A Basic Helix-Loop-Helix Transcription Factor Essential for Cytochrome P450 Induction in Response to Alkanes in Yeast Yarrowia lipolytica*

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When the alkane-assimilating yeast Yarrowia lipolytica is cultivated on n-alkanes, it changes cellular metabolism for adaptation by inducing cytochrome P450 and other genes. From a comparative analysis of promoters of alkane-inducible genes, we identified a cis-acting element, ARE1 (alkane responsive element 1), which provides transcription induction in response to n-alkanes. In a genetic selection for mutants that were defective in ARE1-mediated transcription induction in the presence of n-alkanes, we found that the YAS1 (yeast alkane signaling) gene is essential for alkane response. The YAS1 gene encodes a basic helix-loop-helix (bHLH) family protein. Loss of Yas1p causes defects in n-alkane-dependent transcription induction of the P450 gene and growth on n-alkanes. Yas1p localizes to nuclei and binds to promoters containing ARE1. Yas1p also binds to its own promoter, and the expression of YAS1 is induced by n-alkanes. These features suggest that Yas1p is a novel transcription factor mediating alkane signaling and that it provides an autoregulatory loop.

Cytochromes P450 (P450s) are heme-containing monoxygenases that catalyze diverse reactions in the metabolism of various endogenous and exogenous lipophilic compounds and have been isolated from species as diverse as bacteria and mammals (1). Alkanes are one of the most hydrophobic compounds among substrates of P450s. Alkanes are widespread high energy carbon resources, and many microorganisms, including bacteria, yeasts, and fungi, have developed metabolic systems to utilize those compounds (2, 3). P450s are employed for initial terminal hydroxylation of alkanes in some alkane-utilizing prokaryotic (4) and eukaryotic microorganisms (5). Because many P450s are induced by their substrates, the expression of P450s for alkane terminal hydroxylation is also induced by alkanes (4, 6–14). P450s are employed for initial terminal hydroxylation of alkanes in some alkane-utilizing prokaryotic (4) and eukaryotic microorganisms (5). Because many P450s are induced by their substrates, the expression of P450s for alkane terminal hydroxylation is also induced by alkanes (4, 6–14). P450s are employed for initial terminal hydroxylation of alkanes in some alkane-utilizing prokaryotic (4) and eukaryotic microorganisms (5). Because many P450s are induced by their substrates, the expression of P450s for alkane terminal hydroxylation is also induced by alkanes (4, 6–14). In contrast to the intensive study of the P450 induction mechanism in mammalian cells that has occurred, little is known about P450 induction in other organisms. The question of how very hydrophobic compounds, such as alkanes, are recognized by cells and cause P450 induction in alkane-utilizing microorganisms is of great interest.

P450 induction by n-alkanes has been found in n-alkane-assimilating yeasts such as Candida tropicalis (7–10), Candida maltosa (11, 12), and Yarrowia lipolytica (13, 14), but the regulatory mechanism has not been elucidated in any yeasts. The Candida species that have been used so far for studying the alkane metabolism are mostly diploid or partially diploid, and in such species it is difficult to isolate genes by genetic means. We have chosen Y. lipolytica to study the alkane-inducible transcription mechanism because it has a stable haploid life cycle and, therefore, is advantageous for genetic and molecular analysis (15). Y. lipolytica utilizes n-alkanes efficiently as its sole carbon source, and this property makes the yeast potentially important in fundamental study and also in biotechnological fields (16).

Y. lipolytica has eight ALK genes, from ALK1 to ALK8, encoding P450ALKs, which presumably catalyze the terminal hydroxylation of alkanes to alkanols in the endoplasmic reticulum (13, 14). The ALK1 gene disruptant grew very poorly on n-decane, indicating that ALK1 plays the principal role in n-decane assimilation (13). Alkanes taken up into the cells and oxidized by the cytochrome P450 monooxygenase system are further oxidized by the peroxisomal β-oxidation system. Y. lipolytica has the POX1 to POX5 genes that encode acyl-CoA oxidases, catalyzing the first step of the β-oxidation (17). The POT1 (18) and PAT1 (19) genes encoding 3-oxoacyl-CoA thiolase and acetoacetyl-CoA thiolese, respectively, have also been cloned and characterized. Both thiolases are thought to be involved in the last step of the β-oxidation. The transcription of P450 and acetoacetyl-CoA thiolese genes is highly induced by n-alkanes (13, 14, 19), but the induction mechanism is little understood.

