Identification and Characterization of Three Genes That Affect Expression of *ADH2* in *Saccharomyces cerevisiae*

Larry Karnitz,[1] Michael Morrison and Elton T. Young

Department of Biochemistry, University of Washington, Seattle, Washington 98195

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ABSTRACT

Using a new selection protocol we have identified and preliminarily characterized three new loci (*ADR7, ADR8* and *ADR9*) which affect *ADH2* (alcohol dehydrogenase isozyme II) expression. Mutants were selected which activate *ADH2* expression in the presence of an over-expressed, normally inactive *ADR1* allele. The mutants had very similar phenotypes with the exception that one was temperature sensitive for growth. In the absence of any *ADR1* allele, the mutants allowed *ADH2* to partially escape glucose repression. However, unlike wildtype strains deleted for *ADR1*, the mutants were able to efficiently derepress *ADH2*. The mutations allowed a small escape from glucose repression for secreted invertase, but had no effect on the glucose repression of isocitrate lyase or malate dehydrogenase. The mutations were shown to be nonallelic to a wide variety of previously characterized mutations, including mutations that affect other glucose-repressed enzymes.

THE genome of the yeast *Saccharomyces cerevisiae* encodes at least four different alcohol dehydrogenase (ADH) isozymes (LUTSTORF and MEGNET 1968; PAQUIN and WILLIAMSON 1986). One isozyme, ADHII, is under tight glucose repression. Yeast grown on media containing a fermentable carbon source such as glucose repress the expression of *ADH2* and yeast grown on a nonfermentable carbon source such as glycerol derepress the level of ADHII enzymatic activity several-hundred fold (LUTSTORF and MEGNET 1968). The control of this expression is mediated by the modulation of transcription of the *ADH2* structural gene and requires both *cis*- and *trans*-acting elements. Two cis-acting upstream activating sequences (UASs) have been described for the *ADH2* gene. Both sequences confer glucose-regulated transcriptional regulation upon heterologous genes (BEIER and YOUNG, 1982; SHUSTER *et al.* 1986; BEIER, SLEDZIEWSKI and YOUNG 1985; YU, DONOVIEL and YOUNG 1989). UAS1 is a 22-bp sequence with dyad symmetry which binds the transactivator ADR1 (EISEN *et al.* 1988; YU, DONOVIEL and YOUNG 1989) and UAS2 is a recently identified sequence which binds a less well characterized transactivator (YU, DONOVIEL and YOUNG 1989). Glucose repression of *ADH2* is thought to be modulated, at least in part, by the phosphorylation of ADR1 at one or more cAMP-dependent protein kinase consensus sites (CHERRY *et al.* 1989). Extensive genetic analysis has identified the domain containing serine 230 to be critical in the regulation of ADR1's transcriptional activation properties (DENIS and YOUNG 1983; BEMIS and DENIS 1988; CHERRY *et al.*, 1989; TAYLOR and YOUNG 1990).

Although ADR1 and cAMP-dependent protein kinase-mediated phosphorylation of ADR1 are requisite for *ADH2* transcriptional regulation, these alone do not appear to account for all of the observed catabolite control of *ADH2* expression. Both *cis*- and *trans*-acting genetic loci which affect ADHII levels have previously been described. *Trans*-acting mutations such as *cre1* and *cre2* (DENIS 1984; DENIS and MALVAR 1990), and *ADR1-5C* (CIRIACY 1979) and *cis*-acting mutations in the *ADH2* promoter such as Ty insertions (WILLIAMSON, YOUNG and CIRIACY 1981), deletions (SHUSTER *et al.* 1986), and extensions of a $(dA)_{20}$ tract (RUSSELL *et al.* 1983) allow *ADH2* to partially escape glucose repression. Other *trans*-acting mutations such as *adr6* (TAGUCHI and YOUNG 1987a,b), *ccr1/snf1* (CIRIACY 1979), *ccr4* (DENIS 1984; DENIS and MALVAR 1990), and *bcy1* (CHERRY *et al.* 1989; TAYLOR and YOUNG 1990) prevent complete derepression of *ADH2*. In addition, other genetic loci such as the UAS2 binding factor, a previously reported repressor, ADR4 (CIRIACY 1979), and possibly other signalling mechanisms which interact with ADR1 or other factors have not yet been identified.

The complex regulation of *ADH2*, the stringency of its transcriptional control, and the availability of positive and negative selection protocols have made it an attractive model system for the genetic dissection of its transcriptional control. Previous work has demonstrated that the overexpression of various truncated ADR1 molecules allows escape from glucose repression (BEMIS and DENIS 1988; THUKRAL *et al.* 1989).

[1] Present address: Department of Immunology, Mayo Foundation, Rochester, Minnesota 55905.

