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Isolation and Characterization of the Positive Regulatory Gene *ADR1* from *Saccharomyces cerevisiae*

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The DNA segments containing the *ADR1* gene and a mutant allele, *ADR1-5*^{c}, have been isolated by complementation of function in *Saccharomyces cerevisiae*. The *ADR1* gene is required for synthesis of the glucose-repressible alcohol dehydrogenase (ADHII) when *S. cerevisiae* cells are grown on a nonfermentable carbon source, whereas the *ADR1-5*^{c} allele allows ADHII synthesis even during glucose repression. A plasmid pool consisting of yeast DNA fragments isolated from a strain carrying the *ADR1-5*^{c} allele was used to transform a strain containing the *adr1-1* allele, which prevents ADHII derepression. Transformants were isolated which expressed ADHII during glucose repression. A plasmid isolated from one of these transformants was shown to carry the *ADR1-5*^{c} allele by its ability to integrate at the chromosomal *adr1-1* locus. The wild-type *ADR1* gene was isolated by colony hybridization, using the cloned *ADR1-5*^{c} gene as a probe. The *ADR1-5*^{c} and *ADR1* DNA segments were indistinguishable by restriction site mapping. A partial *ADR1* phenotype could be conferred by a 1.9-kilobase region, but DNA outside of this region appeared to be necessary for normal activation of ADHII by the *ADR1* gene.

In eucaryotic organisms both positive and negative regulatory loci have been identified in the control of gene expression. Included among these regulated genes are those encoding the acid and alkaline phosphatases (9) and quinine-metabolizing enzymes (3) of *Neurospora crassa* and the acid phosphatase (18), galactose-utilizing enzymes (8), cytochrome *c* (16), arginine-metabolizing enzymes (7), and glucose-repressible alcohol dehydrogenase (ADHII) of *Saccharomyces cerevisiae* (5). The principal limitation in all of these genetic systems has been the inability to measure directly the activity of the positive and negative regulatory elements. Recent methods for cloning in yeast genes whose protein counterparts are unknown (12) allow the isolation of the genes encoding some of these regulatory elements. Cloning the DNA comprising a regulatory element would not only serve to characterize the gene's structure, but also aid in developing a direct assay for the product of the regulatory locus.

This paper describes the isolation and characterization of recombinant plasmids containing the positive regulatory locus *ADR1* and its constitutive allele *ADR1-5*^{c}, which control the expression of ADHII of *S. cerevisiae*. The *ADR1* gene was chosen since it is the most well studied of several unlinked regulatory loci controlling ADHII expression (4–6). *ADR1* has been defined genetically by two types of mutations at its locus: *adr1* and *ADR1*^{c}. The recessive *adr1* alleles (e.g., *adr1-1*) do not allow the accumulation of ADHII enzyme activity during growth on nonfermentable carbon sources (4). The semidominant *ADR1*^{c} alleles (e.g., *ADR1-5*^{c}) allow ADHII enzyme expression to escape glucose repression and result in an ADHII enzyme activity which is two- to threefold greater during derepression than is found in cells with the wild-type *ADR1* allele (5). Furthermore, the *adr1-1* allele prevents ADHII mRNA accumulation during derepression, whereas the *ADR1-5*^{c} allele causes an overproduction of ADHII mRNA during both glucose repression and derepression. (6; V. M. Williamson and C. L. Denis, unpublished data).

A more complete understanding of the role of *ADR1* in ADHII expression requires a direct assay for *ADR1*. The first step in developing such an assay would be isolation of the *ADR1* gene on recombinant plasmids. The method and rationale of this isolation are described as follows. If a library of yeast DNA fragments from a strain carrying the *ADR1-5*^{c} allele were prepared in a plasmid vector capable of autonomous replication in *S. cerevisiae* (such as YRp7) and used

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to transform a strain carrying the *adr1-1* allele, those plasmids carrying a functional *ADR1-5*^{c} allele should allow complementation of the defective chromosomal copy of the *adr1-1* allele. By selecting for transformants which expressed ADHII under glucose-repressed conditions, transformants carrying a plasmid with the *ADR1-5*^{c} allele would be isolated. To select for ADHII expression during glucose repression, transformants would be grown in the presence of antimycin A, the respiratory inhibitor (20). Inhibition of respiration by antimycin A forces the *S. cerevisiae* cells to subsist on fermentative energy sources. Since all strains used in this study would lack the enzyme activity for the fermentative ADH (ADHI), only those strains which expressed ADHII during glucose repression would grow in the presence of antimycin A. These transformed strains should contain a plasmid with the *ADR1-5*^{c} allele.

The work presented in this paper describes the isolation of the *ADR1-5*^{c} allele and its use to isolate the wild-type *ADR1* gene. The structure and function of these genes were compared by restriction enzyme analysis and by phenotypic expression in *S. cerevisiae* strains transformed with plasmids containing each of the two loci.

MATERIALS AND METHODS

Strains. The *S. cerevisiae* strains used in this study are described in Table 1. *Escherichia coli* K-12 strain RR1 (*leu pro thi* r_k $m_k$$^+$) was used as the recipient for transformation.

TABLE 1. *S. cerevisiae* strains used

Strain	Genotype
R234	*MATα adc1-11 ADR2-F ADR1-5*^{c} *adm ura1 his4*
500-16	*MATa adc1-11 ADR2-F adr1-1 adm ura1 trp1 his4*
500-11	*MATα adc1-11 ADR2-F adr1-1 adm ura1 trp1 leu2*
502-32	*MATα adc1-11 ADR2 ADR1 ura1 trp1*
200-6	*MATα adc1-11 ADR2 ADR1 trp2*
521-11	*MATα adc1-11 ADR2 ADR1 trp1 leu2 ura1*
530-5	*MATα adc1-11 ADR2 ADR1-5*^{c} *trp1 ade2 ura1*
530-13	*MATa adc1-11 ADR2 ADR1-5*^{c} *trp1 ade2 ura1*
23-17	500-16::YRp7-ADR1-5^c-23A
422	500-16::YRp7-ADR1-5^c-23B
313	500-11::YRp7-ADR1-311
411	500-11::YRp7-ADR1-411
79-72C	*MATα ADC1 ADR2-F ADR1 ADM ura1 trp2*
11-13C	*MATa adc1-11 ADR2-S ADR1 adm trp2 ade2*
505-9	*MATa adc1-11 ADR2 adr1-1 trp2 his4*
505-10	*MATα adc1-11 ADR2 adr1-1 trp2 ura1*
505-11	*MATα adc1-11 ADR2 adr1-1::YRp7-ADR1-5*^{c}*-23A trp1 his4*
505-12	*MATa adc1-11 ADR2 adr1-1::YRp7-ADR1-5*^{c}*-23A trp1 ura1*

