Synergistic Activation of ADH2 Expression Is Sensitive to Upstream Activation Sequence 2 (UAS2) Orientation, Copy Number, and UAS1-UAS2 Helical Phasing

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Received 12 January 1995/Returned for modification 28 February 1995/Accepted 24 March 1995

The alcohol dehydrogenase 2 (ADH2) gene of Saccharomyces cerevisiae is under stringent glucose repression. Two cis-acting upstream activation sequences (UAS) that function synergistically in the derepression of ADH2 gene expression have been identified. UAS1 is the binding site for the transcriptional regulator Adr1p. UAS2 has been shown to be important for ADH2 expression and confers glucose-regulated, ADR1-independent activity to a heterologous reporter gene. An analysis of point mutations within UAS2, in the context of the entire ADH2 upstream regulatory region, showed that the specific sequence of UAS2 is important for efficient derepression of ADH2, as would be expected if UAS2 were the binding site for a transcriptional regulatory protein. In the context of the ADH2 upstream regulatory region, including UAS1, working in concert with the ADH2 basal promoter elements, UAS2-dependent gene activation was dependent on orientation, copy number, and helix phase. Multimerization of UAS2, or its presence in reversed orientation, resulted in a decrease in ADH2 expression. In contrast, UAS2-dependent expression of a reporter gene containing the ADH2 basal promoter and coding sequence was enhanced by multimerization of UAS2 and was independent of UAS2 orientation. The reduced expression caused by multimerization of UAS2 in the native promoter was observed only in the presence of ADR1. Inhibition of UAS2-dependent gene expression by Adr1p was also observed with a UAS2-dependent ADH2 reporter gene. This inhibition increased with ADR1 copy number and required the DNA-binding activity of Adr1p. Specific but low-affinity binding of Adr1p to UAS2 in vitro was demonstrated, suggesting that the inhibition of UAS2-dependent gene expression observed in vivo could be a direct effect due to Adr1p binding to UAS2.

Glucose repression affects a large number of genes in the yeast Saccharomyces cerevisiae (29, 47). ADH2 (the alcohol dehydrogenase 2 gene), which encodes the ADH2 isozyme, is subject to stringent glucose repression at the level of transcription (18). ADH2 repression is mediated in part by REG1, an important regulator of many glucose-repressed genes. Other pleiotropic regulators of glucose repression in S. cerevisiae, such as HXK2 and MIG1, do not participate in repression of ADH2 expression (21, 29, 47). Derepression requires the SNF1 protein kinase, in common with other glucose-repressed genes (5, 8, 16, 17, 40).

Two cis-acting elements that are necessary for complete derepression of ADH2 have been identified in the ADH2 upstream regulatory region (1, 36, 37, 52). Upstream activation sequence 1 (UAS1) is a well-characterized 22-bp palindromic to the transactivator Adr1p binds (9, 21, 36, 44). UAS2 is located upstream of UAS1 and is thought to bind an as yet unidentified transcriptional regulator (51). Both UAS1 and UAS2 confer glucose-regulated expression on a heterologous promoter (52). UAS1-dependent activity requires ADR1, while UAS2-dependent expression is ADR1 independent (52). UAS1 and UAS2 function synergistically to regulate ADH2 expression (52).

Adr1p is a well-characterized zinc finger protein which is the major activator of ADH2 expression (1, 11, 12, 18). Adr1p binds and activates transcription from two TTGGAGA sequences which are present in inverted orientation in UAS1 (22, 45, 52). Similar GGAGA-containing motifs occur at four other positions in the UAS region of the ADH2 upstream regulatory sequences, including once in UAS2 (36, 52). Whether Adr1p binds to these motifs outside UAS1 is unknown since their deletion had a minimal effect on expression of a heterologous reporter (1).

