Adr1 and Cat8 synergistically activate the glucose-regulated alcohol dehydrogenase gene ADH2 of the yeast Saccharomyces cerevisiae

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Glucose-repressible alcohol dehydrogenase II, encoded by the ADH2 gene of the yeast Saccharomyces cerevisiae, is transcriptionally controlled by the activator Adr1, binding UAS1 of the control region. However, even in an adr1 null mutant, a substantial level of gene derepression can be detected, arguing for the existence of a further mechanism of activation. Here it is shown that the previously identified UAS2 contains a distantly related variant of the carbon source-responsive element (CSRE) initially found upstream of gluconeogenic genes. In a mutant defective for the CSRE-binding factor Cat8, derepression of an ADH2-lacZ fusion was reduced to about 12% of the wild-type level. Gene expression in a cat8 adr1 double mutant decreased almost to the basal level of the glucose-repressed promoter. CSREADH2 present in a single copy turned out to be a weak UAS element, while a significant synergism of gene activation was found in the presence of at least two copies. Its importance for regulated gene activation was confirmed by site-directed mutagenesis of the CSRE in the natural ADH2 control region. Direct binding of Cat8 to CSREADH2 could be shown by electrophoretic retardation of the corresponding protein/DNA complex in the presence of a specific antibody. In contrast to what was shown previously for CSRE sequence variants, no significant influence of the isofunctional activator Sip4 on CSREADH2 was detected. In conclusion, these results show a derepression of ADH2 by synergistically acting regulators Adr1 (interacting with UAS1) and Cat8, binding to UAS2 (= CSREADH2).

Keywords: gluconeogenesis, transcriptional regulation, UAS element

INTRODUCTION

Utilization of ethanol as sole carbon source by the yeast Saccharomyces cerevisiae correlates with a more than 100-fold derepression of alcohol dehydrogenase II encoded by the ADH2 gene (Ciriacy, 1975). In contrast, ADH2 is completely repressed in the presence of a fermentable substrate. Two cis-acting elements (UAS1 and UAS2), both of which are necessary for maximal derepression, have been identified in the ADH2 control region (Shuster et al., 1986; Yu et al., 1989; Donoviel et al., 1995). UAS1 turned out to be a binding site of Adr1 (Thukral et al., 1991) which has been genetically identified as a positive regulator of ADH2 (Ciriacy, 1975, 1979). Adr1 contains two zinc finger motifs of the Cys2–His2 type (Blumberg et al., 1987) and interacts as a monomer with each halfsite of the palindromic UAS1 element. Mutagenesis of individual positions within UAS1 allowed derivation of the sequence TTGGRGA or its reverse complement as the preferred Adr1-binding site (Cheng et al., 1994). In addition to its DNA-binding domain, Adr1 also contains four transcription activation domains (TADI–IV) which contact several coactivators and basal transcription factors such as Ada2, Gcn5 and TFIIB as well as subunits of TFIID (Cook et al., 1994; Chiang et al., 1996; Komarnitsky et al., 1998). Although several regulators of UAS1/Adr1-dependent gene expression have been described [the Cat1/Snf1/ Ccr1 protein kinase (Ciriacy, 1977; Denis, 1987), cAMP-dependent protein kinases Tpk1, 2, 3 (Cherry et al., 1989) and the protein phosphatase complex...
Glc7+RegI/Hex2 (Dombek et al., 1993, 1999), the molecular mechanism(s) leading to carbon source control is still controversial (Vallari et al., 1992). At least, regulation of Adr1 activity may occur at the post-translational level, affecting its DNA-binding domain (Sloan et al., 1999).