Media conditions. *S. cerevisiae* strains were grown overnight in YEP medium (1% [wt/vol] yeast extract and 2% [wt/vol] Bacto-Peptone [Difco Laboratories]) supplemented with either 8% (wt/vol) glucose (YD medium) or 3% (vol/vol) ethanol. YD plates contained YEP medium, 2% glucose, and 2.5% agar. Minimal plates lacking either tryptophan or leucine have been previously described (21). All cultures were shaken at 30°C. *E. coli* cultures were grown overnight in L-broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl, pH 7.5) with constant shaking at 37°C.

DNA preparation. Large quantities of high-molecular-weight yeast DNA were prepared as described previously (13). The following procedure was used to prepare small amounts of high-molecular-weight yeast DNA. Cells from a 5-ml overnight culture were incubated for 1 h at 37°C in 0.4 ml of 1 M sorbitol–0.1 M sodium citrate (pH 7.0)–0.06 M EDTA–1 mg of Zymolyase-5000 (Kirin Brewery) per ml–0.08% (vol/vol) β-mercaptoethanol. After incubation, 0.4 ml of a solution containing 2% sodium dodecyl sulfate, 50 mM Tris (pH 8.0), and 10 mM EDTA was added, and the solutions were mixed by inversion for 10 min. To this mixture 0.2 ml of 5 M NaCl was added, and the solution was left at 0°C for 1 to 2 h. The high-molecular-weight DNA was pelleted in a microfuge for 5 min. The supernatant was used for isolation of yeast plasmid DNA after extraction of proteins with an equal volume of phenol-chloroform-isoamyl alcohol (50:50:1), precipitation of the DNA twice with ethanol, and resuspension in 50 μl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). The high-molecular-weight DNA was resuspended gently in 0.4 ml of TE buffer, and the proteins were extracted with the addition of an equal volume of phenol-chloroform-isoamyl alcohol (50:50:1). The aqueous phase was precipitated with 2 volumes of 100% ethanol at −20°C for 30 to 60 min. The pellet was resuspended in 0.2 ml of TE buffer and reprecipitated with ethanol. The pellet was finally resuspended in 0.05 to 0.1 ml of TE buffer. The DNA concentration was approximately 0.1 mg/ml. Plasmid DNA from *E. coli* was prepared as described before (2).

Yeast transformation. A plasmid pool of yeast DNA sequences containing the *ADR1-5*^{c} allele was prepared as described previously (12). The yeast DNA pool containing the *ADR1* gene was provided by K. Nasmyth (12). *S. cerevisiae* strain transformations were conducted as described before (20). Yeast transformations after the initial selection omitted the antimycin A overlay step.

Agarose gels, blotting, and hybridization. DNA samples were cleaved with restriction enzymes obtained from Bethesda Research Laboratories and used as directed. DNA was electrophoresed through 0.7% agarose essentially as described previously (10). DNA was blotted to nitrocellulose filters (17) as described in reference 19. Hybridization conditions have been described before (10).

ADH assays. *S. cerevisiae* cell extracts were prepared and assayed for ADH activity and protein content as previously described (6) except for the following modifications: routinely, 2- or 2.5-ml cultures were washed once with water and resuspended in 0.2 ml of lysis buffer (85 mM KCl, 3 mM $MgOAc_2$,

30 mM Tris-hydrochloride, pH 7.5) to which 1 g of glass beads (diameter, 0.45 to 0.55 mm) was added, and the cells were broken by vortexing; after clarification, the supernatant was withdrawn and reclarified by centrifugation. The ADH isozymes were differentiated by polyacrylamide gel electrophoresis as described previously (20) except that the stacking gel consisted of 4% acrylamide.

Cloning DNA fragments. DNA fragments were ligated into plasmid vectors (YRp7 and YEp13) that had been pretreated with bacterial alkaline phosphatase (Worthington Diagnostics). Treatment with bacterial alkaline phosphatase involved incubating the vectors, cut previously with the appropriate restriction nuclease, for 40 min at 56°C in the following solution: 6 mM $ZnSO_4$, 6 mM EDTA, and 0.24 mg of bacterial alkaline phosphatase per ml. The DNA was purified by phenol-chloroform extraction and ethanol precipitation as described above. The ligated mixture was used to transform *E. coli* RR1 to ampicillin resistance. Ampicillin-resistant colonies were screened by colony hybridization as described before (1), using DNA fragments containing the *ADR1-5*^{c} probe. DNA fragments were isolated after separation by agarose gel electrophoresis of the restriction enzyme-cleaved plasmid DNA. The DNA was electroeluted and purified by DEAE-cellulose chromatography.

Other techniques. Tetrad analysis was carried out as described by Mortimer and Hawthorne (11). Cells in which plasmid had integrated into the yeast genome were isolated as previously described (12).

RESULTS

Isolation of the *ADR1-5*^{c} gene. A library of yeast DNA sequences from strain R234 (*adc1-11 ADR2-F ADR1-5*^{c} *adm*) was prepared in plasmid YRp7 as previously described (12). YRp7 consists of the *E. coli* vector pBR322 and 1.4 kilobases (kb) of DNA, which contains the yeast *TRP1* gene and an adjacent *ars* sequence. An *ars* sequence allows independent replication of the plasmid in yeast, and the *TRP1* gene allows complementation of a defective yeast *TRP1* gene. YRp7 is rapidly lost from the yeast cell under nonselective conditions (12), and the copy number of the plasmid for the cells which do carry it ranges from 10 to 60 copies per cell. DNA from strain R234 was partially digested with the restriction nuclease *Sau*3A, and fragments 2 to 20 kb in size were isolated after centrifugation through a sucrose gradient. These fragments were ligated into the yeast plasmid vector YRp7 which had been previously cut with the enzyme *Bam*HI.