In this study, we isolated mutants that had defects in alkane-dependent transcription induction through a promoter element, ARE1† (alkane responsive element 1). By complementation of a mutant, we identified the YAS1 (yeast alkane signaling) gene, which encodes a basic helix-loop-helix (bHLH) family protein. The YAS1 gene is essential for the n-alkane-dependent transcription induction of the ALK1 gene. Yas1p binds to promoters with ARE1, which contains an E-box motif, the binding site of the bHLH transcription factor. These features suggest that Yas1p is a transcription factor for alkane signaling.

**Experimental Procedures**

**Plasmids—**The LEU2 minimal promoter region (20) was amplified by PCR (primers 5′-AGGTTTTTAGCTGATCTGCTG-3′ and 5′-GTT-CATTGTTCGATGTC-3′; underlined nucleotides indicate the HindIII and Stul sites), digested with HindIII and Stul, and then ligated into the pUC57 vector.

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†The abbreviations used are: ARE1, alkane responsive element 1; bHLH, basic helix-loop-helix; ChIP, chromatin immunoprecipitation; FITC, fluorescein isothiocyanate; HA, hemagglutinin A; ORF, open reading frame; P450, cytochrome P450; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.
ligated into the HindIII-Stul sites of the pSUT5lacZ (21) to yield pS-LZ. ARE1-containing oligonucleotides (5'-gatg TGACAAGTCTGTGGATGA-CACAAGCCAGTTGAAATG-3' and 5'-gatg CTTAAGTCACTGTGGATGA-CACAAGCCAGTTGAAATG-3'), the nucleotides designated in lowercase were added to provide cohesive BglII-BamHI ends, and the conserved nucleotides in ARE1 are underlined) were annealed and ligated into the BglII-BamHI sites of pYOOHC2 (a gift from Dr. Y. Ohtsubo). The plaque containing the desired random copies of ARE1 was selected by sequencing and named pYOOHC2-3. A XbaI-BamHI (filled) fragment of pYOOHC2-3 was inserted into the XbaI-HindIII (filled) site of pS-LZ. pS-LZ was digested in the same way using oligonucleotides bearing mutant ARE1 (5'-gatg TGACAAGTCTGTGGATGA-CACAAGCCAGTTGAAATG-3' and 5'-gatg CTTAAGTCACTGTGGATGA-CACAAGCCAGTTGAAATG-3'), the nucleotides designated in wild-type ARE1 are underlined).

We constructed pA3-3LZ-UTUH, a plasmid designed to integrate 3ARE1s-minimal LEU2 promoter-αM-3 into the ura3 locus, as described below. The Neo-NcoI (filled) fragment of pSUT5 was inserted into the BamHI site (filled) of pBluescript IISK+ (Stratagene) to generate pBS-URA3. pS-URA3 was digested with EcoRV and self-ligated. Into the SaII site (blunted) of the resultant plasmid, a 700-bp EcoRV fragment of pBS-URA3 was inserted to generate pBS-UTUH. pS-LZ was digested with NeoI and SacII, blunted, and then ligated to remove URA3 and ASR1. Into the NotI site (filled) of the resultant plasmid, the ADE1-carrying BamHI fragment (filled) of pSA74 (13) was inserted to generate pA3-3LZ. Into the XbaI site (filled) of pA3-3LZ, the XbaI (filled)-HindIII (filled) fragment of pBS-UTUH was inserted to generate pA3-3LZ-UTUH.