In contrast to UAS1 and its corresponding trans-acting factor, Adr1, UAS2 is much less understood. A DNA-binding factor which is specifically required for UAS2-dependent gene activation and directly interacts with UAS2 has not yet been identified. We have previously analysed the genetic control of structural genes involved in the glyoxylate cycle and gluconeogenesis [ICL1, FBP1 (Schüler & Schüller, 1993, 1994), MLS1 (Caspary et al., 1997) and MDH2 (Roth & Schüller, 2001)]. This led to the identification of a common cis-acting element, designated CSRE (carbon source-responsive element; consensus CCRTYSRNCCG; reviewed by Entian & Schüller, 1997; Gancedo, 1998), which could confer glucose-sensitive gene regulation to a synthetic minimal promoter. CSRE-dependent gene activation requires a functional CAT8 gene (Hedges et al., 1995; Rahner et al., 1996), encoding a transcription factor with a binuclear zinc cluster domain at its N terminus and a C-terminal transcription activation domain. Expression of CAT8, as well as transcription activation by Cat8, is affected by carbon source (Rahner et al., 1996; Randez-Gil et al., 1997). A functional Cat1/Snf1/Ccr1 protein kinase together with its auxiliary factors Cat3/Snf4 (Celenza & Carlson, 1986; Schüler & Schüller, 1987, 1988; Celenza et al., 1989) and Sip1+Sip2+Gal83 (Yang et al., 1994; Schmidt & McCartney, 2000) is absolutely required for Cat8 function. Importantly, Cat8, as well as the related transcription factor Sip4, bind to CSRE$_{ICL1}$, CSRE$_{FBP1}$ and CSRE$_{MDH2}$ motifs (Vincent & Carlson, 1998; Rahner et al., 1999; Roth & Schüller, 2001). A CSRE-driven gene has been shown to be no longer regulated by carbon source with constitutively synthesized Cat8 and Sip4 variants containing heterologous activation domains (Vincent & Carlson, 1998; Rahner et al., 1999; Hiesinger et al., 2001).

Previously, we identified a CSRE motif together with an Adr1-binding site in the upstream region of the acetyl-CoA synthetase gene ACS1 (Kratzer & Schüller, 1997). While characterizing a cat8 null mutant, we obtained evidence for a Cat8-dependent activation of ADH2 (Rahner et al., 1996). However, the hypothesis of coregulation of ACS1 and ADH2 by Cat8 and Adr1 was rejected by Doniovil & Young (1996) who reported a marginal reduction of UAS2-dependent gene expression in a cat8 mutant. In this work we identify a functional and completely Cat8-dependent variant of the CSRE within UAS2. We also demonstrate in vitro binding of Cat8 to UAS2 and therefore suggest a combined derepression of ADH2 by Cat8 and Adr1.

### METHODS

**Yeast strains, media and growth conditions.** Strains of *Saccharomyces cerevisiae* used in this work were derived from the regulatory wild-type JS91.15-23 (MATa ura3 leu2 his3 trp1 can1). Regulatory mutants EMY1 ($\Delta$cat8::HIS3; Rahner et al., 1996), MHY14 ($\Delta$sip4::kanMX4), SRB24 ($\Delta$cat1::LEU2; Hiesinger et al., 2001), JS95.14-1 ($\Delta$adr1::LEU2) and JS95.16-1 ($\Delta$adr1::LEU2 $\Delta$cat8::HIS3; Kratzer & Schüller, 1997) were obtained by introduction of the null alleles indicated. Synthetic complete medium used for selective growth of transformants has been described previously (Schüller & Entian, 1987). For the preparation of protein extracts, yeast transformants were grown in medium under repressing (2% glucose) or derepressing conditions (0.2% glucose + 2% ethanol) until a cell density of about 10$^7$ cells ml$^{-1}$ was obtained.

