Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 329 (2005) 78–86
BBRC
www.elsevier.com/locate/ybbrc

n-Alkane and clofibrate, a peroxisome proliferator, activate transcription of *ALK2* gene encoding cytochrome P450alk2 through distinct *cis*-acting promoter elements in *Candida maltosa*☆

Takahisa Kogure[a,b], Masamichi Takagi[b], Akinori Ohta[a,*]

[a] *Department of Biotechnology, The University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113-8657, Japan*
[b] *Faculty of Applied Life Sciences, Niigata University of Pharmacy and Applied Life Sciences, Higashijima 265-1, Niitsu, Niigata 956-8603, Japan*

Received 18 January 2005
Available online 2 February 2005

Abstract

The *ALK2* gene, encoding one of the *n*-alkane-hydroxylating cytochromes P450 in *Candida maltosa*, is induced by *n*-alkanes and a peroxisome proliferator, clofibrate. Deletion analysis of this gene's promoter revealed two *cis*-acting elements—an *n*-alkane-responsive element (ARE2) and a clofibrate-responsive element (CRE2)—that partly overlap in sequence but have distinct functions. ARE2-mediated activation responded to *n*-alkanes but not to clofibrate and was repressed by glucose. CRE2-mediated activation responded to polyunsaturated fatty acids and steroid hormones as well as to peroxisome proliferators but not to *n*-alkanes, and it was not repressed by glucose. Both elements mediated activation by oleic acid. Mutational analysis demonstrated that three CCG sequences in CRE2 were critical to the activation by clofibrate as well as to the in vitro binding of a specific protein to this element. These findings suggest that *ALK2* is induced by peroxisome proliferators and steroid hormones through a specific CRE2-mediated regulatory mechanism.

Keywords: *Candida maltosa*; Yeast; Cytochrome P450alk; Clofibrate; Peroxisome proliferator; ALK2; Gene expression; Promoter element; CRE2

Cytochromes P450 (P450s) constitute a superfamily of ubiquitous heme-containing monooxygenases that metabolize a wide variety of endogenous and xenobiotic hydrophobic compounds [1,2]. *n*-Alkane-assimilating yeasts, such as *Candida maltosa* [3–6], *Candida tropicalis* [7–10], and *Yarrowia lipolytica* [11,12], have P450s that catalyze the terminal hydroxylation of *n*-alkanes and fatty acids for the assimilation of these compounds. These P450s, designated P450alks, are classified into the CYP52 family. Most alkane-assimilating yeasts have multiple P450alk isozymes encoded by multiple *ALK* genes. It has been reported that individual P450alk isozymes differ from each other in their functions, which suggests that they have evolved to utilize various long-chain hydrocarbons. The growth of *n*-alkane-assimilating yeasts on *n*-alkane as a sole carbon source induces the transcription of a series of genes encoding enzymes involved in *n*-alkane assimilation, including P450alks and β-oxidation enzymes. This transcriptional induction is accompanied by the proliferation of endoplasmic reticulum (ER) and peroxisomes where these enzymes are located [13].

Candida maltosa has eight structurally related P450alk genes: *ALK1* to *ALK8* [6]. Their expression is repressed by glucose, derepressed by glycerol, and induced by *n*-alkane at the transcriptional level (except for *ALK4*, which is assumed to be a pseudogene) [6].

☆ *Abbreviations:* P450, cytochrome P450; P450alk, P450 encoded by an *ALK* gene; P450alk2, P450 encoded by *ALK2*; ARE2, *n*-alkane-responsive element of *ALK2*; CRE2, clofibrate-responsive element of *ALK2*.

* Corresponding author. Fax: +81 3 5841 8015.
E-mail address: aaohta@mail.ecc.u-tokyo.ac.jp (A. Ohta).

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.01.099

Among them, four highly expressed genes—*ALK1*, *ALK2*, *ALK3*, and *ALK5*—encode the primary P450alk species in this yeast; this is inferred because the disruptant in which all four of these genes were simultaneously deleted could not grow on *n*-alkane as a sole carbon source, and because the expression of one of these four genes restored the growth of this disruptant on hexadecane [14]. The individual P450alk isozymes differ in specificity toward various lengths of *n*-alkanes or fatty acids [14–18].

The molecular mechanism underlying the transcriptional induction of *ALK* genes in response to *n*-alkanes or to fatty acids in *n*-alkane-assimilating yeasts has been largely unknown. Recently, a gene encoding a basic helix–loop–helix protein that binds to promoters containing *cis*-elements was identified and was shown to be essential for P450 induction in response to *n*-alkane in *Y. lipolytica* [19]. In *Saccharomyces cerevisiae*, the transcription factor complex Oaf1p-Pip2p activates oleic acid-responsive peroxisomal genes via oleate response elements (OREs) in their promoters [20–27]. However, it is unclear whether these findings are generally applicable to the induction mechanism of yeast P450alk genes or not.

The transcriptional induction properties of individual P450alk isozymes in response to various species of inducers are also diversified. Among the *ALK* genes in *C. maltosa*, the induction of *ALK2* is especially intriguing because this gene is significantly induced not only by alkanes and fatty acids but also by peroxisome proliferators such as clofibrate, which are structurally unrelated to long-chain hydrocarbons [28]. Peroxisome proliferators (PPs) constitute a class of structurally diverse amphipathic chemicals including hypolipidemic fibrate drugs and phthalate ester plasticizers. PPs trigger the transcriptional induction of a wide variety of genes associated with lipid metabolism, including genes coding for the P450 subfamily CYP4A, which catalyzes fatty acid hydroxylation as *ALK2* in *C. maltosa*, leading to proliferation of peroxisomes in mammalian cells [29]. Such a response to PPs is mediated by a member of a nuclear receptor superfamily, called peroxisome proliferator-activated receptor α (PPARα). This particular PPARα binds directly to PPs and activates transcription as a heterodimer with another nuclear receptor, retinoid X receptor (RXR), via *cis*-acting elements called peroxisome proliferator response elements present in the promoters of PP-responsive genes [30–33]. On the other hand, in the bacterium *Bacillus megaterium*, cytochrome P450BM-3, which catalyzes fatty acid hydroxylation, is induced not only by fatty acids but also by some xenobiotics including some PPs. In *B. megaterium*, transcriptional induction is achieved by the release of a repressor from its operator site after the repressor binds inducer chemicals [34]. In lower eukaryotes, the *ALK2* gene of *C. maltosa* is currently the only example of a PP-responsive P450 gene. Although the transcriptional induction mechanism of *ALK2* in response to PPs is still entirely unknown, the common property concerning the catalytic activity of P450s as well as the species of their inducing compounds suggests the possibility that this gene's induction mechanism is evolutionally conserved.