The plasmid p28-1 is from a genomic library of Y. lipolytica in the autonomously replicating Escherichia coli shuttle vector pSUT5 (19). A 1.7-kbp HindIII fragment from p28-1 was inserted into the unique HindIII site of pSUT5 to obtain plasmid p28-1H. The YAS1 deletion cassette was constructed as described below. A 1.4-kbp BglII-EcoRV fragment of p28-1 was inserted into the BamHI and NotI sites of pBluescript IISK+ (Stratagene) to generate pBS-LEU2prom. The resultant plasmid was digested with Spel and XbaI to remove the YAS1 open reading frame (ORF), blunted, and ligated with the ADE1-carrying BamHI fragment (filled) of pSA74 to obtain pBS-dy-Bgl. A 1.4-kbp BglII-EcoRV fragment of p28-1 was inserted into the BamHI and EcoRV sites of pBluescript IISK+ (Stratagene), and the SpeI-KpnI fragment of the plasmid was inserted between the SpeI and KpnI sites of pSUT5 to generate pSYAS1, a plasmid for producing Yas1p. For ARE1s-minimal pYAS1, a HindIII fragment of pSYAS1 was inserted between the SpeI and KpnI sites of pSUT5 to generate p28-1HH. The 1.7-kbp HindIII fragment from p28-1HH was inserted into the unique BamHI site (filled) of pBluescript IISK+ (Stratagene) to yield pSYAS1lacZ. 

**Fig. 1. Identification of ARE1 as a n-alkane-responsive promoter element.** A, ARE1 on the ALK1 (AB010388) promoter and conserved ARE1-like sequences in the promoter regions of PAT1 (AB120846), FOX3 (AJ001301), POT1 (X69898), ALK2 (AB010389), and FOX1 (AJ001299). Accession numbers were indicated in parenthesis. ARE1 was responsible for activation by n-alkane. CXAU1 cells were transformed with the reporter plasmids as indicated, grown in 5·g medium, and then cultivated on each carbon source for 5 h. Crude extracts from the cells were assayed for β-galactosidase activity. The S.E. of six independent experiments is indicated.

**β-Galactosidase Activity Assay and Northern Blot Analysis**—We performed a β-galactosidase activity assay as described (21), except that the cells were incubated with various carbon sources for 5 h. Northern blot analysis was done as described previously (19). A BamHI-BamHI fragment from pSUT5lacZ (21), a PstI-EcoRV fragment from pSAT4-ALK1 (13), and a HindIII-XbaI fragment from p28-1HH were used as probes specific for lacZ, ALK1 and YAS1, respectively. For the PAT1 probe, we used a PCR-amplified fragment with the primers 5'-ACACA-CACCCGTATTTCCGATCC-3' and 5'-GTCTCTGATGTCGGTCTG-3'. 

**Immunoblot Analysis and Indirect Immunofluorescence**—Cells were incubated with or without n-decane for 1 h after OD600 reached 1.5 in 2% (w/v) yeast extract, 0.67% yeast nitrogen base without amino acids; Difco) as follows: 2% (v/v) glucose (SD medium); 2% (w/v) glycerol (SG medium); 2% (w/v) glycerol (NG medium); and 2% (v/v) oleic acid. The growth curve was obtained with an automatically recording incubator TN1506 (Advances).

**Indirect Immunofluorescence—**Cells were incubated with or without n-decane for 1 h after OD600 reached 1.5 in 2% (w/v) yeast extract, 0.67% yeast nitrogen base without amino acids; Difco) as follows: 2% (v/v) glucose (SD medium); 2% (w/v) glycerol (SG medium); 2% (v/v) n-decane; and 2% (v/v) oleic acid. The growth curve was obtained with an automatically recording incubator TN1506 (Advances).

