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Identification of Potential Target Genes for Adr1p through Characterization of Essential Nucleotides in UAS1

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Adr1p is a regulatory protein in the yeast *Saccharomyces cerevisiae* that binds to and activates transcription from two sites in a perfect 22-bp inverted repeat, UAS1, in the *ADH2* promoter. Binding requires two C_2H_2 zinc fingers and a region amino terminal to the fingers. The importance for DNA binding of each position within UAS1 was deduced from two types of assays. Both methods led to an identical consensus sequence containing only four essential base pairs: GG(A/G)G. The preferred sequence, TTGG(A/G)GA, is found in both halves of the inverted repeat. The region of Adr1p amino terminal to the fingers is important for phosphate contacts in the central region of UAS1. However, no base-specific contacts in this portion of UAS1 are important for DNA binding or for *ADR1*-dependent transcription in vivo. When the central 6 bp were deleted, only a single monomer of Adr1p was able to bind in vitro and activation in vivo was severely reduced. On the basis of these results and previous knowledge about the DNA binding site requirements, including constraints on the spacing and orientation of sites that affect activation in vivo, a consensus binding site for Adr1p was derived. By using this consensus site, potential Adr1p binding sites were located in the promoters of genes known to show *ADR1*-dependent expression. In addition, this consensus allowed the identification of new potential target genes for Adr1p.

One of the most common protein motifs in eukaryotic DNA-binding proteins is a zinc finger. The first of these motifs to be discovered, and the most common type, is the Cys_2-$His_2(C_2H_2)$ prototype found in TFIIIA (9, 29, 35, 42). Physical evidence indicates that each finger interacts primarily with a 3-bp subsite on one strand of DNA, and adjacent fingers interact with contiguous 3-bp subsites on the same strand (50). Variations on this theme, however, are beginning to appear (51). Although more than 600 zinc fingers have been identified, the binding sites for relatively few are known, and the importance of each nucleotide within these sites is known for fewer still. Even for closely related fingers, such as those found in Zif268 and Sp1, there are major differences in the tolerance for changes in the binding site that are apparently related to differences in the finger structure (4, 37). Determining the binding sites, and the permissiveness for alterations within these sites, could help identify potential targets for zinc finger proteins.

One well-characterized zinc finger protein is the yeast transcription factor Adr1p (14, 30). Adr1p activates transcription of the *ADH2* gene, which encodes a glucose-repressible alcohol dehydrogenase in *Saccharomyces cerevisiae* (16). Adr1p is also important for derepression of some peroxisomal enzymes, for peroxisome biogenesis (63), and for growth on glycerol (3). Adr1p is inactive as a transcription factor in the presence of glucose (7, 17), although the intact protein is present and DNA binding appears normal (68). Several mechanisms have been proposed to account for the inactivity of Adr1p during glucose repression (12, 20, 73).

Adr1p contains two zinc fingers and a region amino terminal to the fingers, which together are essential for DNA binding (6, 10), and functions through UAS1, a perfect 22-bp repeat in the *ADH2* promoter (21, 61). Each half of the inverted repeat is an independent, functional binding site for one monomer of Adr1p. However, both halves of UAS1 must be present in inverted orientation for transcriptional activation to occur (71, 76).

The binding subsites within UAS1 for fingers 1 and 2 of Adr1p were identified by genetic means (71). The subsites are two adjacent triplets of bases, and the fingers interact with them in an antiparallel fashion; i.e., the amino-terminal finger binds to the 3′ triplet, analogous to the alignment of Zif268 fingers on DNA in the crystal structure (50). The subsite for finger 2 is apparently unusual. Interaction between a single arginine in the finger and the corresponding base in UAS1 makes the major contribution to specific DNA binding affinity by that finger (70). The function, if any, of the remaining 10 bp in UAS1 is unknown. These base pairs constitute part of the perfect inverted repeat, suggesting that there is some selection acting to conserve their identity. They could contact the amino-terminal arm of Adr1p, which is an essential element in binding DNA, or be the binding site for another protein that participates in transactivation in vivo.

Detailed characterization of UAS1 allowed a consensus binding site to be derived and, consequently, the identification of yeast genes which are putative targets for Adr1p transactivation. In addition, identification of the apparent phosphate contacts by ethylation interference revealed the role played by nonspecific contacts between Adr1p and UAS1. Such contacts occur throughout UAS1, including the central region where no base-specific contacts are important. A functional UAS for Adr1p transactivation consists of a pair of binding sites in inverted orientation, with appropriate spacing between them.

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Thus, specificity of Adr1p function requires sequence-, orientation-, and spacing-dependent information in the binding sites.

MATERIALS AND METHODS

Yeast and bacterial strains. The yeast strains used were SSH35 (10) and KDY10 (*MATα adh1Δ adh3 adr1::LEU2 leu2 trp1 ura3*). The bacterial strain used to express the DNA binding domain of Adr1p was *Escherichia coli* MC1061 (40). Cells were transformed and grown and extracts were prepared as described previously (69).

DNA binding and enzyme assay. DNA binding and enzyme assays were performed as described previously (21, 69). Binding site selection and amplification assays (5) used an oligonucleotide with the sequence CTAGATATCCCTGGATCCTA ANNNNNNNNGAATTCAGGCTCAAAGCTCAC (RAN). The two RAN primers used were RAN1 (CTAGATATCCCT GGATCCTAA) and RAN2 (GTGAGCTTTGAGCCTGAAT TC). *Bam*HI and *Eco*RI sites are underlined. For the first round of selection, a radioactive oligonucleotide probe was made with RAN as the template and RAN2 as a primer. A gel shift assay was used to isolate the protein-bound DNA. The bound DNA was eluted from the gel and amplified by PCR using RAN1 and RAN2 as primers. The PCR product was labeled and used as the template for the next round of selection. The PCR conditions consisted of 35 cycles of 1 min at 94°C, 1 min at 52°C, and 1 min at 72°C. PCR-amplified DNA was then digested with *Bam*HI and *Eco*RI and cloned into pUC18 for sequencing. After four rounds of selection, all of the DNA in the binding reaction could be bound by Adr1p.

