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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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 ADHIII 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 *HXX2* 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 palindrome to which 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

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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* DH5 α (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 (*MAT α adh1- Δ 1 adh2 Δ 1::URA3 adh3 trp1 leu2 ura3*) was created by transforming MC71-18B (*MAT α adh1- Δ 1 ADH2 adh3 trp1 leu2 ura3*) (14) with a *Bam*HI fragment from pADH2 Δ 1::URA3 in which *ADH2* sequences between the *Sst*I site at nucleotide -117 and the *Sph*I site at +1418 were removed and replaced by a *Hind*III fragment of URA3 (51). MDY11 (*MAT α adh1- Δ 1 adh2 Δ 1::URA3 adh3 adr1 Δ 1::LEU2 trp1 leu2 ura3*) was made by transforming MDY10 with a *Bam*HI fragment of pADR1 Δ 1::LEU2 in which the *ADR1* 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 CMY215 Δ 1 (44) was used for preparation of extracts for in vitro DNA binding assays.

Plasmid transformations. Plasmids were transformed into DH5 α by using frozen competent cells (26, 33). Yeast strains were transformed by a modified lithium acetate method (23, 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 adenine, and 20 mg of uracil per liter, supplemented with 5% glucose (YPD [repressing medium]) or with 3% ethanol (YPE [derepressing medium]). Agar (Difco) was added at 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. They 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 (Gilford Instruments model 250 spectrophotometer). Cultures were routinely tested for the presence of glucose at the time of harvest by using Diastix (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 1 to 2 and harvested.

Enzyme and protein assays. Preparation of yeast extracts for ADHII enzyme activity assay and the assays themselves were performed as previously described (18). β -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 (46, 52). All reactions contained 1 μ g each of poly(dI-dC) and sheared salmon sperm DNA. Complementary oligonucleotides were labeled with [γ -³²P]ATP, annealed, and then purified from a 15% acrylamide gel. Unlabeled competitors were similarly phosphorylated by using unlabeled ATP, annealed, and gel purified. Yeast whole-cell extracts were prepared as described elsewhere (31, 32, 48). Extracts were prepared from *E. coli* as described elsewhere (4). Binding reactions were incubated for 10 min on ice and then for 5 min at room temperature, and products were analyzed after electrophoresis through a 4% polyacrylamide gel (38:2 acrylamide-bis) in 1 $\times$ 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 *Adr1p*-overexpressing plasmid pWET11 (44). An *Xho*I site was created by insertion of linkers at the *Bsu*36I site of *TRP1* (21). Into this *Xho*I site a 2.2-kb *Sal*I-*Xho*I 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. pMD61 was derived from pMD60 by replacing wild-type *ADR1* sequences between a *Sal*I linker inserted in frame at amino acid 16 (21) and *Sph*I with an analogous *Sal*I-*Sph*I fragment containing the E117A mutation (46, 47). The presence of the E117A mutation was confirmed by digestion with *Bsp* 1286 which cuts DNA containing the mutation but not wild-type DNA.

ADH2-expressing plasmids, pMD20 and pMD21, were constructed by inserting a 3,438-bp *ADH2 Bam*HI-*Xho*I fragment from pADH2 (36) or an analogous 3,418-bp *ADH2* fragment from pBR-*ADH2* Δ UAS2 (52) into *Bam*HI- and *Xho*I-digested pTC3. pTC3 was constructed by inserting a 2-kb CEN3 fragment into the *Pvu*II site of YRp7. 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 *Sma*I 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 *Sma*I-digested pMD21. Degenerate UAS2 oligonucleotides were synthesized on an ABI model 380 automated DNA synthesizer by using a mix composed of 94% of one nucleotide precursor and 2% each of the remaining three precursors for all four bases. The prototypical sequence for the degenerate oligonucleotides was TGATCTCCTC TGCCGGAACACC. Insertion of the oligonucleotide destroyed the *Sma*I site of pMD21. Thus, to enrich for plasmids containing inserts, ligation products were digested with *Sma*I prior to transformation into DH5 α . Plasmids 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 pRS314-II with *Sal*I or *Sma*I, respectively, and ligating the appropriate double-stranded oligonucleotides with the digested (and filled in the case of *Sal*I) 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'-gggTCTCCAAC TATAAGTTGGAGAcc-3' (the UAS1 sequence is underlined); the sequence of the UAS2 oligonucleotide is 5'-TGATCTCCTCTGCCGGAACACCG-3'.

