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Isolation and DNA Sequence of *ADH3*, a Nuclear Gene Encoding the Mitochondrial Isozyme of Alcohol Dehydrogenase in *Saccharomyces cerevisiae*

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The *Saccharomyces cerevisiae* nuclear gene, *ADH3*, that encodes the mitochondrial alcohol dehydrogenase isozyme ADH III was cloned by virtue of its nucleotide homology to *ADH1* and *ADH2*. Both chromosomal and plasmid-encoded ADH III isozymes were repressed by glucose and migrated heterogeneously on nondenaturing gels. Nucleotide sequence analysis indicated 73 and 74% identity for *ADH3* with *ADH1* and *ADH2*, respectively. The amino acid identity between the predicted ADH III polypeptide and ADH I and ADH II was 79 and 80%, respectively. The open reading frame encoding ADH III has a highly basic 27-amino-acid amino-terminal extension relative to ADH I and ADH II. The nucleotide sequence of the presumed leader peptide has a high degree of identity with the untranslated leader regions of *ADH1* and *ADH2* mRNAs. A strain containing a null allele of *ADH3* did not have a detectably altered phenotype. The cloned gene integrated at the *ADH3* locus, indicating that this is the structural gene for ADH III.

The alcohol dehydrogenase (ADH) isozymes of *Saccharomyces cerevisiae* represent a functionally diverse enzyme family. ADH I, the classical fermentative isozyme, is responsible for the last step in the yeast glycolytic pathway, the reduction of acetaldehyde to ethanol (23, 28). ADH II, the oxidative isozyme, is highly repressed by fermentative growth and is derepressed in the absence of a fermentable sugar such as glucose. *ADH2*, the structural gene for ADH II, is regulated at the transcriptional level by catabolite repression through a specific positive effector encoded by the *ADR1* gene (8, 10). The function of ADH II in the cell is to oxidize ethanol, formed during fermentation, to acetaldehyde, which can then be metabolized via the tricarboxylic cycle in the mitochondria and also serves as an intermediate in gluconeogenesis.

ADH III was discovered by Lutsdorf and Megnet (23) and was shown to purify with a particulate fraction from *S. cerevisiae*. Sugar et al. (35) further characterized this activity and confirmed its location in the mitochondrion. Wills and Phelps (45) obtained evidence that the size of the native enzyme was the same as that of ADH I. Despite the same apparent molecular weight, the native ADH III enzyme migrates in an unusual pattern on nondenaturing polyacrylamide gels, suggesting charge heterogeneity on the subunits or formation of heterotetramers synthesized by two different genes or both.

The metabolic function of ADH III is unknown, but indications are that it does not function fermentatively: strains containing ADH III as their only ADH isozyme cannot survive as petites, which must obtain their energy through fermentation (45). A role in respiratory metabolism is thus suggested, which would be consistent with a mitochondrial location. Molecular cloning of the structural gene for ADH III would aid in elucidating its structure and function. The structural genes encoding ADH I and ADH II, *ADH1* and *ADH2*, respectively, have been cloned and sequenced (3, 30, 41, 42), allowing comparisons to be made between the two genes, whose amino acid sequences were already known (18, 44).

The isolation of *ADH3*, the gene encoding ADH III, would allow a comparison of the amino acid sequence of the three isozymes, whose functional roles and cellular locations differ. It is not known whether all three isozymes derive from a common ancestral gene. A comparison of the nucleotide sequences of the three genes might provide some insight into their evolution and the role and cellular location of the progenitor of the three yeast isozymes. The availability of the structural gene would allow it to be overexpressed by using a multicopy yeast plasmid. This would simplify purifying the protein to determine the possible modifications on the enzyme, make it feasible to ultimately prepare sufficient quantities of enzyme for structural comparisons between the three yeast isozymes, and allow a study of the kinetic parameters of the enzyme to infer its metabolic role. Since the ADH III isozyme is apparently transported into mitochondria, characterization of the gene and protein would allow this process to be studied. The ADH III protein has several potential advantages for this work: the structure of yeast ADH I is thought to be very similar to that of horse liver ADH (19, 20), and therefore structural inferences about the position of the residues implicated in mitochondrial transport could be drawn; and the possibility of selecting for the presence or absence of ADH activity allows mutations and their suppressors to be isolated (43), which should be useful in isolating and characterizing yeast strains altered in mitochondrial transport without nonselective screening (47).