Plasmids containing this pool of yeast DNA inserts in YRp7 were used to transform strain 500-16 (*adc1-11 ADR2-F adr1-1 trp1*). The yeast transformants putatively containing the *ADR1-5*^{c} gene in YRp7 were selected on glucose medium which lacked tryptophan and contained the respiratory inhibitor antimycin A. Strain 500-16 is unable to grow in the presence of antimycin A because it lacks ADH activity, whereas strain R234, which contains the *ADR1-5*^{c} allele and expresses ADHII during glucose repression, is able to grow in the presence of antimycin A. Of 10^5 TRP$^+$ transformants, 125 were capable of growing in the presence of antimycin A. Six different yeast transformants were isolated from this group which were able to grow on glucose medium containing antimycin A and from which a plasmid was capable of being isolated after transformation of *E. coli*. Of these six plasmids, one was found to carry sequences homologous to the *ADR2-F* (ADHII) gene (data not shown). The remaining five plasmids were further characterized by restriction site mapping, and only two of these showed any significant homology to each other in their restriction patterns (C. L. Denis, Ph.D. thesis, University of Washington, Seattle, 1982). These results indicate that several different yeast DNA sequences were able to complement the *adr1-1* allele when they were present in YRp7. Since only one of these five yeast transformants grew well in the presence of antimycin A, the plasmid isolated from this transformant was considered to be the one most likely to contain the *ADR1-5*^{c} gene. The restriction pattern of this plasmid, YRp7-ADR1-5^c-23, is shown in Fig. 1.

When this plasmid was used to retransform strain 500-16, a high frequency of antimycin A-resistant transformants resulted: 2,300 of 4,800 total TRP$^+$ transformants per μg of DNA were obtained. Plasmid DNA isolated from these new transformants was shown by restriction site analysis to be identical to plasmid YRp7-ADR1-5^c-23.

Phenotypic expression of plasmid YRp7-ADR1-5^c-23. The ADHII activity of cells transformed with YRp7-ADR1-5^c-23 is given in Table 2 (top line) and can be compared with that for cells containing a chromosomal copy of *ADR1-5*^{c} (bottom line). Cells transformed with YRp7-ADR1-5^c-23 contained high levels of ADHII activity during glucose repression which is consistent with the *ADR1-5*^{c} phenotype. The lack of higher ADHII activities after growth on ethanol-containing medium (derepression) is not understood. Transformed cells grown selectively on ethanol-containing minimal medium lacking tryptophan expressed no higher ADHII activities than when grown nonselectively (data not shown). The actual derepressed ADHII values after nonselective growth were sufficiently high over background (see Table 2) to still allow the presence of a functional *ADR1* gene on the plasmid to be ascertained (see below).

It had been shown previously that a single *ADR1-5*^{c} allele allows the expression of the *ADR2* locus in both parental chromosomes in a diploid strain (5). This ability to act in *trans* can

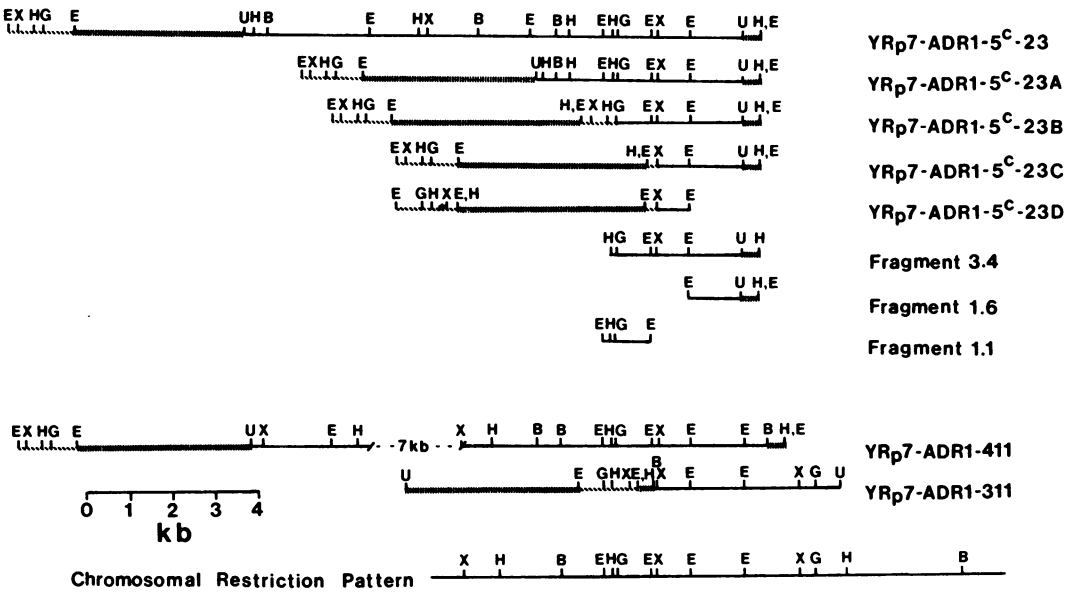

FIG. 1. Restriction enzyme cleavage sites on plasmids containing *ADR1-5*^{c} and *ADR1* alleles. Isolation of plasmids, restriction enzyme digestion, and agarose gel electrophoresis of restriction enzyme DNA fragments are described in the text. Chromosomal restriction patterns for the enzymes *Xba*I, *Hin*dIII, and *Bam*HI were determined by appropriate double digests as described in the legend to Fig. 3 and in the text. The sizes of the *Eco*RI and *Bgl*II fragments were determined as described in the Fig. 3 legend, but their placement was determined by correspondence to the restriction patterns of plasmids YRp7-ADR1-411 and YRp7-ADR1-311. The difference in *Bam*HI sites between plasmid YRp7-ADR1-411 and the chromosomal restriction pattern was due solely to using a probe that could not detect the additional *Bam*HI fragment at the chromosomal location. Enzyme *Eco*RI cuts YRp7-ADR1-411 at additional, undetermined sites in the region of the leftmost two *Bam*HI sites. The cleavage sites of the restriction nucleases are designated as follows: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; X, *Xba*I; G, *Bgl*II; U, *Sau*3A. The DNA sequences are as follows: ——, yeast DNA; ||||||||, pBR322; \\\\\\\\, *TRP1*.

