Gene, 105 (1991) 129–134
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GENE 06042

Glucose-responsive and oleic acid-responsive elements in the gene encoding the peroxisomal trifunctional enzyme of *Candida tropicalis*

(Gene regulation; β-oxidation; oleic acid; heterologous expression; yeast; hydratase-dehydrogenase-epimerase; recombinant DNA; *Saccharomyces cerevisiae*)

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Received by G.C. Shore: 10 June 1991
Accepted: 18 June 1991
Received at publishers: 11 July 1991

SUMMARY

We have investigated the regulation of expression of the gene (*HDE*), encoding the peroxisomal trifunctional enzyme hydratase-dehydrogenase-epimerase (HDE), of the diploid yeast *Candida tropicalis*. Heterologous expression in *Saccharomyces cerevisiae* of constructs containing deletions in the upstream region of the *HDE* gene has allowed for determination of regions responsible for the control of expression of the *HDE* gene. Expression was monitored by immunoblot analysis of yeast lysates with anti-HDE serum. Regions have been identified that are responsible for both repression by glucose and induction by oleic acid. A glucose-responsive region lies between nucleotides (nt) −526 and −393. An oleic acid-responsive region lies between nt −393 and −341. An additional region controlling derepression by nonfermentable carbon sources is located downstream from nt −341. Comparison of the nt sequences of these regions to upstream regions of other oleic acid-responsive genes of *C. tropicalis* has identified possible consensus nt sequences for glucose- and oleic acid-responsive upstream elements in these genes. The regulation of the *HDE* gene in *S. cerevisiae* closely resembles that found in *C. tropicalis*, suggesting that similar mechanisms of transcriptional control operate in both yeasts.

INTRODUCTION

Peroxisomes have many metabolic functions, notably the β-oxidation of fatty acids. Yeasts such as *C. tropicalis* and *S. cerevisiae* are excellent model systems for the study of peroxisome biogenesis. Peroxisomes proliferate and there is a marked increase in the specific activities of the β-oxidation enzymes when these yeasts are grown in carbon sources such as oleic acid (reviewed by Lazarow and Fujiki, 1985; Borst, 1986). We are interested in the mechanisms of regulation of the gene encoding the peroxisomal trifunctional β-oxidation enzyme of *C. tropicalis*, HDE. Although *C. tropicalis* has been instrumental in studying peroxisome biogenesis and function, genetic work with this species has been hampered by the lack of a transformation system and by the asexual, diploid nature of the yeast (Rachubinski, 1990). The amenability of *S. cerevisiae* to genetic manipulation and the fact that peroxisomes proliferate when *S. cerevisiae* is grown on oleic acid (Veenhuis et al., 1987) suggested that *S. cerevisiae* could act as an heterologous expression system for studying the regulation of the *HDE* gene. In our experimental system, the HDE polypeptide could be detected immunologically by a polyclonal antiserum that did not cross-react with the endogenous tri-

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Abbreviations: AOX, acyl-CoA oxidase; *AOX*, gene encoding AOX; bp, base pair(s); *C.*, *Candida*; CATL, catalase; *CATL*, gene encoding CATL; HDE, trifunctional enzyme; *HDE*, gene encoding HDE; kb, kilobase(s) or 1000 bp; nt, nucleotide(s); SDS, sodium dodecyl sulfate; u, unit(s); YNE, see Fig. 1 legend.

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Ex. 1075
Page 1 of 6

functional enzyme of *S. cerevisiae*. Therefore, the *HDE* gene was expressed under the control of its own promoter and functioned as its own reporter.

The aim of the present study was to dissect the regulatory region of a gene encoding a yeast peroxisomal protein and to show that *C. tropicalis HDE* is expressed in *S. cerevisiae* and that its expression is subject to induction by growth in oleic acid.