Genetic studies have identified several genes that act in concert with, or independently of, ADR1 to influence expression of ADH2. One or more of these genes could influence ADH2 expression through a UAS2-dependent pathway. The most likely candidates to act through UAS2 are positively acting factors since UAS2 is an activating sequence. SNF1/CCR1/CAT1, which encodes a serine/threonine protein kinase (6, 7, 15), is necessary for derepression of a large number of glucose-repressed genes, including ADH2 (13, 15). SNF1 affects ADH2 expression in the absence of UAS1 (17). Bcy1 encodes the regulatory subunit of the cyclic AMP-dependent protein kinase, cAPK, and influences ADH2 expression in an ADR1-dependent manner (10, 21). Another protein kinase homologous to cAPK encoded by SCH9 also appears to be needed for full ADH2 expression but in an ADR1-independent manner (17). Several other genes that are necessary for full ADH2 derepression have pleiotropic effects on transcription in S. cerevisiae and could, in principle, influence ADH2 expression in a UAS2-dependent manner. These genes are thought to encode proteins affecting the basic transcriptional machinery, perhaps acting on chromatin to influence expression rather than acting at a regulatory level. These genes include ADR6/SWI1/GAM3 and CCR4/FUN27. Mutations in these genes alter the transcript levels of a large number of otherwise unrelated genes (15, 19, 42, 43, 50). The alterations in ADH2 regulation associated with some of these genes have been shown to be...
either ADR1 independent or independent of ADH2 sequences upstream of the TATA region (17, 19), though an additional UAS1- or UAS2-dependent pathway remains a possibility.

Genes whose products act negatively on transcription, formally behaving as repressors of ADH2 expression, could, in principle, act through UAS2 in an indirect manner. These include REG1/HEX2/SRN1 (which behaves formally as a repressor of glucose-regulated genes and acts on ADH2 expression in both an ADR1-dependent manner and an ADR1-independent manner) (21), CRE1/SPT10 and CRE2/SPT6/SSN20 (which appear to be ADR1 independent) (15, 19), and ADR7 to -9 (which have both ADR1-independent and ADR1-dependent components) (30).

UAS2-dependent ADH2 expression was studied in the intact ADH2 promoter as well as in a new reporter plasmid containing the ADH2 basal promoter and structural gene. This new reporter has several advantages over other commonly used reporter plasmids such as strong negative and positive selection in strains lacking other sources of ADH activity. Differences in the behavior of UAS2 in the intact promoter context compared with the basal-promoter plasmid as well as the effect of Adr1p on UAS2-dependent gene expression suggest that a UAS2 binding factor interacts with Adr1p bound at UAS1.

MATERIALS AND METHODS

Strains. Escherichia coli DH5α (26) was used for the propagation of all plasmids used in this study, except for the selection of LEU2-containing plasmids, pMD60 and pMD61, for which strain RR1 was used (3). S. cerevisiae MDY10 (MATa adh1-11 adh2-11 ura3-11 trp1 leu2 ura3) was created by transforming MC71-18B (which have both ADH1 and ADH2) with a BamHI fragment from pMDU31, a SacII fragment from ADH1 which contains ADH2 sequences between the SacII site at nucleotide -117 and the Spel site at +1418 was removed and replaced by a HindIII fragment of URAS3 (51). MDY11 (MATα adh1-11 adh2-11 ura3-11 trp1 leu2 ura3) was constructed by digesting MDY10 with a BamHI fragment of pADH1::LEU2 in which the ADH1 promoter and 5' half of the coding sequences were removed and replaced by a 2.2-kb fragment of LEU2 (2). The disruptions of ADH2 and ADR1 were confirmed by Southern blot analysis as described elsewhere (34). Yeast strain CMY215A1 (44) was used for preparation of extracts for in vitro DNA binding assays.

Plasmid transformations. Plasmids were transformed into DH5α by using frozen competent cells (26, 33). Yeast strains were transformed by a modified lithium acetate method (25, 28). Transformants were selected by growth on minimal media lacking the appropriate nutrient.