In this study, to investigate the molecular mechanism of transcriptional induction of *C. maltosa ALK2* in response to distinct hydrophobic inducers, including *n*-alkanes, peroxisome proliferators, and oleic acid, we analyzed the gene's promoter structure. We found that *n*-alkane and clofibrate induced *ALK2* transcription through *cis*-acting promoter elements aligned in a partly overlapped arrangement, and that these elements likely mediated distinct transcriptional induction pathways.

Materials and methods

Strains and media. C. maltosa wild-type strain IAM12247 and its derivative CHA1 (*his5*, *ade1*) [35] were used in this study. The media used were YPD [1% yeast extract (Difco), 2% polypeptone (Wako), and 2% glucose] and YNB [0.67% yeast nitrogen base without amino acids (Difco)], the latter of which was supplemented with an appropriate carbon source, such as 2% glucose (SD), 2% glycerol (SG), 0.2% *n*-dodecane, or 0.2% oleic acid. If necessary, adenine or histidine was added to YNB at 24 μg/ml. Peroxisome proliferators, steroid hormones, or other drugs were dissolved in dimethyl sulfoxide and added to SG at the concentration described. *Escherichia coli* HB101 was used for the plasmid preparation.

Construction of ALK2 promoter–reporter fusions. The PCR primers used in this study are listed in Table 1 in the Supplementary data. All *ALK2* promoter–reporter constructs were made on the promoter probe vector pPL1 [36]. Details of the plasmid construction are described in the Supplementary data. The plasmid pPLA2-141 carries the 5′ noncoding region of *ALK2* from nucleotides −141 to −1 (+1 is the first nucleotide of the translation initiation codon) at the site just upstream from the *LAC4* ORF and expresses the reporter *LAC4* at the basal level in *C. maltosa* cells. *ALK2* promoter fragments from −924, −625, −530, −476, −398, and −221 to −142 were each PCR-amplified and inserted between the *Sph*I and *Stu*I sites of pPLA2-141, to yield plasmids pPLA2-924, pPLA2-625, pPLA2-530, pPLA2-476, pPLA2-398, and pPLA2-221, respectively.

Plasmids were constructed to test the functions of the following *ALK2* promoter sub-regions (see the Supplementary data for details). In those plasmids, four tandem copies of each of five *ALK2* promoter sub-regions—from −221 to −139, −149, −167, and −190, and from −189 to −139—were inserted into the blunt-ended *Sph*I site at the −141st nucleotide of the *ALK2* promoter of the plasmid pPLA2-141. The constructed plasmids are pPL4xACRR2, pPL4xACRR2-2, pPL4xARE2, pPL4xARE2-2, and pPL4xCRE2, in the order of the fragments inserted above.

β-Galactosidase assay. CHA1 strains containing the reporter constructs were grown to the late-log phase in 8 ml SD, transferred to 8 ml YNB containing various carbon sources or to 8 ml SG containing various hydrophobic drugs, and cultivated for 6 h. Crude cell extracts were then prepared and assayed for β-galactosidase activity as described previously [36].

Preparation of partially purified cell extracts for DNA binding proteins. C. maltosa IAM12247 were cultivated in SD medium to the late-log phase, transferred to SD, SG, or SG with 1 mM clofibrate, and grown for 6 h. Cells were harvested and suspended in 1 ml/g cells of extraction buffer containing 100 mM Tris–HCl (pH 8.0), 10% glycerol, 400 mM $(NH_4)_2SO_4$, 10 mM $MgCl_2$, 1 mM EDTA, 5 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF),

and 1 μl/ml protease inhibitor cocktail (10 μg/ml each of leupeptin, chymostatin, antipain, and pepstatin). Cells were disrupted using a French pressure cell (SLM Instruments, Rochester, NY). Cell debris and organelles were removed by two successive centrifugations (3000*g* for 10 min, then 100,000*g* for 2 h). The resultant supernatant was diluted with three volumes of binding buffer [20 mM Tris–HCl (pH 8.0), 10% glycerol, 0.05% NP-40, 250 μM $MgCl_2$, 100 μM $ZnCl_2$, 1 mM EDTA, 5 mM DTT, 1 mM PMSF, and 1 μl/ml protease inhibitor cocktail] and passed through a heparin column (Amersham). The column was washed with binding buffer containing 0.1 M NaCl, and the bound proteins were eluted with binding buffer containing 1 M NaCl. The eluted protein fraction was desalted by a PD-10 column (Amersham).

Electrophoretic mobility shift assay. Double-stranded oligonucleotides corresponding to wild-type or mutant CRE2 were end-labeled with ^{32}P using T4 polynucleotide kinase and used as a probe. About 1 ng of probe and 1–10 μg of partially purified cell extracts were incubated in 20 μl binding buffer [20 mM Tris–HCl (pH 8.0), 10% glycerol, 0.05% NP-40, 100 mM NaCl, and 0.2 μg poly(dI–dC)] for 20 min at room temperature. Samples were loaded onto 5% polyacrylamide gel in 1× TBE buffer and electrophoresed for 1.5 h at 160 V. Radioactivity in the gel was analyzed by FLA-3000 (Fuji). For the competition assay, a 10- or 40-fold molar excess of unlabeled wild-type or mutant CRE2 DNA was added to the incubation mixture.