EXPERIMENTAL AND DISCUSSION

(a) Expression of genes encoding HDE, acyl-CoA oxidase and catalase in *Candida tropicalis*

Lysates prepared from *C. tropicalis* cells grown on different carbon sources were analyzed by immunoblotting to assess the effects of carbon source on expression of the genes encoding HDE, AOX and CATL. Although Tween-40 was only needed for the solubilization of oleic acid, it was nevertheless added as an invariant component to all media. The addition of glucose to both high and low concentrations in YNE greatly repressed *HDE* expression (35-fold and 28-fold, respectively) and repressed, although to a lesser extent, *CATL* (7.6-fold and 4.9-fold, respectively) and *AOX* (10-fold and 7.8-fold, respectively) expression (Fig. 1, compare lanes – with lanes G^5 and $G^{0.2}$, respectively). These results suggest that glucose repression and derepression play an important role in the regulation of expression of *HDE*, *CATL* and *AOX* in *C. tropicalis*. The addition of 0.2% oleic acid to low glucose increased expression of *HDE* 6.2-fold, of *CATL* 2.6-fold and of *AOX* 4.5-fold (Fig. 1, compare lanes $G^{0.2}O^{0.2}$ with lanes $G^{0.2}$), demonstrating an induction of the expression of these genes by oleic acid. High levels of expression were seen for all three genes in YNE (contains only Tween-40) (Fig. 1, A and B, lanes –), indicating that Tween-40 can be metabolized by the β-oxidation pathway and used as a carbon source in *C. tropicalis*. The addition of 2% oleic acid to YNE increased *HDE* expression 1.3-fold, *CATL* expression 1.4-fold, and *AOX* expression 1.3-fold (Fig. 1, compare lanes O^2 with lanes –). Taken together, the results indicate that oleic acid and Tween-40 probably act via different mechanisms. *HDE* (3.7-fold), *CATL* (1.1-fold) and *AOX* (1.2-fold) expression was higher when cells were grown in glycerol-ethanol as opposed to growth in low glucose (Fig. 1, compare lanes GY^2E^2 with $G^{0.2}$), demonstrating that a nonfermentable carbon source can influence the level of expression of these genes. The addition of 0.2% oleic acid to nonfermentable glycerol-ethanol further increased expression of *HDE* 3.3-fold, of *CATL* 2.6-fold and of *AOX* 4.7-fold (Fig. 1, compare lanes $GY^2E^2O^{0.2}$ with lanes GY^2E^2), again showing an induction of expression of these genes by oleic acid. Reduction of *HDE*