be demonstrated by observing the synthesis of both electrophoretic variants ADHII-F and ADHII-S in diploid cells grown on glucose-containing medium. To test whether plasmid YRp7-ADR1-5^c-23 displayed this property, the transformant, 500-16/YRp7-ADR1-5^c-23, was mated with a strain carrying the gene for ADHII-S, CH1-50D (*adc1-11 ADR2-S adr1-1*), but defective in ADHII-S activity due to the *adr1-1* allele. Extracts were prepared from this diploid after growth on glucose-containing medium, and the ADH isozymes were identified by polyacrylamide gel electrophoresis. The results are presented in Fig. 2. Lanes a and b contained extracts from strains 11-13C (ADHII-S) and 43-2B (ADHII-F), respectively, grown on medium containing ethanol. Lane c shows that ADHII-F enzyme activity is present in the transformed strain 500-16/YRp7-ADR1-5^c-23 after growth on glucose-containing medium. Lane d displays the pattern for the ADH activity present in the diploid, CH1-50D × 500-16/YRp7-ADR1-5^c-23. A typical heterotetrameric electrophoretic pattern was observed, indicating that both ADHII-S and ADHII-F were synthesized. For the diploid formed between CH1-50D and 500-16 transformed with the plasmid containing the *ADR2-F* gene (see above), only ADHII-F was synthesized (data not shown). These data support the contention that the YRp7-ADR1-5^c-23 plasmid contained a gene which was *trans*-acting to the *ADR2* locus.

Integration of a subclone of YRp7-ADR1-5^c-23 at the *adr1-1* gene locus. To prove that YRp7-ADR1-5^c-23 contained sequences encoding the *ADR1-5*^{c} allele, transformants were selected in which the plasmid carrying the *ADR1-5*^{c} phenotype had integrated into the chromosome. Since YRp7-ADR1-5^c-23 appeared to contain at least two noncontiguous segments of yeast DNA (due to ligation of two different *Sau*3A fragments during the preparation of the DNA library; see below), a smaller DNA segment was sought which conferred the *ADR1-5*^{c} phenotype and lacked the noncontiguous DNA fragment. Such a plasmid could integrate only at *ADR1* or *TRP1*. YRp7-ADR1-5^c-23 was cut with the enzyme *Bam*HI and religated. This resulted in plasmid

TABLE 2. ADHII activities for cells transformed with plasmids[a]

Transformed strain	Relevant genotype	Plasmid	ADHII activity (mean, mU/mg)[b]		% Cells with plasmid
			Glucose	Ethanol	
500-16	*adr1-1*	YRp7-ADR1-5^c-23	540 (510–570)	250 (230–270)	NT[c]
500-16	*adr1-1*	YRp7-ADR1-5^c-23A	200 (86–300)	370 (220–620)	8
500-16	*adr1-1*	YRp7-ADR1-5^c-23B	220 (150–350)	360 (130–740)	7
500-16	*adr1-1*	YRp7-ADR1-5^c-23C	29 (24–43)	440 (370–520)	14
500-16	*adr1-1*	YRp7-ADR1-5^c-23D	8 (4–18)	12 (8–19)	15
500-16	*adr1-1*	YRp7-ADR1-311	9 (6–15)	390 (310–450)	2
500-11	*adr1-1*	YRp7-ADR1-311	13 (7–18)	340 (240–350)	7
500-11	*adr1-1*	YRp7-ADR1-411	19 (11–28)	340 (160–530)	7
500-16	*adr1-1*	None	4	19	
500-11	*adr1-1*	None	10	51	
43-2B	*ADR1*	None	20	2000	
R234	*ADR1-5^c*	None	350	5400	

[a] ADHII activities were determined after growing cells overnight on YD medium supplemented with either 8% glucose or 3% ethanol. Each value represents the average of 4 to 10 determinations except for YRp7-ADR1-5^c-23, whose values are the average of two determinations.

[b] Range is given in parentheses.

[c] NT, Not Tested.

YRp7-ADR1-5^c-23A which was identical to the larger plasmid except for loss of the 4.9- and a 1.8-kb internal *Bam*HI section (Fig. 1). This plasmid still contains about 1 kb of DNA which is not colinear with the chromosomal restriction pattern (Fig. 1; see below). YRp7-ADR1-5^c-23A was able to transform a strain carrying the *adr1-1* allele (500-16) such that it became antimycin A resistant at the same high frequency (1,000 antimycin A transformants of 2,200 TRP$^+$ transformants per μg of DNA) as plasmid YRp7-ADR1-5^c-23 was capable of transforming strain 500-16. The ADHII activity of cells transformed with YRp7-ADR1-5^c-23A is given in Table 2 and is essentially the same as the ADHII activity in cells transformed with YRp7-ADR1-5^c-23. From the yeast transformant 500-16/YRp7-ADR1-5^c-23A, an isolate was obtained which was stable for both the TRP$^+$ and the antimycin A resistance phenotypes. This stable transformant resulted from an integration event in which specific homologous recombination occurred presumably between the *ADR1-5^c* gene on the plasmid and the chromosomal *adr1-1* site, between the *TRP1* gene on the plasmid and the *trp1* chromosomal locus, or between the 1 kb of DNA that is noncontiguous with *ADR1* on the chromosome and its chromosomal site. To test genetically whether this integration had occurred at the *adr1-1* site, the strain containing the stable integrant was crossed with a strain containing the wild-type *ADR1* gene (Table 3). The meiotic products of this cross were subsequently analyzed to determine the amount of meiotic recombination, and hence genetic distance, between the integrated *ADR1-5^c* allele and the *adr1-1* allele. Meiotic recombination between these two loci would give rise to a haploid with the *adr1-1* phenotype. Table 3 presents genetic evidence from this cross which indicates that plasmid YRp7-ADR1-5^c-23A had integrated very near the *adr1-1* locus. Of 58 tetrads analyzed in three separate crosses, none showed a meiotic recombination event between

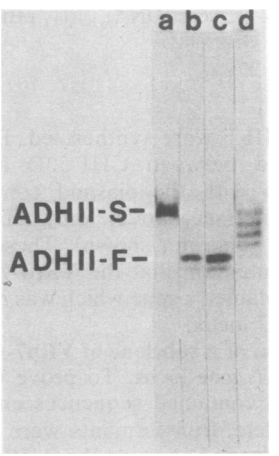

FIG. 2. Polyacrylamide gel identifying the ADH isozymes present in strains transformed with YRp7-ADR1-5^c-23A. Extract preparation, polyacrylamide gel electrophoresis, and staining for ADH activity are described in the text. (Lane a) Strain 11-13C (*ADR2-S*) grown on medium containing ethanol; (lane b) strain 43-2B (*ADR2-F*) grown on medium containing ethanol; (lane c) transformant 500-16/YRp7-ADR1-5^c-23A (*ADR2-F adr1-1*) grown on glucose-containing medium; (lane d) transformant 500-16/YRp7-ADR1-5^c-23A × CH1-50D (*ADR2-S adr1-1*) grown on glucose-containing medium.