TABLE 1

Yeast strains

Strain	Genotype	Source
SSH35	*MATa adh1 adh3 adr1-Δ1::LEU2 trp1 leu2 ura3*	S. CAMIER
SSH46	*MATa adh1 adh3 adr1-Δ1::LEU2 trp1 leu2 ura3*	S. CAMIER
LKY56-1-15	*MATa adh1 ADH2-10 adh3 adr1-Δ1::LEU2 trp1 leu2 ura3*	This study
YM3127	*MATa ura3-52 his3-200 ade2-101 lys2-801::UAS$_{GAL}$-HIS3 met grr1-1829*	M. JOHNSTON
YM3131	*MATa ura3-52 his3-200 ade2-101 lys2-801::UAS$_{GAL}$-HIS3 met hxk2-202*	M. JOHNSTON
YM3153	*MATa ura3-52 his3-200 ade2-101 lys2-801::UAS$_{GAL}$-HIS3 met urr1-1*	M. JOHNSTON
MC1707	*MATa ssn6::URA3 lys2 his4 ade2 ura3-52*	M. CARLSON
373	*MATa ura3-52 his6 gal1 bar1 reg1-501*	M. JOHNSTON
CM1-2	*MATa trp1-Δ1 his3-Δ200 ura3-52 lys2-801^a ade2-101 can1 cyr1-2*	C. MANN
553-4b	*MATa adh1-11 adh3 cre1-1 his4 trp1 ura1*	C. DENIS
500-16-14	*MATa adr1-1 adh1-11 adh3 cre2-1 his4 trp1 ura1*	C. DENIS
MCY831	*MATa his4-539 ade2-101 cid1-226*	M. CARLSON
H250	*MATa SUC2 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 mig1-Δ2::URA3*	H. RONNE

Because the overexpression of ADR1 is a predisposing event toward escape from glucose repression, we reasoned that selection for escape from glucose repression in cells overexpressing a transcriptionally inactive ADR1 might identify new genetic loci that affect *ADH2* expression. For such a selection we utilized an ADR1 molecule truncated at amino acid 220 (ADR1-220) that binds DNA with the affinity of full length ADR1 but is unable to activate *ADH2* transcription in yeast grown on glucose (BEMIS and DENIS 1988; THUKRAL *et al.* 1989). We then selected for suppressor mutations that required the presence of the truncated ADR1-220 for *ADH2* expression to escape from glucose repression. Three complementation groups of partial suppressors were identified. These were demonstrated to be different than a panel of other previously described mutations that affect glucose repression and basal transcription of a variety of unrelated genes. The mutations described in this report appear to be previously undescribed mutations that act in *trans* and may play a regulatory role in *ADH2* expression.

MATERIALS AND METHODS

Yeast strains: The strains used in this study are described in Table 1.

Yeast manipulations: Tetrad analysis was performed essentially as described by SHERMAN, FINK and HICKS (1982), except that tetrads were dissected using the fiber optic procedure of EICHINGER and BOEKE (1990). Yeast were grown in either complete medium (YP) containing 10 g yeast extract, 10 g Bacto-peptone, 20 mg adenine, and 20 mg uracil per liter or synthetic medium (SC) containing 6.7 g yeast nitrogen base, 20 mg tyrosine, 20 mg adenine, and supplemented with 0.4% (w/v) casamino acids per liter. SC, which lacks sufficient tryptophan for growth of tryptophan auxotrophs, was also supplemented as required with 20 mg per liter uracil and/or tryptophan. Media was supplemented with glucose (8% final concentration) or a mixture of nonfermentable carbon sources for derepression (glycerol, lactate, and ethanol, at 2% final concentration). 5′-Fluoroorotic (FOA) acid plates were prepared as described (BOEKE *et al.* 1987) and contained 0.1 mg/ml FOA. Selection for mutants that were resistant to antimycin A (At) was performed as previously described using 1 μg/ml At (CIRIACY 1979). Yeast were transformed using the lithium acetate procedure (ITO *et al.* 1983).

Enzyme assays: For all assays, cells were grown in SC lacking tryptophan and/or uracil. All assays were repeated on different days and for transformed cells at least three different transformants were tested. All assays were performed in at least triplicate. For repressing growth conditions, cells were grown in 8% glucose and harvested during log-phase growth. These cultures were then diluted into 8% glucose and harvested in early log phase growth. For derepressed cultures, the yeast were grown in 2% lactate (pH 5.0), 2% ethanol, and 2% glycerol. Yeast were harvested after reaching stationary phase. ADH assays were performed as previously described (DENIS, CIRIACY and YOUNG 1983). Secreted invertase was assayed using the method of CELENZA and CARLSON (1984), except that cells were grown as described above. Isocitrate lyase assays were as described by DIXON and KORNBERG (1959). Malate dehydrogenase assays were performed as described (POLAKIS and BARTLEY 1965). β-Galactosidase activity was assayed using the protocol of MILLER (1972). Maltase/isomaltase assays using *p*-nitrophenol-α-D-glucopyranoside (PNPG) as substrate were as described (ZIMMERMANN and EATON 1974).