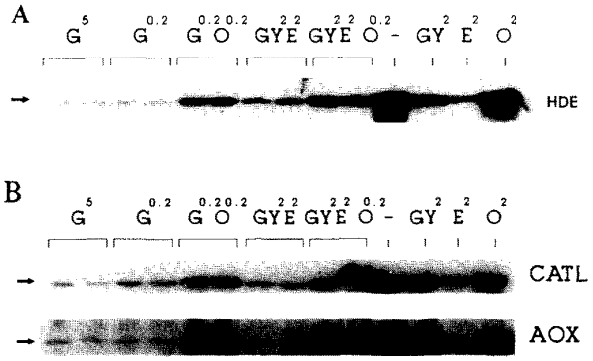

Fig. 1. Effects of various carbon sources on expression of *HDE*, *AOX* and *CATL* in *C. tropicalis*. The yeast *C. tropicalis* Berkhout strain pK233 (ATCC20336) (Tanabe et al., 1966) was grown in minimal medium (YNE; 0.67% yeast nitrogen base/0.05% yeast extract/0.5% (w/v) Tween-40 (Erdmann et al., 1989), containing one of the following as a carbon source: 5% glucose (high glucose); 0.2% glucose (low glucose); 0.2% glucose/0.2% (w/v) oleic acid (glucose-oleic acid); 2% (v/v) glycerol; 2% (v/v) ethanol; 2% (v/v) glycerol/2% (v/v) ethanol (glycerol-ethanol); 2% (v/v) glycerol/2% (v/v) ethanol/0.2% (w/v) oleic acid; or 2% (w/v) oleic acid. 50-ml cultures of cells grown to late exponential phase (A_{600} – 0.4) were pelleted, washed in sterile H_2O, and resuspended in an equal volume of 50 mM Tris · HCl pH 8.0/50 mM NaCl/0.1 mM EDTA/0.1 mM $ZnCl_2$/15 mM phenylmethylsulfonylfluoride. The cells were disrupted by vortexing with glass beads. The crude lysates were clarified by centrifugation. Protein was determined by the method of Bradford (1976) using ovalbumin as a standard. Proteins were analyzed by 0.1% SDS–polyacrylamide gel electrophoresis (Fujiki et al., 1984) followed by immunoblotting using antisera against: (panel **A**) HDE and (panel **B**) CATL and AOX. [^{125}I]Protein A (Amersham, Oakville, ON) was used to detect antigen-antibody complexes (Burnette, 1981). Autoradiography was performed at –70°C with preflashed (Laskey and Mills, 1977) XAR-5 film (Eastman Kodak Co., Rochester, NY). The films were analyzed with a GS-300 scanning densitometer and the GS-350 software (Hoefer Scientific Instruments, San Francisco, CA). Quantitative analyses were the average of at least two independent observations, except for *C. tropicalis* grown in YNE alone or YNE plus one of 2% (v/v) glycerol, 2% (v/v) ethanol, or 2% (w/v) oleic acid. G, glucose; GY, glycerol; E, ethanol; O, oleic acid; [–], YNE. Superscripts refer to % of a particular carbon source. Equal amounts of protein were run in each lane.

(5.1-fold), *CATL* (4.5-fold) and *AOX* (4.7-fold) expression when ethanol is added to glycerol-containing medium (Fig. 1, compare lanes GY^2E^2 to GY^2) indicates that ethanol represses the expression of these genes. This argues that derepression, and not induction by a carbon source (glycerol) in the absence of glucose, is the principal mechanism resulting in increased expression of these genes. It appears that when *C. tropicalis* is grown on carbon sources not metabolized by the β-oxidation pathway, a general negative control mechanism involving repression and derepression effects the expression of *HDE*, *AOX* and *CATL*. The coordinate expression of *HDE*, *AOX* and *CATL* in different carbon sources suggests these genes share common regulatory elements.

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Ex. 1075
Page 2 of 6

(b) Expression of *HDE* deletion constructs in *Saccharomyces cerevisiae*

Determination of the upstream elements involved in the regulation of the *HDE* gene necessitated its introduction into the genetically manipulable yeast *S. cerevisiae*. The *HDE* gene was inserted into the *CEN*-containing plasmid YCp50 producing plasmid YCp505H (Fig. 2A), which was used to transform *S. cerevisiae*. The YCp50 vector assures a stable average gene dosage of one copy per cell (Struhl, 1983). Deletions (Fig. 2B) in the upstream region of *HDE* (Fig. 3) were constructed to determine the 5′ elements mediating the responses detailed above. *HDE* expression in *S. cerevisiae* was assayed by immunoblotting of total lysates with anti-HDE serum which is specific for the heterologously synthesized HDE.