TABLE 3. Genetic linkage of integrated plasmids[a]

Integrated plasmid	Gene pair	Ascus type (no.)			Maximum map distance (centi morgan)
		PD	NPD	T	
YRp7-ADR1-5^c-23A[b]	*ADR1-5^c-adr1-1*	24	0	0	2.0
YRp7-ADR1-5^c-23A[c]	*ADR1-5^c-adr1-1*	15	0	0	3.1
YRp7-ADR1-5^c-23A[b]	*TRP1-adr1-1*	24	0	0	2.0
YRp7-ADR1-5^c-23A[d]	*ADR1-5^c-adr1-1*	18	0	0	2.6
YRp7-ADR1-411[e]	*ADR1-adr1-1*	8	0	0	5.6
YRp7-ADR1-411[e]	*TRP1-adr1-1*	7	0	1	6.2
None[f]	*TRP1-ADR1*	1	3	16	>50

[a] Those cells which contained the *ARD1* gene were detected by being able to grow on YD plates containing antimycin A after replication from YD plates but unable to grow on YD plates containing antimycin A after replication from YD plates whose glucose concentration had been increased to 8%. PD, Parental ditype; NPD, nonparental ditype; T, tetratype tetrads.

[b] Test cross: 23-17 (*trp1 adr1-1*) (500-16::YRp7-ADR1-5^c-23A) × 502-32 (*ADR1 trp1*).

[c] Test cross: 23-17 × 200-6 (*ADR1*).

[d] Test cross: 23-17 × 530-5 (*ADR1-5^c trp1*).

[e] Test cross: 411 (500-11::YRp7-ADR1-411) (*trp1 adr1-1*) × 530-13 (*ADR1-5^c trp1*).

[f] From several crosses.

the *ADR1-5^c* allele and the *adr1-1* allele; i.e., no meiotic products displayed the *adr1-1* phenotype. Sixteen of these progeny containing the *ADR1-5^c* allele were analyzed for ADHII activity, and in all cases they contained activity nearly commensurate with the activity from a strain containing a chromosomal copy of *ADR1-5^c* (Table 4). From one of these test crosses the meiotic products were scored for linkage of the *TRP1* gene, present on YRp7-ADR1-5^c-23A, to that of the *ADR1-5^c* integrant. In all 24 tetrads analyzed, the TRP$^+$ phenotype segregated with the *ADR1-5^c* allele, whereas the chromosomal *TRP1* gene is unlinked to *ADR1* (Table 3, bottom line).

The map distance between the integrated *ADR1-5^c* allele and the *adr1-1* allele was estimated to be not more than 2.0 centimorgans. Based on current estimates of the relationship between map distance and kilobases of DNA (16), the *ADR1-5^c* allele carried by plasmid YRp7-ADR1-5^c-23A had integrated at a maximum of 4 to 10 kb from the *adr1-1* locus. This is consistent with the expected distance after an integration event at the *adr1-1* locus for a plasmid of this size.

If plasmid YRp7-ADR1-5^c-23A had integrated at the *adr1-1* locus, then plasmid DNA should be present at that site. The DNA from three of the tetrads described above was analyzed for pBR322 sequences. The DNA was cut with the restriction enzyme *Bam*HI and analyzed by Southern transfer analysis, using hybridization to nick-translated pBR322. In all cases, only the strains carrying the *ADR1-5^c* integrant displayed a segment about 10 kb in size which hybridized to pBR322 sequences. One such tetrad analysis is shown in Fig. 3. The expected size for this segment would be about 10 kb if the whole of plasmid YRp7-ADR1-5^c-23A had integrated into the yeast genome. These results imply that detectable loss of DNA from plasmid YRp7-ADR1-5^c-23A had not occurred when it integrated at the *adr1-1* locus.

Isolation and characterization of the *ADR1* gene. To compare the isolated *ADR1-5^c* allele with the wild-type *ADR1* gene and to compare the phenotypes of strains transformed with the respective alleles, the *ADR1* gene was isolated. A library of yeast plasmids containing the *ADR1* gene was grown in *E. coli* and screened by

TABLE 4. ADHII activities of integrated plasmids[a]

Strain	Relevant genotype	ADHII activity (mU/mg)					
		Glucose			Ethanol		
		Mean	SD	*n*	Mean	SD	*n*
Segregants[b]	*adr1-1::YRp7-ADR1-5^c-23A*	230	77	8	3,760	1,920	14
422	*adr1-1::YRp7-ADR1-5^c-23B*	180	(170–190)	3	3,860	(3,700–3,900)	3
313	*trp1::YRp7-ADR1-311 adr1-1*	9	10	9	850	270	9
Segregants[c]	*adr1-1::YRp7-ADR1-411*	27	(6–51)	6	3,020	(1,300–4,200)	6

[a] ADHII activities were measured as described in Table 2, footnote *a*.

[b] Segregants were derived from the crosses 23-17 × 502-32 and 23-17 × 200-6.

[c] Segregants were derived from the cross 411 × 530-13.

colony hybridization, using nick-translated segments of YRp7-ADR1-5^c-23A as specific hybridization probes (21). Of 5,000 colonies screened, 2 hybridized strongly to the probe. Plasmid DNA prepared from these two colonies was characterized by restriction site mapping and hybridization to segments of YRp7-ADR1-5^c-23A. Both of the resultant plasmids, YRp7-ADR1-311 and YRp7-ADR1-411, contained restriction site homology to YRp7-ADR1-5^c-23A (Fig. 1). In addition, the expected restriction fragments of YRp7-ADR1-311 and YRp7-ADR1-411 hybridized strongly to the corresponding segment from YRp7-ADR1-5^c-23A (Denis, Ph.D. thesis). These results support the alignment of the two plasmids shown in Fig. 1. They also indicate that the *ADR1-5^c* and *ADR1* alleles are identical within the limit of the above analysis. This suggests that the *ADR1-5^c* mutation is a point mutation. At most, the *ADR1* and *ADR1-5^c* alleles differ by about 30 bases, the limit for determining by agarose gel electrophoresis the sizes of the *Eco*RI fragments of the two plasmids.