Results

Identification of an n-alkane- and clofibrate-responsive region in ALK2 promoter

To identify the *cis*-acting promoter elements necessary for the transcriptional induction of *ALK2* in response to *n*-alkane and/or clofibrate, deletion analysis of the *ALK2* promoter was carried out using *Kluyveromyces lactis LAC4* as a reporter gene [36] (Fig. 1). *LAC4* encodes β-galactosidase and can be expressed in *C. maltosa*, where the CUG codon is assigned for L-serine [37]. Both the *n*-dodecane- and clofibrate-induced β-galactosidase activities gradually decreased by successive deletion from the 5′ end of the promoter up to −222 (the nucleotide immediately before the initiation codon is −1), and the ratios between these two activities were kept roughly constant. By further deletion up to −142, the responsiveness to both inducers was abolished and the β-galactosidase activities were at the levels of no induction. This result indicated that a region necessary for the response to *n*-alkane and clofibrate is present around the sequence from −221 to −142. To confirm the function of this region, the four tandem copies of the region from −221 to −139 were placed upstream from the position −141. The resultant promoter induced about 4-fold and 14-fold greater reporter activity in response to *n*-alkane and clofibrate, respectively, in comparison with the induction of the reporter construct with a single copy of this region (pPLA2-221). This suggests that the region from −221 to −139 contains promoter elements for the response to *n*-alkane and clofibrate. We named this 83-bp region ACRR2 (alkane and clofibrate responsive region of *ALK2*).

Identification of elements responsive to n-alkane and/or clofibrate within ACRR2

The nucleotide sequence of ACRR2 is AT-rich and contains two types of motifs: CATGTG at the 5′-end

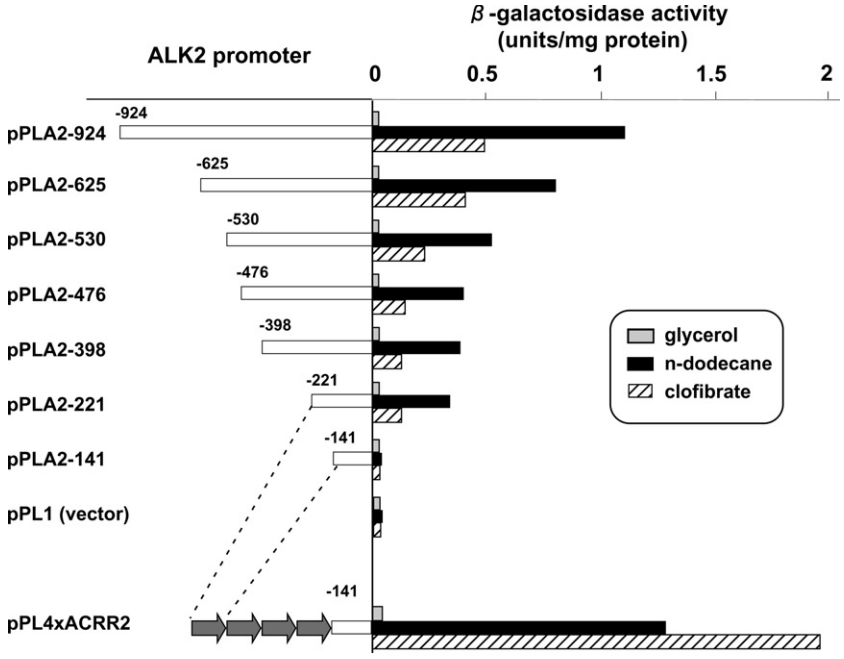

Fig. 1. Deletion analysis of *ALK2* promoter. *C. maltosa* CHA1 cells that carry partly deleted *ALK2* promoter-*LAC4* fusion constructs were grown on SG, YNB plus 0.2% *n*-dodecane, or SG plus 1 mM clofibrate for 6 h. β-Galactosidase activities in the cell extracts were measured. Indicated activities are each the average of at least three independent assays.

and three direct repeats of T(T/A)TCCG (Fig. 1). The former sequence matches the E-box (CANNTG) sequence that could be the target of basic helix–loop–helix (bHLH) transcription factors [38]. To analyze the functions of these motifs in response to the representative inducers—*n*-dodecane, oleic acid, and clofibrate—four tandem copies of each of various sub-regions of ACRR2 were combined with the basal promoter–reporter construct (pPLA2-141 in Fig. 1), and the responsiveness of each sub-region to each inducer compound was evaluated (Fig. 2A). Removal of the 3′-end of the T(T/A)TCCG sequence from ACRR2 by deleting 10 base pairs resulted in approximately 3.1-fold and 2.6-fold increases in reporter activity in response to *n*-dodecane and oleic acid, respectively, but resulted in no increase in response to clofibrate (pPL4xACRR2-2 in Fig. 2) in comparison with the response of full-length ACRR2 (pPL4xACRR2). However, under the noninducing condition where glycerol was the sole carbon source, the reporter activity by this deletion construct also increased by about 3-fold, suggesting that this small deletion caused a general increase in promoter activity. From this point of view, the removal of the 3′-terminal 10-bp sequence of ACRR2 might impair the induction in response to clofibrate.

In the case of a 28-bp deletion from the 3′-end of ACRR2 (pPL4xARE2), which lost two of three direct repeats containing CCG triplets, the induction level by *n*-dodecane was almost the same as that of the complete ACRR2, but the induction levels by oleic acid and clofibrate were approximately 50% and 10% of those of ACRR2, respectively. These results indicate that the deleted 3′-end 28-bp sequence of ACRR2 is not necessary for the response to *n*-alkane, but is partially necessary for the response to oleic acid and almost essential for the response to clofibrate. The 51-bp deletion from the 3′-end of ACRR2 (pPL4xARE2-2) almost abolished the induction of reporter activity in response to any of the inducers, except for weak activity by *n*-dodecane. These results suggested that the region from −189 to −167 is important for the response to *n*-alkane and oleic acid. The importance of the 51-bp region from −189 to −139 of ACRR2 for the response to clofibrate was indicated by the construct pPL4xCRE2, which gave very high reporter activity in response to clofibrate—even higher than that shown by the complete ACRR2. This region also responded weakly to oleic acid and not at all to *n*-dodecane. From these results we concluded that the upstream 55-bp region from −221 to −167 in