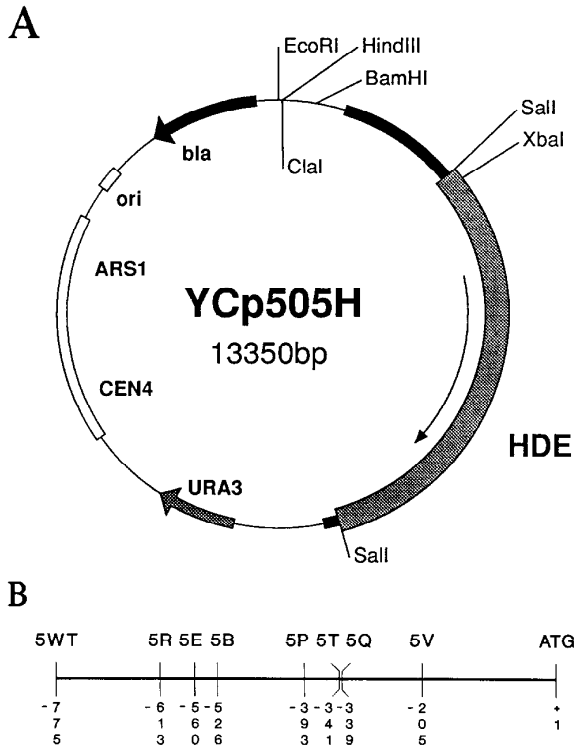

Fig. 2. Constructions for analysis of the *HDE* gene. (Panel **A**) Subcloning of the *HDE* gene for expression in *S. cerevisiae*. A 4.2-kb *Sal*I fragment containing the *HDE* gene (Aitchison et al., 1990) was ligated into *Sal*I-digested pGEM5Zf(+) to produce recombinant plasmid 3T. YCp505H was constructed by ligating the *Sca*I-*Sac*I fragment containing the *HDE* gene from 3T into the *Sal*I site of YCp50. Both the fragment and the vector were treated with T4 DNA polymerase to produce blunt ends. 1.8 kb of pGEM5Zf(+) (blackened box) flanked the 5′ end of the *HDE* gene to act as a buffer for subsequent BAL 31 digestion. The thin arrow indicates the direction of transcription of the *HDE* gene. *E. coli* DH5-α cells were transformed as described by Bethesda Research Laboratories (Burlington, ON) and grown in Luria broth supplemented with 100 μg ampicillin/ml. Recombinants were selected by colony hybridization (Grunstein and Hogness, 1975) and characterized by restriction endonuclease digestion. (Panel **B**) Generation of 5′ deletion constructs. BAL 31 exonuclease was used to produce deletions in the upstream region of the *HDE* gene. Digestions were performed in K·glutamate buffer (Hanish and McClelland, 1988) at 37°C with 1 u of BAL 31/pmol of *Xba*I-linearized YCp505H, which removed approx. 35 bp/min from each end. The digested DNA ends were made blunt using T4 DNA polymerase, and *Bam*HI linkers (GGGATCCC) were added. The plasmids were then digested with *Bam*HI and recircularized. BAL 31 deletion end points were determined by dideoxy sequencing (Sanger et al., 1977). Numbering (all minus) is as in Fig. 3.

The removal of sequences from nt −775 (5WT; see Fig. 2B) to nt −613 (5R) had essentially no effect on the levels of *HDE* expression in either low glucose or glucose-oleic acid (Fig. 4, compare lanes 5WT and 5R). Deletion of the next 53 nt to −560 (5E) showed a threefold and twofold increase in *HDE* expression when cells were grown in low glucose and glucose-oleic acid, respectively, suggesting the presence of a repressive control element between nt −613 and −560 (Fig. 4, compare lanes 5E with lanes 5R). Sequences between nt −560 (5E) and −526 (5B) positively regulate *HDE* expression, because when removed, levels of *HDE* expression decreased to approx. 40% of 5WT in glucose and glucose-oleic acid (Fig. 4, compare lanes 5B with lanes 5E). Removal of the next 133 nt to −393 caused a 6.6-fold increase and a 36-fold increase in *HDE* expression relative to 5WT in glucose and glucose-oleic acid, respectively (Fig. 4, compare lanes 5P with lanes 5WT). Therefore, the region between nt −526 and −393 contains a negative regulatory element. There are two sequences within this region that are conserved in a number of oleic acid-responsive genes of *C. tropicalis* (Table I; Fig. 3). One of these sequences is similar to the consensus sequence [ATTTC$^{CC}_{TT}$] for regulation of *SUC2* by glucose in *S. cerevisiae* (Sarokin and Carlson, 1986). It is interesting to speculate that these sequences may be similarly involved in the glucose response of all these genes.

HDE expression in 5P (nt −393) and 5T (nt −341) is 6.6-fold and 2.1-fold that of 5WT, respectively, in low glucose, and 36-fold and 4-fold that of 5WT, respectively, in glucose-oleic acid (Fig. 4, compare lanes 5P, 5T, and 5WT). This region contains an oleic acid-responsive element, because the ratios of 5P/5T (relative to 5WT) is threefold higher in glucose-oleic acid than in low glucose. Therefore, sequences unique to 5P, i.e., nt −393 to −341, respond positively to oleic acid. A nonanucleotide sequence [consensus = $^{T}_{C}$GGTT$^{A}_{G}$TTA] between nt −393 and −341 of *HDE* is conserved in oleic acid-responsive genes of *C. tropicalis* (Table I; Fig. 3). This sequence may be a binding site for an oleic acid-responsive factor.