Growth media and culture conditions. Yeast complete medium contained 10 g of yeast extract (Difco), 20 g of Bacto Peptone (Difco), 20 mg of uracil per liter, supplemented with 5% glucose (YPD [repressing medium]) or with 3% ethanol (YPE [derepressing medium]). C. elegans (Difco) was added to 2% to make YPD or YPE plates. Yeast synthetic medium, SM (52), containing the appropriate drop-out solution and supplemented with 5% glucose or with 3% ethanol was used. Strains were grown at 30°C. Liquid cultures were inoculated with a single colony from plates and grown for 12 h in YPD or SM-glucose. Yeasts were then diluted 1:50 into fresh medium of the same type and grown for a further 12 to 20 h before being harvested at an optical density at 600 nm of 1 to 2 (Gillord Instruments model 520 spectrophotometer). Cultures were routinely tested for the presence of glucose at the time of harvest by using Dianstix (Miles Inc., Diagnostics Division). Derepression was carried out by growth of cultures as described above, washing once in sterile water, and resuspending the pelleted cells in derepressing medium to which 0.05% glucose was added. These cultures were then grown to an optical density at 600 nm of 0.1 to 2 and harvested.

Enzyme and protein assays. Preparation of yeast extracts for ADH3 enzyme activity assay and the assays themselves were performed as previously described (18). b-Galactosidase activity was assayed in whole cells by the method described by Miller and modified by Guarente (24, 34). Protein concentrations were quantitated by the Coomassie blue dye-binding assay supplied by Bio-Rad Inc.

DNA migration assay. Binding reactions were carried out essentially as previously described (4, 48). All reactions contained 40 µg of poly(A) + RNA and sheared salmon sperm DNA. Complementary oligonucleotides were labeled with [y-32P]ATP, annealed, and then purified from a 15% acrylamide gel. Unlabeled competitors were similarly phosphorylated by using unlabeled ATP, annealed, and then sheared. Whole-cell extracts were used as described elsewhere (31, 32, 48). Extracts were prepared from E. coli as described elsewhere (44). Binding reactions were incubated for 10 min on ice and then for 5 min at room temperature, and products were analyzed after electrohoresis through a 4% polyacrylamide gel (38:2, acrylamide-bis) in 1 x Tris-glycine buffer at 200 V at 4°C.

Plasmid constructions. The LEU2-containing plasmid overexpressing wild-type Adr1p, pMD60, was created from pKD58 (20), a modified version of the ADR1-overexpressing plasmid pWET11 (44). An XhoI site was created by insertion of linkers at the BsrFl site of TRP1 (21). Into this XhoI site a 2.2-kb SalI-XhoI fragment from YEp13 containing the LEU2 gene was inserted. Leucine prototrophs were selected in E. coli RR1. The resulting plasmid, pMD60, converted S. cerevisiae to leucine prototrophy but would not complement a trp1-1 mutation. pMD60 was derived from pMD60 by replacing wild-type ADH1 with the E117A linker inserted in frame at amino acid 16 (21) and Spel with an analogous SacII-Spel fragment containing the E117A mutation (46, 47). The presence of the E117A mutation was confirmed by digestion with Bgl II which cuts DNA containing the mutation but not wild-type DNA. ADH2-expressing plasmids, obtained with pMD12 and pMD21, were constructed by inserting a 3.438-bp ADH2 BamHI-XhoI fragment from pADH2 (36) or an analogous 3.418-bp ADH2 fragment from pBR-ADH2/URAS2 (52) into BamHI- and XhoI-digested pFC3. pFC3 was constructed by inserting a 2-kb CEN3 fragment into the pN11 site of YR6. In constructing the UAS2 deletion, the ADH2 upstream sequences between a C·G base pair immediately 5' of UAS2 and a CCGGG sequence flanking the 3' end of UAS2 were removed. Thus, the UAS2 deletion created a UAS1 site at the former UAS2 locus. Derivatives of pMD21 containing wild-type UAS2 or UAS2 point mutations were created by ligation of degenerate, double-stranded UAS2 oligonucleotides with SacII-digested pMD21. Degenerate UAS2 oligonucleotides were synthesized on an ABI 3500 automated DNA synthesizer by using a mix composed of 94% of one nucleotide precursor and 2% of each of the remaining three precursors for all 4 bases. The prototypical sequence for the degenerate oligonucleotides was TGATCTCCTC TGCCGGAAACCC. Insertion of the oligonucleotide destroyed the SacII site of pMD21. Thus, to enrich for plasmids containing inserts, ligation products were digested with SacII prior to transformation into intact yeast. Plasmid containing inserts were sequenced to determine the number, orientation, and sequence of the UAS2 oligonucleotide present.

