

## ADRI-Mediated Regulation of ADH2 Requires an Inverted Repeat Sequence

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DNA sequence analysis of wild-type and mutant *ADH2* loci suggested that two unusual features 5' of the promoter, a 22-base-pair perfect dyad sequence and a (dA)<sub>20</sub> tract, were important for regulation of this gene (D. W. Russell, M. Smith, D. Cox, V. M. Williamson, and E. T. Young, *Nature [London]* 304:652-654, 1983). Oligonucleotide-directed mutagenesis was used to construct *ADH2* genes lacking the 22-base-pair dyad or the (dA)<sub>20</sub> tract (V.-L. Chan and M. Smith, *Nucleic Acids Res.* 12:2407-2419, 1984). These mutant genes and other *ADH2* deletions constructed by BAL 31 endonuclease digestion were studied after replacing the wild-type chromosomal locus with the altered alleles by the technique of gene transplacement (T. L. Orr-Weaver, J. W. Szostak, and R. S. Rothstein, *Proc. Natl. Acad. Sci. USA* 78:6354-6358, 1981), using canavanine resistance as the selectable marker. Deletions lacking the dyad failed to derepress normally and did not respond to mutations at the *ADRI* locus, which encodes a protein necessary to activate *ADH2*. Deletions of the (dA)<sub>20</sub> tract did not have a detectable phenotype. A small deletion located just 3' to the (dA)<sub>20</sub> tract (between positions -164 and -146) had a low amount of *ADRI*-dependent transcription during repressed growth conditions, indicating that the regulatory protein encoded by *ADRI* is present in a potentially active form during repression and that alterations of a DNA sequence in the promoter region can unmask its latent activity.

Studies of genes which are coordinately controlled in response to a single physiological event have revealed a striking variety of regulatory mechanisms in procaryotes. The mechanisms of coordinate gene control in eucaryotes have not been elucidated in as fine detail. One model system for the study of coordinate gene expression in eucaryotes is glucose catabolite repression in yeasts. The synthesis of many enzymes in yeasts is repressed when the cells are grown in a glucose-based medium and derepressed when the cells are grown in medium containing a nonfermentable carbon source such as ethanol or pyruvate (36). It was originally believed that cyclic AMP was involved in this effect (30), but later data suggested that this was not the case (33).

The glucose-repressible genes encoding iso-1-cytochrome *c* (50), invertase (6), alcohol dehydrogenase II (17), maltase (20), and the enzymes involved in galactose metabolism (23, 26) are regulated at the transcriptional or posttranscriptional level. The DNA sequences located 5' to the structural genes have been shown to be involved in mediating the glucose repression-derepression response (reviewed in reference 22). One hypothesis is that genes under coordinate regulation will have DNA sequences in common which respond to the same effector molecules (14). Many amino acid biosynthetic enzymes in yeasts are regulated coordinately (28), and several of the genes encoding these enzymes have a 6-base-pair (bp) sequence in common (19).

The alcohol dehydrogenase II enzyme (ADH II) of *Saccharomyces cerevisiae* is absent when cells are grown in medium containing glucose as the carbon source and derepressed over 100-fold when cells are shifted to a nonferment-

able carbon source (9, 10, 29; see reference 49 for a review). The gene which codes for ADH II, *ADH2*, has been shown to be transcriptionally regulated (48; H. Blumberg, personal communication).

Genetic analysis of ADH II regulation revealed that derepression of ADH II activity and mRNA accumulation requires the product of an unlinked regulatory locus *ADRI*, which appears to be specific for *ADH2* (11, 13, 17); in the absence of a functional *ADRI* gene product ADH II activity is not significantly derepressed. Full derepression also requires the activity of at least three other genes which are involved in pleiotropic carbon catabolite repression (12). A gene corresponding to a classical repressor locus for *ADH2* has not been identified (13, 16).

Two classes of *cis*-acting mutations which allow *ADH2* to escape glucose repression have been isolated and characterized (13, 37, 47, 48). The first class is due to the insertion of a 5.6-kilobase (kb) yeast transposon, Ty, into the 5' nontranscribed region of *ADH2* (48). Ty insertion places the adjacent *ADH2* locus under the regulatory system of the transposon so that normal glucose repression and *ADRI*-mediated activation are replaced by mating-type control (45). The other class of *cis*-acting mutations is represented by an extension of and alterations adjacent to a poly(dA) tract located 168 bp upstream of the transcription start site of the *ADH2* gene (37). This increase, from 20 to 54 or 55 dA residues, and adjacent nucleotide changes result in hyperderepression of ADH II activity when cells are grown in ethanol-base media and in partial loss of glucose repression. These mutations still respond to alterations at the *ADRI* regulatory locus (13).

Since Ty insertions are the most frequently recovered *cis*-acting mutations which alter regulation of *ADH2*, it was necessary to perform in vitro mutagenesis of the 5'-flanking region of *ADH2* to localize sites essential for regulation of the gene. Previous work showed that a 1-kb region contain-

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TABLE 1. *S. cerevisiae* strains used

Strains	Genotype
900-17-1Acan <sup>r</sup> .....	<b>a</b> <i>adh1Δ1 adh2-601 adh3 ade2 trp1 can<sup>r</sup>1</i>
521-6can <sup>r</sup> .....	<b>a</b> <i>adh1-11 ADH2 adh3 leu2 trp1 ural can<sup>r</sup>1</i>
JSVII .....	<b>a</b> <i>adh1-11 ADH2-7 adh3 leu2 trp1 ural</i>
JSIX .....	<b>a</b> <i>adh1-11 ADH2-9 adh3 leu2 trp1 ural</i>
JSIX 1119 .....	<b>a</b> <i>adh1-11 ADH2-9 adh3 adr1Δ1::LEU2 leu2 ural</i>
JSIX 1100 .....	<b>α</b> <i>adr1-11 ADH2-9 adh3 ADR1-5<sup>c</sup> ural</i>
JSX .....	<b>a</b> <i>adh1-11 ADH2-10 adh3 leu2 trp1 ural</i>
JSX 1122 .....	<b>a</b> <i>adh1-11 ADH2-10 adh3 adr1Δ1::LEU2 leu2 trp1 ural</i>
JSX 1126c .....	<b>α</b> <i>adh1-11 ADH2-10 adh3 ADR1-5<sup>c</sup> trp1 ural</i>
RC17a .....	<b>a</b> <i>adh1Δ1 ADH2-17a adh3 ade2 trp1 can<sup>r</sup>1</i>
RC8a .....	<b>a</b> <i>adh1Δ1 ADH2-8a adh3 ade2 trp1 can<sup>r</sup>1</i>
RC40a .....	<b>a</b> <i>adh1Δ1 ADH2-40a adh3 ade2 trp1 can<sup>r</sup>1</i>
RC11a .....	<b>a</b> <i>adh1Δ1 ADH2-11a adh3 ade2 trp1 can<sup>r</sup>1</i>
RC11a 1123 .....	<b>α</b> <i>adh1 ADH2-11a adh3 adr1Δ1::LEU2 leu2</i>
RC11a 1124 .....	<b>α</b> <i>adh1-11 ADH2-11a adh3 ADR1-5<sup>c</sup> ade2 ural</i>
DCB1E .....	<b>a</b> <i>adh1Δ1 ADH2 adh3 ade2 trp1 can<sup>r</sup>1</i>
11-1 .....	<b>a</b> <i>adh1Δ1 adh2-601 ADH2 ADH2-11a adh3 ade2 CAN<sup>r</sup>1</i>
521-6-Δ1 .....	<b>a</b> <i>adh1-11 ADH2 adh3 adr1Δ1::LEU2 leu2 trp1 ural</i>
R234.1-7A <sup>a</sup> .....	<b>α</b> <i>adh1-11 ADH2 adh3 ADR1-5<sup>c</sup> ade2 trp2 ural</i>
JY1000 .....	<b>α</b> <i>adh1-11 ADH2-10 adh3 leu2 ural</i>
JY1001 .....	<b>α</b> <i>adh1-11 ADH2 adh3 adr1Δ1::LEU2 leu2 ural</i>