Plasmid construction: All plasmids were propagated in the *E. coli* strain DH5 α. The 2μ-based overexpression plasmid pGYEP-OX contains the *ADH1* promoter and the CYC1 termination fragment. The vector was constructed from the base plasmid pGEM3zf- (Promega, Madison, Wisconsin) by the insertion of a YEp24 *Eco*RI (nt 105)-*Hin*dIII (nt 2241) 2μ fragment into the *Eco*RI site of pGEM3zf-. All fragment ends were rendered blunt by treatment with Klenow fragment and ligation did not recreate the *Eco*RI site. The *URA3* gene was then inserted into this 2μ plasmid. The *Hin*dIII (nts 2272–3438) YEp24 *URA3* containing fragment was rendered blunt and ligated into the *Sma*I site of the 2μ plasmid to yield pGYEP. The *ADH1* promoter and CYC1 terminator (*Sal*I-*Hin*dIII fragment) from plasmid pYC-DE2 (TAYLOR and YOUNG 1990, derived from pMA56 (AMMERER 1985)) was then inserted into the *Sal*I and *Hin*dIII sites of pGYEP to give pGYEP-OX. This plasmid (pGYEP-OX) contains a unique EcoRI site between the *ADH1* promoter and CYC1 terminator. Either a full-length (*Nci*I-*Bst*EII) or an amino-terminal 220 amino acid fragment (*Nci*I-*Sca*I) of *ADR1* was ligated into the *Eco*RI site after rendering

all 5′ overhangs blunt with the Klenow fragment. The plasmid, expressing the full-length ADR1, was designated pGYEP-ADR1 and the truncated version was designated pGYEP-ADR1-220. The fusion of the *Sca*I site with the blunted *Eco*RI site (pGYEP-ADR1-220) generated an in-frame stop codon, providing for a perfect truncation after amino acid 220 of ADR1.

The centromere containing plasmids pRS314-ADR1-220 and pRS316-ADR1-220 were constructed by ligating the *ADH1* promoter/ADR1-220/*CYC1* terminator fragment (*XbaI-HindIII*) of pGYEP-ADR1-220 into the unique *XbaI* and *HindIII* sites of pRS314 or pRS316 (SIKORSKI and HIETER 1989). pRS314-ADHII$^{2.2}$ was constructed by the introduction of the *Sph*I *ADH2* structural gene fragment (which contains the *ADH2* TATA box, but no UAS elements) into the *Sph*I site of pSP72 (Promega). The 5′ *BamHI* (nt −1148) to *HpaI* (nt −749) fragment upstream of the *ADH2* structural gene (which also contains no known regulatory sequences) was then inserted into the *EcoRI* site which had been rendered blunt with the Klenow fragment. Into the SmaI site (located between the 5′ *ADH2* sequences and the TATA sequences of the *ADH2*), four head-to-tail copies of an IBF binding site (KARNITZ *et al.* 1990) were inserted. The expression from the plasmid-borne *ADH2* gene is very low (<10 milliunits/mg protein) in the absence of UAS elements. The entire polylinker, containing the new *ADH2* transcription unit, was then transferred into pRS314.

The plasmids pKD8, pKD14, and pKD16 were generously supplied by K. DOMBEK. The SPT plasmids were a gift from F. WINSTON (Harvard University).

Mutant isolation: To identify suppressor mutations, ten colonies of the doubly transformed SSH35 strain, containing pRS314-ADR1-220 and pHH70 (an *ADH2/lacZ* fusion gene), were inoculated into small cultures and grown to saturation. Cells (2×10^7) from each culture were plated onto Ura$^-$, Trp$^-$, At$^+$ plates. Five plates were subjected to UV radiation (254 nm) using conditions which resulted in approximately 50% cell viability. Spontaneous mutations were isolated from the five other plates. All the plates were incubated at 30° for 3–4 days and a large and small colony were picked from each plate. The plates were incubated again for 2 weeks and two more colonies were picked from each plate. Approximately 50 colonies from all 10 plates were picked and used for further analysis. The selection protocol yielded suppressors at a frequency of 2.5×10^{-6}. To test for increased expression of the ADHII/β-galactosidase fusion protein, yeast were streaked onto Ura$^-$, Trp$^-$ plates (pH 7.0), containing the chromogen 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). Mutants harboring *trans*-acting mutations turned dark blue, whereas the doubly transformed starting strain (SSH35), which was used for the selection, turns only light blue on such plates after a few days incubation at 30°.

Complementation assays: The previously characterized mutant strains were mated to the mutant strains described in this report and the diploid was selected using the appropriate auxotrophic markers. The diploids were then assayed for secreted invertase activity under glucose repressing growth conditions.