Removal of nt downstream from nt −341 (5T) to give 5Q and 5V progressively reduced *HDE* expression to the limits of detection in both glucose and glucose-oleic acid (Fig. 4), suggesting that the minimal length for a functional *HDE* promoter lies between nt −341 and −205.

To extend the above results, 5P and 5T, which delineate

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Ex. 1075
Page 3 of 6

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        -770       -760       -750       -740       -730       -720       -710       -700       -690
     GTCGA CGTTTTGCCG CCACTTGTGA AGGGAAGAAG AGTCGTTGAG TTGATGTAAT TAAGCTGGCA CGTAGATACC AGAAGGTTCT AGAGTAGAGC
-680       -670       -660       -650       -640       -630       -620       -610       -600       -590
 TTGGGTGGTG TTTGGCCCTG TTTGGACCAC GGATAGAGAT GGAGAATCCC TTGGTTAGAG CGGAGAGGAA AAAATTGAAA CTTTGCATAT CCCACTTCAT
-580       -570       -560       -550       -540       -530       -520       -510       -500       -490
 TATCCTTGAT GTAACCGTTT TATGGGGTAA TTAAAGTGTG GAAAAATAAT CAGGGAGACA TATTCCCGAT CAATTGGGTG GTGGTCGCTC AATTTCTGTG
-480       -470       -460       -450       -440       -430       -420       -410       -400       -390
 AGTAGTAGGC TCAGTGGTGT GTATTGGGAT TGGTAGTAGT CTGTATAAGC AGTGTTATAT AACCCATTGC TTGTTGATTC CTATTTTGCT GGCAAAAGTG
-380       -370       -360       -350       -340       -330       -320       -310       -300       -290
 ACAACTGTAG TTGTGAGATA ATCCTCGGTT ATTACGCCTG GGGGGGCAGA CAGCCAAAGT TGTGCCCGTG CGACAATGGC ATCAGAAGAA ACAGAAAAAA
-280       -270       -260       -250       -240       -230       -220       -210       -200       -190
 AAAACACAGG CATTTTTATC CACATGCACA CTACCCCCAC TATTCCTGTC TGCAGTGTGC TTGTGTGTGG CCCCCCGCAG AATCAACAGG GCAAACTCTG
-180       -170       -160       -150       -140       -130       -120       -110       -100       -90
 GAGCCTGAAT CTTTATATAA ACTTCAGGCA TTGGCCCCCC TTTTCACAAT TCTTCACATC CACCATTTTT TTTCTTCTTT CCTACCATAT TAGTTTTTTT
-80        -70        -60        -50        -40        -30        -20        -10        +1
 TTATTCTTTT CCTACCTATC TGATTATTAT CAAACATCTG GTCATCCTCA AAAGAAAGAA AGAAACTATA ACAATCAATC ATG
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Fig. 3. Nucleotide sequence of the upstream region of the *HDE* gene. The nt sequence of *HDE* has been determined previously (Aitchison et al., 1991). The first nt of the start codon is designated + 1. The first digits of numerals are aligned with the corresponding nt. Nucleotides 5′ to this nt are designated by negative values. The double-underlined nt sequence corresponds to a consensus nt sequence for response to glucose. The sequence in bold type and underlined is similar to the glucose-responsive element of *SUC2*. The single-underlined sequence corresponds to a consensus nt sequence for response to oleic acid. The sequence in bold type corresponds to a TATA consensus sequence.

