element involved in proliferation of peroxisomes in yeast in response to oleate. *Eur. J. Biochem.* **214**:323–331.
- Einerhand, A. W., T. M. Voorn-Brouwer, R. Erdmann, W. H. Kunau, and H. F. Tabak. 1991. Regulation of transcription of the gene coding for peroxisomal 3-oxoacyl-CoA thiolase of *Saccharomyces cerevisiae*. *Eur. J. Biochem.* **200**:113–122.
- Haas, L. O., J. M. Cregg, and M. A. Gleeson. 1990. Development of an integrative DNA transformation system for the yeast *Candida tropicalis*. *J. Bacteriol.* **172**:4571–4577.
- Hara, A., M. Ueda, T. Matsui, K. Furuhashi, N. Kanayama, and A. Tanaka. 1999. Construction of an autonomously replicating plasmid in *n*-alkane-assimilating yeast, *Candida tropicalis*. *J. Biosci. Bioeng.* **87**:717–720.
- Hwang, C. W., K. Yano, and M. Takagi. 1991. Sequences of two tandem genes regulated by carbon sources, one being essential for *n*-alkane assimilation in *Candida maltosa*. *Gene* **106**:61–69.
- Igual, J. C., B. C. Gonzalez, L. Franco, and O. J. Perez. 1992. The *POT1* gene for yeast peroxisomal thiolase is subject to three different mechanisms of regulation. *Mol. Microbiol.* **6**:1867–1875.
- Kanayama, N., Y. Himeda, H. Atomi, M. Ueda, and A. Tanaka. 1997. Expression of acetoacetyl-CoA thiolase isozyme genes of *n*-alkane-assimilating yeast, *Candida tropicalis*: isozymes in two intracellular compartments are derived from the same genes. *J. Biochem. (Tokyo)* **122**:616–621.
- Kanayama, N., M. Ueda, H. Atomi, T. Kurihara, J. Kondo, Y. Teranishi, and A. Tanaka. 1994. Comparison of molecular structures and regulation of biosynthesis of unique thiolase isozymes localized only in peroxisomes of *n*-alkane-utilizable yeast, *Candida tropicalis*. *J. Ferment. Bioeng.* **78**:273–278.
- Kanayama, N., M. Ueda, H. Atomi, and A. Tanaka. 1998. Genetic evaluation of physiological functions of thiolase isoenzymes in the *n*-alkane-assimilating yeast *Candida tropicalis*. *J. Bacteriol.* **180**:690–698.
- Karpichev, I. V., Y. Luo, R. C. Mariani, and G. M. Small. 1997. A complex containing two transcription factors regulates peroxisome proliferation and the coordinate induction of β -oxidation enzymes in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **17**:69–80.
- Karpichev, I. V., and G. M. Small. 1998. Global regulatory functions of Oaf1p and Pip2p (Oaf2p), transcription factors that regulate genes encoding peroxisomal proteins in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **18**:6560–6570.
- Kawamoto, S., C. Nozaki, A. Tanaka, and S. Fukui. 1978. Fatty acid β -oxidation system in microbodies of *n*-alkane-grown *Candida tropicalis*. *Eur. J. Biochem.* **83**:609–613.
- Kurihara, T., M. Ueda, N. Kamasawa, M. Osumi, and A. Tanaka. 1992. Physiological roles of acetoacetyl-CoA thiolase in *n*-alkane-utilizable yeast, *Candida tropicalis*: possible contribution to alkane degradation and sterol biosynthesis. *J. Biochem. (Tokyo)* **112**:845–848.
- Kurihara, T., M. Ueda, N. Kanayama, J. Kondo, Y. Teranishi, and A. Tanaka. 1992. Peroxisomal acetoacetyl-CoA thiolase of an *n*-alkane-utilizing yeast, *Candida tropicalis*. *Eur. J. Biochem.* **210**:999–1005.
- Kurihara, T., M. Ueda, H. Okada, N. Kamasawa, N. Naito, M. Osumi, and A. Tanaka. 1992. β -Oxidation of butyrate, the short-chain-length fatty acid, occurs in peroxisomes in the yeast *Candida tropicalis*. *J. Biochem. (Tokyo)* **111**:783–787.
- Kurihara, T., M. Ueda, and A. Tanaka. 1988. Occurrence and possible roles of acetoacetyl-CoA thiolase and 3-ketoacyl-CoA thiolase in peroxisomes of an *n*-alkane-grown yeast, *Candida tropicalis*. *FEBS Lett.* **229**:215–218.
- Kurihara, T., M. Ueda, and A. Tanaka. 1989. Peroxisomal acetoacetyl-CoA thiolase and 3-ketoacyl-CoA thiolase from an *n*-alkane-utilizing yeast, *Candida tropicalis*: purification and characterization. *J. Biochem. (Tokyo)* **106**:474–478.
- Leuker, C. E., A. M. Hahn, and J. F. Ernst. 1992. β -Galactosidase of *Kluyveromyces lactis* (Lac4p) as reporter of gene expression in *Candida albicans* and *C. tropicalis*. *Mol. Gen. Genet.* **235**:235–241.
- Leuker, C. E., A. Sonneborn, S. Delbruck, and J. F. Ernst. 1997. Sequence and promoter regulation of the *PCK1* gene encoding phosphoenolpyruvate carboxykinase of the fungal pathogen *Candida albicans*. *Gene* **192**:235–240.