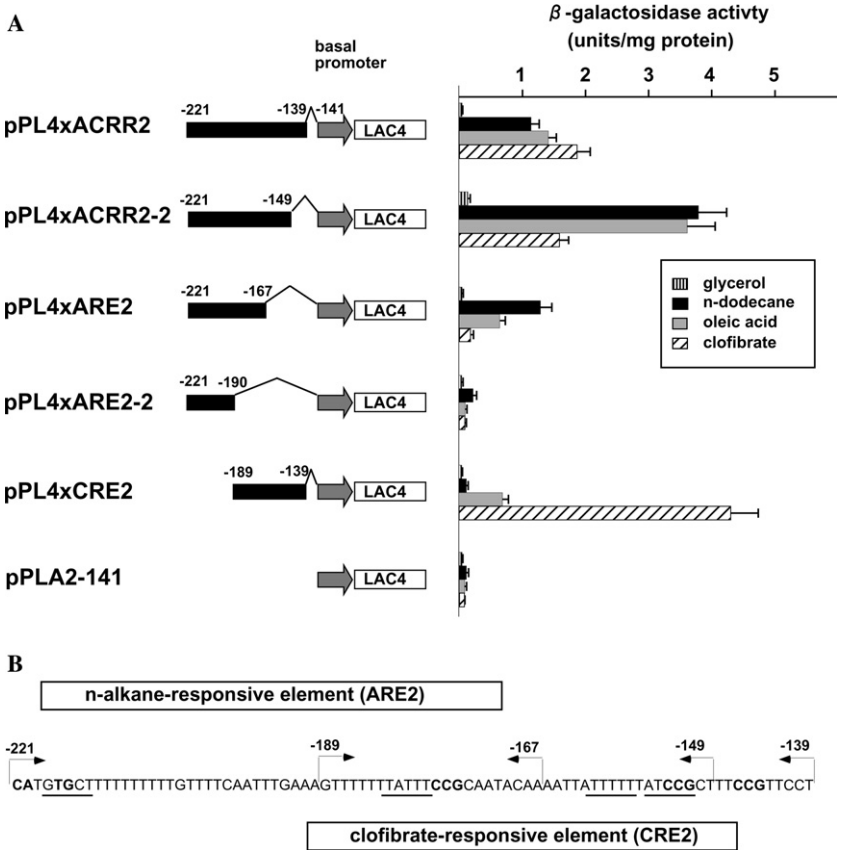

Fig. 2. Responsiveness of ACRR2 sub-regions to inducers. (A) *C. maltosa* CHA1 cells with plasmid pPL4xACRR2, pPL4xACRR2-2, pPL4xARE2, pPL4xARE2-2, pPL4xCRE2, or pPLA2-141 were grown on SG, YNB plus 0.2% *n*-dodecane, YNB plus 0.2% oleic acid, or SG plus 1 mM clofibrate for 6 h. β-Galactosidase activities in the cell extracts were measured. (B) The functions of ACRR2 sub-regions. Arrows indicate the end points of tested DNA regions. E-box and CCG triplet-containing motifs are underlined. ARE2 (−221 to −167) and CRE2 (−189 to −139) are indicated.

ACRR2 is specifically involved in induction by *n*-alkane and that the downstream 51-bp region from −189 to −139 in ACRR2 is specifically involved in induction by clofibrate. Therefore, we named the former region ARE2 (*n*-alkane-responsive element of *ALK2*) and the latter CRE2 (clofibrate-responsive element of *ALK2*). Induction by oleic acid is probably mediated by both elements.

CRE2-mediated induction is responsive to peroxisome proliferators, polyunsaturated fatty acids, and steroid hormones, and is not affected by glucose

We examined CRE2's responsiveness to hydrophobic chemicals other than clofibrate and oleic acid by using the reporter construct pPL4xCRE2 (Fig. 3). As shown in Fig. 3A, two other peroxisome proliferators, 1 mM mono-(2-ethyl-hexyl)phthalate (MEHP), and 100 μM Wy14643, induced CRE2-mediated reporter activity. Polyunsaturated fatty acids, including linoleic acid (18:2), linolenic acid (18:3), and arachidonic acid (20:6), which are known to highly activate PPARα, also induced reporter activity. Moreover, steroid hormones such as β-estradiol and testosterone at a concentration of 100 μM induced higher reporter activity than what clofibrate did. However, neither phenobarbital nor dexamethasone, known as potent inducers for mammalian drug-metabolizing P450s, induced CRE2-mediated transcription (data not shown). Without CRE2 on the reporter construct (pPLA2-141 in Fig. 2), neither of these chemicals induced reporter activity (data not shown). These results indicate that CRE2 mediates transcriptional induction in response to certain types of peroxisome proliferators, unsaturated fatty acids, and steroid hormones.

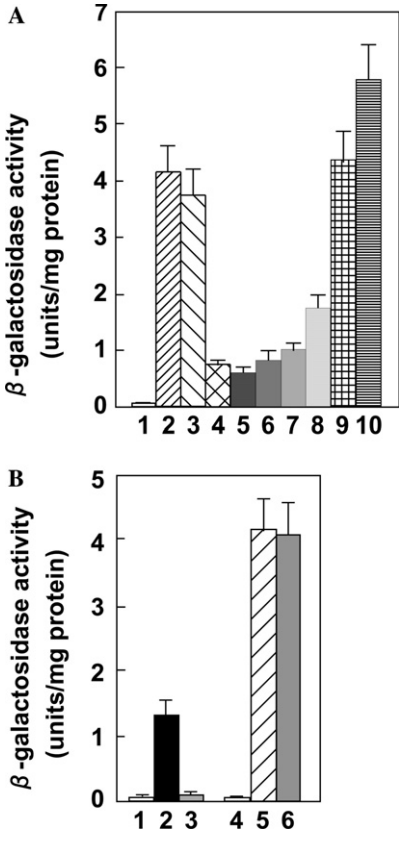

Fig. 3. The effects of various compounds and glucose on the CRE2-mediated reporter induction. (A) *C. maltosa* CHA1 cells with pPL4xCRE2 were grown on SG (bar 1), SG plus 1 mM clofibrate (bar 2), SG plus 1 mM MEHP (bar 3), SG plus 100 μM Wy14643 (bar 4), YNB plus 0.2% oleic acid (bar 5), YNB plus 0.2% linoleic acid (bar 6), YNB plus 0.2% linolenic acid (bar 7), YNB plus 1 mM arachidonic acid (bar 8), SG plus 100 μM testosterone (bar 9), or SG plus 100 μM β-estradiol (bar 10). (B) *C. maltosa* CHA1 cells with pPL4xARE2 (bars 1, 2, and 3) or pPL4xCRE2 (bars 4, 5, and 6) were grown on SG (bars 1 and 4), YNB plus 0.2% *n*-dodecane (bar 2), SD plus 0.2% *n*-dodecane (bar 3), SG plus 1 mM clofibrate (lane 5), or SG plus 1 mM clofibrate and 2% glucose (bar 6).