**Plasmid constructions.** To obtain pKW7 variants, the synthetic DNA fragment ADH2-UAS2 (5’-ctcaTGATCTCCTCTGCCGGAACACGCGCGCATgtcacc-3’, together with the
complementary oligonucleotide; authentic ADH2 upstream sequence in capital letters) was inserted into promoter test plasmid pJS401 (ΔICL1-lacZ URA3 2µ; Caspary et al., 1997), cut at its single XhoI site. Copy number and orientation of inserts were determined by DNA sequencing. The complete ADH2 control region (−926/+3) was amplified as a HindIII/BamHI fragment by PCR and subsequently inserted into lacZ fusion vector YEp356R (Myers et al., 1986) to give pKW13 (ADH2-lacZ URA3 2µ). Point mutations within UAS2 (alteration of 4 nt essential for a functional CSRE) were generated with the QuickChange site-directed mutagenesis kit (Stratagene), using mutagenic primers ADH2-Mut1 (5′-CTCTCTGCGTGACACCGGCIgATC-C-3′; artificial SalI site underlined) and ADH2-Mut2 (reverse complement). The desired mutation in plasmid pKW14 (ADH2-(CSRE)_3-lacZ URA3 2µ) was confirmed by DNA sequencing. Plasmids pAR33, pAR34 and pMH33, encoding deregulated variants of CAT8 and SIP4, have been described previously (carbon source-independent MET25 promoter, constitutive Ino2 or VP16 activation domains together with a FLAG epitope; Rahner et al., 1999; Hiesinger et al., 2001).

**Gel retardation assay.** For the study of protein/DNA interactions, probe ADH2-UEAS2 was radioactively end-labelled by a fill-in reaction using [α-32P]dATP and Klenow enzyme. The synthetic DNA fragment OAS2 (containing CSRE_{E telA} (Schöler & Schüller, 1994) was used as competitor. Fusion proteins GST-Gal4 (aa 1–300 of Gal4, pMH25-GAL4), GST-Sip4 (aa 1–300, pMH25-SIP4; Hiesinger et al., 2001) and GST-Cat8 (aa 1–281, pAR1; Rahner et al., 1996) were prepared from *Escherichia coli* lysates by affinity chromatography with glutathione Sepharose as recommended by the manufacturer (Amersham Pharmacia). Yeast protein extract containing Cat8(1–281)-Ino2-FLAG was prepared from derepressed transformants of mutant strain SRH24 (carrying plasmid pAR34) as described previously (Schöler & Schüller, 1994). For supershift experiments, 200 ng monoclonal anti-FLAG antibody M2 (Kodak/IBI) was used.

**Miscellaneous procedures.** Strains of *S. cerevisiae* were transformed according to an established procedure (Son et al., 1993). β-Galactosidase assays were performed with yeast crude extracts as described previously (Schöler & Schüller, 1994). Synthetic oligonucleotides were purchased from Gibco-BRL Life Technologies. For the amplification of DNA fragments by PCR, the expand high fidelity system (Roche Molecular Biochemicals) was used.

**RESULTS**

**UAS2 of ADH2 contains overlapping CSRE-related sequences**

Activation of the glucose-regulated alcohol dehydrogenase gene ADH2 under derepressing conditions is mediated by Adr1, binding to the UAS1 promoter motif (Yu et al., 1989; Thukral et al., 1991). However, deletion of either UAS1 (ADH2) or ADR1 still allowed a substantial derepression of ADH2, arguing for an additional UAS element and trans-activator. Although UAS2 was identified immediately upstream of UAS1 (Yu et al., 1989), the corresponding regulatory factor remained unknown. Since our previous characterization of a *cat8* deletion mutant provided evidence for reduced expression of an ADH2-lacZ fusion (Rahner et al., 1996), we looked for a Cat8-binding site (= CSRE) within UAS2. Indeed, UAS2 contains two sequence motifs on complementary DNA strands (Fig. 1a) which almost completely overlap and are distantly related to the previously defined CSRE consensus (CCRTYNNRCGG; Schöler & Schüller, 1994; Caspary et al., 1997; Roth & Schüller, 2001). We thus inserted the synthetic DNA fragment ADH2-UAS2 into the upstream region of an ICL1-lacZ reporter gene, devoid of its natural regulatory sequences (pJS401; Caspary et al., 1997). As is shown in Fig. 1(b), only a twofold stimulation of basal gene expression was detected in the presence of a single copy insertion in either orientation. In contrast, strong activation was found with at least two copies, arguing for a marked synergism (> 300-fold activation with three copies; cf. plasmid pKW7NNR). Importantly, UAS2-dependent gene activation absolutely required a functional CAT8 gene, supporting our view of UAS2 as a functional CSRE variant (Table 1). The Cat8-related activator Sip4, which partially contributes to activation by CSRE_{FBP}, and CSRE_{IFL1} (Vincent & Carlson, 1998; Hiesinger et al., 2001), was dispensable for CSRE_{ADH2}-mediated gene derepression. A decrease in gene expression to about half of the wild-type level was also found in the *adr1* mutant. Absence of the Cat1 (Snf1) protein kinase which acts as a positive upstream regulator of Cat8 also led to a total loss of activity.