**Transcription Factor for Alkane Response**—The underlined sections indicate the promoter element. The transcriptional activity of ARE1s-minimal pSUT5lacZ (21) was measured using ARE1s-minimal pLEU2-lacZ-AVED, ade1, and CAXAU1 (ura3, ade1::ADE1), respectively. Ade+ transformants were analyzed for correct integration by Southern blot analysis. Mutagenized CXUS3-4LZ1 cells were grown on 1/3YPD-gal plates (0.33% yeast extract, 0.67% peptide, 0.67% glucose, 20 mM sodium phosphate (pH 7), 0.01% X-gal, and 2% agar) and screened for white or pale blue colonies in the presence of n-decane. The yeast strain was obtained by UV mutagenesis from CXU3-4LZ1. The YAS1 deletion cassette was liberated by digesting pBS-dy-Bgl with SacII and Smal and introduced into the CXAU1 strain to obtain the yas1Δ strain. Ade+ transformants were analyzed for correct integration by Southern blot analysis. Media and growth conditions were described previously (19). An appropriate carbon source was added to YNB (0.67% yeast nitrogen base without amino acids; Difco) as follows: 2% (v/v) glucose (SD medium); 2% (v/v) glycerol (SG medium); 2% (v/v) n-decane; and 2% (v/v) oleic acid. The growth curve was obtained with an automatically recording incubator TN1506 (Advances).

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**Immunoblot Analysis and Indirect Immunofluorescence**—Cells were incubated with or without n-decane for 1 h after OD600 reached 1.5 in 2% (w/v) yeast extract, 0.67% yeast nitrogen base without amino acids; Difco) as follows: 2% (v/v) glucose (SD medium); 2% (v/v) glycerol (SG medium); 2% (v/v) n-decane; and 2% (v/v) oleic acid. The growth curve was obtained with an automatically recording incubator TN1506 (Advances).
Cells were treated with n-decane for 1 h after OD$_{600}$ reached 1.5 in SD medium. Immunoprecipitation of Yas1p-HA was carried out with an anti-HA antibody at a dilution of 1:100. Specific primer pairs were used to amplify the promoter region of the ALK1 (5'-H11032-CTTCAATGTTGCGGCTC-3' and 5'-H11032-GGTTCTGATGAGTTAACAC-3'), PAT1 (5'-H11032-CCTTCGGGCACATCACGTG-3' and 5'-H11032-TTGTATTTGTGTCCCGAGC-3'), YAS1 (5'-H11032-TAATCATCACGGGCTGCAG-3' and 5'-H11032-GACCCTAGTCCAAATGGCG-3'), or LEU2 (5'-H11032-CACCAACACTATTAGTGGCAG-3' and 5'-H11032-CGACAGCAACTACTCCTTTC-3') genes from the extracted DNA.

RESULTS

Identification of ARE1—Because both ALK1 and PAT1 are induced at the transcription level by n-alkanes (13, 19), we analyzed their promoter regions and found a highly conserved sequence, CTTGTGNxCATGTG (Fig. 1A). We found similar sequences in the promoters of other genes, namely ALK2, POX1, POX3, and POT1, which are involved in the cytochrome P450 and β-oxidation systems (Fig. 1A). The sequences were found in the opposite direction on the promoters of ALK2, whose induction by n-alkanes was shown previously (14), and POX1 (Fig. 1A). The TGTG repeat (CACA in the opposite direction) is strictly conserved and seems to be important. We named the 24-bp sequence between CTTGTG and CATGTG on the ALK1 promoter the alkane responsive element 1 (designated ARE1) and further analyzed its function. The importance of ARE1 on the ALK1 promoter in alkane response was suggested by deletion analysis of the ALK1 promoter (21).

To analyze the function of ARE1 outside the ALK1 context, we inserted three copies of ARE1 upstream of the minimal LEU2 promoter directing the expression of the reporter lacZ gene. The promoter with 3×ARE1s insertion was activated in response to n-decane (Fig. 1B) or n-hexadecane (data not shown), but not to oleic acid (Fig. 1B). Insertion of mutant ARE1 did not, however, result in n-alkane-dependent activation (Fig. 1B). These findings indicate the specific involve-
ment of ARE1 in alkane-responsive transcription. Because PAT1 is also induced by oleic acid (19), another element that is responsive to fatty acids should be present on its promoter.