pRS314-II (30, 39) containing UAS1 or UAS2 oligonucleotides was constructed by cutting pRS14-B with SacII or SalI, respectively, and ligating the appropriate double-stranded oligonucleotides with the digested (in the case of SalI) vector. DNA sequence analysis confirmed the number and orientation of the inserts. pRSU1 contains UAS1 inserts, and pRSU2 contains UAS2 inserts. The sequence of the UAS1 oligonucleotide is 5'-gggTCTCCAACT TATAAAGTTGGAGAccc-3' (the UAS2 sequence is uppercase); the sequence of the UAS2 oligonucleotide is 5'-TGATCTCCTCTGGCCGGAAACCC-3'.

RESULTS

UAS2 is orientation dependent in the native ADH2 promoter. UAS2 was originally defined by the ability of a sequence (5'-TGATCTCCTCTGGCCGGAAACCC-3') located immediately upstream of UAS1 to confer glucose-regulated, ADR1-independent expression on a heterologous reporter gene (52). Like most enhancer-like elements in S. cerevisiae and other organisms, UAS2 was orientation independent and its stimulatory activity increased with an increase in the number of copies present. To facilitate study of the role of UAS2 in its normal promoter context, a series of centromeric plasmids containing the intact ADH2 promoter, including upstream regulatory sequences, and the structural gene was constructed. Plasmid pMD20 carries the wild-type ADH2 structural gene, 1.147 bp of the 5' flanking sequence, and about 1,100 bp of 3' flanking DNA. An analogous plasmid, pMD21, carries a deletion of UAS2 (52) (Fig. 1A). These plasmids were introduced into the ADH-null strain MDY10, and the ADH111 activity was measured. The deletion of UAS2 reduced derepressed ADH2 expression about 10-fold and had no effect on repression (Fig. 1B). Insertion of an oligonucleotide having a wild-type UAS2 sequence restored ADH2 expression to levels indistinguishable from that of a plasmid containing an unaltered promoter. The plasmid containing a wild-type insert differs from a native promoter by the addition of two extra C·G base pairs 5' of UAS2 that were introduced during the construction of the UAS2 deletion. Since the plasmid with the extra base pairs 5' of UAS2 did not exhibit the same ADH2 activity as did the plasmid with a native promoter, this result demonstrated that altering the spacing between the UAS region (UAS1 and UAS2) and sequences upstream of this region by 2 bp did not significantly alter ADH2 expression. The sequence between
UAS2 and UAS1 is the same as that in the wild-type promoter for all plasmids containing reinserted UAS2 oligonucleotides. UAS2 functions differently in constructs containing the entire ADH2 upstream regulatory region compared with its function in the context of the UAS2/CYC1/lacZ reporter. This was first indicated by the response to UAS2 elements inserted in reverse orientation or as multiple head-to-tail repeats in reporter plasmids compared with plasmids containing the wild-type ADH2 upstream regulatory region. A plasmid, pMD21-4, containing a single UAS2 element inserted in an orientation opposite to that in the normal ADH2 upstream regulatory region had a level of ADHII activity only twofold higher than that of the UAS2 deletion construct, pMD21 (Fig. 2). Thus, in the context of a complete ADH2 upstream regulatory region, UAS2 is orientation dependent. This result was unexpected given the orientation-independent function of UAS elements in general (41) and of UAS2 in particular (52). An equally unexpected result was seen when wild-type UAS2 elements were multimerized (Fig. 2). ADH2 promoters containing two or three UAS2 elements as head-to-tail repeats in the normal orientation were significantly less active than those containing only one copy of UAS2. In contrast, a reporter gene whose promoter contained multiple UAS2 elements was more active than the same gene whose promoter contained a single UAS2 element (52).