Because glucose repressed *n*-alkane's ability to induce P450alk genes, we investigated the effects of glucose on ARE2- and CRE2-mediated transcription using cells with pPL4xARE2 or pPL4xCRE2 (see Fig. 2) [6]. Fig. 3B indicates that, while glucose severely repressed ARE2-mediated transcriptional induction by *n*-alkane (bars 1, 2, and 3), it did not repress CRE2-mediated induction by clofibrate (bars 4, 5, and 6).

CCG triplets in the directly repeated motifs are critical to CRE2-mediated transcriptional induction

To examine the importance of the CCG triplets in the repeated motif T(T/A)TCCG, the CCG triplets of CRE2 were replaced with AAT singly or in combination, and four copies of the resultant mutant CRE2 were placed, in a tandem array, upstream from the basal promoter (Fig. 4A; the corresponding wild-type construct was pPL4xCRE2). The mutation in any of the three CCG triplets in CRE2 dramatically decreased the clofibrate-induced β-galactosidase activity of the cells with these constructs (Fig. 4B). The mutation of the CCG triplet at the 3′ end (CRE2M3) was less effective than those of the other two CCG triplets. The simultaneous replacement of all three CCG triplets with AAT (CRE2M4) abolished the induction of reporter activity. The same CRE2M4 also abolished the induction by oleic acid and estrogen (data not shown). These results indicate that these CCG triplets are critical for the CRE2-mediated response to inducer compounds.

CCG triplets in CRE2 are critical for the binding of specific proteins to CRE2 in vitro

To investigate the binding of specific proteins to CRE2, we carried out electrophoretic mobility shift assays (EMSA) using the labeled CRE2 or mutant CRE2 DNA as a probe along with partially purified cell extracts from cells grown under various conditions

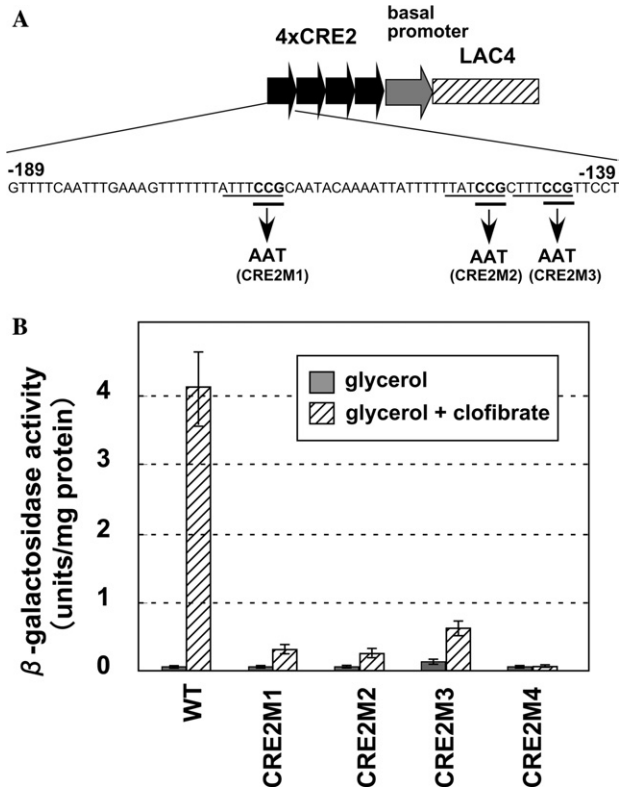

Fig. 4. Effects of the mutations in CCG triplets on CRE2-mediated reporter induction. (A) Mutant CRE2 and the reporter constructs. A single CCG triplet was replaced with AAT (pPL4xCRE2M1, pPL4xCRE2M2, and pPL4xCRE2M3) or all three were replaced (pPL4xCRE2M4). (B) *C. maltosa* CHA1 cells with reporter constructs were grown on SG plus or minus 1 mM clofibrate for 6 h, and β-galactosidase assay was performed.

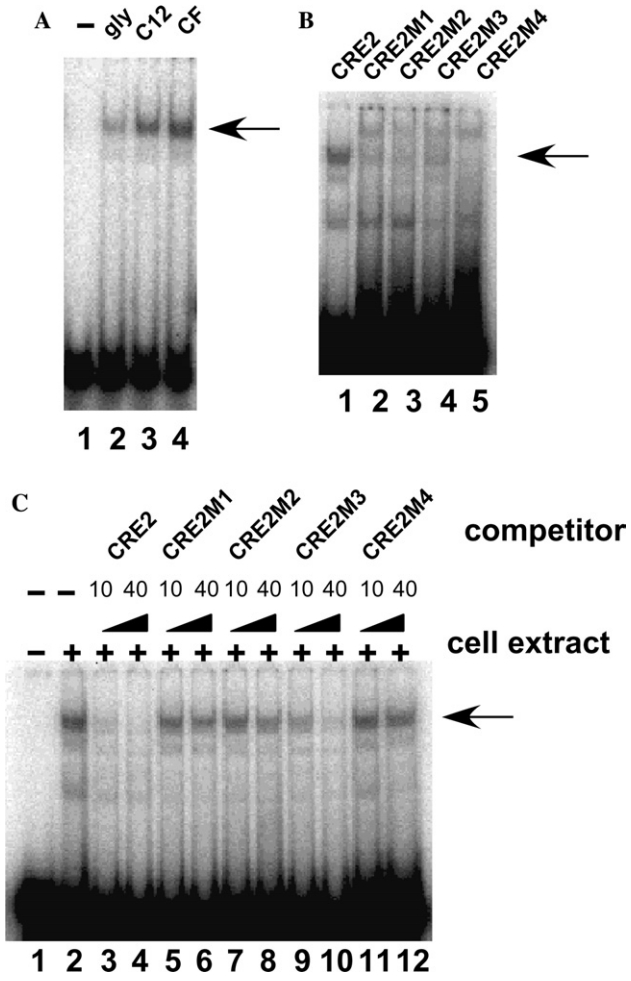

Fig. 5. Analysis of the binding of specific proteins to CRE2 in vitro by EMSA. (A) ^{32}P-labeled CRE2 was incubated with partially purified cell extracts from the cells grown on SD (lane 2), SG (lane 3), or SG plus 1 mM clofibrate (lane 4). The sample in lane 1 contains no extracts. (B) ^{32}P-labeled CRE2 (lane 1), CRE2M1 (lane 2), CRE2M2 (lane 3), CRE2M3 (lane 4), or CRE2M4 (lane 5) was incubated with partially purified extracts from the cells grown on SG plus 1 mM clofibrate. (C) A 10- or 40-fold molar excess of unlabeled CRE2 (lanes 3 and 4) or mutant CRE2 (CRE2M1, lanes 5 and 6; CRE2M2, lanes 7 and 8; CRE2M3, lanes 9 and 10; and CRE2M4, lanes 11 and 12) was added to the reaction of (A, lane 4). Arrows indicate bands of the CRE2–protein complex.