Transformation of *S. cerevisiae* strains carrying the *adr1-1* allele with plasmids YRp7-ADR1-311 and YRp7-ADR1-411 resulted in equivalent amounts of ADHII activity (Table 2). The ADHII activity was repressed after growth on medium containing glucose but became derepressed after growth on medium containing ethanol. The low ADHII activity during growth on glucose-containing medium is consistent with the phenotype expected for the *ADR1* gene as compared with that found for the *ADR1-5^c* allele (Table 2). The similarity of the activities for cells transformed with YRp7-ADR1-311 and YRp7-ADR1-411 despite the much larger size of YRp7-ADR1-411 suggests that the maximum amount of DNA required for expressing *ADR1* resides in the overlap region between the two plasmids. This region corresponds to about 2.6 kb of DNA.

Further proof that a functionally intact *ADR1* gene was present on YRp7-ADR1-411 was obtained by isolating transformants which had become stable for the TRP$^+$ phenotype after integration of the plasmid at the *adr1-1* allele (see Table 3). This integrant, *adr1-1::YRp7-ADR1-411*, had an ADHII phenotype during glucose repression and derepression that was identical to a strain with a chromosomal copy of the *ADR1* gene (see Table 4). Another stable transformant was found in which plasmid YRp7-ADR1-311 was integrated at the *trp1* allele (Denis, Ph.D. thesis). This integrant, *trp1::YRp7-ADR1-311 adr1-1*, was capable of derepressing ADHII during growth on medium containing ethanol (Table 4). However, the degree of derepression was only one-half to one-third of that found in a strain carrying the wild-type *ADR1* gene, such as strain 43-2B (see Table 2). This low derepression could result from either an effect on *ADR1* expression by its presence at the *trp1* locus or a lack of sequences required for normal *ADR1* expression.

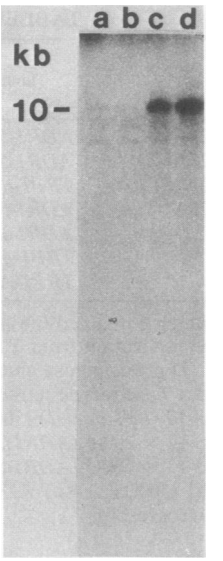

FIG. 3. Hybridization patterns of genomic DNA from strains carrying the *adr1-1::YRp7-ADR1-5^c-23A* locus. Preparation of DNA, restriction site analysis, Southern gel analysis, and conditions for hybridization to nick-translated pBR322 are described in the text. DNA from plasmid YRp7-ADR1-5^c-23A digested with *Bam*HI was used as the molecular weight marker. The DNA in lanes a to d was isolated from each of the progeny colonies derived from one complete tetrad of the cross 23-17 (*adr1-1::YRp7-ADR1-5^c-23A*) × 200-6 (*ADR1*). Each of the DNAs was cut with the restriction enzyme *Bam*HI. (Lane a) Strain 505-9 (*ADR1*); (lane b) strain 505-10 (*ADR1*); (lane c) strain 505-11 (*adr1-1::YRp7-ADR1-5^c-23A*); (lane d) strain 505-12 (*adr1-1::YRp7-ADR1-5^c-23A*).

Restriction site mapping of the genomic *ADR1* region. The chromosomal *ADR1* region was mapped by restriction site analysis to determine whether the plasmid DNA sequences corresponded to the chromosomal DNA. Various internal segments of plasmid YRp7-ADR1-5^c-23A were used as hybridization probes to map the genomic region around the *ADR1* gene. DNA from two strains containing the wild-type *ADR1* gene was cut with several restriction nucleases, and the fragments were subjected to Southern transfer analysis. Figure 4 depicts an autoradiogram of several such digestions and analyses. The nick-translated probe in this case was the 3.4-kb *Hin*dIII fragment (fragment 3.4) from YRp7-ADR1-5^c-23A. With each restriction nuclease, only the expected number of hybrid-

ization bands appeared, and their sizes corresponded to the restriction site map of the isolated plasmids. That is, when the genomic DNA was cut with *Xba*I only two fragments resulted which hybridized to the probe (Fig. 4, lanes a and b); *Eco*RI digestion produced three small fragments of the same size as those previously found on the isolated plasmids (lanes c and d), and a *Hin*dIII digestion produced only one band (lanes e and f). These results indicate that the region on fragment 3.4 corresponds directly to a segment of genomic DNA. These results also show that only one copy of the *ADR1* gene exists in the yeast cell, for otherwise other hybridization bands would have been observed. From these data and similar analyses with other restriction enzymes the genomic restriction pattern shown in Fig. 1 was deduced. This pattern corresponds to that found for plasmids YRp7-ADR1-311 and YRp7-ADR1-411. Partial characterization of the restriction sites surrounding the chromosomal *adr1-1* and *ADR1-5*^{c} alleles resulted in the same restriction pattern as for the *ADR1* region (data not shown). Because the plasmid YRp7-ADR1-5^c-23 restriction site map differs significantly from the genomic pattern leftward of the *Hin*dIII site which is adjacent to the *Bgl*II site, plasmid YRp7-ADR1-5^c-23 was probably formed by the insertion of two noncontiguous yeast DNA segments into YRp7. This is supported by Southern analysis of *Hin*dIII digestions of genomic DNA from strains carrying either the *ADR1* or the *ADR1-5*^{c} allele. In neither case did a 1.0-kb *Hin*dIII fragment of genomic DNA, corresponding to the 1.0-kb *Hin*dIII fragment of either YRp7-ADR1-5^c-23 or -23A, hybridize to nick-translated YRp7-ADR1-5^c-23A (data not shown). Therefore, the juncture of the two noncontiguous yeast DNA segments must be within the 1.0-kb *Hin*dIII fragment of plasmid YRp7-ADR1-5^c-23.