To confirm the specific contribution of Cat8 to UAS2-dependent gene activation, we assayed the influence of a regulatory CAT8 variant (MET25-CAT8-INO2*_{TAD}* fusion gene, plasmid pAR33; promoter and activation domain not affected by carbon source) on reporter plasmid pKW7NNR. When introduced into the *cat8 sip4* double mutant SRH24, the modified Cat8 activator could strongly stimulate the reporter gene even under conditions of glucose repression (> 50-fold, compared to wild-type; Table 2). Under derepressing conditions, the wild-type level of gene expression was completely restored. We also investigated the influence of a deregulated SIP4 variant (MET25-SIP4-VP16 fusion gene, plasmid pMH33) which could efficiently activate a CSRE_{IFL1}-driven reporter gene (Hiesinger et al., 2001). However, with this construct, UAS2-dependent gene activation remained at 8% of the derepressed wild-type level (Table 2). In conclusion, these results suggest that Cat8 is indeed an important activator of UAS2 (= CSRE_{ADH2})-dependent gene expression, while Sip4 at best mediates some minor influence on this CSRE variant.

**Site-directed mutagenesis of CSRE_{ADH2}**

In addition to UAS2-containing synthetic promoter constructs, we also investigated *ADH2-lacZ* fusion genes containing either the complete natural upstream region (−926/+3) or a mutant CSRE_{ADH2} variant (pKW13 and pKW14, respectively; cf. Fig. 2). With the ADH2 wild-type promoter, deletion of either ADR1 or CAT8 led to a significant decrease of β-galactosidase activity (to 19.5 and 12.5% of the reference, respectively; Fig. 2). In the *adr1 cat8* double mutant, almost no
Table 1. Influence of regulatory mutations on UAS2 (= CSRE<sub>ADH2</sub>)-dependent gene activation

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Relevant genotype Specific <i>β</i>-galactosidase activity of pKW7NNR transformants

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<td>MET25-SIP4-VP16</td>
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derepression occurred. The same strains were also transformed with an ADH2-lacZ fusion, deviating at four UAS2 nucleotide positions which are critical for CSRE function. With this reporter construct (pKW14), gene expression in the wild-type strain decreased to 50% of the activity found for the natural control region.

Fig. 2. Influence of Adr1 and Cat8 on wild-type and mutant ADH2 control regions. Strains J591.15-23 (wild-type), J595.14-1 (adr1), EMY1 (cat8) and J595.16-1 (cat8 adr1) were transformed with the episomal reporter plasmids shown. Plasmids pKW13 and pKW14 differ with respect to 4 nt of the CSRE core sequence within UAS2 (underlined). Transformants were grown under repressing (rep.; 2% glucose) and derepressing conditions (der.; 0-2% glucose + 2% ethanol). Specific <i>β</i>-galactosidase activities are given in nmol ONPG hydrolysed min<sup>-1</sup> (mg protein)<sup>-1</sup>. SD, Standard deviation of the mean value; T, TATA box.
Importantly, a further decrease (to 14.5%) was observed in the cat8 mutant, arguing for the existence of an additional Cat8-dependent UAS element in the ADH2 promoter. In the absence of Adr1, mutation of CSRE_{ADH2} led to a reduction of derepressed activity by a factor of eight. Again, derepression was almost completely defective in the adr1 cat8 double mutant. These findings show that Adr1 and Cat8 together mediate >98% of total ADH2 gene derepression. Since the defects observed with adr1 and cat8 single mutants are more than additive, a synergistic derepression mediated by both activators is suggested.