<sup>a</sup> From M. Ciriacy; other strains were constructed in this laboratory.

ing the flanking sequences of *ADH2* 5' to the promoter was sufficient for conferring glucose repression on the related yeast *ADH1* promoter and structural gene (2). Deletion analysis of this region suggested that glucose repression was due to the absence of positive activation mediated by *ADR1* (1). Derepression of a hybrid *ADH2-ADH1* gene required DNA sequences located between the TATA box (-110 with respect to the transcription start site which is designated +1 [38]) and position -260, probably within the interval -220 to -260. This region contains several unusual nucleotide sequences: a 22-bp perfect dyad; seven repeats with the consensus AGGAGA, which is present in the dyad and as part of other direct and indirect repeats; and a tract of (dA)<sub>20</sub> whose amplification alters *ADH2* regulation (37). To study the consequences of removing these sequences from the normal *ADH2* allele, we made deletions within this region by BAL 31 treatment or by oligonucleotide mutagenesis (8), and the expression from the mutant *ADH2* alleles was analyzed after replacement of the wild-type chromosomal *ADH2* locus by the mutated genes.

## MATERIALS AND METHODS

**Strains and medium.** *S. cerevisiae* strains used are listed in Table 1. *Escherichia coli* RR1 was used for all *E. coli* transformations (31). Basic yeast medium was as described by Hartwell (24) and Johnston et al. (27), and yeast

transformation was performed as described by Hinnen et al. (25). Antimycin A was obtained from Boehringer Mannheim Biochemicals (Indianapolis, Ind.) and used at 1 μg/ml.

**Growth of strains and ADH assays.** Cells were grown overnight at 30°C in complete (YPD) medium (24) containing 3% glucose. One portion of the culture was diluted into fresh medium also containing glucose. Another portion was centrifuged, washed with sterile water, and suspended in complete medium containing 3% ethanol. The cells were then grown for an additional 24 h. Cell extracts and ADH assays were performed as described previously (18). All ADH assays were performed at 25°C.

**Construction of deletions within *ADH2* 5'-flanking sequences by BAL 31 digestion.** DNA fragments containing variable amounts of the 5'-flanking sequence adjacent to the *ADH2* structural gene were constructed as follows. Plasmid YCpDXX was constructed from plasmid pBC3T1 (2) by partial digestion of 10 μg of DNA with *Xba*I, creation of blunt-ended DNA with the Klenow enzyme, and the addition of phosphorylated *Xho*I linkers (31). The YCpDXX plasmid contains a single *Xho*I site located at the position of a small deletion between the two *Xba*I sites in the 5'-flanking region of *ADH2* located at -1017 and -1055. Supercoiled YCpDXX plasmid (45 μg) was cut with *Xho*I and treated with 5 U of BAL 31 (200-μl volume) in a medium containing 600 mM NaCl, 12 mM CaCl<sub>2</sub>, 13 mM MgCl<sub>2</sub>, and 20 mM Tris hydrochloride (pH 8.0) for 40 min at 30°C, which resulted in an average digestion of approximately 400 bp per end. *Xho*I linkers were added by ligation, and the resultant plasmids were used to transform *E. coli* RR1.

DNA fragments containing different amounts of 5'-flanking sequence, all commencing at a site located at position -1146 relative to the transcription start site and increasing in length toward the transcription initiation site, were created from plasmid pBC3T1. A 20-μg sample of the plasmid was cut with *Eco*RV, which cuts within the coding region of *ADH2* 67 bp from the initiation codon, and treated with 2.5 U of BAL 31 in 200 μl of BAL 31 buffer (31) for 20 min at 30°C. *Xho*I linkers were ligated to the BAL 31-treated DNA (31), and the plasmids were introduced into *E. coli* RR1 by transformation to ampicillin resistance.

Plasmids containing internal deletions located in the 5'-nontranscribed *ADH2* sequences were constructed by combining the appropriate 5' piece with the appropriate 3'-piece. The reconstructed plasmids differ from the pBC3T1 plasmid only by the deletions in the *ADH2* flanking sequence and the insertion of the *Xho*I octamer linker 5'-CCTCGAGG at the site of the deletion.

**Oligonucleotide mutagenesis.** The construction of *ADH2* alleles with a deletion of the dyad or with deletions and alterations of the (dA)<sub>20</sub> tract has been described previously (8). These deletions were characterized by DNA sequence analysis. Restriction enzyme fragments containing the mutation were ligated into yeast-*E. coli* shuttle plasmids containing the *ADH2* gene lacking the same restriction enzyme fragment.