Synthesis of mutant Adr1p binding sites. The double-stranded DNA probes used to determine the binding site preferences for Adr1p were synthesized from a template containing 53 nucleotides (SK-wild) and a primer (SK-primer) containing 18 nucleotides that is complementary to the 3′ end of the longer oligonucleotide, with a 4-bp overhang for cloning purposes. Their sequences are 5′-GTTCTCCAACTTATAA GTTGGAGATGCCCGGTGTTCCGGCAGAGGAGA GGTAC-3′ and 5′-CTCTCCTCTGCCGGAACA-3′. UAS1 is underlined. Manipulation of these oligonucleotides has been described previously (71). The mutant binding sites contained symmetric changes at the 11 positions in each half of UAS1 to the other three possible bases. They are referred to by the wild-type (WT) base, its position in the 3′ binding site (top strand), followed by the new base. The first base 3′ of the center of dyad symmetry, which is a T, is referred to as position one; the first base 5′ of the center of dyad symmetry, which is an A, is referred to as 1′. Mutant oligonucleotide SK36 (TAA123CGG) contains changes of the central 6 bp from TTATAA to CCGCGG. UAS1Δ2, UAS1Δ4, and UAS1Δ6 are oligonucleotides in which the central 2, 4, and 6 bp, respectively, have been deleted from SK-wild. The sequence of UAS1-half (SK-half), top strand, is 5′-GTCATGACTCAGG TAAGTTGGAGATGCCCGGTGTTCCGGCAGAGGAG AGGTAC-3′ (the 3′ half of UAS1 is underlined).These oligonucleotides were cloned in the expression vector pLG669Z(K) as described previously (72).

Ethylation interference. The oligonucleotides used for ethylation interference studies had either a full UAS1 (Sush-9; UAS1-centered [UAS1-c]) or one half of UAS1 (SK-half; see above). The sequence of the top strand of UAS1-c is 5′-AA ATTCTCTTATTTCTCCAACTTATAAGTTGGAGAATG CCCGTCTGACCC-3′. Oligonucleotides were labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase, annealed to the appropriate unlabeled strand, and purified on a 12% polyacrylamide gel (38:2 acrylamide/bisacrylamide).

Ethylation interference experiments were performed by using a modification of procedures described previously (62). Labeled double-stranded oligonucleotides in 100 μl of sodium cacodylate (pH 8.0) were mixed with an equal volume of freshly prepared ethanol saturated with ethylnitrosourea. The samples were incubated for 30 min at 51°C. DNA was precipitated in sodium acetate and ethanol, rinsed with 70% ethanol, dried in vacuo, and then used for binding reactions. Binding reactions and gel electrophoresis were performed as described above. Bound and free DNA fragments were eluted from the gel and purified by chromatography on a Schleicher & Schuell Elutip. The DNA was cleaved in 100 μl of 0.3M KOH–10 mM EDTA and incubated at 90°C for 30 min. The reaction was neutralized by addition of 80 μl of 0.3 N HCl and 20 μl of 3 M sodium acetate (pH 5.2). The DNA was precipitated by adding tRNA and ethanol, rinsed with 70% ethanol, dried, and resuspended in sequencing gel buffer. Cleaved fragments were separated on a 8 M urea–8% polyacrylamide sequencing gel.

Analysis of GenBank sequences for potential Adr1p binding sites. The yeast DNA sequences of GenBank 77 were searched by using Quest (release 5.4) from the IntelliGenetics Suite of programs (IntelliGenetics, Inc., Mountain View, Calif.) with the following key: NC(T/C)CC(A/G)(A/T/G)$N_{2-18}N_{2-18}$(T/A/C)(T/C)GG(A/G)GN. This key was derived from previously reported Adr1p-UAS1 binding data (71) and the data presented in Fig. 3 and Table 1.

RESULTS

Critical UAS1 determinants for WT ADR1 binding. A binding site selection-amplification assay (5) was used to identify binding sites for Adr1p. To enhance the efficiency of binding, a truncated version of Adr1p containing the complete DNA binding domain, amino acids 17 to 229, was expressed in *E. coli* and used in the reactions. An oligonucleotide whose central 8 bp were degenerate was used as the binding substrate (Fig. 1A). Eight base pairs were chosen for degeneracy because preliminary results indicated that the Adr1p binding site was no larger than 8 bp. After four rounds of binding and amplification, the selected oligonucleotides could be detected by gel mobility shift assay (data not shown). The amplified products were cloned into pUC18, and 16 individual clones were sequenced (Fig. 1B). From these 16 clones, a consensus sequence was derived (Fig. 1C). The essential requirements for binding are contained within the 4-bp sequence 5′-GG(A/G)G-3′. The flanking nucleotides on the 5′ and 3′ sides were essentially random, although there did appear to be a bias toward T at the 3′ side. Finding a 4-bp consensus sequence appears to justify using an oligonucleotide with an 8-bp degeneracy rather than a larger one. The sequence GGAG is present in each half of UAS1. Moreover, purines at these positions were shown to be involved in Adr1p binding by methylation interference studies (69).

Another type of assay was used to investigate the binding site in more detail. All possible paired, symmetric mutations were made at each of the 22 positions in palindromic UAS1. The ability of Adr1p to bind to a 53-bp oligonucleotide containing these potential binding sites was tested by gel retardation analysis using extracts made from *E. coli* expressing the same DNA binding domain of Adr1p that was used in the previous study. A nonsaturating amount of Adr1p was used to ensure that differences in affinity between the different probes could be detected. Some of these UAS1 elements were used in a previous study to identify altered specificity mutants in finger

A. Degenerate oligonucleotide:

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5'---tcctaaNNNNNNNNgaattc----3'
3'---aggattNNNNNNNNcttaag----5'
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B. Selected sequences for ADR1(5' to 3'):

cT	G	G	A	G	GGGt
cAT	G	G	A	G	TAt
cCGGC	G	G	A	G	t
cG	G	G	G	G	TTAt
aAGGGT	G	G	A	g	a
cA	G	G	A	G	GGGt
aGGG	G	G	G	G	Gg
cGAGC	G	G	A	G	t
c	G	G	G	G	CGCGt
cGGTT	G	G	G	G	t
cAGA	G	G	G	G	Tt
cGGT	G	G	G	G	Tt
cAATT	G	G	G	G	t
cGGTC	G	G	G	G	t
cGTA	G	G	G	G	t
cGGGC	G	G	G	G	t

C. Consensus: G G (A/G) G

Frequency: 16/16 16/16 (6/10)/16 15/16

FIG. 1. Selection and amplification of the binding sites for Adr1p. (A) Sequence of the degenerate oligonucleotide RAN. The residues at the randomized positions are shown in capital letters. (B) Binding of the randomized oligonucleotide was carried out as described in Materials and Methods. After four rounds of binding and selection, the bound oligonucleotides were eluted from the gel, amplified, digested with *Eco*RI and *Bam*HI, and cloned into pUC18 digested with the same two restriction enzymes. The sequences of the G-rich strands for 16 cloned fragments are shown. Residues at the originally randomized position are shown in capital letters. The 5′ lowercase nucleotide is either a c or an a, depending on which degenerate strand was G rich. (C) Consensus of the 16 sequences. The frequency shows the number of occurrence of that residue in the 16 sequences.