RESULTS

UAS2 is orientation dependent in the native *ADH2* promoter. UAS2 was originally defined by the ability of a sequence (5'-TGATCTCCTCTGCCGGAACACCG-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 ADHII 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 had virtually the same ADHII 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

FIG. 1. *ADH2* promoter sequences. (A) Comparison of wild-type UAS2 and the flanking sequence (upper sequence), the same sequence with UAS2 deleted (middle sequence), and the UAS2 region of *ADH2* promoter plasmids created by insertion of UAS2 oligonucleotides at the *Sma*I site of pMD21 (lower sequence). The 23-bp sequence originally tested in the UAS2/*CYC1/lacZ* reporter (52) is underlined. The sequence deleted from the promoter in plasmid pMD21 is underlined. An overview of the point mutations tested in pMD21-X derivatives is shown on the lower strand (see Table 2 for results). Squares, positions of mutations with a relatively large effect on *ADH2* derepression; circles, bases at which mutations had a lesser effect; asterisks, two extra C·G base pairs resulting from the insertion of UAS2 oligonucleotides into the *Sma*I site, which replaces UAS2, in pMD21. These C·G base pairs are present in all plasmids derived from pMD21 but do not occur in the wild-type *ADH2* 5' sequence or the wild-type plasmid pMD20. The sequences flanking the 3' end of UAS2, between UAS2 and UAS1, in the derivatives of pMD21 are identical to those in the normal *ADH2* promoter. The *Sma*I site of pMD21 into which the UAS2 oligonucleotides were inserted is doubly underlined. (B) ADHII activities from plasmids expressing *ADH2* from a wild-type promoter (pMD20), a promoter carrying a 20-bp deletion of UAS2 (pMD21), and a promoter into which a wild-type UAS2 had been inserted (pMD21-1). Schematic representations of the *ADH2* promoter region are shown. Double-headed arrows, UAS1 (present in all constructs); arrowhead, UAS2.

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 *adr1Δ1::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 to 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-

FIG. 2. Effect of *ADR1* on ADHII activities from plasmids containing one, two, three, or no copies of UAS2 or one reversed copy of UAS2 in the context of the entire *ADH2* upstream regulatory region. A schematic representation of the *ADH2* promoter region is given for each construct. Double-headed arrows, UAS1 (present in all plasmids); open rectangles, UAS2 oligonucleotides found to have a wild-type sequence inserted into the *Sma*I site of pMD21 either in the native orientation (rightward-pointing arrowhead) or in the opposite orientation (leftward-pointing arrowhead); Δ UAS2, deletion of 20 bp of the UAS2 element from pMD21. Plasmids were transformed into isogenic yeast strains, MDY10 (wild type [wt] *ADR1*) and MDY11 (*adr1* Δ ::*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%. a, reproduced from Fig. 1 to facilitate comparison with activities in the *adr1* Δ ::*LEU2* strain.

quence for proper *ADH2* expression, a pool of double-stranded, degenerate UAS2 oligonucleotides were inserted into the *Sma*I 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, and derepressed ADHII activities were assayed. Single point mutations throughout UAS2 reduced *ADH2* expression 1.5- to 10-fold. Several mutations (in plasmids pMD21-5, pMD21-6, pMD21-7, and pMD21-8) reduced ADHII activity to levels

similar to those observed with the UAS2-deletion construct (Table 2). No point mutations that decreased ADHII activity to a level below that of pMD21 were found. Other mutations (represented by pMD21-9, pMD21-10, pMD21-11, pMD21-12, and pMD21-13) had a less profound effect on *ADH2* expression. These results are consistent with the interpretation that the specific sequence within UAS2 is important for its activity.

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 ele-

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

Plasmid ^a	Insert ^b	ADHII activity ^c (mU/mg)
pMD20	None (wild type)	2,600
pMD21	None (Δ UAS2)	380
pMD21.1	UAS2 + 1 bp	900
pMD21.5	UAS2 + 5 bp	590
pMD21.10	UAS2 + 10 bp	1,900

^a Plasmids pMD20 and pMD21 are described in the legend to Fig. 1. Plasmids pMD21.1, pMD21.5, and pMD21.10 are derived from pMD20 by insertion of oligonucleotides containing an additional 1, 5, or 10 bp, respectively, between UAS1 and UAS2.

^b The additional nucleotides were present after nucleotide 22 of the UAS2 sequence shown in Fig. 1. The additional nucleotides were (top strand in Fig. 1) C (pMD21.1), CACTC (pMD21.5), and CACACCTGAA (pMD21.10). These nucleotides were generated by a random number generator on a pocket calculator, assigning G, A, T, and C to numbers ending in 1, 2, 3, and 4, respectively.