Adr1p alters UAS2-dependent ADH2 expression in the context of the entire ADH2 upstream regulatory region. The different effects of multimerization and reversal of UAS2 on gene expression could arise from the different basal promoter elements used or the presence of the upstream regulatory region which is present in the native promoter but not in the reporter gene. For example, interactions between a regulatory protein bound to UAS2 and other transactivators bound to other upstream regulatory sequences, such as Adr1p bound to UAS1, which would be absent from the UAS2/CYC1/lacZ reporter could be responsible for the unexpected activity of UAS2 in the whole-promoter constructs.

To test whether Adr1p was involved in the diminished expression from the whole-promoter constructs containing multiple UAS2 elements or a single reversed UAS2 element, ADH2 expression from these plasmids was assayed in an isogenic adr1Δ::LEU2 strain, MDY11. In the absence of Adr1p, a promoter lacking UAS2 or containing a single UAS2 element was inactive whereas a promoter containing two or three UAS2 elements had significant activity (Fig. 2). This difference was most easily detected and measured during derepression because the ADHII activities were higher, but the ADHII activities were also reproducibly higher during repressed conditions for the constructs containing multiple UAS2 inserts. In the absence of Adr1p a promoter containing a single reversed UAS2 element had reproducibly higher ADH2 expression than did a promoter lacking UAS2, suggesting that the lack of Adr1p allowed UAS2 to function in an orientation-independent manner. Thus, the absence of Adr1p converted the native ADH2 promoter into a simple UAS2-dependent promoter. These results suggest that there is an interaction between Adr1p and a factor bound at UAS2 that is dependent on the number of copies of UAS2 and on its orientation.

Altered helical phasing between UAS1 and UAS2 affects ADH2 expression. If Adr1p bound to UAS1 interacts with a factor bound to UAS2 to synergistically activate ADH2 expression, the interaction, and hence ADH2 expression, might be disrupted by altering the distance and helical phase between UAS1 and UAS2. A phase-sensitive promoter activity is usually interpreted as indicating that two bound proteins must interact with a specific orientation on the DNA (27). UAS1-UAS2 oligonucleotides containing extra base pairs were inserted into the UAS2 deletion plasmid pMD21. Plasmids were identified by sequence analysis in which oligonucleotide insertion reconstructed UAS2 and altered the spacing between the elements by 1, 5, or 10 bp relative to the wild-type sequence. These plasmids were introduced into the ADH-null strain MDY10, and ADHII activities were assayed under repressing and derepressing conditions (Table 1). The ADHII activity varied in a phase-sensitive manner. The activity derived from the construct in which the spacing was increased by 5 bp was lower than any of the others. When the spacing was increased by 10 bp, nearly wild-type levels of ADH2 expression were restored. These results support the idea that an interaction between the putative UAS2-binding factor and Adr1p is important for synergistic activation of ADH2 expression and that this interaction is dependent on the relative positions of the two factors.