1 of Adr1p (71). In that study, saturating amounts of Adr1p were used to detect binding by different mutant proteins.

The results of this directed binding-site mutagenesis confirmed the results of the binding-site selection experiments. High-affinity binding required only four specific base pairs within each half of UAS1 (Fig. 2). Changing guanines 7, 8, and 10 to any other base resulted in loss of all detectable binding. Position 9 was equally important, but either purine was acceptable at this position. Reduced but significant binding was detected with all changes at positions 5, 6, and 11. At positions 1 to 4, there appeared to be no nucleotide preference, suggesting that base-specific contacts are not important at these positions.

UAS1 (3' half): 5'- T A A G T T G G A G A -3'

Position: 1 2 3 4 5 6 7 8 9 10 11

Relative binding affinity, A*

Position 5		Position 6		Position 11	
T	1.0	T	1.0	A	1.0
C	0.01	C	0.1	G	0.4
A	0.1	A	0.01	T	0.7
G	0.3	G	0.01	C	0.6

FIG. 3. Binding of Adr1p to UAS1 mutated at positions 5, 6, and 11. Positions in boldface indicate the positions at which alterations were tested. A binding curve was generated for each probe by using various amounts of extract protein containing WT Adr1p. The binding reactions were analyzed by gel electrophoretic mobility shift assays and were quantitated by densitometry. A* is the inverse of the amount of protein necessary to bind 30% of the DNA, normalized to the amount needed to bind 30% of the WT probe. Values of 0.01 are very approximate.

Positions 5 and 6 are normally occupied by T residues in the primary, G-rich strand. Since reduced but detectable DNA binding was observed with all changes at these positions, the binding affinity for these mutant sites was determined more quantitatively. For the same reason, the binding affinities for mutant sites containing changes at position 11 were quantified. These data are summarized in Fig. 3. The apparent binding affinity of Adr1p for the mutant sites was 3- to 100-fold lower than its affinity for the WT site.

These results vindicate the use of the binding site selection assay. The sequence GG(A/G)G was present in all binding sites selected from the degenerate oligonucleotide pool. This sequence was also contained within the best binding sequence, TTGG(A/G)GA, deduced from the collection of mutant sites in which one base pair was changed at a time. Additionally, comparison of these two approaches suggests that it would be important to use a low concentration of binding protein in the

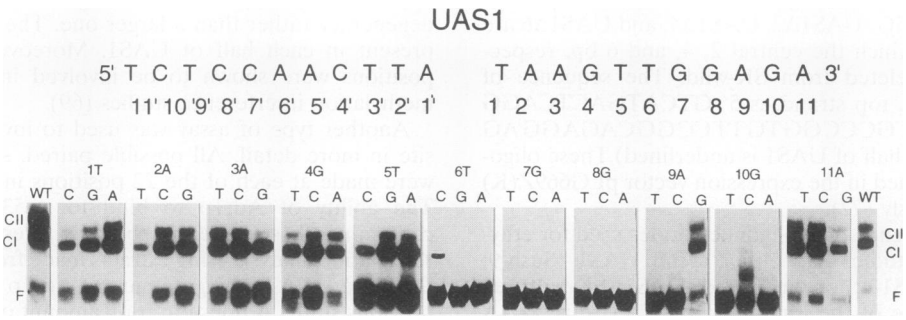

FIG. 2. Binding site specificity of *ADR1*. Gel retardation assays using mutant UAS1 probes (based on SK-wild) and an extract of *E. coli* MC1061 carrying plasmid pCQ-ADR1(17–229) were carried out as described in Materials and Methods. F, free probe; CI, monomer Adr1p-DNA complex; CII, two monomers of Adr1p bound to DNA.

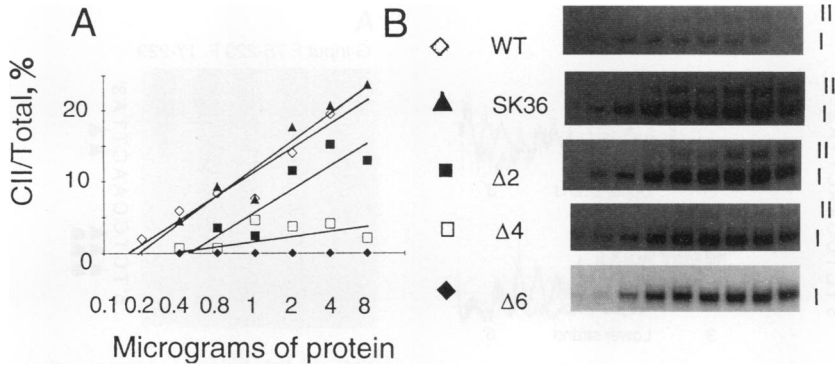

FIG. 4. Effect of deletions and mutations in the center of UAS1 on the binding of two monomers of Adr1(17–229)p. Gel retardation assays using different UAS1 probes (based on UAS1-wild [see Materials and Methods]) and extracts of *E. coli* MC1061 expressing Adr1(17–229)p were performed as described in Materials and Methods. (A) The percentage of complex CII was determined from PhosphorImager analysis of a gel retardation analysis similar to that shown in panel B. (B) Lanes from left to right correspond to increasing amounts of *E. coli* extract (0.05, 0.1, 0.2, 0.4, 0.8, 1, 2, 4, and 8 μg of total protein). Positions of the probe with one and two molecules of Adr1(17–229)p bound are indicated by I and II, respectively.

selection procedure to obtain an array of binding sites that faithfully reflects the affinity between the protein and the sites selected.