MATERIALS AND METHODS

Strains. The strains of *S. cerevisiae* used in this study are listed in Table 1.

Media, cell growth, and tetrad analysis. Yeast cells were grown with shaking in liquid medium at 30°C as previously described (34, 42). Glucose, glycerol, or glycerol-lactate was used as the carbon source. Yeast asci were dissected with a micromanipulator as described previously (34).

Plasmid construction, DNA manipulation, and yeast transformation. DNA fragment purification, restriction endonu-

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TABLE 1. *S. cerevisiae* strains used

Strain	Relevant genotype	Source or comments
301.10a	**a** *adh1-11 adh2-40 leu2 his4 trp2*	This laboratory
302-21	α *adh1-11 adh2-43 adh3 his4 trp1*	This laboratory
701.1	**a**/α *adh1-11 adr1-1 his4/HIS4 leu2/LEU2 trp1 ura1*	Diploid between 500-11 and 500-16, this laboratory
900-17	**a** *adh1-Δ1 adh2-601 ade2 trp1 ura1*	Derivative of D7aa (40)
900-17-1A	**a** *adh1-Δ1 adh2-601 adh3 ade2 trp1 ura1*	Selected from 900-17 as resistant to allyl alcohol
900-17-101a	**a** *adh1-Δ1 adh2-601 adh3::TRP1 ade2 ura1*	This work
SF4-1B	α *adh2-43 adh3 his4*	M. Ciriacy

clease digestion, cloning, and yeast transformation procedures have been described previously (17, 43).

Southern and colony hybridization analyses. Total chromosomal DNA was isolated from lysed spheroplasts and analyzed as described previously (42). Colony hybridization was performed on 3 MM paper by standard techniques (14) with nick-translated or end-labeled DNA probes (27).

ADH activity analysis. Cell extracts were prepared from log-phase yeast cells lysed with glass beads and assayed for ADH activity with ethanol and NAD^+ as the susbtrates (11).

Nondenaturing polyacrylamide gels. Nondenaturing polyacrylamide gels were prepared and stained for enzyme activity as previously described (13, 41).

DNA sequence analysis. (i) Dideoxynucleotide sequencing. Restriction enzyme fragments of ADH3 were ligated into vector mp8 or mp9 cleaved with the appropriate endonuclease. Dideoxy sequencing reactions with a synthetic M13-specific 12-base primer (Collaborative Research, Inc.) and DNA polymerase I (Klenow fragment; New England Nuclear Corp.) were performed as previously described (32).

(ii) Chemical sequencing. Restriction enzyme fragments of *ADH3* were end labeled by filling in and sequenced by the method of Maxam and Gilbert (24; A. C. Christensen, Ph.D. thesis, University of Washington, Seattle, 1982).

(iii) Electrophoresis. Gel electrophoresis was performed on 40-cm gels (0.4 mm thick) of either 6% acrylamide–0.3% bisacrylamide or 8% acrylamide–0.4% bisacrylamide in 135 mM Tris–45 mM borate–2 mM EDTA, pH 8.8, at 65 W for 2 to 9 h.