**Fig. 3. Cloning and analysis of the YAS1 gene.** A, restriction enzyme map and strategy for disruption of the YAS1 gene. The ability (+) or inability (−) to complement the growth defect of the yas1−1 mutant is indicated. B, the nucleotide sequence of the YAS1 gene and the deduced amino acid sequence of Yas1p. The CACA motifs are underlined. C, amino acid sequence alignment by Clustal W (38) of the bHLH region of Yas1p, S. cerevisiae Ino4p (P13902), human Mlx (Q9UH92), frog L-myel (Q05404), and mouse S-myel (Q9Z304). SWISS-PROT accession numbers are shown in parentheses. Identical (asterisks) and conserved (dots) amino acids are indicated.
under the control of 3×ARE1s, to isolate mutants with defects in the activation of ARE1 by n-decane on plates containing glucose as a supplementary carbon source (Fig. 2A). Glucose was chosen as a supplementary carbon source because it does not repress the expression of ALK1 (13). Of 150,000 mutagenized cells screened, we finally isolated three mutants that had lower levels of β-galactosidase activity and ALK1 mRNA in the presence of n-decane than did the parental strain.

One of the mutants failed to induce the expression of lacZ under the promoter with 3×ARE1s and ALK1 in the presence of n-alkanes (Fig. 2B), whereas the induction of PAT1 by oleic acid was not affected (Fig. 2B). The mutant strain did not grow on n-decane or n-hexadecane, but it did grow on glucose and glycerol, as did the wild-type strain (Fig. 2C). This mutant was expected to have a mutation in the signaling pathway for n-alkane-dependent transcription induction, and we named it the yeast alkane signaling mutant strain, designated yas1-1. To our surprise, induction of PAT1 by n-alkanes was not impaired in this mutant (Fig. 2B).

The YAS1 Gene Encodes a bHLH Family Protein and Is Essential for Alkane Response—The YAS1 gene was isolated from a Y. lipolytica genomic library (19) by functional complementation of the growth defect on n-decane of the yas1-1 mutant. Of ~22,000 transformants screened, one showed positive growth on n-decane. Analysis and sequencing of the complementing plasmid revealed an ORF of 414 bp (Fig. 3, A and B) that did not contain Y. lipolytica splice site motif sequences (5′ donor site, branch site, and 3′ acceptor site) (16, 24). Sequencing of the ORF region of the original mutant strain yas1-1 revealed an adenine nucleotide insertion at the position 202 from the first nucleotide of the translation initiation ATG codon and the occurrence of a frameshift mutation. The potential TATA box, TATAA, was found at nucleotides –86 to –82. The deduced product of YAS1, Yas1p, is 137 amino acids in length and has a predicted molecular mass of 15,085 Da. The entire amino acid sequence of Yas1p did not show high homology to any known proteins, but a motif search (25) revealed that Yas1p has a Mys-type helix-loop-helix domain preceded by a basic region. A PSI-BLAST search (26) showed that the bHLH domain of Yas1p was most similar to that of Ino4p, which is a transcription factor involved in phospholipid biogenesis in the yeast Saccharomyces cerevisiae (27) (Fig. 3C). The bHLH family includes transcription factors that are involved in the metabolism of lipophilic compounds such as AhR, ARNT, and SREBP (28).

We deleted the YAS1 gene from the CXAU1 wild-type strain by replacing the ORF by the ADE1 gene to obtain the yas1Δ strain. The yas1Δ strain was unable to grow on n-decane as the original mutant (Fig. 2B). The yas1Δ strain was also unable to grow in liquid medium containing n-decane as the sole carbon source, but it grew in medium with oleic acid (Fig. 2D). Although alkanes are thought to be metabolized via fatty acids, the YAS1 gene is specifically essential for alkane metabolism, but not for fatty acid utilization. ALK1 induction by n-alkanes was also lost in the yas1Δ strain, as it was in the original mutant (Fig. 2B). These findings indicate that the YAS1 gene is essential for the alkane-responsive induction of ALK1 and for alkane utilization.