Role of the central base pairs in UAS1. The specific nucleotide sequence of the central 8 bp separating the two Adr1p binding sites in UAS1 did not appear to contribute significantly to Adr1p-DNA binding specificity (Fig. 1 and 2). To provide further support for this interpretation, the central 6 bp were changed en bloc from TTATAA to CCGCGG. Adr1p bound to this altered sequence with high affinity. Both complex I (CI) and complex II (CII), representing binding of one and two Adr1p monomers (69), respectively, were observed at the same protein concentrations as when WT UAS1 was used (33) (Fig. 4). This result confirms that the specific nucleotide sequence of the central 6 bp does not make a major contribution to Adr1p binding specificity.

Although the nucleotide sequence of the central positions in UAS1 appears to be unimportant, the spacing between the individual Adr1p binding sites might be important for allowing two monomers of Adr1p to simultaneously occupy UAS1. To test this possibility, mutant UAS1 sites in which 2, 4, and 6 bp were deleted from the middle of UAS1 were made. When these binding sites were tested in gel retardation assays, the apparent binding affinity, determined as total DNA bound, was not detectably altered (33). However, the ability to form CII was significantly affected by deletions of the central base pairs. The relative abilities of Adr1p to form CII with WT and mutant UAS1 probes are shown in Fig. 4. The amount of CII was decreased when UAS1Δ2 and UAS1Δ4, which contain deletions of the central 2 and 4 bp of UAS1, respectively, were used as probes. No CII was detected when UAS1Δ6, in which 6 bp were deleted from the center of UAS1, was used as the probe. This finding indicates that more than 2 bp separating the two Adr1p binding sites are required for the simultaneous occupation of each site. Additionally, the formation of CI was not inhibited by substitution of positions 1 to 6 of UAS1 (Fig. 4). This finding further supports the view that the central base pairs of UAS1 do not play a role in sequence-specific contacts between Adr1p and DNA.

Phosphate contacts between Adr1p and UAS1. These results can be explained in at least two, non-mutually exclusive ways. The central base pairs could serve a spacing function, preventing steric interference between the two monomers of Adr1p but not making direct contact with the protein. Alternatively, or perhaps in addition, the central base pairs could contribute nonspecifically to DNA binding affinity through phosphate backbone contacts with Adr1p. Ethylation interference analysis of Adr1p-UAS1 binding was used to test the latter possibility. Analysis of Adr1p binding to a complete UAS1 element is shown in Fig. 5. Since UAS1 can be bound by either one or two molecules of Adr1p, forming two complexes, CI and CII, respectively, CII was isolated after gel electrophoresis. Analysis of CII yields clearer information about interference because CI consists of a mixture of DNA-protein complexes containing one molecule of Adr1p bound to either the left or right half of the UAS1 palindrome. Thus, if the left half is unable to bind Adr1p, the right half can be bound, and vice versa, leading to at most a twofold reduction in signal at positions where ethylation interferes with binding. Ethylation of phosphates at positions 10, 9, 8, 5, 10′, 9′, 8′, 3′, and 2′, on both the top and bottom strands (see Fig. 8), interfered with binding, suggesting that Adr1p makes important contacts with about 18 phosphates in UAS1.

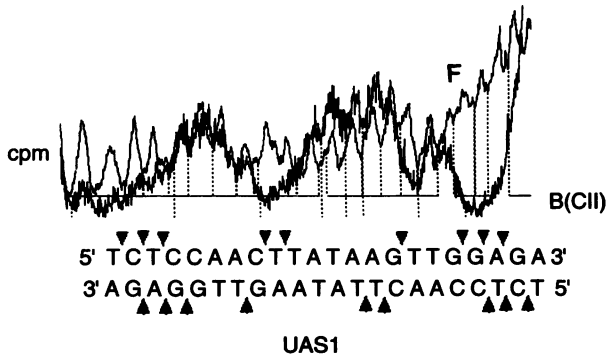

FIG. 5. Ethylation interference of two molecules of Adr1p binding to UAS1. The 50-bp oligonucleotide UAS1-c, ^{32}P labeled on either the upper or lower strand (data not shown), was modified with ethylnitrosourea. Samples were cleaved with KOH and analyzed on a sequencing gel. The gel was analyzed on a PhosphorImager, and the data were converted into counts per minute and plotted as shown. Arrowheads show positions in the sequence where modification interfered with binding. The UAS1 sequence is aligned approximately with the tracing of the autoradiogram shown at the top. F, free probe; B(CII), bound probe.

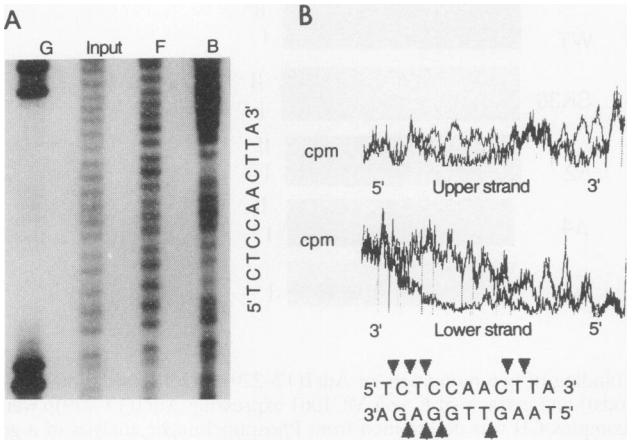

FIG. 6. Ethylation interference of one molecule of Adr1p binding to a single site. (A) The 53-bp oligonucleotide UAS1-half with a single Adr1p binding site, ^{32}P labeled on either the top or bottom strand, was modified with ethylnitrosourea. Samples were cleaved with KOH and analyzed on a sequencing gel. G, Maxam-Gilbert DNA sequence ladder for G residues; F, free probe; B, bound probe. (B) Summary of the data. The positions at which modification interfered with binding are indicated with arrowheads.

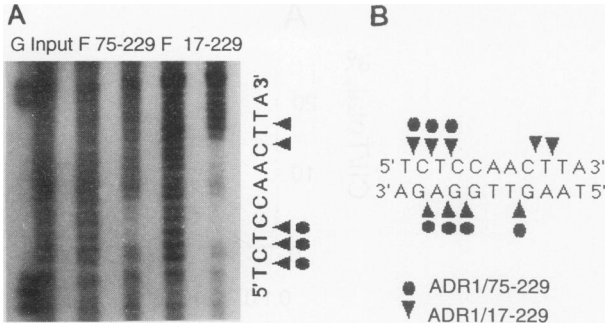

FIG. 7. Ethylation interference of an Adr1p amino-terminal deletion mutant binding to a single site. (A) The 53-bp oligonucleotide UAS1-half with a single Adr1p binding site, ^{32}P labeled on either the top or bottom strand, was modified with ethylnitrosourea and then used for gel retardation assay with Adr1(17–229)p and the amino-terminal deletion mutant Adr1(75–229)p. Samples were eluted from the gel, cleaved with KOH, and analyzed on a sequencing gel. G, Maxam-Gilbert DNA sequence ladder for G residues; F, free probe. (B) Summary of the data. The positions at which ethylnitrosourea modification interfered with binding are indicated by arrowheads for Adr1(17–229)p and with circles for Adr1(75–229)p.