(Fig. 5). As shown in Fig. 5A, a shift band of weak intensity was given by the wild-type CRE2 DNA and the extract of glucose-grown cells. The intensity of the shift band was improved by the extract from glycerol-grown cells and further improved by the extract from the cells grown on glycerol plus clofibrate. These results suggest that the derepressive and inducing conditions increase the cellular amount or binding efficiency of CRE2-binding protein(s).

When the mutant CRE2s (see Fig. 4) were used as probes in conjunction with the clofibrate-induced cell extract, the shift bands were very weak or undetectable (especially in the case of CRE2M4) (Fig. 5B). The competition assay showed that the mutant CRE2s were much less competitive than the wild-type CRE2 (Fig. 5C). Among those mutants, cold CRE2M3 was competitive to some extent and gave a faint shift band at 40-fold excess concentration (Fig. 5C, lane 10), suggesting that the third 3′-end CCG of CRE2 is less essential than the other two in binding specific proteins to CRE2. This seems to correspond well to the moderate response of CRE2M3 to clofibrate (Fig. 4B). Thus, the observed correlation between the specific DNA–protein complex formation and clofibrate-responsive transcriptional induction by CRE2 or its mutant derivatives strongly suggests that the protein(s) that bound to CRE2 in vitro is the transcription factor involved in clofibrate response. The CCG triplets should have a critical role(s) in the binding of this transcription factor to CRE2.

Discussion

In this study, we have successfully identified two small regions, ARE2 and CRE2, on the promoter of the *ALK2* gene encoding CYP52 family cytochrome P450alk2: ARE2 functions in response to *n*-alkane and

oleic acid, while CRE2 functions in response to some peroxisome proliferators, unsaturated fatty acids, and steroid hormones. The differences between the two, in both their inducer chemicals and the degree to which glucose suppresses them, suggest that these two elements work on distinct transcriptional induction pathways.

The profile of inducer chemicals that activate transcription via CRE2 is similar to that for the mammalian nuclear receptors involved in the transcriptional induction of P450s [39]. One such nuclear receptor, PPARα, is involved in the transcriptional activation of fatty acid–metabolizing P450s and is highly activated by both peroxisome proliferators and unsaturated fatty acids; moreover, another nuclear receptor PXR is involved in the transcriptional activation of drug-metabolizing P450s of the CYP3A family and is highly activated by steroids [33]. These nuclear receptors activate transcription as heterodimers with RXR and recognize *cis*-acting promoter elements consisting of direct repeats of closely located unit motif sequences [30]. CRE2 also contains direct-repeat motifs, suggesting the involvement of transcription factors similar to the mammalian nuclear receptors mentioned above. However, the two most essential CCG motifs are 21 bp apart, and the mutation in any of the three CCG motifs seriously damaged the function of CRE2 in vivo and in vitro. These findings suggest that the mechanism underlying transcriptional activation through CRE2 is not necessarily the same as the mammalian nuclear receptor system mentioned above.

Mutational analysis of the CCG triplets in CRE2 strongly suggests that the CRE2-bound protein(s) in EMSA is the clofibrate-responsive transcription activator, and that the CCG triplets might be the targets of such a transcription factor. CCG (or CGG in the opposite direction) triplet-containing direct repeats are also found in promoters of other *ALK* genes of *C. maltosa*, although the sequences around the CCG triplets are variable. Among such *ALK* genes, *ALK3* also has the *n*-alkane- and clofibrate-responsive region with CCG triplets containing direct repeats on its promoter (unpublished data). Hence, this motif could be one of the common *cis*-acting clofibrate-responsive elements in *C. maltosa*. CCG triplet-containing motifs are known as the target sites of transcription factors that belong to the fungal binuclear Cys(6)–Zn(2) cluster family [40]; thus, the transcription factor involved in clofibrate response through CRE2 might also belong to this Cys(6)–Zn(2) transcription factor family.

Recently, a CRE2-like sequence, containing a direct repeat of CGGA(A/T)A accompanied by an AT-rich stretch (5′-CGGA(A/T)ATCGGATATTTTTTTT-3′), was identified as an estradiol-responsive element (drug-responsive element, DRE) of genes encoding ATP-binding cassette transporters in *C. albicans*, a yeast closely related to *C. maltosa* [41]. Furthermore, it is intriguing that a Cys(6)–Zn(2) transcription factor has been identified as the specific DNA-binding protein to the pisatin-responsive element in the filamentous fungus *Nectria haematococca*. The pisatin-responsive element also had a CCG triplet-containing direct repeat and was found to be responsible for the induction of a P450 that functions in the detoxification of this fungistatic isoflavonoid [42]. These findings suggest that a similar transcription factor(s) functions on CRE2 and on these drug-responsive elements of these fungal species.

We also examined the importance of the E-box sequence (CATGTG) and a CCG triplet in the function of ARE2 by introducing mutations into these two sequences. The alteration of the former sequence to ATTGCA resulted in an approximately 50% or 30% reduction in reporter activity upon induction by *n*-dodecane or oleic acid, respectively, suggesting that the E-box sequence is involved in the responses to both inducers, though more to *n*-dodecane. The alteration of the latter sequence to AAT resulted in an approximately 5% or 55% reduction in reporter activity upon induction by *n*-dodecane or oleic acid, respectively (data not shown), suggesting that the CCG motif is involved in the response to oleic acid but not to *n*-alkane. These results imply that ARE2 could be further dissected into functional elements, although the removal of the 3′-end 23 bp that includes the CCG motif largely reduced the responsiveness to both *n*-dodecane and oleic acid (Fig. 2, pPL4xARE2-2).