**DNA sequencing.** The locations of *Xho*I linkers in the plasmids were determined by the technique of Maxam and Gilbert (34). Plasmid DNA (20 to 50 μg) was cut with *Xho*I, ethanol precipitated, and suspended in 20 μl of buffer consisting of 6.7 mM Tris hydrochloride (pH 7.5), 6.7 mM MgCl<sub>2</sub>, 6.7 mM 2-mercaptoethanol, 0.05 mM dTTP, 10 to 30 μCi of [α-<sup>32</sup>P]dCTP, and 2 U of Klenow enzyme. Fragments labeled at a single end were created by cutting the plasmid at another restriction enzyme site (*Bam*HI) and purified by agarose gel electrophoresis. After the sequencing reactions,

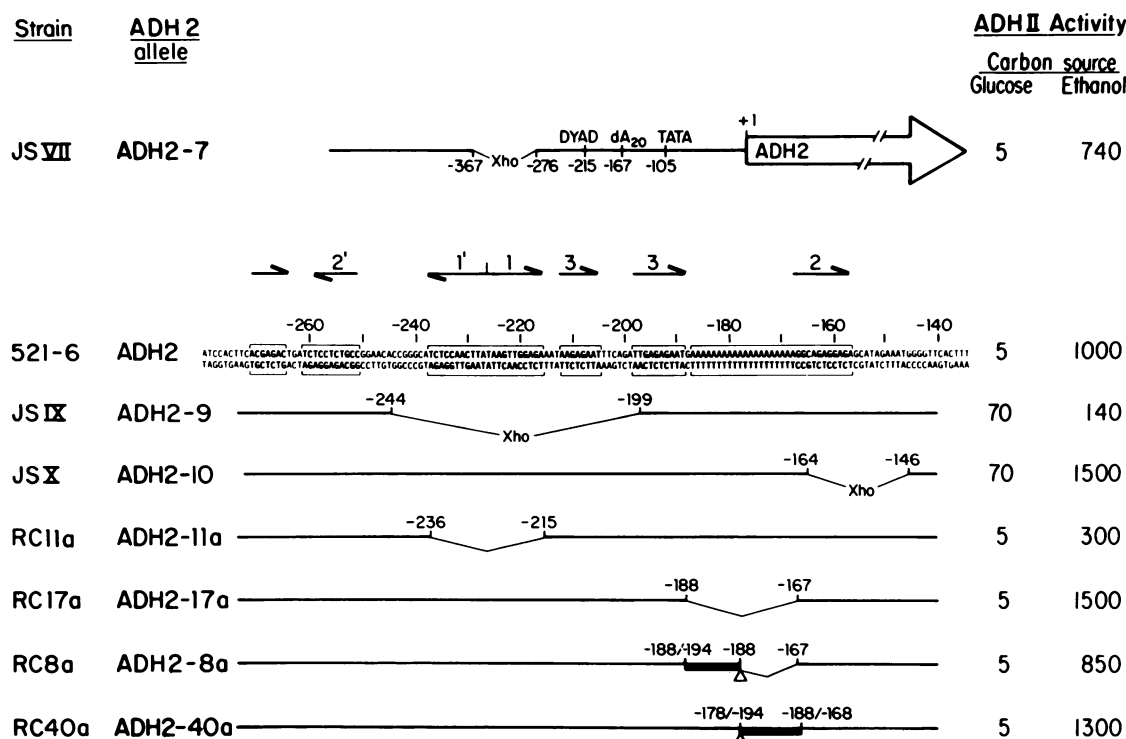


FIG. 1. Schematic diagram and nucleotide sequence of 5'-flanking region of *ADH2* wild-type and mutant genes. The positions of the nucleotides shown above are relative to the transcription start site. The major transcription initiation sites are located 54 and 56 nucleotides from the A of the initiator methionine codon, and a eucaryotic promotor consensus sequence is located at position -115 (TATA). The position of the 22-bp dyad is indicated by the double-headed arrow. The nucleotides enclosed in brackets are discussed in the text. B, *Bam*HI; S, *Sph*I. The ADH activities are given in milliunits per milligram of protein in extracts prepared as described in Materials and Methods.

the DNA was denatured and run on 15% acrylamide gels for 1.5 h at 65 W. The position of the *Xho*I linker was determined by comparison with the known *ADH2* sequence (38).

**S1 nuclease mapping.** The determination of the 5' ends of the *ADH2* mRNA was performed by the S1 mapping technique described by Berk and Sharp (3) as modified by Taguchi et al. (45). Single-stranded DNA probe labeled at the *EcoRV* site at position +66 in the *ADH2* gene was hybridized to 25  $\mu$ g of total yeast RNA (isolated by the method of Schultz [41]) for glucose-grown cultures of 5  $\mu$ g of total yeast RNA from ethanol-grown cultures. After hybridization, single-stranded nucleic acid was digested with 400 U of S1 nuclease in the presence of 4  $\mu$ g of single-stranded salmon sperm DNA for 30 min at 37°C. The remaining double-stranded nucleic acid was denatured in 90% formamide and run on a 6% acrylamide-7 M urea sequencing gel.

**Gene replacement.** An integrating vector, YIpTC', containing *TRP1* from YRp7 (44), most of *CAN<sup>S</sup>1* (4), and all of pBR322 was constructed. The *TRP1* gene was obtained as an *EcoRI*-*Bgl*III DNA fragment of 0.8 kb; the *EcoRI* site was filled in with Klenow polymerase. *CAN<sup>S</sup>1* was on a 1.7-kb *Bam*HI-*Clal* fragment lacking its 3' terminus and probably part of its promoter (obtained from J. Broach, personal communication). These two fragments were ligated in the presence of pBR322 which had been digested with *EcoRI* and *Clal* and filled in with Klenow polymerase to eliminate the *EcoRI* site. A 3.4-kb *Bam*HI fragment containing *ADH2* was isolated from a plasmid containing the appropriate *ADH2* allele and ligated with *Bam*HI-digested YIpTC' to obtain YIpTC'-ADH2. These plasmids have a unique *Sac*I site upstream of the *ADH2* structural gene which can be used to target integration to the *ADH2* locus (38). A *trp1 can<sup>r</sup>1*

*adh1 adh3* strain (900-17-1A *can<sup>r</sup>1* is shown) was transformed to Trp<sup>+</sup> with the *Sac*I-digested plasmid, the Trp<sup>+</sup> Can<sup>s</sup> transformants were grown up in YPD, and 10<sup>4</sup> to 10<sup>5</sup> cells were plated on plates lacking arginine and containing 2 mg of canavanine sulfate in a well in the center of the plate. Resistant colonies that grew up were picked and tested for Trp auxotrophy and those that had become Trp<sup>-</sup> were tested further by Southern hybridization analysis and ADH assays.