To determine which phosphate contacts are made by a single monomer of Adr1p, ethylation interference was carried out on a single Adr1p binding site. A probe containing one binding site forms only complex CI, indicating that Adr1p binds to it as a monomer (69). The results of this analysis were similar to those obtained when the complete UAS1 element was used. Ethylation of four phosphates on the upper strand, at positions 10, 9, 8, and 5, and five on the lower strand, at positions 10′, 9′, 8′, 3′, and 2′, interfered with binding (Fig. 6). Ethylation of one or two other phosphates may interfere weakly with binding. Comparison of the apparent phosphate contacts made with the complete UAS1 and the single Adr1p binding site (Fig. 5 and 6) indicates that the phosphate contacts made by one monomer of Adr1p are confined to one half of UAS1.

It has previously been shown that amino acid residues amino terminal to the zinc fingers of Adr1p influence DNA binding (10, 72). Therefore, it is possible that the contacts on the C-rich strand of the binding site could be due to nonfinger contacts. To test this possibility, the DNA binding of an Adr1 polypeptide lacking part of the amino-terminal region, Adr1(75–229)p, was analyzed by ethylation interference. The interference pattern for this polypeptide was slightly different than for Adr1(17–229)p. Specifically, ethylation of phosphates 2′ and 3′ no longer interfered with binding (Fig. 7A). To test whether amino acids carboxy terminal to the zinc fingers make additional phosphate contacts, another deletion mutant was made. This mutant contains Adr1p amino acids 75 to 175 and is thus lacking amino acids 176 to 229 that were included in the mutant studied above. The interference pattern for Adr1(75–175)p was the same as that for Adr1(75–229)p (33). Thus, the amino-terminal region of Adr1p is important for phosphate contacts made with the C-rich strand of the binding site in the center of UAS1, while residues carboxy terminal to the fingers do not contribute additional contacts. Whether the amino-terminal region contacts DNA directly or is acting through the finger region is unknown.

Figure 8 summarizes the expected phosphate contacts with the mutant UAS1 elements containing deletions of the central base pairs. It is possible for two monomers of Adr1p to bind simultaneously to a UAS1Δ2, UAS1Δ4, or WT UAS1 element without competition for the same phosphate residues. However, such competition would be expected to occur with UAS1Δ6. Thus, loss of a phosphate contact might be a sufficient explanation for the loss of CII formation in UAS1Δ6. Steric interference might also play a role in preventing joint occupancy of UAS1Δ6 by two monomers of Adr1p.

Transactivation from mutant UAS1 elements. In addition to participating in Adr1p binding, the central region of UAS1 might also serve as a binding site for another protein involved in *ADH2* expression. Such a hypothetical binding protein might either repress transcription or facilitate transactivation. A

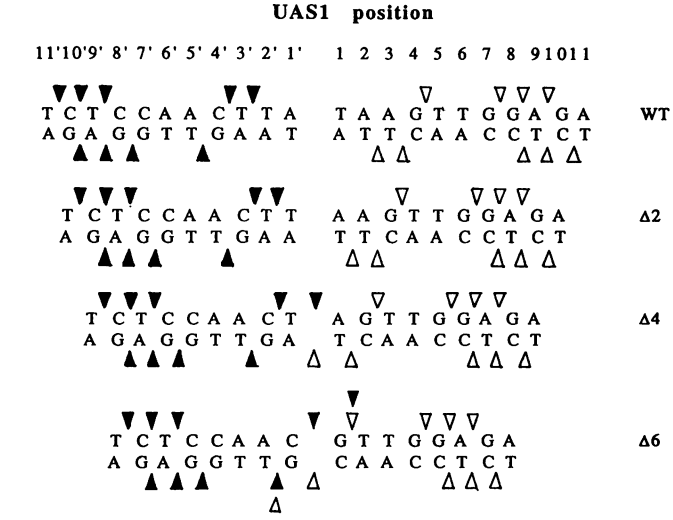

FIG. 8. Phosphate contacts expected in mutant UAS1 elements. The positions of phosphate contacts which interfere with two molecules of Adr1(17–229)p bound to WT UAS1 (based on ethylation interference data) are shown on the first line. Lines Δ2, Δ4, and Δ6 summarize predicted phosphate contacts for mutated UAS1 with 2, 4, and 6 bp deleted from its center.

TABLE 1. Mutant UAS1 elements and transactivation[a]

UAS1 element[b]	β-Galactosidase activity[c]					
	adr1::Δ*1*		*ADR1* (CEN)		*ADR1*[c] (CEN)	*ADR1* (2μm), R
	R	DR	R	DR	R	
None	0.2	0.4	0.1	0.8	0.1	NM
WT	0.5	0.5	2.1	20	40	170
SK36	0.9	9.9	2.9	28	NM	77
Δ2	0.5	0.8	2.1	21	37	140
Δ4	0.6	1.1	3.0	16	87	280
Δ6	0.4	1.4	1.0	5.0	9.2	11
Half	0.5	2.0	1.0	1.8	1.1	9.0

[a] The *ADR1* genotype of SSH35 and KDY10 (both are *adr1Δ1::LEU2*) was manipulated by introducing a plasmid containing *TRP1* and either wild-type *ADR1*, *ADR1*[c] (Ser-230-to-Ala change), or the same plasmid lacking *ADR1*. Cultures of cells were grown overnight in synthetic medium lacking uracil and tryptophan for plasmid selection and containing ethanol, glycerol, and lactate (2% each) and 0.1% glucose (derepressing conditions [DR] were used for the CEN plasmid-containing cell cultures) or containing 2% glucose (repressing conditions [R] were used for the 2μm plasmid- and *ADR1*[c]-containing cell cultures), harvested, and assayed.