^c Measured in MDY10 transformants under derepressing growth conditions. The data are means for three independent transformants. The standard error of the mean was generally about 25%. Repressed ADHII activity was also measured and did not differ significantly between transformants containing the various plasmids.

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

Plasmid ^a	Mutation ^b	ADHII activity ^c (mU/mg)
pMD21-1	None (wild type)	1,600
pMD21-5	C21-G	200
pMD21-6	A20-T	340
pMD21-7	G16-A	360
pMD21-8	C5-T	370
pMD21-9	C8-G	570
pMD21-10	G12-C	810
pMD21-11	A18-G	870
pMD21-12	A18-T	1,200
pMD21-13	T4-G	1,200

^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							
Plasmid	Inserts	A.		B.		C.		D.	
		MDY11 (<i>adr1::LEU2</i>)		MDY10 (wt <i>ADR1</i>)		MDY10 (wt <i>ADR1</i> +pMD60)		MDY10 (wt <i>ADR1</i> +pMD61)	
		r	dr	r	dr	r	dr	r	dr
pRS314-II	NONE	<5	<5	<5	<5	<5	<5	<5	<5
pRSU1-1	↔	<5	<5	<5	20	ND	ND	ND	ND
pRSU1-2	↔↔↔	<5	<5	<5	600	ND	ND	ND	ND
pRSU2-3	▶	20	100	20	70	10	30	30	100
pRSU2-10	▶▶	90	300	50	200	20	40	100	700
pRSU2-1	▶▶▶▶	400	800	200	800	200	400	200	800
pRSU2-11	◀	50	300	20	80	ND	ND	ND	ND

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 *SalI* 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 (LRRR, LLRR, LLLR, and LPLLLR, where R is the native orientation). These constructs behaved similarly to pRSU2-1 (RRRR), 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 (*adr1Δ1::LEU2*), MDY10 (wild-type [wt] *ADR1*), MDY10 + pMD60, and MDY10 + pMD61. pMD60 is a 2-μm-based plasmid overexpressing 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.

ments might also contribute to different effects of reversal or multimerization in the two situations. To study the characteristics of UAS2-dependent gene expression in isolation from other *ADH2* regulatory sequences, a reporter gene containing the *ADH2* basal-promoter elements driving expression of *ADH2* was utilized. In this construct, pRS314-II, all known *ADH2* regulatory sequences have been removed (1, 37, 52) and the basal promoter is protected from transcription-activating vector sequences by 400 bp of the *ADH2* far 5' sequence (38). In essence, the *ADH2* promoter in this plasmid contains an internal deletion extending from -117 to -743 with respect to the start site of transcription.

Two well-characterized UAS sequences were tested to determine whether reporter activity derived from pRS314-II responded appropriately to the UAS element present. Oligonucleotides containing the binding site for the yeast homolog of the Rous sarcoma virus internal enhancer binding factor, yIBF (30, 31), were inserted into the *SmaI* site of pRS314-II. The yIBF-containing plasmids were introduced into the *ADH*-null strain MDY10 and an isogenic *adr1Δ1::LEU2* strain, MDY11. Both repressed and derepressed ADHII activities were then assayed (data not shown). These plasmids gave high levels of ADHII activity that was unaffected by carbon source or *ADR1* genotype, as would be predicted for an IBF-dependent gene (30-32). Insertion of one or two copies of UAS1-containing oligonucleotides into the *SalI* site of pRS314-II yielded reporters pRSU1-1 and pRSU1-2, respectively. When the UAS1-containing reporters were tested in MDY10 and MDY11, *ADH2* expression was found to be tightly repressed by the presence of glucose in the growth media and derepression was completely *ADR1* dependent (Fig. 3), consistent with the be-

havior of other UAS1-dependent reporter genes (37, 52). With no UAS sequence inserted 5' of the *ADH2* promoter, pRS314-II was transcriptionally inactive, as demonstrated by lack of ADH activity in cells containing the plasmid (Fig. 3 and data not shown).

UAS2-containing oligonucleotides were inserted into the *ADH2* basal-promoter plasmid, pRS314-II, yielding the pRSU2 reporter constructs. The UAS2 elements chosen for study were present in single copy in normal and reversed orientations and also as tandem repeats of two and four copies with each in its normal orientation. UAS2-dependent *ADH2* expression from pRSU2 constructs (Fig. 4) was not as strongly repressed by glucose as was expression from the UAS2/*CYC1/lacZ* reporters (52). In other ways expression levels from the two reporters were similar. Expression was *ADR1* independent and increased upon multimerization of UAS2. UAS2 was orientation independent, but, in contrast with the results for the UAS2/*CYC1/lacZ* reporter, expression was significantly higher when UAS2 was in the reversed orientation. The similarity of UAS2 function in the pRSU2 constructs compared with the UAS2/*CYC1/lacZ* reporters (52) confirms that the presence of *ADH2* upstream regulatory sequences, specifically UAS1, rather than the *ADH2* basal-promoter elements, is responsible for the decreased activity in plasmids containing reversed or multiple UAS2 elements in an otherwise intact *ADH2* promoter.