## RESULTS

**Construction of deletions in 5' regulatory region of *ADH2*.** Deletions were constructed in the region of -150 to -1100 by BAL 31 digestion (previous studies [1] showed that this region of *ADH2* was sufficient to confer *ADR1*-mediated glucose control on a different promoter). Internal deletions of variable length in the region -300 to -1100 had no detectable effect on *ADH2* expression from a centromere-containing plasmid (data not shown). Two plasmids containing *ADH2* alleles with deletions between positions -244 and -199 (*ADH2-9*) and -164 and -146 (*ADH2-10*) were associated with altered *ADH2* expression in transformants. *Bam*HI DNA fragments containing these two alleles were ligated with an integrating vector, YIpTC', containing *TRP1* and *CAN1* yeast DNA fragments. A third DNA fragment containing *ADH-7* was ligated with the same vector to serve as a control. This *ADH2* allele (deleted between positions -367 and -276) was normally regulated when present on a plasmid containing *CEN3* (data not shown). Each of these *ADH2* alleles contains an 8-bp *Xho*I linker in place of the deleted sequences. Four other *ADH2* alleles were constructed by oligonucleotide mutagenesis (8). *ADH2-11a* lacks the 22-bp dyad (-215 to -236); *ADH2-17a* lacks the

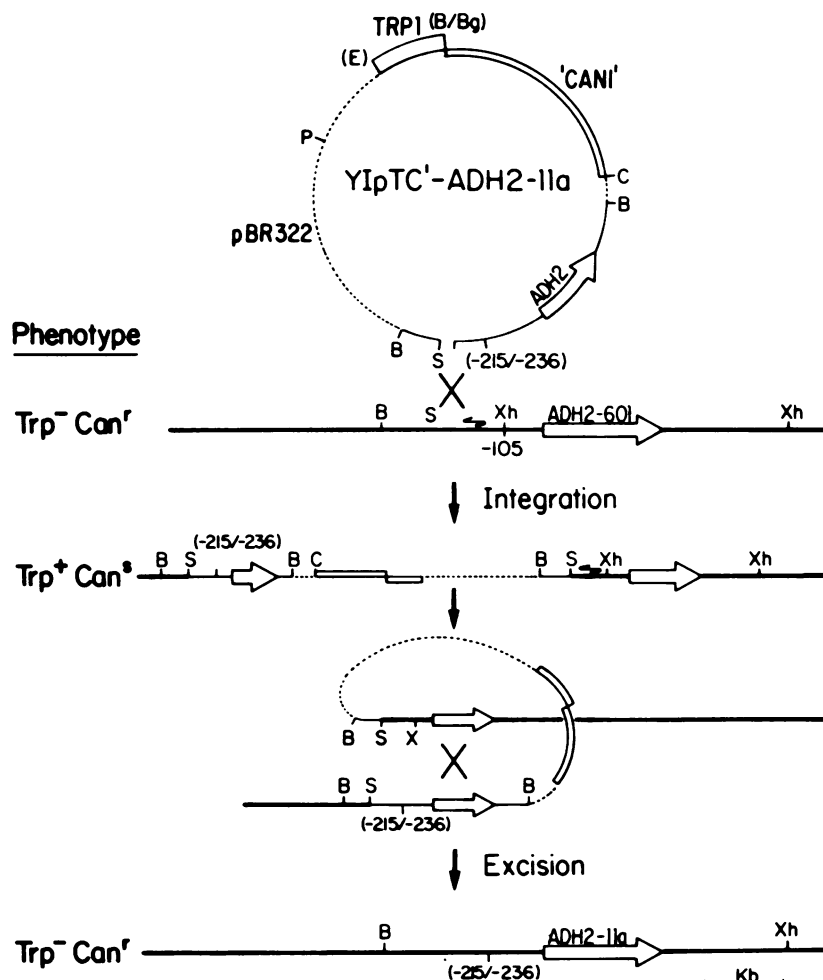


FIG. 2. Gene replacement. The integrating plasmid YIpTC'-ADH2-11a is shown during *SacI* targeting of integration at the *ADH2*-601 locus of strain 900-17-1A *can<sup>r</sup>*. The Trp and Can phenotypes that are associated with the original strain, the integrant, and the strain arising by looping-out of the plasmid DNA and recombination downstream of the targeting site are shown on the left of the figure. B, *Bam*HI; S, *Sac*I; Xh, *Xho*I; Bg, *Bgl*II; E, *Eco*RI; C, *Cla*I. Sites in parentheses have been destroyed by filling-in before ligation.

(dA)<sub>20</sub> tract (-167 to -188); *ADH2-40a* retains 10 of the 20 dA residues originally present and has a 3' duplication of the 9 bp 5' to the (dA)<sub>10</sub>; *ADH2-8a* lacks the (dA)<sub>20</sub> tract and has a tandem duplication of the 9 bp 5' to the original (dA)<sub>20</sub> tract (positions -187 to -195). These latter two alterations arose by mispairing during oligonucleotide mutagenesis (8). Figure 1 shows a schematic diagram of the *ADH2* wild-type and mutant loci and the wild-type DNA sequence from -140 to -275.

**Gene replacement.** Yeast strains containing a single copy of the mutant allele at the *ADH2* locus were isolated by gene replacement (5, 40). A vector, YIpTC', containing the wild-type or mutant *ADH2* allele, the yeast *TRP1* gene (lacking *ARS1* activity), the arginine permease locus (*CAN<sup>S</sup>1*), and pBR322 sequences was constructed (Fig. 2). A *can<sup>r</sup>1 trp1* yeast strain (521-6 *can<sup>r</sup>*) lacking ADH I and ADH III activity was transformed to Trp<sup>+</sup> with each of the plasmids. These transformants are sensitive to canavanine due to the *CAN<sup>S</sup>1* gene on the plasmid. Transformants containing a single plasmid integrated at the *ADH2* locus were subjected to canavanine selection to isolate recombinants which had lost the plasmid and one *ADH2* allele. DNA isolated from Trp<sup>-</sup> Can<sup>r</sup> isolates was screened by Southern hybridization anal-

ysis to identify strains in which the mutant *ADH2* allele had replaced the original *ADH2* allele. Strains containing *ADH2* alleles 7, 9, and 10 could be readily identified because they introduced a new *Xho*I restriction site 5' to the wild-type *ADH2* locus. The other mutant alleles did not introduce a new restriction site. To show that the resident *ADH2* locus had been replaced by these mutant alleles, we used strain 900-17-1A *can<sup>r</sup>* for the replacement. The *ADH2* locus in strain 900-17-1A *can<sup>r</sup>* has an *Xho*I site in a delta sequence derived from Ty6<sup>c</sup> at position -106 (48) which would be lost together with the delta sequence after gene replacement as shown in Figure 2. To confirm that the loss of the delta sequence was accompanied by the replacement of the wild-type dyad sequence with the mutant *ADH2-11a* allele, which lacks the dyad, a Southern transfer experiment was performed with the mutagenic 23-bp oligonucleotide as a probe. The 23-mer probe contains sequences flanking the dyad but lacks the dyad itself and thus forms more stable hybrids with the mutant *ADH2-11a* allele with the *ADH2* allele (8). The expected restriction enzyme fragments were observed (Fig. 3), and the stability of the hybrids formed with DNA isolated from strain RC11a was greater than the stability of the hybrids formed with the wild-type *ADH2* DNA.