[b] UAS1 elements were present in single copy in the *CYC-lacZ* reporter gene in plasmid pLG669Z(K), a *URA3*, 2μm-based plasmid (71, 76). The sequences of the UAS1 oligonucleotides are given in Materials and Methods.

[c] Measured for several transformants of SSH35 (CEN plasmid) or KDY10 (2μm plasmid) and reported in Miller units (43). Average values, which generally had an error of ±25%, are reported. NM, not measured.

precedent for the former possibility exists in the interaction between the yeast transcriptional repressor α2 and its palindromic operator site. The central part of the operator site for α2 is the binding site for Mcm1p/PRTF/GRF (34, 58), whose participation is necessary for repression of genes under mating-type control. To address this possibility, mutant UAS1 elements containing changes in the central base pairs were tested in vivo for the ability to activate and regulate expression of a *CYC1-lacZ* reporter gene. A protein whose binding site included the central 6 bp of UAS1 would presumably not bind to UAS1-SK36, UAS1Δ2, UAS1Δ4, or UAS1Δ6, in which some or all of the central 6 bp have been mutated or deleted. If the central base pairs play a role in repression, then a reporter gene containing one of these mutant UAS1 elements should be active during growth under repressing conditions. To the contrary, however, the activities of the reporters containing the mutant UAS1 elements were as low as that of the WT reporter (Table 1, *ADR1* [centromeric {CEN}]). This finding suggests that the central base pairs of UAS1 play at most a very minor role in repression.

If the central base pairs are the binding site for a protein required for transactivation, then the reporter genes containing mutant UAS1 elements should not be active during growth in derepressing medium. Activation of the reporter genes was tested in several different contexts: no Adr1p, normal levels of WT Adr1p or Adr1[c]p, and high levels of WT Adr1p. Adr1[c]p was tested because it is a particularly potent activator of transcription (17, 20) and thus might allow the detection of weak binding. The results of this experiment are presented in Table 1. All three UAS1 deletion mutants, as well as the 6-bp substitution mutant, were active, and their activities were *ADR1* dependent. Deletion of 2 or 4 bp from the center of UAS1 had no significant effect on transactivation, but deletion of 6 bp reduced transactivation from 6- to 30-fold, depending on the source of Adr1p. However, the reporter containing UASΔ6 still activated to levels significantly above background in each case. Even though some of the activity of the SK36 substitution mutant was *ADR1* independent, it also exhibited a substantial *ADR1* dependence. The results obtained with these mutant UAS1 elements suggest that the central base pairs do not constitute an important part of a binding site for a protein other than Adr1 that is required for transactivation. The reduced transactivation from the reporter containing UAS1Δ6 is consistent with the reduced ability of Adr1p to form a dimeric complex with this binding site (Fig. 4).

The residual activity of UASΔ6 was surprising since no complex CII was detected with UAS1Δ6 in vitro. In previous experiments, mutant UAS1 elements able to bind only an Adr1p monomer or single Adr1p binding sites, even when repeated in tandem arrays, were unable to activate transcription (76). This finding suggests that CII may be able to form on the mutant UAS1Δ6 element in vivo, or that Adr1p may be able to activate transcription from a single binding site, which is contrary to previous results (76). The latter interpretation appears not to be the correct explanation for Adr1p expressed at low levels since a reporter with UAS1-half was inactive with either WT Adr1p or Adr1[c]p (Table 1). Thus, it seems likely that two molecules of Adr1p can weakly bind to UASΔ6 and that joint occupancy was not detected in the gel shift assay.

Activation of the UAS-half reporter gene was observed, however, when Adr1p was overexpressed. The failure to observe activation when Adr1p is expressed at its normal level was not due to lack of sensitivity of the reporter gene assay since Adr1[c]p, which is significantly more active that WT Adr1p with all of the other UAS1 elements, did not show any activity with UAS1-half. The implication of this result is that a single monomer of Adr1p is competent to activate transcription. The failure of Adr1p expressed at normal levels to activate from UAS1-half may be due to failure to bind efficiently in vivo, a defect that may potentially be overcome by a higher concentration of Adr1p. Although no cooperativity of Adr1p binding in vitro has been demonstrated (69), this finding suggests that it may occur in vivo.

Identification of other potential Adr1p target genes in *S. cerevisiae*. A computer-aided search for potential Adr1p target genes in *S. cerevisiae* was made by using the consensus Adr1p binding site (T/G/A)(T/C)GG(A/G)G as described in Materials and Methods. The search protocol was designed to locate two binding sites in inverted orientation spaced at least 4 bp apart. This arrangement of sites allows optimal *ADR1*-dependent transcriptional activation (76) and is thus more likely than other arrangements to yield binding sites with enhancer activity. The search examined 2,582 sequences and yielded 455 matches. This number of matches is not significantly different from the number predicted on the basis of the nucleotide composition of the yeast genome. Sequences identified by the search were then screened for UAS1 homology in the 5′ noncoding region of genes transcribed by RNA polymerase II. Because the maximum distance that UAS1 can be located upstream from the transcriptional start site and still retain enhancer activity is not known, any match within approximately 1,000 nucleotides 5′ to the translational start site and not in a known upstream open reading frame was considered. These criteria further narrowed the number of matches to 53 and the number of genes with matches to 43. The potential binding sites located in this search will be referred to as UAS1 homologs and are listed in Table 2.

The nucleotide sequences of the UAS1 homologs are consistent with our observation that the central spacer DNA of UAS1 appears to provide no base-specific contacts for Adr1p. Except for the minimum requirement of 4 nucleotides, which was suggested by the data presented in Table 1, and a maximum allowance of 36 nucleotides, which was set arbitrarily because the maximum size of the spacer DNA allowable