RESULTS

Allele-specific activation by truncated ADR1: The overexpression of wild-type ADR1 allows glucose-resistant, constitutive *ADH2* expression in strains with either a wild-type *ADH2* or an *ADH2–10* allele (Table 2). Such glucose-resistant *ADH2* expression is manifest *in vivo* as growth on glucose-containing media that is resistant to the respiratory inhibitor At and it is assessed *in vitro* as increased ADH enzymatic activity. In agreement with previously published results, a plasmid (pRS314-ADR1-220) which overexpresses the amino-terminal 220 amino acids of ADR1 (ADR1-220) was unable to activate transcription of the wild-type *ADH2* allele in yeast grown under glucose-repressing conditions (BEMIS and DENIS 1988; THUKRAL *et al.* 1989) (Table 2). Such strains are At-sensitive (At^s) and have very low levels of ADH activity when assayed *in vitro*. In contrast, the same ADR1-220 deletion was able to efficiently activate *ADH2–10* expression in strains grown under the same glucose-repressing conditions. The *ADH2–10* allele contains an 18-bp deletion between nucleotides −146 to −164. This deletion disrupts no known *cis*-acting sequences, but it allows partial escape from glucose repression and the constitutive activity of *ADH2–10* is dependent upon *ADR1* (SHUSTER *et al.* 1986). Thus, the strain is At^r and has high levels of ADH enzymatic activity. These results have been obtained for several different *ADH2* and *ADH2–10* allele-containing strains.

TABLE 2

Effect of overexpression of wild-type and truncated ADR1 on glucose-repressed *ADH2* expression

Relevant genotype	At^r	ADH activity (milliunits/mg protein)
ADH2 (ADR1)	+	2100
ADH2 (ADR1-220)	−	<5
ADH2-10 (ADR1)	+	2900
ADH2-10 (ADR1-220)	+	800

Plasmids which overexpress wild-type ADR1 (YEp1-ADR1) and an ADR1 truncated at amino acid 220 (pRS316-ADR1-220) were introduced into either SSH35 (wild-type *ADH2*) or LKY56-1-15 (*ADH2-10*). The transformed strains were tested for At^r on glucose-containing plates or grown in liquid culture under repressing conditions (8% glucose). Glass bead extracts were prepared from yeast grown in liquid culture and assayed for ADH activity.

The observation that ADR1-220 was able to efficiently activate *ADH2–10* expression on glucose was unexpected. Like the full-length ADR1, overexpressed ADR1-220 has the capacity to allow bypass of glucose repression. In those strains harboring a wild-type *ADH2* allele, overexpression of full-length ADR1, but not ADR1-220 allowed *ADH2* expression to escape from glucose repression. These results demonstrated that ADR1-220 is both stably produced and able to bind DNA and activate transcription *in vivo*, however its activation potential was dependent upon the promoter context.

Isolation of mutations which allow *ADR1–220*-dependent *ADH2* expression: The overproduction of truncated ADR1-220 in an *ADH2–10* allele background allowed *ADH2* expression to partially bypass glucose repression. Presumably, ADR1 overexpres-

sion was also a predisposing event to the bypass of glucose repression in a strain with a wild-type *ADH2* allele. Therefore, we reasoned that selection for suppressor mutations that allowed *ADH2* expression to escape glucose repression only in the presence of ADR1-220 might identify new genetic loci involved in *ADH2* expression.

To identify such suppressor mutations, colonies from strain SSH35 (At^s) were selected that could grow on At plates containing 8% glucose as the carbon source. Because this selection would be expected to generate both intragenic and extragenic suppressors that would allow the escape from glucose repression, a second *ADH2* allele was utilized in the mutant isolation scheme. To this end, the strain SSH35 containing pRS316-ADR1-220 (a $Ura3^+$ plasmid) was transformed with a plasmid (pHH70, $Trp1^+$) that contains an *ADH2* promoter plus DNA encoding the first 22 amino acids of ADHII fused in-frame to the *lacZ* gene. The production of the fusion protein is glucose regulated and only those mutants that affect the production of ADHII in a *trans*-acting manner will also contain increased levels of β-galactosidase activity. Because we were only interested in *trans*-acting mutations, only those suppressors with increased expression of the ADHII-β-galactosidase fusion protein (plasmid pHH70) were followed. Of the original 56 colonies, 22 were significantly more blue than were wild-type strains harboring pRS316-ADR1-220 and pHH70 when plated on X-gal-containing plates. These mutants were selected for further study. To confirm the phenotype of increased *ADH2* expression and β-galactosidase activity, cultures of each mutant were grown and glass bead extracts were prepared. The ADHII and β-galactosidase activities in the extracts were then assayed. All At^r mutants that showed increased β-galactosidase activity on plates also contained increased intracellular β-galactosidase and ADH activities as compared to parental wild-type strains (results not shown).