the oleic acid-responsive region, along with the full-length *HDE* (5WT), were chosen to investigate the effects of various carbon sources. Since only 5P showed detectable levels of *HDE* expression in high glucose (Fig. 5, panel G^5), sequences upstream from nt −393 contain elements involved in glucose repression, in agreement with results presented above showing a negative regulatory region between nt −526 and −393. As there is no detectable *HDE* expression seen with 5T, the positive elements responsible for the expression seen with 5P are located between nt −393 and −341 (Fig. 5, panel G^5). Low glucose increased *HDE* expression due to derepression in both 5P (3.9-fold) and 5T (Fig. 5, compare lanes $G^{0.2}$ with G^5), indicating that elements responsible for derepression are located downstream

TABLE I

Sequences of the responsive regions of the *HDE* gene conserved in other *Candida tropicalis* genes encoding peroxisomal proteins[a]

Genes[b]	Glucose[c] (distance)[c]				Oleate[d] (distance)[e]	
	A		**B**			
HDE	GGGAGACATA		TTTCTGTGAGT		CGGTTATTA	
	−528	−519	−488	−478	−355	−347
POX4	GAGAGAGAGA		ATTGTCTGAGT		CGGTTATTC	
	−482	−473	−330	−320	−359	−351
POX18	GGGAGAGAGA		TTTTTGTATGT		CGGTAGTTA	
	−396	−387	−194	−184	−223	−215
P450alk	GGTAGACTTA		TTCATGTGAAA		TGGTTGTTG	
	−580	−571	−437	−427	−253	−261
CATL	GGGAAACAGA		TTTGTGTGAGG		TGGTTATTA	
	−422	−431	−600	−590	−695	−687
Consensus	GGGAGA$^{G}_{C}$A$^{T}_{G}$A		TTTNTGTGAG$^{T}_{A}$		$^{T}_{C}$GGTT$^{A}_{G}$TT$^{C}_{A}$	

[a] The sequences of the glucose-responsive (nt −526 to −393) and the oleic acid-responsive (nt −393 to −341) regions of the *HDE* gene were compared to the sequences of the upstream regions of other oleic acid-responsive genes of *C. tropicalis*.

[b] *HDE*, trifunctional enzyme; *POX4*, fatty acyl-CoA oxidase (Okazaki et al., 1986); *POX18*, peroxisomal 18-kDa protein (Szabo et al., 1989); *P450alk*, alkane-inducible cytochrome P450 (Sanglard and Loper, 1989); *CATL*, catalase (Murray and Rachubinski, 1989).

[c] Sequences in the glucose-responsive region of *HDE* conserved in other oleic acid-responsive genes of *C. tropicalis*. Column **A**, conserved sequence **A**; column **B**, conserved sequence **B** similar to the *SUC2* glucose-responsive sequence of *S. cerevisiae*.

[d] Sequence in the oleic acid-responsive region of *HDE* conserved in other oleic acid-responsive genes of *C. tropicalis*.

[e] Distance in nt upstream from the A (+ 1) of the start codon. Last digits of numbers are aligned with corresponding nt.

Nucleotides denoted in bold type deviate from the derived consensus sequences.

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Page 4 of 6

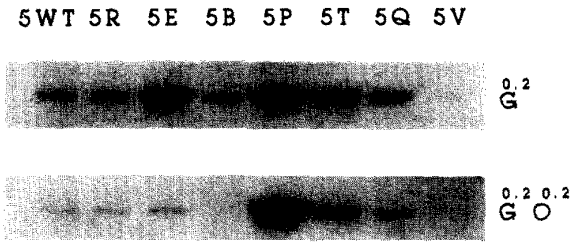

Fig. 4. Expression of deletion constructs in *S. cerevisiae*. *S. cerevisiae* DL-1 (Van Loon et al., 1983) was transformed by the method of Ito et al. (1983). Transformants harboring various deletion constructs were grown in YNE supplemented with leucine (30 μg/ml) and histidine (20 μg/ml) containing either low glucose (upper panel, $G^{0.2}$) or glucose-oleic acid (lower panel, $G^{0.2}O^{0.2}$; see Fig. 1 for details of media composition, gel, and abbreviations), lysed and analyzed for *HDE* expression by immunoblotting (see Fig. 1 for details). Lanes of each panel contain equal amounts of protein.