The specific sequence of UAS2 is important for complete derepression of ADH2 expression in the context of the entire ADH2 upstream regulatory region. If UAS2 is bound by a transcriptional regulatory protein, the specific sequence of that region would be predicted to be important for activation by such a factor. To determine the importance of the UAS2 se-
sequence for proper ADH2 expression, a pool of double-stranded, degenerate UAS2 oligonucleotides were inserted into the SmaI site of pMD21. The UAS2/UAS1 region of plasmids containing UAS2 oligonucleotide inserts was analyzed by DNA sequence analysis to determine the number, orientation, and sequence of UAS2 inserts. Plasmids of interest were transformed into an ADH-null yeast strain, MDY10, (wild type [wt] ADR1) and MDY11 (adr1Δ1::LEU2). Cultures were grown under repressing (r) or derepressing (dr) conditions. The data are means for at least three independent transformants. The standard error of the mean for all values was less than 15%.

Characterization of a new reporter gene containing the ADH2 basal promoter region and structural gene. Although an interaction with UAS1-bound Adr1p could account for the properties of UAS2 in the native ADH2 promoter compared with the CYC1/ lacZ reporter plasmid, the basal promoter element (Fig. 1) was characterized.

**TABLE 1. Effect of altering UAS1-UAS2 spacing on ADH2 expression**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Insert</th>
<th>ADHII activity (mU/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMD20</td>
<td>None (wild type)</td>
<td>2,600</td>
</tr>
<tr>
<td>pMD21</td>
<td>None (ΔUAS2)</td>
<td>380</td>
</tr>
<tr>
<td>pMD21.1</td>
<td>UAS2 + 1 bp</td>
<td>900</td>
</tr>
<tr>
<td>pMD21.5</td>
<td>UAS2 + 5 bp</td>
<td>590</td>
</tr>
<tr>
<td>pMD21.10</td>
<td>UAS2 + 10 bp</td>
<td>1,900</td>
</tr>
</tbody>
</table>

a All constructs contain a single UAS2 oligonucleotide inserted in the same orientation as that in the normal ADH2 promoter (Fig. 1).

b Measured in MDY10 transformants under derepressing growth conditions. The data are means for three independent transformants. The standard errors of the means were less than 15%.

**TABLE 2. Single base pair changes in UAS2 decrease ADH2 expression**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Mutation</th>
<th>ADHII activity (mU/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMD21-1</td>
<td>None (wild type)</td>
<td>1,600</td>
</tr>
<tr>
<td>pMD21-5</td>
<td>C21-G</td>
<td>200</td>
</tr>
<tr>
<td>pMD21-6</td>
<td>A20-T</td>
<td>340</td>
</tr>
<tr>
<td>pMD21-7</td>
<td>G16-A</td>
<td>360</td>
</tr>
<tr>
<td>pMD21-8</td>
<td>C5-T</td>
<td>370</td>
</tr>
<tr>
<td>pMD21-9</td>
<td>C8-G</td>
<td>570</td>
</tr>
<tr>
<td>pMD21-10</td>
<td>G12-C</td>
<td>810</td>
</tr>
<tr>
<td>pMD21-11</td>
<td>A18-G</td>
<td>870</td>
</tr>
<tr>
<td>pMD21-12</td>
<td>A18-T</td>
<td>1,200</td>
</tr>
<tr>
<td>pMD21-13</td>
<td>T4-G</td>
<td>1,200</td>
</tr>
</tbody>
</table>

a All constructs contain a single UAS2 oligonucleotide inserted in the same orientation as that in the normal ADH2 promoter (Fig. 1).

b For example, C5-T means that cytosine at position 5 of UAS2 was changed to thymidine. See Fig. 1.

c Measured in MDY10 transformants under derepressing growth conditions. The data are means for three independent transformants. The standard errors of the means were less than 15%.
ADHII activity mU/mg