To determine which mutants were dependent upon ADR1-220 for At-resistant (At^r), the suppressor strains were grown on nonselective media and then plated on 5′-fluoroorotic acid-containing plates to select for cells lacking pRS314-ADR1 ($Ura3^+$). After scoring for the loss of the Ura^+ phenotype, the yeast were scored for growth on At. Twelve of the remaining 22 mutants failed to grow on At, indicating dependance of the mutant phenotype upon the truncated ADR1. To determine if the suppressors were intragenic or extragenic, the cured strains were retransformed with the original "wild-type" ADR1-220 plasmid and again scored for At^r. Eight of 12 regained At^r, indicating that the suppressor mutations were not on the ADR1-220 plasmid.

Preliminary genetic characterization: To test for dominance, the remaining eight extragenic suppressor mutants, were mated to a congenic strain (SSH46) maintaining selection for the ADR1-220 expression plasmid in the diploid. In all cases the At^r phenotype was lost in the diploid strain, indicating that the mutations were recessive. Those suppressors which had the highest β-galactosidase and ADH activities and that appeared to be ADR1-220-dependent, *trans*-acting mutations were selected for further study. These were S35, S41, S45, S54 and S55. S35 arose spontaneously and the others were derived from the UV mutagenesis. These strains were also tested for temperature and cold sensitivity. No cold-sensitive mutations were found. Two of the mutants (S41 and S54) were found to be temperature-sensitive for growth (ts) at 37°, but not at 35°. A summary of the ts character as well as the ADH and β-galactosidase activities found in both the wild-type starting strain and the selected suppressor mutants grown under glucose-repressing conditions is shown in Table 3.

TABLE 3

Characterization of suppressor mutations

Strain	ADH activity	β-Galactoside activity	Growth at 37°
SSH35	<5	0.1	+
S35	120	1.1	+
S41	200	2.1	−
S45	320	2.4	+
S54	190	1.0	−
S55	220	2.2	+

The wild-type starting strain (SSH35) and the suppressor strains (S35, S41, S45, S54 and S55), transformed with the *ADH2* promoter-*lacZ* fusion plasmid (pHH70) and the ADR1-220 overexpressing plasmid (pRS316-ADR1-220) were grown in liquid culture under glucose repressing conditions. Glass bead extracts were prepared and assayed for ADH and β-galactosidase activities. ADH activities are expressed as milliunits/mg protein. β-Galactosidase activityes are expressed as ΔA_{420}/min/mg protein.

To ensure that the observed mutant phenotypes were all due to a single genetic lesion, three mutants, representing the three complementation groups (see below) were mated to a congenic wild-type strain (SSH46). Diploids were selected and sporulated, and the resulting tetrads were dissected. Since At^r was the only way to score for the mutation and ADR1-220 was required for the At^r phenotype, it was useful to use an ADR1-220 overexpression plasmid based on a 2μ vector (pGYEP-ADR1-220) that would generally segregate 4:0. ADR1-220 expressed from the 2μ-based plasmid gave similar levels of ADHII activity from wild-type *ADH2* and *ADH2–10* alleles as did ADR1-220 expressed from the pRS413-ADR1-220-containing plasmid (data not shown). The cured suppressor mutants were transformed with pRS314 (Trp1+) and mated to SSH46 transformed with pGYEP-ADR1-220 (Ura^+). The dissected tetrads (>5) were scored for At^r, mating type, ts growth, and

uracil, leucine, and tryptophan prototrophy. The only spores analyzed were from tetrads in which uracil prototrophy (pGYEP-ADR1-220 containing) segregated 4:0. At^r segregated 2:2 for all tetrads and the phenotype correlated well with the ADH activity in glass bead extracts prepared from repressed cells. The ts phenotype of S41 and S54 also cosegregated with the At^r phenotype in all tetrads analyzed, indicating a linkage between the two phenotypes. Loss of glucose repression of secreted invertase (see later) also cosegregated with the ADH phenotype (data not shown). These data demonstrated that, for all of the suppressor mutations, all of the observed phenotypes were due to mutations of the same or a tightly linked gene, and that each phenotype was likely a consequence of a single mutation.

The number of complementation groups that these mutants represent was determined by mating a Trp$^+$, At^r spore product derived from the tetrads described above, with the original suppressors containing pRS314-ADR1-220 (Ura3$^+$ plasmid). The diploid strains were grown under repressing conditions and were tested for ADH and secreted invertase activities. The five mutants were found to represent three complementaion groups (data not shown). S35 and S55 comprise one group. The two ts mutants S41 and S54 comprise another group and S45 represents the last group. S41, S45 and S55 were chosen as representatives of each group and further characterized. These have been designated *a*lcohol *d*ehydrogenase *r*egulatory (*adr*) genes; *ADR7* (S41 and S54), *ADR8* (S45) and *ADR9* (S35 and S55).