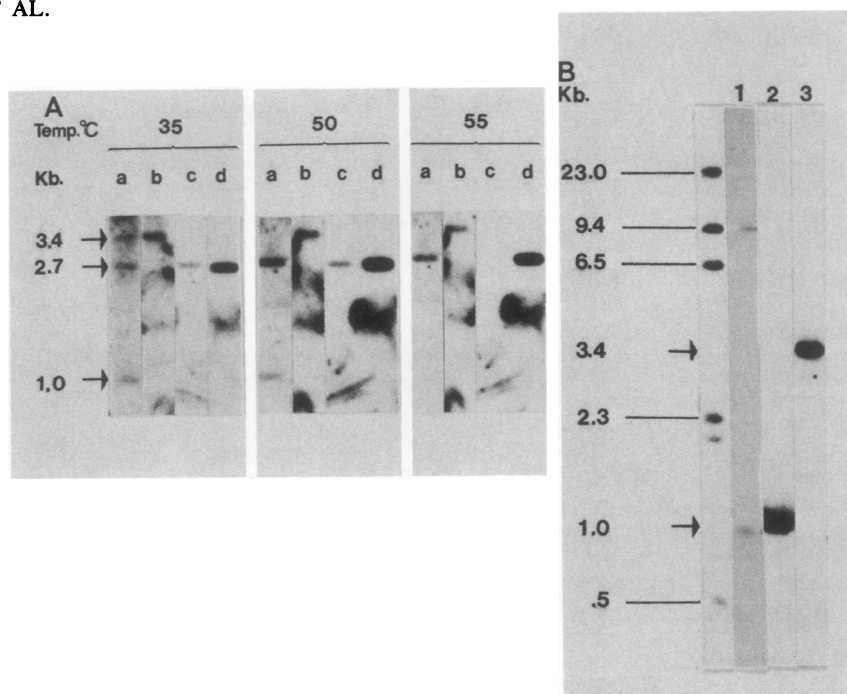


FIG. 3. Southern hybridization analysis. DNA was extracted from yeast strains by detergent lysis after conversion of the cells to spheroplasts (15). Purified DNA was digested with restriction enzymes (*Bam*HI and *Xho*I double digests are shown) that would allow *ADH2* to be distinguished from *ADH2-9* and *ADH2-10* or *ADH2-601* to be distinguished from *ADH2-11a*. The digested DNA (usually 2  $\mu$ g) was analyzed on 1% agarose gels, transferred to nitrocellulose, and hybridized with either a nick-translated probe (a 1-kb *Bam*HI-*Xho*I fragment derived from positions -1100 to -199 5' of *ADH2-9*) or the kinase-labeled oligonucleotide that was used to generate *ADH2-11a* (8). The 23-mer oligonucleotide lacks the dyad sequence at positions -215 to -236 in *ADH2* but has the sequences flanking it on both sides; hence, it forms more stable hybrids with the *ADH2-11a* allele than with *ADH2*. (A) Thermal stability of hybrids formed between the dyad-mutagenic oligonucleotide and *ADH2* or *ADH2-11a*. Strain 11-1 is the original *Trp*<sup>+</sup> *Can*<sup>s</sup> transformant containing both *ADH2* alleles, and RC11A is the *Trp*<sup>-</sup> *Can*<sup>r</sup> strain which retains only the *ADH2-11a* allele. The plasmid DNAs were derived from YIpTC'-*ADH2* and YIpTC'-*ADH2-11a* (Fig. 1) which had been digested with *Bam*HI. Lanes: a, strain 11-1; b, strain RC11A; c, YIpTC'-*ADH2*; d, YIpTC'-*ADH2-11a*. (B) Identification of *ADH2-9*, *ADH2-10*, and *ADH2-11a* in recombinants containing various *ADR1* alleles. The ADH activity of segregants in the resulting tetrads was determined, and then appropriate tetrads were chosen for DNA analysis to confirm the genotype that was predicted on the basis of the Leu phenotype and the ADH assays. DNA was isolated from segregants of crosses between strains JSIX (*ADH2-9 ADR1*), JSX (*ADH2-10 ADR1*), or RC11A (*ADH2-11a ADR1*) and 521-6 $\Delta$ 1 (*ADH2 adr1 $\Delta$ 1::LEU2*) or R234.1-7A (*ADH ADR1-5<sup>c</sup>*). The *ADR1* genotype was confirmed by Southern hybridization for *adr1 $\Delta$ 1::LEU2* and by backcrosses for *ADR1-5<sup>c</sup>*. The results with one representative segregant are shown for *ADH2-9 ADR1-5<sup>c</sup>* (lane 1) and *ADH2-10 adr1 $\Delta$ 1::LEU2* (lane 2) and *ADH2 ADR1* (lane 3). The probe was a *Bam*HI-*Xho*I fragment from *ADH2-9* (Fig. 1) that contains only 5'-flanking sequences. The genomic DNA was digested with *Bam*HI and *Xho*I. Both *ADH2-9* and *ADH2-10* produced a band of approximately 1 kb and *ADH2* produced a 3.4-kb band, all as predicted. The 9-kb band in lane 1 is apparently a partial digestion product.

**ADH II activities in strains with altered *ADH2* 5'-flanking region.** The ADH II activity in strains containing the mutant *ADH2* alleles was determined after growth in medium containing a repressing (glucose) or derepressing (ethanol) carbon source (Fig. 1). Strains JSIX and JSX, which contain mutant alleles *ADH2-9* and *ADH2-10*, respectively, showed a modestly elevated level of glucose-insensitive ADH II expression: the glucose-insensitive ADH II activity was about 10-fold higher than in an isogenic *ADH2* strain but was only 3 to 10% of the fully derepressed enzyme activity in the isogenic *ADH2* strain (Fig. 1 and 4). ADH II activity in the other strains was as strongly repressed as in a strain with a control wild-type *ADH2* locus.

After depression, strains JSVII, JSX, RC17a, RC8a, and RC40a (see Fig. 1 for the *ADH2* alleles present in these strains) had as much ADH II activity as the isogenic wild-type control, strain DCB1E. Strains JSIX and RC11a, which lack the dyad sequence (-215 to -236), derepressed to about 10 to 20% of the wild-type level of ADH II activity. Since the ADH II activity of strains JSIX and RC11a after depression was very similar, it seems likely that the poor derepression of strain JSIX is caused by the loss of the dyad. The low-level constitutive expression in strain JSIX must

not be a consequence of losing this sequence since strain RC11a is fully repressed. The (dA)<sub>20</sub> is not required for either repression or full derepression, but an alteration adjacent to it, as in *ADH2-10*, can affect repression.