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Inserts</th>
<th>A. MDY11 (adr1::LEU2)</th>
<th>B. MDY10 (wt ADRI)</th>
<th>C. MDY10 ADR1+pMD60 (wt ADRI)</th>
<th>D. MDY10 ADR1+pMD61</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>r</td>
<td>dr</td>
<td>r</td>
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<tr>
<td>pRS314-II</td>
<td>NONE</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td>pRSU1-1</td>
<td></td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>20</td>
<td>ND</td>
</tr>
<tr>
<td>pRSU1-2</td>
<td></td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>600</td>
<td>ND</td>
</tr>
<tr>
<td>pRSU2-3</td>
<td></td>
<td>20</td>
<td>100</td>
<td>20</td>
<td>100</td>
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<tr>
<td>pRSU2-10</td>
<td></td>
<td>90</td>
<td>300</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>pRSU2-11</td>
<td></td>
<td>50</td>
<td>300</td>
<td>200</td>
<td>200</td>
</tr>
</tbody>
</table>

FIG. 3. ADHII activities from pRS314-II derivatives containing single or multiple copies of UAS1 or UAS2 oligonucleotides. Double-headed arrows, palindromic UAS1 oligonucleotide inserts that were cloned into the SmaI site of pRS314-II; open rectangles, UAS2 oligonucleotide inserts cloned into the SmaI site of pRS314-II; arrowheads within the rectangles, orientation of UAS2 elements relative to the site of transcription initiation (rightward-pointing arrowheads, native orientation; leftward-pointing arrowheads, opposite orientation). We also tested several other combinations of multiple elements in various relative orientations (LRR, LLLR, LLLL, and LPLLLR, where R is the native orientation). These constructs behaved similarly to pRSU2-1 (RLLL), although repressed activity increased with the number of opposite elements (for clarity of presentation, they are not shown). Plasmids were transformed into yeast strains MDY11 (adr1::LEU2), MDY10 (wild-type [wt] ADRI), MDY10 + pMD60, and MDY10 + pMD61. pMD60 is a 2µm-based plasmid expressing wild-type Adr1p from the ADH1 promoter. pMD61 is similar to pMD60, except that it expresses a mutant Adr1p with reduced DNA binding affinity that cannot transactivate. Strains were grown in synthetic media lacking tryptophan and supplemented with 5% glucose (r) or 3% ethanol (dr). The data are means for at least three independent transformants. Standard errors of the means were less than 17% for all values.
ADH2-expressing reporters were generally lower in MDY10 (ADR1) than in MDY11 (adr1) (Fig. 2). To confirm that this effect was due to Adr1p, the activities of prSU2 plasmids were measured in MDY10 containing a second, 2µm-based plasmid, pMD60, in which Adr1p is expressed at high levels from the ADH1 promoter. Overexpression of Adr1p resulted in significantly decreased ADH2 expression from prSU2 constructs containing one or two copies of UAS2 (Fig. 3) under both repressing and derepressing growth conditions. Expression from prSU2-1 (four copies of UAS2) was not affected to the same extent by the ADR1 gene dosage. This result implies that increasing UAS2 copy number in the promoter can lessen the deleterious effects of Adr1p on UAS2-dependent expression.

To determine whether the ability of Adr1p to bind DNA is necessary for the decrease in UAS2-dependent ADH2 expression, a plasmid containing a mutation (glutamate 117 to alanine) in the ADR1 gene was introduced into MDY10. The E117A mutation in the first zinc finger of Adr1p reduces RNA binding about 20-fold and abolishes transactivation (46). Overexpression of Adr1p-E117A did not reduce expression from the prSU2 reporters as did overexpression of wild-type Adr1p (Fig. 3). This result indicates that DNA binding by Adr1p is necessary for diminished UAS2-dependent expression and makes it unlikely that an indirect effect such as squelching by overexpressed Adr1p is responsible for the effect.