- Luo, Y., I. V. Karpichev, R. A. Kohanski, and G. M. Small. 1996. Purification, identification, and properties of a *Saccharomyces cerevisiae* oleate-activated upstream activating sequence-binding protein that is involved in the activation of *POX1*. *J. Biol. Chem.* **271**:12068–12075.
- Masuda, Y., S. M. Park, M. Ohkuma, A. Ohta, and M. Takagi. 1994. Expression of an endogenous and a heterologous gene in *Candida maltosa* by using a promoter of a newly-isolated phosphoglycerate kinase (*PGK*) gene. *Curr. Genet.* **25**:412–417.
- Miller, J. H. 1972. Assay of β -galactosidase, p. 352–355. In J. H. Miller (ed.), *Experiments in molecular genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Ohkuma, M., Y. Masuda, S. M. Park, R. Ohtomo, A. Ohta, and M. Takagi. 1995. Evidence that the expression of the gene for NADPH-cytochrome P-450 reductase is *n*-alkane-inducible in *Candida maltosa*. *Biosci. Biotechnol. Biochem.* **59**:1328–1330.
- Osumi, M., N. Miwa, Y. Teranishi, A. Tanaka, and S. Fukui. 1974. Ultrastructure of *Candida* yeasts grown on *n*-alkanes. Appearance of microbodies and its relationship to high catalase activity. *Arch. Microbiol.* **99**:181–201.
- Poch, O., H. L'Hôte, V. Dallery, F. Debeaux, R. Fleer, and R. Sodoyer. 1992. Sequence of the *Kluyveromyces lactis* β -galactosidase: comparison with prokaryotic enzymes and secondary structure analysis. *Gene* **118**:55–63.
- Rachubinski, R. A., Y. Fujiki, and P. B. Lazarow. 1987. Isolation of cDNA clones coding for peroxisomal proteins of *Candida tropicalis*: identification and sequence of a clone for catalase. *Biochim. Biophys. Acta* **909**:35–43.
- Raleigh, E. A., K. Lech, and R. Brent. 1994. *E. coli*, plasmids, bacteriophages, p. 1.1–1.15. In F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), *Current protocols in molecular biology*. John Wiley & Sons, Inc., New York, N.Y.
- Rottensteiner, H., A. J. Kal, M. Filipits, M. Binder, B. Hamilton, H. F. Tabak, and H. Ruis. 1996. Pip2p: a transcriptional regulator of peroxisome proliferation in the yeast *Saccharomyces cerevisiae*. *EMBO J.* **15**:2924–2934.
- Rottensteiner, H., A. J. Kal, B. Hamilton, H. Ruis, and H. F. Tabak. 1997. A heterodimer of the Zn₂Cys₆ transcription factors Pip2p and Oaf1p controls induction of genes encoding peroxisomal proteins in *Saccharomyces cerevisiae*. *Eur. J. Biochem.* **247**:776–783.
- Schjerling, P., and S. Holmberg. 1996. Comparative amino acid sequence analysis of the C₆ zinc cluster family of transcriptional regulators. *Nucleic Acids Res.* **24**:4599–4607.
- Sloots, J. A., J. D. Aitchison, and R. A. Rachubinski. 1991. Glucose-responsive and oleic acid-responsive elements in the gene encoding the peroxisomal trifunctional enzyme of *Candida tropicalis*. *Gene* **105**:129–134.
- Soga, O., H. Kinoshita, M. Ueda, and A. Tanaka. 1997. Evaluation of peroxisomal heme in yeast. *J. Biochem. (Tokyo)* **121**:25–28.
- Szabo, L. J., G. M. Small, and P. B. Lazarow. 1989. The nucleotide sequence of *POX18*, a gene encoding a small oleate-inducible peroxisomal protein from *Candida tropicalis*. *Gene* **75**:119–126.
- Tan, H., K. Okazaki, I. Kubota, T. Kamiryo, and H. Utiyama. 1990. A novel peroxisomal nonspecific lipid-transfer protein from *Candida tropicalis*. Gene structure, purification and possible role in β -oxidation. *Eur. J. Biochem.* **190**:107–112.
- Tanaka, A., M. Ueda, H. Okada, and S. Fukui. 1987. Formation of several enzymes associated with alkane utilization by yeast. *Ann. N. Y. Acad. Sci.* **501**:449–453.
- Todd, R. B., and A. Andrianopoulos. 1997. Evolution of a fungal regulatory gene family: the Zn(II)₂Cys₆ binuclear cluster DNA binding motif. *Fungal Genet. Biol.* **21**:388–405.
- Ueda, M., H. Okada, A. Tanaka, M. Osumi, and S. Fukui. 1983. Induction and subcellular localization of enzymes participating in propionate metabolism in *Candida tropicalis*. *Arch. Microbiol.* **136**:169–176.
- Yamada, T., A. Tanaka, and S. Fukui. 1982. Properties of catalase purified from whole cells and peroxisomes of *n*-alkane-grown *Candida tropicalis*. *Eur. J. Biochem.* **125**:517–521.
- Yamada, T., A. Tanaka, S. Horikawa, S. Numa, and S. Fukui. 1982. Cell-free translation and regulation of *Candida tropicalis* catalase messenger RNA. *Eur. J. Biochem.* **129**:251–255.
- Young, D. C., S. D. Kingsley, K. A. Ryan, and F. J. Dutko. 1993. Selective inactivation of eukaryotic β -galactosidase in assays for inhibitors of HIV-1 TAT using bacterial β -galactosidase as a reporter enzyme. *Anal. Biochem.* **215**:24–30.