The kinetics of derepression in strains RC11a, JSIX, and DCB1E were compared to assess more accurately the effect of the dyad deletion on *ADH2* derepression (Fig. 4). ADH II activity began to increase at 60 min in all three strains, but the rate of increase in enzyme activity in strains RC11a and JSIX was about 20-fold lower than the rate of increase in enzyme activity in the isogenic strain DCB1E. As expected from the previous results, RC11a had essentially no activity before derepression, and JSIX had a low but significant amount, about 25 mU/mg of protein. The ADH II activity continued to increase slowly for 24 h in strains JSIX and RC11a. Thus, loss of the dyad affects both the rate of derepression of ADH II activity and the ultimate enzyme level.

**Transcription mapping.** The glucose-insensitive ADH II activity of JSIX and JSX mutant strains could be a result of either an increase in transcription from the normal transcription initiation site or an increase in transcription from an alternative site. To determine which of these possibilities

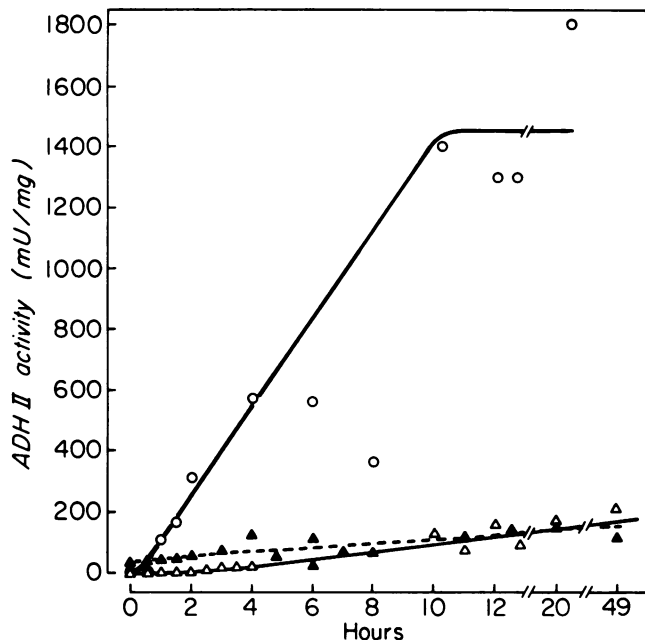


FIG. 4. Kinetics of ADH II derepression. Strains DCB1E (*ADH2*) JSIX (*ADH2-9*), and RC11a (*ADH2-11a*) were grown in YPD medium to about  $5 \times 10^6$  cells per ml at 30°C. The cells were pelleted by centrifugation, washed once with 1% yeast extract–2% peptone, and suspended in 1% yeast extract–2% peptone–2% ethanol at the original cell concentration. The cultures were shaken vigorously at 30°C, and 10-ml samples were withdrawn at the times indicated for ADH assays as described in the text. At 5, 11, and 20 h the cells were diluted into fresh YPE to maintain the cells in the exponential phase of growth. Symbols: ○, DCB1E (*ADH2*); ▲, JSIX (*ADH2-9*); △, RC11a (*ADH2-11*).

was occurring, we determined the 5' ends of *ADH2* mRNAs from strains JSVII (*ADH2-7*), JSIX (*ADH2-9*), and JSX (*ADH2-10*) (Fig. 1) by S1 mapping (see Materials and Methods). The results (Fig. 5A) showed that the increase in glucose-insensitive ADH II activity of mutants JSIX and JSX mutants is due to a corresponding increase in *ADH2* mRNA concentration and that these mRNAs initiate in the same place as the *ADH2* mRNA of wild-type cells. No *ADH2* mRNA was detected in either the *ADH2* control strain, 521-6, or the JSVII mutant, as expected since ADH II activity in these strains was strongly repressed by growth in glucose-based medium (Fig. 1). Since the *ADH2* mRNAs made in strains JSIX, JSX, and 521-6 have the same 5' ends, it seems unlikely that increased stability could account for the increased *ADH2* message levels in strains JSIX and JSX.

*ADH2* mRNA levels and 5' end mapping after derepression were also determined in strains containing mutant alleles *ADH2-7*, *-9*, and *-10*. An S1 nuclease protection experiment was performed with mRNA isolated from yeasts grown in medium containing ethanol as a carbon source (Fig. 5B). Strains JSVII (*ADH2-7*) and JSX (*ADH2-10*) produced *ADH2* mRNA having the same 5' ends and in approximately the same amounts as the wild-type control. This is consistent with the observation that both strains were capable of derepressing ADH II to approximately wild-type levels after a shift from glucose- to ethanol-based medium (Fig. 1). No *ADH2* mRNA was detected for the ethanol-grown JSIX (*ADH2-9*) strain, but less RNA isolated from derepressed cells was loaded onto the gel than when RNA isolated from glucose-grown cells was analyzed. The low level of RNA in

strain JSIX is consistent with the observation that this strain showed only a small increase in ADH II activity after a shift from glucose- to ethanol-based medium (Fig. 4).

**Response of mutant *ADH2* alleles to mutations at the *ADR1* locus.** *ADR1* is a *trans*-acting regulatory locus, apparently specific for *ADH2* derepression (13). Both recessive *adr1* and semidominant *ADR1<sup>c</sup>* alleles have been isolated that prevent *ADH2* derepression or allow some glucose-insensitive *ADH2* expression and hyperderepression, respectively (13). Deletion of the *ADR1* locus results in a repressed, but nonderepressible ADH II phenotype, indicating that *ADR1* is required for positive activation of *ADH2* but does not encode a repressor function (Blumberg, personal communication). *ADR1* acts at the transcriptional or posttranscriptional level (2, 17).

The response of four of the mutant *ADH2* alleles to the absence of *ADR1* function or the presence of a constitutive *ADR1-5<sup>c</sup>* allele was assessed by measuring ADH II activity in strains containing wild-type and mutant *ADR1* alleles. The defective *ADR1* allele, *adr1Δ1*, is a deletion of most of the gene, including its promoter region and 2 kb of coding sequence, which has been replaced by the yeast *LEU2* gene (Blumberg, personal communication).