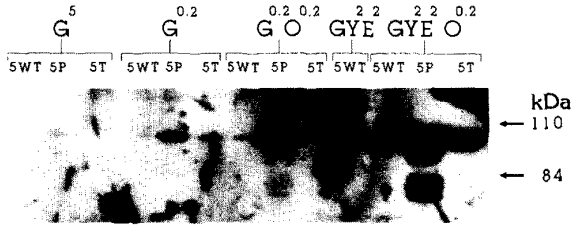

Fig. 5. Effects of various carbon sources on *HDE* expression by constructs 5WT, 5P, and 5T. Transformants harboring constructs 5WT, 5P and 5T were grown in various carbon sources. Yeast lysates were prepared and analyzed for *HDE* expression by immunoblotting. Carbon sources, gel, and methods are as in Fig. 1. Equal amounts of protein were run in each lane.

from nt −341. Addition of oleic acid to low glucose increased expression of 5WT to detectable levels, 5P 14-fold and 5T 1.3-fold (Fig. 5, compare lanes $G^{0.2}O^{0.2}$ with lanes $G^{0.2}$), providing further evidence for an oleic acid-responsive element between nt −393 and −341.

Substitution of nonfermentable glycerol-ethanol for low glucose in media containing oleic acid resulted in increased expression for 5WT (9.3-fold), 5P (3.4-fold) and 5T (45-fold) (Fig. 5, compare lanes $GY^2E^2O^{0.2}$ with $G^{0.2}O^{0.2}$), indicating that sequences responsive to glycerol-ethanol (or the absence of glucose) are downstream from nt −341 (5T) (Fig. 5, compare lanes $GY^2E^2O^{0.2}$ with $G^{0.2}O^{0.2}$).

The addition of 0.2% oleic acid to glycerol-ethanol increased *HDE* expression 1.4-fold (Fig. 5, 5WT, compare lanes $GY^2E^2O^{0.2}$ with GY^2E^2). This increase is approx. 40% of that seen in *C. tropicalis* (3.3-fold; Fig. 1). Switching from a fermentable to a nonfermentable carbon source increased *HDE* expression of 5WT at least eightfold (Fig. 5, compare lanes GY^2E^2 with lanes $G^{0.2}$), (about twice that seen under the same conditions in *C. tropicalis*; 3.5-fold; Fig. 1). These results suggest that *HDE* expression in *S. cerevisiae* is regulated in a fashion similar to that seen in *C. tropicalis*, except that the extent of repression, derepression and activation differs. The *HDE* gene is subject to greater repression and derepression but to lower activation by oleic acid when expressed in *S. cerevisiae*, which is consistent with the reduced ability of *S. cerevisiae* vis-à-vis *C. tropicalis* to metabolize fatty acids. Removal of sequences upstream from 5P (nt −393) results in a restoration of high levels of expression of *HDE* in *S. cerevisiae*, indicating that negative control involving repression and derepression is involved in the regulation of the *HDE* gene. Since the expression of the *HDE* gene is similar in *C. tropicalis* and in *S. cerevisiae*, these two yeasts probably possess similar mechanisms for the control of expression of this gene.

(c) Conclusions

(*1*) Carbon sources regulate the expression of *HDE*, *AOX* and *CATL* in *C. tropicalis* and *S. cerevisiae* by inductive, repressive and derepressive mechanisms.

(*2*) The mechanism of regulation of expression of *HDE* is similar in *C. tropicalis* and *S. cerevisiae*.

(*3*) A glucose-responsive region lies between nt −526 and −393 of *HDE*, and two sequences within this region are conserved in a number of oleic acid-responsive genes of *C. tropicalis*.

(*4*) An oleic acid-responsive region lies between nt −393 and −341 of *HDE*, and a sequence within this region is conserved in a number of oleic acid-responsive genes of *C. tropicalis*.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Natural Sciences and Engineering Research Council of Canada. J.D.A. is a recipient of an Ontario Graduate Studentship. R.A.R. is a recipient of a Medical Research Council of Canada Scholarship. We thank Gillian Regoeczi for expert technical assistance.

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Page 5 of 6

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Page 6 of 6