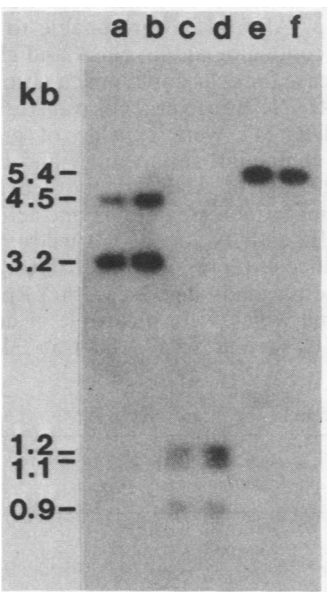

FIG. 4. Hybridization patterns of genomic DNA in the *ADR1* region. Restriction nuclease cleavage, agarose gel electrophoresis, and conditions for hybridization are described in the text. Nick-translated fragment 3.4 was used as the hybridization probe. Genomic DNA shown in lanes a and b was cut with restriction nuclease *Xba*I; genomic DNA shown in lanes c and d was cut with the restriction enzyme *Eco*RI; and genomic DNA shown in lanes e and f was cut with the restriction nuclease *Hin*dIII. (Lanes a, c, and e) DNA from strain 11-13C (*ADR1*); (lanes b, d, and f) DNA from strain 79-72C (*ADR1*).

Identification of the *ADR1* functional region on plasmid YRp7-ADR1-5^c-23A. To delineate the location of the *ADR1* functional region on plasmid YRp7-ADR1-5^c-23A, various plasmids were prepared that contained only part of the yeast insert from plasmid YRp7-ADR1-5^c-23A. These plasmids were created by excising sections of YRp7-ADR1-5^c-23A and inserting them into the plasmid vector YRp7. They were then used to transform a strain carrying the *adr1-1* allele from a TRP$^-$ to a TRP$^+$ phenotype. The amount of ADHII activity in the transformants was determined after growth on glucose- or ethanol-containing medium. Figure 1 gives the restriction site pattern for each of the newly created plasmids. Table 2 provides the ADHII activities of cells transformed with the various plasmids. For comparison, the activities in untransformed yeast strains containing the three relevant alleles of *ADR1* are given.

Plasmid YRp7-ADR1-5^c-23B was created by excising the 4.1-kb *Bgl*II segment from YRp7-ADR1-5^c-23A and ligating it into YRp7 cut at its single *Bgl*II site. The DNA insert in plasmid YRp7-ADR1-5^c-23B is a subset of fragment 3.4 which was shown above to contain one contiguous piece of yeast DNA. Cells transformed with YRp7-ADR1-5^c-23B had the same amount of ADHII activity as did cells transformed with YRp7-ADR1-5^c-23A (Table 2). The function of YRp7-ADR1-5^c-23B was further analyzed after a transformant was isolated which was stable for both TRP$^+$ and antimycin A-resistant phenotypes. Plasmid YRp7-ADR1-5^c-23B was shown to have integrated at or near the *adr1-1* allele by crossing the strain with the integrated plasmid YRp7-ADR1-5^c-23B to a strain carrying the wild-type *ADR1* gene (Denis, Ph.D. thesis). The ADHII activity of the strain with the *adr1-1::YRp7-ADR1-5^c-23B* locus was nearly identical to that for a strain carrying a chromosomal copy of the *ADR1-5*^{c} allele (Table 4).

Further subcloning of YRp7-ADR1-5^c-23A into the YRp7 plasmid resulted in partial or

complete loss of *ADR1* activity. Plasmid YRp7-ADR1-5^c-23C was formed by ligating the 2.5-kb *Xba*I segment of YRp7-ADR1-5^c-23C into the *Xba*I site of YRp7. Plasmid YRp7-ADR1-5^c-23C has about 1.0 kb less DNA than plasmid YRp7-ADR1-5^c-23B (Fig. 1). When cells transformed with YRp7-ADR1-5^c-23C were grown on glucose-containing medium, they expressed about one-tenth the ADHII activity as did cells transformed with YRp7-ADR1-5^c-23B (Table 2). Upon growth on ethanol-containing medium, cells transformed with YRp7-ADR1-5^c-23C expressed an ADHII activity comparable to that for cells transformed with YRp7-ADR1-5^c-23B. Since YRp7-ADR1-5^c-23C allowed the derepression of ADHII, this plasmid presumably contained at least the functional segment for the *ADR1* gene. Plasmid YRp7-ADR1-5^c-23D, which contains only the 0.7-kb *Xba*I-*Eco*RI segment of the yeast insert in YRp7-ADR1-5^c-23C, no longer contained a functional *ADR1* gene. When strain 500-16 was transformed with it, the resultant ADHII activity was no greater than the ADHII activity present in untransformed 500-16 (Table 2).

Fragments 1.1 and 1.6 derived from YRp7-ADR1-5^c-23A (see Fig. 1) were separately subcloned into plasmid YEp13 (which contains the yeast *LEU2* gene, 2μ yeast DNA segments, and pBR322). When strain 500-11 was transformed with these plasmids, the transformed cells displayed ADHII activities that were no greater than the activity found in untransformed 500-11 (Denis, Ph.D. thesis). However, when fragment 3.4 (from YRp7-ADR1-5^c-23A) was subcloned into YEp13, cells transformed with this plasmid allowed ADHII to be expressed during growth on glucose-containing medium (50 mU/mg) and on ethanol-containing medium (2,000 mU/mg).

From these results the smallest functional *ADR1-5^c* gene sequence is that which is represented on plasmid YRp7-ADR1-5^c-23C. This region contains 1.9 kb of yeast DNA and is a subset of the DNA region which is common to both plasmids YRp7-ADR1-411 and YRp7-ADR1-311.

Only about 2 to 15% of the cells transformed with the YRp7 plasmids contained plasmid at the time of assaying for ADHII activity due to the loss of the YRp7 plasmid by nonselective growth (Table 1). By growing the transformed cells in the presence of antimycin A, continuous selection for ADH-positive cells could be maintained. When cells transformed with plasmid containing the *ADR1-5^c* allele were grown in medium containing glucose and antimycin A, they expressed about fivefold-greater ADHII activity than transformed cells grown without antimycin A (Table 5; cf. Table 2). Cells transformed with plasmids YRp7-ADR1-5^c-23A and YRp7-ADR1-5^c-23B when grown in the presence of antimycin A expressed two- to fourfold more ADHII activity than did cells containing a chromosomal copy of *ADR1-5^c* (Table 5). This result suggests that ADHII expression is limited by the number of copies of the *ADR1-5^c* gene. Cells transformed with YRp7-ADR1-411 were unable to grow on medium containing antimycin A and glucose as was the case for cells containing a chromosomal copy of *ADR1*. However, cells transformed with YRp7-ADR1-311 were capable of growth on medium containing antimycin A and contained about 120 mU of ADHII activity per mg, an order of magnitude greater enzyme activity than the ADHII activity found in a wild-type strain during glucose repression (cf. Tables 2 and 5). All of the plasmids derived from YRp7-ADR1-5^c-23A and which were incapable of derepressing ADHII, such as YRp7-ADR1-5^c-23D and the