**In vitro interaction of Cat8 with CSRE_{ADH2}**

Previous work demonstrated binding of Sip4 and Cat8 to CSRE sequence variants (Vincent & Carlson, 1998; Rahner et al., 1999; Roth & Schüller, 2001; Hiesinger et al., 2001). To confirm the direct influence of Cat8 on UAS2, gel retardation experiments with protein extracts prepared from *E. coli* lysates, were performed. GST fusion proteins containing the zinc cluster domains of Gal4 (negative control), Sip4 and Cat8 were affinity-purified and subsequently incubated with a CSRE_{ADH2}-containing probe. While strong retardation signals were observed with GST-Cat8 (Fig. 3, lane 4), only a weak signal could be obtained with an identical amount of GST-Sip4 (lane 3). In contrast, signals of similar intensity for both binding factors were previously found with CSRE_{ADH2} probes (Roth & Schüller, 2001). Obviously, Cat8 and Sip4 bind to CSRE sequence variants with different affinities.

In addition to bacterially synthesized binding factor, gel retardation experiments were also done with yeast protein extract containing an epitope-tagged Cat8-Ino2-FLAG variant (Fig. 4). Extracts were prepared from a cat8 sip4 double mutant (SRH24), transformed with the corresponding fusion gene (on plasmid pAR34). Compared to the vector control, a strong retardation signal could be observed (lane 3) which was further shifted in the presence of specific anti-FLAG antibody (lane 4). Competition by an excess of competitor migrates close to the position of Cat8-Ino2-FLAG. Together, these band
shift assays show that CSRE<sub>ADH2</sub> specifically interacts with Cat8, but not with the related transcriptional activator Sip4.

**DISCUSSION**

For the utilization of ethanol, alcohol dehydrogenase (ADH), aldehyde dehydrogenase (ALD) and acetyl-CoA synthetase (ACS) are required to finally produce acetyl-CoA which is subsequently used for mitochondrial oxidation and cytoplasmic gluconeogenesis. We have previously shown that ACS1 of *S. cerevisiae* is activated additively by Cat8 and Adr1, together being responsible for about 80% of its promoter strength (Kratzer & Schüller, 1997). This result prompted us to look for a similar genetic control of ADH2 which encodes the first enzyme of ethanol oxidation. In addition to Adr1 and its corresponding binding site in the ADH2 control region (US1), the existence of an additional activation sequence (US2) is well known (Yu et al., 1989; Thukral et al., 1991). However, the nature of the transcriptional regulator responsible for US2-dependent derepression has remained unclear. In this work, we identified a functional CSRE variant within US2 which specifically interacts in vitro with the Cat8 (but not the Sip4) activator and contributes to transcriptional derepression of ADH2 synergistically with the Adr1-dependent US1 element.