The Expression Induction of YAS1 by n-Alkanes—To determine whether the expression of YAS1 itself is also regulated by n-alkanes, we examined the level of YAS1 mRNA. The YAS1 mRNA was induced when cells were incubated with n-decane or n-hexadecane (Fig. 4A). We found CACA repeats in the 5′ upstream region of YAS1 (underlined in Fig. 3B). To examine the expression level of Yas1p, we introduced three repeats of a HA epitope into the carboxyl terminus of Yas1p. Expression of Yas1p-HA complemented the growth defect of the yas1-deleted mutant on n-decane (Fig. 4B), indicating that Yas1p-HA is functional. The increase of Yas1p-HA in media containing n-decane was confirmed by immunoblot analysis (Fig. 4C). Yas1p-HA migrated more slowly on SDS-PAGE than expected from the amino acid sequence, probably because of its high proportion of basic amino acids (predicted pl of Yas1p-HA is 9.51) (29).

Yas1p-HA Localizes to the Nucleus—We investigated the localization of Yas1p-HA by indirect immunofluorescence using an anti-HA monoclonal antibody in cells cultivated in medium with or without n-decane. Accumulation of Yas1p-HA was detected in the nuclei of the cells incubated with n-decane (Fig. 5). Clear localization could not be observed in the cells incubated without n-decane because of the lower level of Yas1p-HA (Fig. 5).

The Localization of Yas1p-HA Does Not Depend on n-Decane—To test whether the nuclear localization of Yas1p-HA is regulated by n-alkanes, we expressed Yas1p-HA from the LEU2 minimal promoter (Fig. 6A). To change the promoter, a restriction enzyme site was introduced upstream of the initia-
expression of Yas1p-HA from the native promoter. Expression of Yas1p-HA from the LEU2 minimal promoter complemented the growth defect of the yas1-deleted mutant on n-decane (Fig. 6B), indicating that the Yas1p-HA expressed by the promoter is functional. Then, the localization of Yas1p-HA in cells incubated with or without n-decane was investigated (Fig. 6C). Yas1p-HA localized in the nuclei independently of n-decane (Fig. 6C).

Yas1p-HA Binding to Promoters Containing ARE1—ARE1 contains an E-box (CANNTG) motif, the consensus sequence with which bHLH proteins interact (30). Nuclear localization and the bHLH motif of Yas1p prompted us to test whether Yas1p binds to ARE1. We performed ChIP experiments and found that immunoprecipitation of Yas1p-HA yielded many PCR products of ALK1 and PAT1 but not of LEU2, indicating specific associations of Yas1p-HA with ALK1 and PAT1 promoter regions that contain ARE1 (Fig. 7). Approximately the same amounts of PCR product were obtained from whole extracts of cells with or without Yas1p-HA (Fig. 7). Because the expression of YAS1 was induced by n-alkanes and the YAS1 promoter contained CACA repeats, we also tested whether Yas1p-HA binds to its own promoter. Yas1p-HA binding to the YAS1 promoter was also detected (Fig. 7), which suggests an autoregulation of YAS1.

FIG. 7. Yas1p-HA associated specifically with the promoters of ALK1, PAT1 and YAS1. ChIP was performed by anti-HA antibody using cells expressing Yas1p or Yas1p-HA incubated in the presence of n-decane for 1 h. DNA of whole cell extract (WCE) and immunoprecipitation (IP) was amplified by using primers for ALK1, PAT1, YAS1, and LEU2 promoters.

DISCUSSION

The present findings suggest that Yas1p is a novel bHLH transcription factor that binds to ARE1 and is necessary for the transcription induction of the P450ALK1 gene in response to n-alkanes. The bHLH transcription factors are known to work as homo- or heterodimers (30). We speculate that Yas1p requires another bHLH protein for its DNA binding, because purified His6-tagged Yas1p expressed in bacteria did not show shift bands toward ARE1 in an electrophoresis mobility shift assay (data not shown). Identification of the putative binding partner of Yas1p and elucidation of the entire alkane response pathway are our planned work.

We propose that the expression of YAS1 is regulated by its product, Yas1p. This positive feedback regulation mechanism leads to a rapid increase of YAS1 transcription in the presence of alkanes and subsequently allows a quick activation of the P450 and β-oxidation systems and adaptation to exposure to alkanes.