TABLE 5. ADHII activities for strains grown in presence of antimycin A[a]

Transformed strain	Plasmid	ADHII activity (mean, mU/mg)[b]	% Cells with plasmid
500-16	YRp7-ADR1-5^c-23A	760 (670–1,050)	88
500-16	YRp7-ADR1-5^c-23B	1,190 (900–1,570)	100
500-16	YRp7-ADR1-5^c-23C	220 (170–280)	100
500-16	YRp7-ADR1-5^c-23D	No growth	
500-16	YRp7-ADR1-411	No growth	
500-16	YRp7-ADR1-311	100 (69–148)	95
500-16 (*adr1-1*)	None	No growth	
43-2B (*ADR1*)	None	No growth	
R234 (*ADR1-5^c*)	None	340 (280–430)	

[a] Activities were determined as described in Table 2, footnote *a*. Antimycin A was added to the concentration of 0.1 μg per ml of culture. All transformed strains were tested for TRP$^+$ and antimycin A resistance phenotypes after growth on nonselective media. All strains were unstable for both phenotypes, indicating that integration had not occurred before or during growth on antimycin A-containing media.

[b] Glucose + antimycin A; range is given in parentheses.

YEp13 plasmids into which fragments 1.1 and 1.6 had been subcloned, were unable to grow on YD medium containing antimycin A (Table 5; data not shown). These results confirm that the smallest functional segment capable of expressing *ADR1* function is 1.9 kb as represented on plasmid YRp7-ADR1-5^c-23C.

DISCUSSION

The results reported here describe the isolation of recombinant plasmids containing the *ADR1* and *ADR1-5^c* alleles. The *ADR1-5^c* allele was shown to be present on plasmids YRp7-ADR1-5^c-23A and YRp7-ADR1-5^c-23B by isolating stable transformants in which these two plasmids had integrated near the *adr1-1* locus. Since integration of a plasmid at a particular site occurs by recombination between homologous sequences (14), this is strong evidence that the plasmids contained the *ADR1-5^c* allele. The *ADR1* gene was shown to be present on plasmids YRp7-ADR1-411 and YRp7-ADR1-311 by hybridization of this plasmid to DNA fragments present on plasmid YRp7-ADR1-5^c-23A, by sharing restriction site homology to YRp7-ADR1-5^c-23A, and by the integration of YRp7-ADR1-411 at the *adr1-1* locus. Furthermore, the ADHII phenotypes of cells transformed with the plasmids containing the *ADR1-5^c* and *ADR1* alleles after growth on medium containing glucose were consistent with the ADHII phenotype of the respective chromosomal alleles from which the plasmids were derived.

The procedure for isolating the *ADR1-5^c* allele also selected for several other plasmids carrying DNA sequences capable of complementing the *adr1-1* allele and which did not carry either the ADHI or the ADHII structural gene. Since no other gene in the DNA from the original donor strain (R234) should have been capable of complementing the *adr1-1* allele, these phenotypes of the cloned DNAs in transformed cells could have arisen by mutation of these gene loci during the plasmid library preparation, by activation of the yeast insert due to its position on the plasmid, or by the presence of the DNA insert in multiple copies in the cell. Because two of these plasmids retained their ability to complement the *adr1-1* allele after integration into the yeast genome, the first two of these possibilities are preferred (unpublished data).

The *ADR1* and *ADR1-5^c* DNA segments contained extensive restriction site homology. Partial restriction site mapping of the chromosomal *ADR1-5^c* and *adr1-1* alleles indicated that both shared restriction site homology with the chromosomal copy of the *ADR1* gene. These results indicate that the *ADR1-5^c* and *adr1-1* mutations did not arise from gross rearrangements of the *ADR1* gene or its surrounding DNA regions. These mutations probably represent point mutations or small insertions or deletions. The *ADR1-5^c* allele did not contain any repetitive yeast elements since fragment 3.4 hybridized to the expected number of genomic DNA fragments.

The smallest DNA fragment capable of derepressing ADHII activity was 1.9 kb. This fragment of DNA as represented on plasmid YRp7-ADR1-5^c-23C is a subset of the 2.6-kb fragment of DNA which was shown to be sufficient for ADHII derepression in the plasmids containing the *ADR1* gene. Whereas plasmid YRp7-ADR1-5^c-23C expressed *ADR1* function, it did so to a significantly lower degree during the growth on medium containing glucose than did plasmids YRp7-ADR1-5^c-23A and -23B. The latter plasmid carried about 1.0 kb more yeast DNA than was present on plasmid YRp7-ADR1-5^c-23C. YRp7-ADR1-5^c-23B was also shown to carry the *ADR1-5^c* mutation, for when it integrated at the *adr1-1* locus, the resultant strain gave rise to ADHII activities during glucose repression and derepression that were nearly equivalent to those found for a chromosomal copy of the *ADR1-5^c* allele. Whether the site of the *ADR1-5^c* mutation lies within the yeast insert of plasmid YRp7-ADR1-5^c-23C cannot be determined. The lack of comparable ADHII expression for cells transformed with YRp7-ADR1-5^c-23C compared with YRp7-ADR1-5^c-23B can be explained by a deficient expression of the *ADR1* gene or by a lack of sequences containing the *ADR1-5^c* mutation.

The isolation of the *ADR1* gene allows the development of a specific assay for *ADR1* mRNA. However, due to very low abundance of mRNAs which share homology to the *ADR1* cloned DNA and to the presence of overlapping RNA transcripts in the *ADR1* functional region, the *ADR1* mRNA was not positively identified (Denis, Ph.D. thesis). No difference in the amounts of any of the transcripts was observed when the RNA from a strain carrying the *ADR1* gene was compared with RNA from a strain carrying the *ADR1-5^c* allele. Sequencing of the *ADR1* functional region and attachment of this region to a promoter which would allow higher levels of *ADR1* gene products to be made in the cell will be helpful in detecting the *ADR1* mRNA and polypeptide.

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