TABLE 2. Yeast genes with 5′ noncoding sequence homology to UAS1

Function	Gene	Location[a]	5′ site	No. of bp of spacer DNA	3′ site	Reference
Utilization of nonfermentable carbon sources	*9SF*	−206	CCCCAC	14	ACGGAG	2
	ADH2	−271	CTCCAA	8	TTGGAG	56
	CIT1	−285	CCCCGC	26	TTGGAG	67
	CTA1	−158	CTCCGA	20	TTGGAG	15
	CTA1	−158	CCCCAC	10	TTGGAG	15
	CYB2	−114	CTCCAA	8	TTGGAG	28
	FOX2	−163	CTCCAA	33	ATGGGG	31
	GUT2	−333	CCCCAC	19	ACGGGG	64
	MRPL31	−135	CTCCGT	14	TCGGAG	26
	POX1	−246	CTCCGA	36	TTGGGG	18
	PPA2	−108	CTCCGC	8	ACGGAG	39
	RPM2	−473	CTCCGA	27	TCGGGG	44
Carbohydrate metabolism	*CDC68*	−587	CCCCAA	12	TTGGAG	55
	GAL2	−276	CTCCGA	32	ATGGGG	8
	MAL61	−193	CTCCAC	9	GTGGGG	47
	RAG1	−442	CCCCAT	22	TTGGGG	25
	RAG1	−442	CCCCGC	12	TTGGGG	25
	SUC1	−418	CTCCGC	34	GTGGAG	32
	SUC1	−418	CTCCGC	22	GTGGAG	32
	SUC1	−418	CTCCGC	10	GTGGAG	32
	SUC2	−378	CCCCAC	15	ATGGAG	32
	SUC3	−423	CTCCGC	10	GTGGAG	32
	SUC4	−423	CTCCGC	10	GTGGAG	32
	SUC5	−423	CTCCGC	10	GTGGAG	32
	SUC7	−423	CTCCGC	11	GTGGAG	32
Nitrogen metabolism and protein synthesis	*ARO3*	−427	CCCCAC	34	TCGGAG	49
	DPH2	−279	CTCCAT	9	TCGGAG	11
	GDH1	−699	CTCCGC	33	GTGGGG	45
	GDH1	−603	CTCCAT	23	ATGGGG	45
	GDH1	−474	CTCCAT	4	ACGGGG	45
	L12EIIB	−1013	CTCCGA	34	ACGGGG	46
	L12EIIB	−939	CCCCAC	14	ACGGAG	46
	PUT1	−892	CCCCGC	32	TTGGAG	74
	PUT3	−165	CTCCGC	19	TTGGAG	41
	RPL2A	−254	CTCCAA	21	TCGGAG	53
	RPL37A	−247	CTCCGC	12	ACGGAG	57
	RPL44	−137	CTCCAA	36	GCGGAG	54
	UBI1	−290	CTCCGT	11	GTGGAG	48
	UBI3	−420	CCCCAT	28	TTGGGG	48
	UBI3	−565	CCCCAT	4	GTGGAG	48
Cell cycle and differentiation	*CLB4*	−256	CCCCAT	20	TCGGAG	23
	CMP2	−587	CTCCGC	28	TTGGAG	38
	IME1	−395	CCCCAA	33	TTGGGG	65
	ISC2	−165	CCCCAC	7	GTGGAG	36
	RAD52	−682	CTCCAT	32	GTGGGG	1
	SIR2	−914	CTCCAC	4	ATGGAG	60
	SPO7	−412	CTCCAA	10	TCGGAG	75
	SPX18	−153	CTCCAA	5	ACGGAG	25
Miscellaneous	*SNQ2*	−656	CTCCAC	22	ACGGAG	59
	SNQ2	−656	CCCCAT	11	ACGGAG	59
	TTR	−96	CTCCAA	20	ATGGAG	24

[a] Number of nucleotides 5′ to the ATG codon at the translational start site.

for UAS activity has yet to be determined experimentally, no other constraints were placed on the composition of the spacer DNA. Therefore, biases in the length or composition of this DNA should reflect Adr1p binding requirements. The spacing between the binding sites of each match varied randomly, with three new matches being detected for every two-nucleotide increase in spacer DNA length. Thus, the spacer DNA of the UAS1 homologs showed no bias for length. In addition, the nucleotide composition of the spacer DNA, on average, reflected the average composition of the yeast genome. This finding suggests that the spacer DNA also shows no bias in nucleotide composition. The genes whose promoter region contains a UAS1 homolog are discussed below.

DISCUSSION

UAS1 is a 22-bp inverted repeat that is the binding site for two monomers of Adr1p in the *ADH2* promoter. Activation of transcription by Adr1p requires two binding sites in inverted orientation and hence is presumed to require binding of two monomers. Two monomers bind independently of one another in vitro to the two identical halves of the palindrome. The positions and orientations of the two zinc fingers of Adr1p on their binding sites in UAS1 were determined by analyzing change-of-specificity mutants of *ADR1* (71). Two monomers of Adr1p bind to UAS1 in a tail-to-tail orientation, with each monomer of Adr1p interacting with bp 7 to 10 in each identical

half of UAS1. The present studies extend our understanding of the role of the remaining 7 bp in each Adr1p binding site.

From the crystal structure of Zif268 bound to DNA (50), positions 5, 6, and 7 in UAS1 were expected to be contact sites for residues R-149, L-146, and R-143, respectively, of finger 2. As discussed elsewhere (71), only position 7 appears to be involved in a strong, base-specific contact. This contact occurs with R-143 of finger 2. The other residues of finger 2 expected to contact DNA, Leu-146 and Arg-149, can be changed to Ala with little loss in DNA binding affinity (70). This finding suggests that Leu-146 and Arg-149 do not make base-specific contacts at positions 6 and 5, respectively, in UAS1 and is consistent with the observed tolerance for base pair substitutions at positions 5 and 6.

A comparison of the crystal structures of protein-DNA complexes for three different C_2H_2 class zinc finger proteins reveals different arrangements of protein-DNA phosphate contacts. Zif268 makes phosphate contacts with the same DNA strand on which base-specific contacts were made (50). The zinc fingers of Gli and TTK, however, contact the phosphate backbone and specific bases on both strands (22, 51). Adr1p-DNA contacts appear to be different from any one of these three. Base-specific contacts with UAS1 are presumed to be made on the G-rich strand of the binding site, but extensive phosphate contacts are made on both strands of the DNA. Several of the phosphate contacts are in the central region of UAS1 where no base-specific contacts are important. The amino-terminal region of Adr1p is important in establishing at least two of these phosphate contacts. Thus, the central region of UAS1 has at least one important function, to provide non-base-specific contacts for Adr1p. Another function may be to prevent steric interference between two molecules of Adr1p bound in close proximity to one another. The central base pairs do not seem to play an important role in gene expression other than in allowing simultaneous occupancy of UAS1 by two monomers of Adr1p. That is, it does not appear to be the binding site for another protein involved in *ADH2* gene expression.