The PAT1 gene was still induced by n-alkanes in yas1 mutants. We considered two possible reasons for this phenomenon. First, some functional homologues of Yas1p might be present and function preferably in the alkane-dependent induc-
tion of PAT1. Based on the almost entire loss of the alkane-depen-
dent induction of the ALKI gene in yas1 mutants, this conclu-
sion is somewhat unlikely; yet, it cannot be completely ruled out. Second, n-alkane-derived fatty acids might induce the expression of PAT1, the expression of which is induced by fatty acids as well (Ref. 19 and Fig. 2B). The P450ALK1 gene was found to be expressed in slight amounts without n-alkanes or even in the yas1 mutants (Fig. 2B) unless it was repressed by glycerol (13). This finding suggests that Yas1p is necessary for the active induction of the ALKI gene by n-alkanes but that the lack of Yas1p does not cause a complete loss of the expression of ALKI (see Fig. 2B). Thus, n-alkanes might have been oxidized to fatty acids in the yas1 mutants considered here, and the metabolites may have induced the PAT1 gene expression, although such oxidation would not have sufficed to support the growth of the yas1 mutant cells.

Repeats of the sequence CATGGTAA were found in the pro-
moter regions of the alkane-inducible cytochrome P450-encoding
genes CYP52A1 and CYP52A2 in the alkane-assimilating yeast Candida tropicalis (9). Recently, a 29-bp region, which contains the CACA motif, was identified as a sequence that responds to n-alkane and oleic acid on the promoter of the peroxisomal 3-ketoacyl CoA thiolase gene of C. tropicalis (31). In another alkane-assimilating yeast, C. maltosa, ATGTTG repeats were also found in promoters of the P450ALK genes and the NADPH-cytochrome P450 reductase gene (11, 12, 32). From this conservation of the TGTG (or CACA) motif, we speculate that the alkane response mediated by Yas1p-like bHLH family proteins is conserved among alkane-assimilating yeasts.

Cytochromes P450 are widely distributed in species as di-
verse as bacteria and mammals and play the central role in the
metabolism of hydrophobic compounds (1). The expression of P450s, particularly those involved in the metabolism of exoge-
 nous compounds, is induced by their substrates in many cases (33). The regulation mechanism of P450 expression has been investigated intensively in mammalian cells (34) but is poorly understood in lower eukaryotes. Recently, Khan et al. (35) identified a 40-bp pisatin (a fungalistic isolavonoid)-responsive element upstream of the pisatin demethylase, a cyto-
chrome P450 gene in a plant-pathogenic fungus, and isolated a gene encoding a binuclear zinc (Cys4-Zn4) (36) transcription factor that binds to the 40-bp element. However, the involvement of the DNA-binding protein to the pisatin-dependent P450 induction has not yet been clarified. In mammalian cells, the expres-
sion of P450s for xenobiotics metabolism is mediated by mem-
nbers of the nuclear receptor (NR) family and by the dioxin
receptor AhR and Arnt, which belong to the bHLH/PAS family (34). Nuclear receptor family proteins have not been identified outside metazoans (36), and it has been a long unanswered question of how yeast P450ALKs are induced by n-alkanes. We are interested in the evolutionary relationship between Yas1p and AhR/Arnt, each of which has a bHLH motif. Upon ligand binding, AhR translocates from cytoplasm to nuclei and forms a heterodimer with its partner Arnt, which localizes in the nuclei (37). Yas1p is a small molecule and does not seem to contain a ligand-binding domain, although the possibility of such a domain cannot be ruled out. The constitutive nuclear localization of Yas1p suggests that Yas1p carries out a different function than AhR does. It will be interesting to consider in future studies whether or not Yas1p functions in a similar manner as Arnt. In addition, it will be important to determine whether a putative partner of Yas1p can bind to alkanes and function as AhR. It is also possible that some other receptors sense alkane and transduce the signal to Yas1p and its part-
ner. This study provides the first clue for the elucidation of P450 induction mechanisms by exogenously hydrophobic com-
 pounds in yeasts and for a comparative understanding of the regulatory mechanisms in species from yeasts to mammals.

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