UAS2-dependent *ADH2* expression in the pRSU2 reporter genes is decreased in the presence of overexpressed *ADR1*. One unexpected result was observed when the effect of the *ADR1* genotype on expression of the pRSU2 constructs was tested. The ADHII activities from the UAS2-dependent

FIG. 4. (A) Sequences of oligonucleotides used as probes and competitors in DNA migration assays. 1, 41-mer containing UAS2 and flanking sequences. The 23 bp of UAS2 used in the in vivo experiments are underlined. 2, 26-mer used in constructing UAS2 deletions. 3, UAS2 23-mer used in all in vivo assays. 4, UAS2 23-mer containing a point mutation changing C-5 to T. The mutated base is underlined. 5, UAS2 23-mer containing a point mutation changing C-8 to A. (B) DNA migration assays using the UAS2 23-mer probe. B, Adr1p (17-229)-containing complex; F, free probe. Lane 1, about 0.1 μ g of protein from an extract of *E. coli* expressing Adr1p (17-229) (the extract was heated to 100°C for 5 min immediately prior to the binding reaction); lane 2, about 0.1 μ g of heat-treated protein derived from cells containing no Adr1p-expressing plasmid; lane 3, 4 μ g of the extract lacking Adr1p that was not heat treated; lane 4 no protein added. (C) Gel retardation assays using a UAS2 41-mer as a probe. The lanes correspond to those in panel B. (D) DNA migration assays showing the effect of UAS2 point mutations on Adr1p binding. Odd-numbered lanes, no protein; even-numbered lanes, about 0.1 μ g of the heat-treated, Adr1p-containing extract. Lanes 1 and 2, probe 3 (wild type UAS2); lanes 3 and 4, probe 5 (C-8 to A); lanes 5 and 6, probe 4 (C-5 to T). The upper complex(es) in lanes 3 of panels B and C is due to a heat-sensitive *E. coli* DNA-binding protein.

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, 2 μ m-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 *ADR1* reduces DNA 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.

Binding of Adr1p (17-229) to UAS2 in vitro. The presence of a sequence in UAS2 that is related to the binding site for Adr1p in UAS1 was noted previously (1, 36, 52), and some evidence that Adr1p (17-229) could bind to UAS2 was deduced from footprinting experiments (22). However, the footprint over UAS2 was seen only at high concentrations of Adr1p

(17-229) after UAS1 had been fully occupied, suggesting that if there was a binding site for Adr1p in UAS2 it was much weaker than the sites in UAS1 and might require cooperativity with monomers bound to UAS1 in order to be occupied. More-recent studies of the DNA binding properties of Adr1p allowed a consensus binding site to be deduced. The preferred sequence is TTGG(A/G)GA (9). TTGGAGA is found in both halves of the inverted repeat of UAS1. The sequence GAGGAGA in UAS2 (on the upper strand in Fig. 1A the sequence is TCTCCTC) was not directly tested but is predicted to bind Adr1p with an affinity reduced 100-fold compared with the optimal binding site (9).

To test directly the possibility that Adr1p can bind to UAS2 in the absence of UAS1, DNA migration assays were done with a truncated version of Adr1p expressed in *E. coli* and oligonucleotides containing wild-type UAS2 or UAS2 containing point mutations as probes (Fig. 4A). Binding of Adr1p to the wild-type UAS2 probes was detected in extracts from Adr1p (17-229)-expressing bacteria. A single complex that is specific for Adr1p-expressing extracts is indicated (B) in Fig. 4B and C. In a parallel experiment, a similar amount of the UAS1 probe was completely shifted to the bound state, suggesting that Adr1p binds to UAS2 with a significantly lower affinity than UAS1, as was predicted (data not shown). To determine whether Adr1p (17-229) was binding to the predicted sequence in UAS2, probes containing point mutations that are predicted to decrease Adr1p binding affinity (9) were tested. These probes showed no detectable binding to Adr1p (Fig. 4D). This result implies that Adr1p binds to UAS2 via the predicted GAGGAGA motif.

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.

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