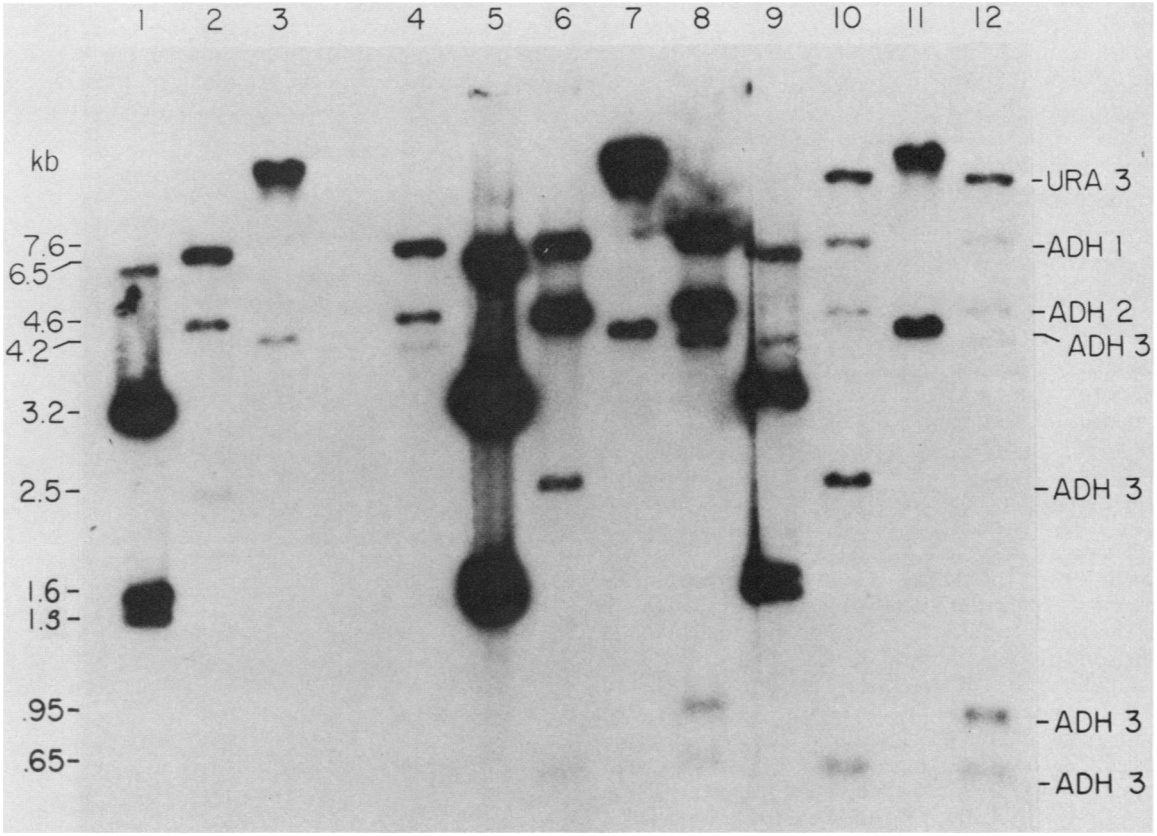


FIG. 1. Identification of *ADH3* DNA fragments by Southern hybridization analysis. Yeast DNA was isolated as described in Materials and Methods and digested with the restriction enzymes indicated below, and samples were electrophoresed on a 1% agarose gel. DNA fragments were transferred to nitrocellulose and then hybridized with nick-translated DNA fragments: *ADH1* 1.6-kb *Sph*I fragment (lanes 1 through 4), *ADH2* 1.6-kb *Sph*I fragment (lanes 5 through 8), or YIpADH3-b1 (lanes 9 through 12). Lanes 1, 5, and 9 contain a mixture of *ADH1* and *ADH2* plasmid DNAs digested with restriction enzymes to yield *ADH1* DNA fragments of 1.6 and 3.2 kb and *ADH2* DNA fragments of 1.4 and 6.5 kb. Yeast DNA (10 μg) was digested with *Eco*RI (lanes 2, 6, and 10), *Sal*I (lanes 3, 7, and 11), or *Eco*RI-*Sal*I (lanes 4, 8, and 12). The genomic *ADH1* and *ADH2* *Eco*RI DNA fragments are 6.5 and 4.6 kb long, respectively.

TABLE 2. ADH activity in *S. cerevisiae* transformants[a]

Strain	ADH genotype	Plasmid	ADH activity (mU/mg of protein)
301.10a	*adh1-11 adh2-43 ADH3*		450
302-21	*adh1-11 adh2-43 adh3*		<10
302-21	*adh1-11 adh2-43 adh3*	YEpADH3-3	<10
302-21	*adh1-11 adh2-43 adh3*	YEpADH3-9	3,200

[a] ADH specific activity was measured as described in Materials and Methods. Cell cultures were grown in complete medium containing 3% glycerol–3% lactate as the carbon source.