**DISCUSSION**

These and previous studies show that UAS2 plays an important role in regulating ADH2 expression in *S. cerevisiae* and suggest that it is the binding site for a protein that interacts with Adr1p bound to UAS1. The interaction between these two sites synergistically enhances expression (52). Synergism between UAS1 and UAS2 accounts for most if not all of the activity of the ADH2 promoter since these two elements alone were as active as a much larger fragment of the ADH2 promoter in a reporter gene (52). In agreement with this conclusion, deleting UAS2 and ADR1 eliminated all of the expression from the native promoter (Fig. 2). In addition to acting as a binding site for another glucose-regulated transcription factor, in vitro DNA binding data (22) and the GAGGAGA motif in UAS2 (9, 36) suggested that Adr1p might bind to this site itself.

The interaction between UAS2 and sequences downstream of it, in particular UAS1, was studied in the native ADH2 promoter by inverting UAS2, by adding extra copies of UAS2, and by changing the helical phasing between UAS1 and UAS2. We interpret the results in the following way. There is an interaction between Adr1p and a factor bound to UAS2 which is orientation dependent. An inverted orientation, or improper phasing of UAS2 inappropriately positions the UAS2 binding factor such that it cannot interact optimally with Adr1p bound to UAS1. Additional copies of UAS2 increase the likelihood that the UAS2 factor does not occupy the UAS2 element adjacent to UAS1, resulting in reduced interaction between the two factors and, thus, reduced ADH2 expression.

An alternative explanation, that altered UAS2 copy number, orientation, or phasing affects interaction with factors binding to the ADH2 promoter downstream of UAS1, or to some factor other than Adr1p binding to UAS1, seems unlikely for several reasons. If a factor binding downstream of UAS1-
UAS2, such as TBP for example, were sensitive to helical phasing, this would most likely have been detected when insertions or deletions were made in the middle of UAS1, and it was not (9, 45). There is no evidence for another factor binding to UAS1: its activity is completely dependent on ADR1 (9, 45, 46, 52). In addition, no evidence for a UAS in this region was found (1, 37).

The reduced ADH2 expression caused by reversed orientation or multimerization of UAS2 was not seen in a promoter containing the basal ADH2 promoter elements and no other UAS. In this promoter context, as in the heterologous CYC1 promoter (52), UAS2 was orientation independent and its activity increased with copy number, as is the case with most UAS elements tested. However, most UAS elements have been tested only for orientation dependence and multimerization in heterologous promoters. An orientation-dependent UAS from CDC9 differs from UAS2 in that it is orientation dependent in a heterologous reporter gene (49).

It seems unlikely that UAS2 is unique in being orientation dependent in the context of its native promoter. In most promoters studied in detail there is evidence for a multitude of binding factors. Proper interaction between these factors bound to adjacent sites probably requires a specific orientation. On the other hand, when the factors are acting alone, their distance from the TATA box may allow them to interact, albeit inefficiently, with general transcription factors by DNA looping or bending.

Most studies of isolated UAS elements indicate that they are less active in single copy in a reporter gene than when located in their native promoter. For example, a synthetic pheromone response element functioned better when imbedded in the native FUS1 promoter, replacing the native pheromone response element, than when tested alone in a reporter gene (25). This was also true of UAS2 and probably reflects the ability to synergize with other transcription factors bound to a native promoter.

ADR1 had an unexpected inhibitory effect on UAS2-dependent activity using the basal ADH2 promoter. This inhibitory effect was dependent on the DNA-binding activity of ADR1p and was increased by increasing the amount of ADR1p in the cell, suggesting that it might be due to a direct effect of ADR1p binding to UAS2. This interpretation is consistent with the in vitro binding data showing that ADR1p (17-229) binds to UAS2, most likely at the AGGAGA sequence, with low affinity. Other interpretations, such as an indirect effect or a direct protein-protein interaction are also possible but seem less likely.

ACKNOWLEDGMENTS

We thank L. Karnitz for construction and initial characterization of pRS314-II and other members of our laboratory, especially K. Dombek, for materials, advice, and criticism. This work was supported by NIH grant GM26079.

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