Because the mutations allowed *ADH2* expression to partially escape glucose repression, other glucose repressible enzymes were also assayed to determine if the mutations had more global effects. In wild-type cells, the synthesis of secreted invertase, isocitrate lyase (ICL), malate dehydrogenase (MDH), and maltase is repressed by glucose. Glass bead extracts prepared from yeast grown under repressed and derepressed conditions were assayed for MDH, ICL and maltase/isomaltase activities (Table 4). MDH and ICL activities were unaffected in either *adr7* or *adr8* strains when compared to wild type. However, secreted invertase activities partially escaped glucose repression in the mutant strains when compared to wild type, although the increases were not large enough to allow the mutants to become resistant to the gratuitous inducer, 2-deoxyglucose, using sucrose as a carbon source (results not shown). Unexpectedly, the wild-type SSH35 strain did not derepress maltase under the derepressing growth conditions of 2% lactate, 2% glycerol and 2% ethanol. Such growth conditions have previously been reported to partially derepress maltase synthesis (ENTIAN and ZIMMERMANN 1980). While neither *adr7* or *adr8* had an effect on the glucose-repressed levels of maltase activity, both mutations allowed derepression under growth conditions which did not permit derepression of the wild type.

Many mutations which allow invertase activity to escape glucose repression have previously been isolated and characterized. These include *cid1* (NEIGEBORN and CARLSON 1987), *hex1/hxk2* (ZIMMERMANN and SCHEEL 1977; FLICK and JOHNSTON 1990), ssn6 (CARLSON *et al.* 1984), *reg1/hex2* (ENTIAN and ZIMMERMANN 1980), and *mig1* (NEHLIN and RONNE 1990). Mutations which affect glucose repression of the *GAL1* promoter, such as *urr1* and *grr1* (FLICK and JOHNSTON 1990), and mutations which affect glucose repression of the *ADH2* gene, such as *cre1* and *cre2* (DENIS 1984) have also been isolated. Strains harboring these mutations were obtained (Table 1) and mated to the mutants and their wild-type parent, SSH35. In addition, we also tested a *cyr1* allele, since it allows *ADH2* expression to escape glucose repression (K. DOMBEK, personal communication). Complementation tests were performed with these mutants to determine if the mutations in S41, S45 and S55 might be allelic to any of these. Since all of the previously identified mutations are recessive and constitutive for secreted invertase activity, this assay was used to test for allelism to adr7, adr8 and adr9. All of the previously characterized mutations gave rise to high levels of constitutive secreted invertase expression on glucose. However, secreted invertase expression for all of these mutants, except *cid1,* was completely repressed by glucose after mating to the wild-type SSH35 strain and to each of the suppressor mutants (data not shown), indicating that *adr7, adr8* and *adr9* are not allelic to the previously characterized mutants which were tested. Secreted invertase expression did slightly escape repression by glucose in diploids of *cid1* with wild-type and adr7, adr8, and adr9 strains. There was a slight increase in secreted invertase expression when *cid1* was mated to *adr7* and *adr8* strains when compared to the mating with SSH35. This could be the result of a slight interplay between these mutations. *Adr7, adr8* and *adr9* were also shown to be different from *SPT4, SPT5, SPT11/12* (WINSTON *et al.* 1984) and *SPT6/CRE2/SSN20* (CLARK-ADAMS and WINSTON 1987; NEIGEBORN, CELENZA and CARLSON 1987; DENIS and MALVAR 1990 [results not shown]). None of the mutations which were dependent on overexpressed ADR1-220 was found to be allelic to *CRE1* (DENIS 1984; DENIS and MALVAR 1990).

***ADR1* dependence:** *Adr7, adr8* and *adr9* were originally selected to be dependent upon the truncated, overexpressed ADR1-220 protein for the expression of the At^r phenotype. Therefore we assessed the interplay between *ADR1* and these mutations. The mutant strains were transformed with plasmids express-

TABLE 4

***Adr7, adr8* and *adr9* effects on other glucose-repressed enzyme activities**

Relevant genotype	Invertase activity		ICL activity		MDH activity		Maltase/isomaltase activity	
	r	dr	tr	dr	r	dr	r	dr
Wild type	2 ± 0.2	183 ± 46	0 ± 0	62 ± 12	400 ± 57	910 ± 144	9 ± 0	10 ± 3
adr7	16 ± 1	266 ± 14	0 ± 0	47 ± 8	320 ± 38	1140 ± 103	9 ± 1	58 ± 9
adr8	9 ± 1	171 ± 15	0 ± 0	71 ± 1	340 ± 33	860 ± 80	10 ± 3	65 ± 5
adr9	17 ± 2	106 ± 3	ND	ND	ND	ND	ND	ND