In *Y. lipolytica*, another *n*-alkane-assimilating yeast, promoter sequence ARE1, containing a direct repeat of two E-box–like motifs, was specified as an *n*-alkane-responsive *cis*-acting element [19,43]. Furthermore *YAS1*, encoding a protein with a basic helix–loop–helix motif, was identified as an essential gene for ARE1-mediated gene expression [19]. These findings imply the involvement of a similar protein in the function of *C. maltosa* ARE2, although the E-box-like motif is not repeated in ARE2.

Our efforts are presently focused on the identification of *trans*-acting factors that function on the *cis*-acting elements described here.

Acknowledgments

We thank Drs. H. Horiuchi and R. Fukuda for helpful discussions. This work was performed using the facilities of the Biotechnology Research Center at the University of Tokyo.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2005.01.099.

References

[1] D.R. Nelson, L. Koymans, T. Kamataki, J.J. Stegeman, R. Feyereisen, D.J. Waxman, M.R. Waterman, O. Gotoh, M.J. Coon, R.W. Estabrook, I.C. Gunsalus, D.W. Nebert, P450 superfamily: Update on new sequences, gene mapping, accession numbers and nomenclature, Pharmacogenetics 6 (1996) 1–42.

[2] D.R. Nelson, Cytochrome P450 and the individuality of species, Arch. Biochem. Biophys. 369 (1999) 1–10.

[3] M. Takagi, M. Ohkuma, N. Kobayashi, M. Watanabe, K. Yano, Purification of cytochrome P450alk from *n*-alkane-grown cells of *Candida maltosa*, and cloning and nucleotide sequencing of the encoding gene, Agric. Biol. Chem. 53 (1989) 2217–2226.

[4] M. Ohkuma, T. Hikiji, T. Tanimoto, W.H. Schunck, H.G. Muller, K. Yano, M. Takagi, Evidence that more than one gene encodes *n*-alkane-inducible cytochrome P-450s in *Candida maltosa*, found by two-step gene disruption, Agric. Biol. Chem. 55 (1991) 1757–1764.

[5] M. Ohkuma, T. Tanimoto, K. Yano, M. Takagi, CYP52 (cytochrome P450alk) multigene family in *Candida maltosa*: Molecular cloning and nucleotide sequence of the two tandemly arranged genes, DNA Cell Biol. 10 (1991) 271–282.

[6] M. Ohkuma, S. Muraoka, T. Tanimoto, M. Fujii, A. Ohta, M. Takagi, CYP52 (cytochrome P450alk) multigene family in *Candida maltosa*: Identification and characterization of eight members, DNA Cell Biol. 14 (1995) 163–173.

[7] D. Sanglard, C. Chen, J.C. Loper, Isolation of the alkane inducible cytochrome P450 (P450alk) gene from the yeast *Candida tropicalis*, Biochem. Biophys. Res. Commun. 144 (1987) 251–257.

[8] D. Sanglard, A. Fiechter, Heterogeneity within the alkane-inducible cytochrome P450 gene family of the yeast *Candida tropicalis*, FEBS Lett. 256 (1989) 128–134.

[9] W. Seghezzi, D. Sanglard, A. Fiechter, Characterization of a second alkane-inducible cytochrome P450 encoding gene, CYP52A2, from *Candida tropicalis*, Gene 106 (1991) 51–60.

[10] W. Seghezzi, C. Meili, R. Ruffiner, R. Kuenzi, D. Sanglard, A. Fiechter, Identification and characterization of additional members of the cytochrome P450 multigene family CYP52 of *Candida tropicalis*, DNA Cell Biol. 11 (1992) 767–780.

[11] T. Iida, A. Ohta, M. Takagi, Cloning and characterization of an *n*-alkane-inducible cytochrome P450 gene essential for *n*-decane assimilation by *Yarrowia lipolytica*, Yeast 14 (1998) 1387–1397.

[12] T. Iida, T. Sumita, A. Ohta, M. Takagi, The cytochrome P450ALK multigene family of an *n*-alkane-assimilating yeast, *Yarrowia lipolytica*: Cloning and characterization of genes coding for new CYP52 family members, Yeast 16 (2000) 1077–1087.

[13] M. Ohkuma, S.M. Park, T. Zimmer, R. Menzel, F. Vogel, W.H. Schunck, A. Ohta, M. Takagi, Proliferation of intracellular membrane structures upon homologous overproduction of cytochrome P-450 in *Candida maltosa*, Biochim. Biophys. Acta 1236 (1995) 163–169.

[14] M. Ohkuma, T. Zimmer, T. Iida, W.H. Schunck, A. Ohta, M. Takagi, Isozyme function of *n*-alkane-inducible cytochromes P450 in *Candida maltosa* revealed by sequential gene disruption, J. Biol. Chem. 273 (1998) 3948–3953.

[15] T. Zimmer, K. Kaminski, U. Scheller, F. Vogel, W.H. Schunck, In vivo reconstitution of highly active *Candida maltosa* cytochrome P450 monooxygenase systems in inducible membranes of *Saccharomyces cerevisiae*, DNA Cell Biol. 14 (1995) 619–628.

[16] T. Zimmer, M. Ohkuma, A. Ohta, M. Takagi, W.H. Schunck, The CYP52 multigene family of *Candida maltosa* encodes functionally diverse *n*-alkane-inducible cytochromes P450, Biochem. Biophys. Res. Commun. 224 (1996) 784–789.

[17] T. Zimmer, T. Iida, W.H. Schunck, Y. Yoshida, A. Ohta, M. Takagi, Relation between evolutionary distance and enzymatic properties among the members of the CYP52A subfamily of *Candida maltosa*, Biochem. Biophys. Res. Commun. 251 (1998) 244–247.

[18] U. Scheller, T. Zimmer, E. Kargel, W.H. Schunck, Characterization of the *n*-alkane and fatty acid hydroxylating cytochrome P450 forms 52A3 and 52A4, Arch. Biochem. Biophys. 328 (1996) 245–254.

[19] S. Yamagami, D. Morioka, R. Fukuda, A. Ohta, A basic helix-loop-helix transcription factor essential for cytochrome P450 induction in response to alkanes in yeast *Yarrowia lipolytica*, J. Biol. Chem. 279 (2004) 22183–22189.

[20] H. Rottensteiner, A.J. Kal, M. Filipits, M. Binder, B. Hamilton, H.F. Tabak, H. Ruis, Pip2p: A transcriptional regulator of peroxisome proliferation in the yeast *Saccharomyces cerevisiae*, EMBO J. 15 (1996) 2924–2934.