Appropriate genetic crosses were performed, and strains with the desired genotype were isolated. The *ADH2* and *adr1Δ1* genotypes were confirmed by Southern hybridization analysis since the mutant and wild-type alleles can be distinguished by restriction enzyme site differences and by hybridization to different probes. Figure 3 shows representative examples of Southern blots of DNA from strains containing or lacking just the dyad (*ADH2* and *ADH2-11a*,

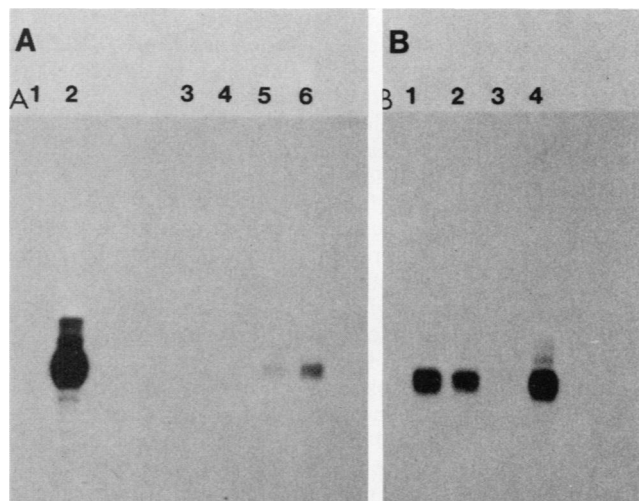


FIG. 5. Mapping the 5' ends of *ADH2* mRNA. The 5' ends of *ADH2* mRNA were determined by S1 protection (see the text) for *ADH2* strain 521-6 and the strains carrying deletions in the *ADH2* 5'-flanking regions JSVII (deletion from -367 to -276), JSIX (deletion from -244 to -199), and JSX (deletion from -164 to -146). (A) There is 25 μg of total RNA per lane. Lanes 1, no RNA control; 2, strain 521-6 grown in ethanol; 3, strain 521-6 grown in glucose; 4, strain JSVII grown in glucose; 5, strain JSIX grown in glucose; 6, strain JSX grown in glucose. (B) There is 5 μg of total RNA per lane as follows: 1, strain 521-6 grown on ethanol; 2, strain JSVII grown on ethanol; 3, strain JSIX grown on ethanol; 4, strain JSX grown on ethanol. In this experiment no RNA was detected in nucleic acid isolated from JSIX even though the cells contained significant ADH II enzyme activity. In other experiments *ADH2* RNA was detectable at a low level in this strain.

TABLE 2. Effect of *ADR1* regulatory gene mutations on *ADH2* wild-type and mutant alleles

<i>ADH2</i> allele	Deletion endpoints	ADH II activity <sup>a</sup> with the following <i>ADR1</i> allele:					
		<i>ADR1</i>		<i>ADR1-5<sup>c</sup></i>		<i>adr1Δ1</i>	
		Glucose	Ethanol	Glucose	Ethanol	Glucose	Ethanol
<i>ADH2</i>		30 (20–40)	1,500 (1,200–1,900)	380 (210–680)	2,000 (1,600–2,900)	12 (4–30)	20 (10–40)
<i>ADH2-9</i>	–244/–199	50 (30–70)	300 (110–380)	100 (90–140)	100 (90–240)	30 (10–40)	100 (50–100)
<i>ADH2-11a</i>	–236/–215	10 (4–10)	440 (410–460)	10 (9–10)	300 (240–360)	<20	420 (340–490)
<i>ADH2-10</i>	–164/–146	70 (50–80)	2,600 (2,300–2,800)	550 (300–650)	2,800 (1,900–4,600)	10 (7–30)	370 (290–510)

<sup>a</sup> ADH II activities are in milliunits per milligram of protein in cell extract. The values shown are the average from different experiments using at least four different segregants with the same genotype; the numbers in parentheses represent the range of values obtained.

respectively) and strains containing alleles *ADH2-9* and *ADH2-10* (Fig. 1). The *ADR1-5<sup>c</sup>* allele was shown to be present by appropriate backcrosses, sporulation, and tetrad analysis.

Strains lacking the dyad sequence did not respond to different *ADR1* alleles (Table 2). The ADH II activity after derepression of strains with *ADH2-9* and *ADH2-11a* was approximately the same in strains with either *ADR1*, *ADR1-5<sup>c</sup>*, or *adr1Δ1* alleles, indicating that the sequences necessary for *ADR1*-mediated activation are absent. In addition, *ADH2-9* and *ADH2-11a* did not respond to *ADR1-5<sup>c</sup>* during growth on glucose-containing medium.

Strains with the *ADH2-10* allele showed a response to the *ADR1-5<sup>c</sup>* allele that was similar to the response shown by wild-type *ADH2*. Its activity was increased to the same extent as *ADH2* by *ADR1-5<sup>c</sup>* during growth on both repressing and derepressing carbon sources. Expression of *ADH2-10* showed two important differences from *ADH2* that allowed its regulation by *ADR1* to be distinguished from that of the wild-type allele: its constitutive activity on glucose-containing medium was dependent on *ADR1* function, and it derepressed 20% as well in the *adr1Δ1* strain as in the *ADR1* strain, whereas *ADH2* showed no significant derepression in the *adr1Δ1* strain. The former property of *ADH2-10* was surprising since there was no previous indication that the *ADR1* allele encoded a product that was functional during growth on glucose-containing medium.

That the enzyme activities measured in vitro were representative of the enzyme activity in vivo was shown by testing the strains listed in Table 2 for growth on complete medium containing glucose and antimycin A. Antimycin A is a respiratory inhibitor that prevents the growth of cells that do not contain at least 50 mU of ADH activity per mg. In all cases the antimycin A phenotype was consistent with the amount of ADH activity measured in extracts. In particular, the strain with the genotype *ADH2-10 adr1Δ1* did not grow in the presence of antimycin A, whereas strain *ADH2-10 ADR1* did grow, confirming the *ADR1* dependence of *ADH2-10* during repressing growth conditions.

## DISCUSSION

Catabolite repression and derepression of *ADH2* expression require specific sequences 5' to the TATA box as well as the function of several *trans*-acting regulatory loci. Other studies of an *ADH2-ADH1* hybrid gene suggested that catabolite repression and derepression of *ADH2* was mediated primarily by positive activation acting through sequences located between –150 and –250. Of the two most

unusual features of this region, a (dA)<sub>20</sub> tract and a 22-bp dyad, only loss of the dyad had a discernible effect on *ADH2* expression. Extension of the (dA)<sub>20</sub> tract to (dA)<sub>54</sub> or (dA)<sub>55</sub> and associated adjacent nucleotide changes are associated with partial loss of glucose repression and a strong promoter-up effect (13, 37), suggesting that loss of the (dA)<sub>20</sub> tract might have a promoter-down phenotype. The results presented here indicate that loss of the (dA)<sub>20</sub> has no effect on *ADH2* expression. Although more complicated explanations are possible, this suggests that the (dA)<sub>20</sub> tract is not an important component of the *ADH2* promoter or regulatory region.