The wild-type and mutant strains (containing the low copy number plasmid, pKD16, which expresses wild-type ADR1) were grown under repressing (r) and derepressing (dr) conditions. Secreted invertase activity (labeled invertase) was measured in intact cells as described in MATERIALS AND METHODS. Maltase/isomaltase activities were determined using PNPG as the substrate in permeabilized cells. Isocitrate lyase (labeled ICL) and malate dehydrogenase (labeled MDH) activities were determined in glass bead extracts. All values are means of triplicate determinations ± standard deviations. ICL, MDH, and PNPG catalytic activities are reported as μmol/min/mg protein. Invertase activities are reported as μmol glucose/min/100 mg dry weight of cells. ND, not determined.

ing either no *ADR1* allele (pKD8), the over-expressing *ADR1–220* allele (pRS314-ADR1-220), or a wild-type, full-length *ADR1* allele from a single-copy vector (pKD16). The transcription of the ADR1 gene on pKD16 is driven by its own promoter and does not appear to overexpress ADR1 or allow *ADH2* to escape from glucose repression (K. DOMBEK, personal communication).

Surprisingly, when the mutants were grown under glucose-repressing conditions in the absence of ADR1, *ADH2* expression was able to slightly escape glucose repression when ADH enzymatic activity was assayed in glass bead extracts (the yeast were still At^s). In addition all three mutants efficiently derepressed *ADH2* expression in the absence of ADR1 and expression of the *ADH2-lacZ* fusion gene faithfully mirrored these findings (Table 5). These results demonstrated that the mutations were indeed functioning in *trans* and that while ADR1-220 was required for At^r, it was not completely obligatory for *ADH2* to escape glucose repression.

In order to confirm the original ADH and β-galactosidase phenotypes of the mutations, mutant strains containing no plasmids were transformed with both pRS314-ADR1-220 and pHH70 (*ADH2-lacZ* fusion). As can be seen in Table 5, *adr7, adr8* and *adr9* strains grown under derepressing conditions and harboring both plasmids, expressed increased levels of both ADH and β-galactosidase enzymatic activities when compared to wild type. These results confirm the *trans*-acting nature of the mutations.

We also examined what effect full-length, wild-type ADR1 would have on the expression of ADH2 in the *adr7, adr8* and *adr9* strains. Unlike overexpressed ADR1-220, low level expression of the wild-type ADR1 in the mutants did not significantly increase ADH2 expression above the levels observed in the absence of ADR1 in yeast grown under glucose-repressing conditions (Table 5). In addition, ADR1 had only a small effect on complete *ADH2* derepression. These results demonstrate that *adr7, adr8* and *adr9*, while allowing *ADH2* expression to become slightly constitutive in the absence of ADR1, have a synergistic effect in combination with the overexpressed ADR1-220.

To rule out the possibility that the mutations are mediating their effects through alterations in *ADH2* mRNA or protein stability, a plasmid that expresses *ADH2* under the control of a heterologous UAS element was employed (pRS314-$ADHII^{2.2}$). In the plasmid, the *ADH2* structural gene along with the *ADH2* TATA box has been placed under the control of an unrelated, glucose-unresponsive UAS (IBF, KARNITZ *et al.* 1990). This plasmid-borne ADH2 gene was not responsive to glucose repression (our unpublished data). When we tested this construct we observed that the mutations had no effect on this *ADH2* allele (Table 5, labeled pRS314-$ADHII^{2.2}$), indicating that the mutations are functioning through elements upstream of the *ADH2* coding sequence,

DISCUSSION

We have identified three new genetic loci (*adr7, adr8* and *adr9*) which affect *ADH2* expression under both repressing and derepressing growth conditions. These suppressor mutants were uncovered by selecting for an At^r phenotype that was dependent upon the expression of a truncated, normally inactive ADR1 transcriptional activator and then screening for those that were *trans*-acting but unlinked to the ADR1-expressing plasmid. While all three mutants were completely dependent upon the plasmid-borne *ADR1–220* allele for expression of the At^r phenotype *in vivo, in vitro* assays of ADH enzymatic activity revealed that *ADH2* expression in *adr7, adr8* and *adr9* strains did slightly escape glucose repression. However, the mutations exhibited their greatest effect under derepressing growth conditions. *Adr7, adr8* and *adr9* strains, unlike wild-type strains, allowed *ADH2* expression to efficiently derepress in the ab-

TABLE 5

Effect of *ADR1* alleles on *ADH2* expression in conjunction with *adr7*, *adr8* and *adr9*