[21] Y. Luo, I.V. Karpichev, R.A. Kohanski, G.M. Small, 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 (1996) 12068–12075.

[22] I.V. Karpichev, Y. Luo, R.C. Marians, G.M. Small, A complex containing two transcription factors regulates peroxisome proliferation and the coordinate induction of β-oxidation enzymes in *Saccharomyces cerevisiae*, Mol. Cell. Biol. 17 (1997) 69–80.

[23] I.V. Karpichev, G.M. Small, Global regulatory functions of Oaf1p and Pip2p (Oaf2p), transcription factors that regulate genes encoding peroxisomal proteins in *Saccharomyces cerevisiae*, Mol. Cell. Biol. 18 (1998) 6560–6570.

[24] H. Rottensteiner, A.J. Kal, B. Hamilton, H. Ruis, H.F. Tabak, A heterodimer of the Zn_2Cys_6 transcription factors Pip2p and Oaf1p controls induction of genes encoding peroxisomal proteins in *Saccharomyces cerevisiae*, Eur. J. Biochem. 247 (1997) 776–783.

[25] A.W. Einerhand, W.T. Kos, B. Distel, H.F. Tabak, Characterization of a transcriptional control element involved in proliferation of peroxisomes in yeast in response to oleate, Eur. J. Biochem. 214 (1993) 323–331.

[26] M. Filipits, M.M. Simon, W. Rapatz, B. Hamilton, H. Ruis, A *Saccharomyces cerevisiae* upstream activating sequence mediates induction of peroxisome proliferation by fatty acids, Gene 132 (1993) 49–55.

[27] H. Rottensteiner, A. Hartig, B. Hamilton, H. Ruis, R. Erdmann, A. Gurvitz, *Saccharomyces cerevisiae* Pip2p-Oaf1p regulates *PEX25* transcription through an adenine-less ORE, Eur. J. Biochem. 270 (2003) 2013–2022.

[28] R. Ohtomo, K. Kobayashi, S. Muraoka, M. Ohkuma, A. Ohta, M. Takagi, Peroxisome proliferators activate cytochrome P450 genes in an alkane-assimilating yeast, *Candida maltosa*, Biochem. Biophys. Res. Commun. 222 (1996) 790–793.

[29] E.A. Lock, A.M. Mitchell, C.R. Elcombe, Biochemical mechanisms of induction of hepatic peroxisome proliferation, Annu. Rev. Pharmacol. Toxicol. 29 (1989) 145–163.

[30] K. Motojima, Peroxisome proliferator-activated receptor (PPAR): Structure, mechanisms of activation and diverse functions, Cell Struct. Funct. 18 (1993) 267–277.

[31] J. Auwerx, Regulation of gene expression by fatty acids and fibric acid derivatives: An integrative role for peroxisome proliferator activated receptors, The Belgian Endocrine Society Lecture 1992, Horm. Res. 38 (1992) 269–277.

[32] K.L. Gearing, M. Gottlicher, E. Widmark, C.D. Banner, P. Tollet, M. Stromstedt, J.J. Rafter, R.K. Berge, J.A. Gustafsson, Fatty acid activation of the peroxisome proliferator-activated receptor, a member of the nuclear receptor gene superfamily, J. Nutr. 124 (1994) S1284–S1288.

[33] S.A. Kliewer, J.M. Lehmann, M.V. Milburn, T.M. Willson, The PPARs and PXRs: Nuclear xenobiotic receptors that define novel hormone signaling pathways, Recent Prog. Horm. Res. 54 (1999) 345–367.

[34] N. English, V. Hughes, C.R. Wolf, Common pathways of cytochrome P450 gene regulation by peroxisome proliferators and barbiturates in *Bacillus megaterium* ATCC14581, J. Biol. Chem. 269 (1994) 26836–26841.
[35] S. Kawai, T. Hikiji, S. Murao, M. Takagi, K. Yano, Isolation and sequencing of a gene, *C-ADE1*, and its use for a host-vector system in *Candida maltosa* with two genetic markers, Agric. Biol. Chem. 55 (1991) 59–65.
[36] M. Ohkuma, Y. Masuda, S.M. Park, R. Ohtomo, A. Ohta, M. Takagi, Evidence that the expression of the gene for NADPH-cytochrome P-450 reductase is *n*-alkane-inducible in *Candida maltosa*, Biosci. Biotechnol. Biochem. 59 (1995) 1328–1330.
[37] H. Sugiyama, M. Ohkuma, Y. Masuda, S.M. Park, A. Ohta, M. Takagi, In vivo evidence for non-universal usage of the codon CUG in *Candida maltosa*, Yeast 11 (1995) 43–52.
[38] W.R. Atchley, W.M. Fitch, A natural classification of the basic helix-loop-helix class of transcription factors, Proc. Natl. Acad. Sci. USA 94 (1997) 5172–5176.
[39] M.S. Denison, J.P. Whitlock Jr., Xenobiotic-inducible transcription of cytochrome P450 genes, J. Biol. Chem. 270 (1995) 18175–18178.
[40] R.B. Todd, A. Andrianopoulos, Evolution of a fungal regulatory gene family: The Zn(II)2Cys6 binuclear cluster DNA binding motif, Fungal Genet. Biol. 21 (1997) 388–405.
[41] M. de Micheli, J. Bille, C. Schueller, D. Sanglard, A common drug-responsive element mediates the upregulation of the *Candida albicans* ABC transporters *CDR1* and *CDR2*, two genes involved in antifungal drug resistance, Mol. Microbiol. 43 (2002) 1197–1214.
[42] R. Khan, R. Tan, A.G. Mariscal, D. Straney, A binuclear zinc transcription factor binds the host isoflavonoid-responsive element in a fungal cytochrome p450 gene responsible for detoxification, Mol. Microbiol. 49 (2003) 117–130.
[43] T. Sumita, T. Iida, S. Yamagami, H. Horiuchi, M. Takagi, A. Ohta, *YlALK1* encoding the cytochrome P450ALK1 in *Yarrowia lipolytica* is transcriptionally induced by *n*-alkane through two distinct *cis*-elements on its promoter, Biochem. Biophys. Res. Commun. 294 (2002) 1071–1078.