Loss of the dyad was associated with a poorly derepressed phenotype; both the rate of derepression and the ultimate level of *ADH2* RNA and enzymatic activity were 20-fold lower in the two strains lacking the dyad sequence (Fig. 4). The defect in these strains could be attributable to either the loss of the dyad which acts positively or to an upstream inhibitory sequence which, having been brought closer to the gene, inhibits its expression. Since strains JSX and RC17a had *ADH2* deletions almost identical in size to the deletion in strain RC11a but had normal *ADH2* derepression, it seems more likely that loss of the dyad rather than an upstream inhibitory sequence caused the poorly derepressed *ADH2* phenotype. Moreover, strains lacking the dyad sequence also failed to respond normally to mutations in the regulatory gene *ADR1*. *ADR1<sup>c</sup>* alleles allow expression of *ADH2* in glucose-containing media. Strains lacking the dyad sequence showed essentially no response to the *ADR1-5<sup>c</sup>* allele, as was also observed for deletions of the dyad sequence when the mutant allele was on a plasmid (2). The phenotype of the *ADR1-5<sup>c</sup>* allele can be mimicked by the presence of multiple copies of *ADR1*, and deletion of the dyad results in loss of the ability to respond to this copy number effect (Blumberg, personal communication). Loss of the dyad sequence also prevents the 5'-flanking region of *ADH2* on a multicopy plasmid from competing effectively in vivo with derepression of a wild-type chromosomal *ADH2* gene, whereas the intact 5'-flanking region of *ADH2* on a multicopy plasmid lowers the rate and ultimate level of derepression of *ADH2* (M. Irani, personal communication).

Taken together, these results provide strong evidence, albeit indirect, that *ADR1* mediates *ADH2* activation through the dyad sequence. An inverted repeat sequence has been implicated in regulating other yeast genes (21, 32). The results do not distinguish between the two obvious possibilities for the role these sequences play: structural or sequence specific. There is some evidence that the dyad confers a different structure on both plasmid and chromosomal DNA:



purified plasmid DNA containing the dyad is cleaved by nuclease S1 very near or within the dyad (7; A. Sledziewski, personal communication), and the 5'-flanking region of *ADH2* chromosomal DNA contains a DNase I- and S1-hypersensitive site very near the dyad sequence (42; unpublished data).

The importance of the sequences within the dyad, rather than a DNA structure created by this inverted repeat, is suggested by two observations: loss of the dyad still allows derepression to about 5% of the wild-type level, suggesting that other sequences play a minor role in redepression, independent of the dyad; and sequences present at the end of the dyad are present seven times in the region implicated in *ADH2* control (Fig. 1). This sequence, with the consensus (A/T)GGAGA, is present twice in the dyad as part of the 22-bp inverted repeat (1 and 1'), as part of another 22-bp indirect repeat separated by 87 bp (2 and 2'), and as part of a 7-bp direct repeat (3). Loss of three of these conserved sequences by deletion between the dyad and the TATA box did not effect *ADH2-ADH1* derepression on a plasmid (1), whereas loss of five GGAGA sequences, including the dyad region, between -256 and the TATA box led to poor derepression of *ADH2-ADH1*. These results suggest a major role for the consensus sequence in the dyad and a minor role for this sequence located elsewhere. However, the GGAGA repeats not in the dyad do not respond to either the *ADR1-5<sup>c</sup>* allele or multiple copies of *ADR1*. A role for repeated eucaryotic genes has been demonstrated directly for other genes (19). A synthetic oligonucleotide containing the sequence GGAGA, which is also present at each end of the dyad upstream of *ADH2*, allowed galactose induction of a hybrid *GAL1-lacZ* gene in yeasts (21).

Two mutant loci, *ADH2-9* and *ADH2-10*, were partially active on glucose and showed different responses to mutant alleles of *ADR1*. The low, constitutive ADH II expression was due to transcription initiating at the normal start site, indicating that new promoters had not been created by the deletions. The constitutive expression of *ADH2-10*, but not *ADH2-9*, was dependent on *ADR1*: in strain JSX1122 (*adr1*) the *ADH2-10* allele was inactive on glucose. This was the first indication that the *ADR1* gene product is active, or at least partially so, during glucose-repressed growth conditions. Surprisingly, the *ADH2-10* allele derepressed to about 20% of the wild-type level in the same strain. These results showed that *ADH2-10* behaved aberrantly in two ways. First, it was able to respond to *ADR1* on glucose whereas the wild-type gene could not; and second, it showed a decreased dependence on *ADR1* for derepression. On the other hand, *ADH2-10* responded normally to *ADR1-5<sup>c</sup>* presumably because it has the intact dyad region. The *ADH2-9* allele, although having some glucose-insensitive expression, did not respond to mutations at the *ADR1* locus (Table 2) or to multiple copies of *ADR1* (Blumberg, personal communication). Like the *ADH2* allele lacking just the dyad (*ADH2-11a*), *ADH2-9* derepressed slowly to about 5% of the wild-type level for ADH II activity (Fig. 4), and its derepression was independent of *ADR1* (Table 2). Deletion analysis of the 5'-flanking region in carbon catabolite-repressed genes *GAL1* (46) and *SUC2* (39) has shown that removal of upstream sequences can result in 10 to 30% of the derepressed level of expression under glucose growth conditions. The deletion analysis of *ADH2* indicates that similar sequences may exist in the *ADH2* 5'-flanking region, as shown by alleles *ADH2-9* and *ADH2-10*. Struhl (43) has proposed that glucose repression may occur by a negative control

mechanism involving a repressor protein. Such a mechanism may contribute to the glucose repression of *ADH2*, but our deletion analyses suggest that it can account for only a minor amount of repression.

We imagine that glucose repression of *ADH2* is primarily due to a lack of positive activation rather than a true negative mechanism acting through DNA-binding repressor proteins. This conclusion is based on several observations: the inability to derepress *ADH2* in an *adr1* strain (13) (Table 2); the failure to identify deletions resulting in high-level constitutive expression (1; this work); the absence of genes which have the property of efficiently relieving glucose repression of *ADH2* (13, 16); and the ability to titrate a positive activator but not a putative repressor in vivo (Irani, personal communication). The absence of a fully functional *ADR1* gene product presumably accounts for the lack of positive activation of *ADH2* during glucose repression. However, glucose repression of *ADH2* may also be influenced secondarily by a negative control mechanism as indicated by *ADH2* deletions which have a weak constitutive phenotype and the isolation of mutants which allow some glucose-insensitive *ADH2* expression (13, 16). Thus, glucose repression of *ADH2* may involve multiple regulatory controls.

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