Although both almost completely overlapping CSRE motifs within US2 deviate from the previously defined consensus sequence, a regulated, 80-fold activation of basal gene expression was found in the presence of at least two CSRE<sub>ADH2</sub> copies (Fig. 1). Recent data derived from the saturation mutagenesis of a strong CSRE suggest that the sequence CCGGTGTTCCG is closer to the modified consensus than the CCGGAAACCG motif on the complementary strand (S. Roth & H.-J. Schüller; unpublished). Gel retardation experiments showed that Cat8 and the related zinc cluster protein Sip4 differ with respect to binding affinity to CSRE sequence variants. Thus, Cat8 must be considered as the dominating regulator acting via US2. This conclusion is in agreement with a recently published microarray analysis, comparing mRNA levels from wild-type and cat8 mutant strains on a genome-wide scale (Haurie et al., 2001). A minor role, even of Adr1, can be derived from the reduction of US2-dependent gene expression in an adr1 mutant (Table 1) as well as from the previous demonstration of low-affinity binding of Adr1 to US2 (Donoviel et al., 1995). Synergistic gene activation is not only observed in the presence of multiple US2 copies in a synthetic promoter (Fig. 1) but also with US1 and US2 in the natural ADH2 control region (Yu et al., 1989). Presumably, Cat8 and the US1-binding factor Adr1 cooperate in maximal derepression of an ADH2-lacZ reporter gene. This conclusion derived from the analysis of cat8 and adr1 single mutants is in agreement with previous results, comparing US2 promoter mutants in the absence or presence of Adr1 (Donoviel et al., 1995). Synergistic activation of ADH2 by Cat8 and Adr1 contrasts to the additive contribution of these regulators to derepression of ACS1 (Kratzer & Schüller, 1997). This may simply result from varying distances of Cat8- and Adr1-binding sites upstream of ACS1 (separated by 200 bp) and ADH2 (3 bp apart). Synergistic derepression of ADH2 by Cat8 and Adr1 could be the result of recruiting a common coactivator/mediator of transcriptional activation. Possible candidates may be Ada2 and the histone acetyltransferase Gcn5, as well as basal transcription factors TFIIIB and components of TFIIID (TPB, Taf90 and Taf130/145) for which direct contacts to activation domains within Adr1 have been demonstrated (Chiang et al., 1996; Komarnitsky et al., 1998).

The influence of Cat8 on US2-dependent gene activation is further confirmed by the obvious deregulation of a reporter gene by the MET25-CAT8-INO2<sup>TAD</sup> variant, encoding a protein with a constitutive activation domain (INO2<sup>TAD</sup>) driven by a carbon source-independent promoter (MET25). Compared to a wild-type control, gene expression was clearly increased under repressing, but not under derepressing conditions (Table 2). An increased expression of US2-dependent ADH2 was previously shown in the presence of multicopy plasmids containing SPT6 and MEU1 genes (Donoviel & Young, 1996). While the essential SPT6 gene may be involved in chromatin modification during transcriptional elongation (Hartzog et al., 1998), the function of MEU1 (multicopy enhancer of US2) remained unclear. A high copy number of MEU1 increased ADH2 expression about three- to fivefold under either condition, whilst its deletion led to a twofold reduction (Donoviel & Young, 1996). Our data on the contribution of Cat8 (which are in agreement with the findings of Haurie et al., 2001) completely differ from results reported by these authors. In contrast to the complete loss of US2-driven gene expression documented in this work (Table 1), Donoviel & Young (1996) merely describe a slight reduction by a factor of two. The reason for this discrepancy remains unclear.

Assuming US2 is the sole Cat8-dependent activating element, equivalent and non-additive effects of *cis* and *trans* mutations (CSRE<sub>ADH2</sub> and cat8) on ADH2 gene expression should be expected. However, in the absence of Cat8, gene expression was clearly more affected than with a mutant US2 (Fig. 2). Possibly, the site-directed mutagenesis did not abolish all US2 activity. This appears unlikely since positions critical for a functional CSRE had been altered. Instead, we suggest the existence of (at least) one additional CSRE motif in the ADH2 upstream region. We were able to identify the sequence motif CCGTCTCTCCG (−478/−468) which fits the weakly stringent consensus CCN<sub>N</sub>C GG typical of a subfamily among zinc cluster proteins. This element may be considered as a weak CSRE, being responsible for Cat8-dependent ADH2 derepression in the absence of US2.

We finally asked whether at least one among five aldehyde dehydrogenase genes (ALD2, ALD3, ALD4/
Dombek results were taken from Denis et al. as well as post-translational regulation of Adr1 by Tpk1,2,3/Bcy1 may antagonistically influence Adr1. It is unknown whether and Adr1. Various protein kinase and phosphatase complexes may also contribute to the regulation of Adr1. Ciriacy, F., Hartig, A. & Schüller (1987).

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