Relevant genotype	Plasmid	ADH activity (milliunits/mg protein)		β-Galactosidase activity	
		r	dr	r	dr
Wild type	pKD8 (vector)	<5	40 ± 8	2.1 ± 0.6	19 ± 3.5
	pRS314-ADR1-220	20 ± 8	730 ± 73	4.8 ± 0.5	92 ± 0
	pKD16 (ADR1)	34 ± 6	1300 ± 190	—	—
	pRS314-ADH	1500 ± 600	—		
adr7	pKD8 (vector)	50 ± 14	270 ± 60	14 ± 4.9	140 ± 12
	pRS314-ADR1-220	280 ± 44	930 ± 38	25 ± 5	160 ± 46
	pKD16 (ADR1)	72 ± 10	620 ± 95	—	—
	pRS314-ADHII$^{2.2}$	1700 ± 200	—		
adr8	pKD8 (vector)	62 ± 5	580 ± 190	14 ± 5	91 ± 0
	pRS314-ADR1-220	190 ± 11	580 ± 34	32 ± 2	64 ± 19
	pKD16 (ADR1)	81 ± 28	1118 ± 56	—	—
	pRS314-ADHII$^{2.2}$	1100 ± 290	—		
adr9	pKD8 (vector)	50 ± 12	260 ± 90	—	—
	pRS314-ADR1-220	90 ± 15	800 ± 80	—	—
	pKD16 (ADR1)	72 ± 23	1080 ± 110		
	pRS314-ADHII$^{2.2}$	1400 ± 665	—		

Strains were transformed with the indicated *ADR1*- or *ADH2*-containing plasmids and grown as described in the MATERIALS AND METHODS section under repressed (r) and derepressed (dr) conditions. Glass bead extracts were prepared and ADH activity was measured. For β-galactosidase assays, strains containing the plasmids pKD8 and pRS314-ADR1-220 were doubly transformed with pHH70. β-Galactosidase activities were determined in permeabilized cells and are reported in Miller units. Values are the mean of triplicate determinations, ± standard deviation. —, Not determined.

sence of ADR1 as do *cre1/spt10* and *cre2/spt6/ssn20* (DENIS 1984). These results indicate that *ADR7*, *ADR8* and *ADR9* may play important roles in the regulation of *ADH2* expression and that *ADR7*, since it has a ts phenotype, has an essential function in the cell.

The weak bypass of glucose repression of secreted invertase activity led us to test for allelism between our mutations and a wide variety of other previously described mutations that lead to an escape of secreted invertase expression. All of the tested mutations (*reg*1, *mig*1, *cyr1*, *ssn6*, *grr1*, *hxk2*, *urr1* and *cid*1) complemented *adr7*, *adr8* and *adr9*, indicating that they were not allelic. Two other possible mutations which were not tested are *cat80* (ENTIAN and ZIMMERMAN 1980) and *flk1/tup1/cyc9/umr7* (STARK, FUGIT and MOWSHOWITZ 1980). However, their phenotypes are not consistent with the phenotypes described for these *adr* mutations. Based on these data the mutations described here are believed to be previously undescribed. Cloning and genetic mapping will be needed to determine their uniqueness.

The preliminary genetic characterization of these mutants provided very few clues as to how they might affect *ADH2* expression. They had no effect on the expression of a cyc1-lacZ fusion gene driven by multiple UAS1 or UAS2 elements (our unpublished results). In addition, they did not affect either *ADH2* mRNA or protein stability, nor did they alter the expression of *ADR1-lacZ* fusion gene constructs (M. MORRISON, unpublished data). Because they function in *trans*, and do not appear to function through any known *cis*-acting element, it is possible that one or more of the mutations exerts its effects through an unknown *cis*-acting element. This element might be partially defined by the *ADH2–10* deletion. Such a *cis*-acting sequence may be in a repressor pathway. The observed phenotypes of the mutants are consistent with the mutations being in a glucose-regulated repressor pathway. *Mig1* (NEHLIN and RONNE 1990) and *urr1* (FLICK and JOHNSTON 1990, 1991) have recently been reported to be in glucose-regulated repression pathways for both *SUC2* and *GAL1*, respectively. Possibly, *ADR7*, *ADR8* and *ADR9* may play roles in an analogous glucose-mediated repressor pathway for *ADH2*.

Alternatively, since *ADH2* derepression is also influenced by adr7, adr8 and adr9, it seems more likely that the mutations are having a more generalized effect on transcription. Mutations in genes such as *ADR6/SWI1* (TAGUCHI and YOUNG, 1987a,b; PETERSON and HERSKOWITZ 1992), *SPT6/SSN20/CRE2* and *SSN6* and *TUP1* (KELEHER *et al.* 1992) and other *SPT* (WINSTON *et al.* 1984, 1987) and *SNF* (NEIGEBORN, RUBIN and CARLSON 1986; LAURENT, TREITEL and CARLSON 1990; HAPPEL, SWANSON and WINSTON 1991) alleles affect the expression of a variety of unrelated genes. Even though *adr7*, *adr8* and *adr9* appear to be relatively specific for the *ADH2* gene, they may have more pleiotropic effects and they may be functioning in an analogous fashion to these mutations having rather generalized effects on transcription. Current studies are focused on the cloning of

the genes in order to gain a better understanding of their role in *ADH2* expression.

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