It is difficult to reconcile the conservation of the perfectness of the palindromic nature of UAS1 with the fact that the central base pairs do not appear to play an important role in binding or transactivation. Perhaps our method of analysis is too crude. For example, maybe these base pairs play a role in gene expression during some particular growth regimen or only when the gene is present at its normal chromosomal location. The circular nature of the plasmid DNA on which these mutant UAS1 elements were tested might obviate the need for a particular DNA sequence that facilitated DNA binding and transcription.

Alternatively, perhaps the palindromic nature of the central base pairs is fortuitous. This explanation is consistent with the fact that UAS1 homologs in other *ADR1*-dependent genes do not have a palindromic nature except within the core sequence which interacts with Adr1p in a base-specific manner.

ADR1 was discovered by Ciriacy (13) because it was an activator of *ADH2* expression. Recently, several other genes whose expression is *ADR1* dependent have been discovered serendipitously (63). Of the genes known to exhibit *ADR1*-dependent expression, three were detected by the homology search conducted with data from the characterization of UAS1 reported here. These genes were *ADH2*, as expected, *FOX2*, which codes for the peroxisomal multifunctional β-oxidation protein (31), and *CTA1*, which codes for the peroxisomal isozyme of catalase (63). *ADR1* is required for maximal expression of *CTA1* and *FOX2* during growth with ethanol as the sole carbon source (63). In addition, Adr1p binds in vitro with relatively high affinity to *CTA1* promoter sequence containing a UAS1 homolog. This finding suggests that the UAS1 homolog in the 5′ noncoding sequence of the *CTA1* gene may be functioning as an enhancer element. Two of the genes not detected by the homology search but which show *ADR1*-dependent expression were *POT1* (*FOX3*), which codes for peroxisomal 3-oxoacyl coenzyme A thiolase, and *PAS1*, which is essential for peroxisomal assembly (63). The 5′ noncoding sequences of these genes each contain a weak match with the UAS1 consensus. These matches were considered weak because one of the binding sites in each UAS1 homolog deviates from the experimentally derived consensus by one nucleotide. This deviation should reduce binding of one Adr1p monomer to the nonconsensus binding site by 100-fold (Fig. 3). Since activation of *ADH2* expression from UAS1 normally requires the binding of two Adr1p monomers (69), it is possible that binding in vivo to the WT site of these weak homologs increases the stability of binding to the nonconsensus site. Alternatively, the promoter context of these genes might be such that Adr1p activates expression when bound as a monomer since overexpression of *ADR1* does allow a low level of activation from a single Adr1p binding site. If either is true, then this suggests that the UAS1 consensus sequence may not have detected all of the functional UAS1 homologs in the data base. Currently, however, it also cannot be ruled out that the imperfect UAS1 homologs may not be functional and the effect of *adr1* mutations on expression of these genes is indirect.

Other genes whose promoter sequences contain UAS1 homologs that have been shown to bind Adr1p in vitro are *CYB2*, which codes for glucose-repressible cytochrome b_2 (27, 28), and *SUC2*, which codes for invertase (19, 32). The expression of these genes, however, does not show a clear dependence on *ADR1* (19, 27). The UAS1 homolog in the *CYB2* promoter is located only 8 bp 5′ to the TATA element (28), while the *SUC2* homolog is much further removed at 245 nucleotides 5′ to the TATA element (32). In comparison, UAS1 in the *ADH2* promoter is located 110 nucleotides 5′ to the TATA element (56), while the UAS1 homologs of *CTA1* and *FOX2* are located from 30 to 120 bp 5′ to TATA elements (15, 31). These findings suggest that the positioning of a UAS1 homolog relative to the TATA element may be important for determining whether the homolog has enhancer activity. Because of this possible positioning requirement, a number of the UAS1 homologs detected in our search may not have enhancer activity.

Previously, it had been demonstrated that Adr1p is required for optimal growth with glycerol as the sole carbon source (3). Also required for growth on glycerol are the *GUT1* gene, which codes for glycerol kinase, and the *GUT2* gene, which codes for glycerol phosphate dehydrogenase (66). A UAS1 homolog was located at −332 in the 5′ noncoding sequence of the *GUT2* gene. Although the binding of Adr1p to this sequence and the requirement of *ADR1* for *GUT2* expression have not been tested, these observations suggest that the UAS1 homolog in the *GUT2* promoter may be functional in vivo. Recently, Pavlik et al. (52) reported that expression of the *GUT1* gene is *ADR1* dependent. The 5′ noncoding sequence of this gene contains two Adr1p binding sites. However, they are oriented in tandem and therefore were not identified by our homology search. It cannot be ruled out that these single Adr1p binding sites function as enhancer elements in the context of the *GUT1* promoter. An alternative possibility is that a UAS1 homolog exists upstream of the sequenced 5′ noncoding region. However, this would place the homolog more than 270 nucleotides 5′ to the TATA elements, a distance farther upstream than the UAS1 homolog of *SUC2*, which does not appear to exhibit

enhancer activity. It seems more likely that the *ADR1* dependence of *GUT1* is indirect, perhaps reflecting coordinate regulation with *GUT2* expression.

Given the specific classes of genes identified in this UAS1 homology search, Adr1p may play an even broader role in yeast cell physiology and growth than had been previously suspected. In general, the genes containing UAS1 homologs could be grouped into four main categories: those involved in the utilization of nonfermentable carbon sources, those involved in carbohydrate metabolism, those involved in nitrogen metabolism and protein synthesis, and those involved in cell cycle and differentiation. Most of the UAS1 homologs were located in the promoters of nonessential genes, which is consistent with the observation that *ADR1* is not an essential gene. The genes involved in carbohydrate metabolism included three that code for sugar transporters: *RAG1*, which codes for the *S. cerevisiae* homolog of the *Kluyveromyces lactis* low-affinity glucose transporter; *GAL2*, which codes for the galactose permease (8); and *MAL61*, which codes for the maltose permease (47). The UAS1 homologs in the 5′ flanking sequence of the *GAL2* and *MAL61* genes partially overlap previously defined upstream activation sequences. It was also particularly interesting to note that six of the identified genes code for protein subunits of ribosomes; five are subunits of the 60S subunit, and one is a subunit of the 40S subunit. In addition, *GDH1*, which codes for a key enzyme involved in ammonia utilization (45), and *IME1*, which is required for activating the expression of genes required for sporulation and the initiation of meiosis (60), were also identified in the search. If the expression of these and other key genes containing UAS1 homologs is altered by Adr1p binding, then Adr1p may be involved in coordinating the control of carbon source utilization, growth rate, and differentiation with carbon and nitrogen source availability.

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