RESULTS

Identification of restriction enzyme fragment containing *ADH3* and molecular cloning of *ADH3*. *ADH3* was cloned by taking advantage of its homology to *ADH1* and *ADH2*. A DNA fragment capable of hybridizing to *ADH1* and *ADH2* probes was identified by Southern hybridization analysis (Fig. 1). In addition to the strong hybridization to their genomic counterparts, *ADH1* and *ADH2* cross-hybridized with each other and, more weakly, to a third band. This was most obvious in the *Sal*I digest of genomic DNA, in which a 4.2-kilobase (kb) band hybridized to both *ADH1* and *ADH2*. DNA in this region of the gel was excised, cleaved with *Eco*RI, and ligated with YIp5, an *S. cerevisiae-Escherichia coli* shuttle vector, which had been digested with *Eco*RI and *Sal*I. Ampicillin-resistant transformants were screened by colony hybridization with *ADH1* or *ADH2* DNA as the probe, and colonies that were positive with both were tested further. Two types of plasmids were recovered. The first type contained a 0.7-kb *Eco*RI fragment, and the second type contained this *Eco*RI fragment plus a 1-kb *Eco*RI-*Sal*I fragment. These are the sizes of the yeast DNA fragments that hybridized weakly to *ADH1* and *ADH2* DNA (Fig. 1).

The putative *ADH3*-containing plasmid that contained both the *Eco*RI and *Eco*RI-*Sal*I fragments, designated YIpADH3-b1, hybridized weakly to DNA fragments from *ADH1* and *ADH2* but strongly to fragments of the appropriate size in the digests (Fig. 1). A fragment of DNA containing *URA3* was also evident because the hybridization probe was a plasmid that contained *URA3*. A DNA fragment of 4.0 kb was revealed in the *Sal*I-*Eco*RI digest probed with *ADH1*, *ADH2*, or *ADH3*. In the *Eco*RI and *Eco*RI-*Sal*I digest DNA fragments of ca. 4.2 and 3.8 kb, respectively, were revealed when probed with *ADH3* but not when probed with *ADH1* or *ADH2*. These DNA fragments could contain other ADH-related genes or pseudogenes.

Functional activity of *ADH3*. To demonstrate that the cloned fragments represent an active structural gene for an ADH enzyme, their ability to stimulate ADH activity in yeast transformants was measured. To enhance the ADH activity stimulated by the cloned gene, the *Eco*RI and *Eco*RI-*Sal*I fragments were recloned by using the yeast 2μ-TRP1 plasmid pMW5 (1), which is present in many copies per cell. Two types of plasmids were obtained due to the two orientations possible for the *Eco*RI fragment. These plasmids, YEpADH3-3 and YEpADH3-9, were introduced into an ADH-null *S. cerevisiae* strain, 302-21. Increased sensitivity to allyl alcohol indicated the presence of ADH activity in Trp^+ transformants that contained YEpADH3-9 but not in those that contained YEpADH3-3. When cell extracts were assayed for ADH enzyme activity, strain 302-21(YEpADH3-9) contained 3,200 mU of ADH activity per mg of protein, whereas strain 302-21(YEpADH3-3) and untransformed strain 302-21 contained less than 10 mU/mg, indicating the presence of an intact, active ADH gene in YEpADH3-9 but not in YEpADH3-3 (Table 2).

ADH III can be distinguished from ADH I and ADH II by its electrophoretic mobility on nondenaturing gels; ADH III migrates more slowly than ADH I or ADH II and is present in a diffuse set of five bands (13, 39, 45) (Fig. 2). The enzyme in extracts of strains 302-21(YEpADH3-9) and 301.10a (*adh1-11 adh2-43 ADH3*) migrated slowly and heterogeneously, as expected for ADH III. In contrast, the ADH II standards on the gel migrated as single bands.

Glucose repression of cloned *ADH3*. To determine whether the intact *ADH3* gene, including its regulatory sequences, had been cloned, the ADH III activities of an ADH-null *S. cerevisiae* strain, 900-17-1A(YEpADH3-9), growing on a repressing (glucose) or nonrepressing (glycerol plus lactate) carbon source were measured. The cloned *ADH3* gene in YEpADH3-9 was glucose repressed (Table 3). There was a threefold increase in specific activity in response to a derepressing carbon source over that with a repressing carbon source. An *ADH3* wild-type strain, 301.10a, lacking the plasmid, showed a threefold derepression as well. The total activity in the transformed strain was enhanced about fourfold in response to the plasmid, so the total ADH activity was about one-half of that in a wild-type strain containing all three isozymes.

DNA sequence analysis. The strategy used to obtain the nucleotide sequence of *ADH3* is shown in Fig. 3. The complete nucleotide sequence of the *ADH3* gene and its 5′ and 3′ flanking sequences are shown in Fig. 4, as is the amino acid sequence of ADH III deduced from the nucleotide sequence. Overlapping sequences were obtained through-

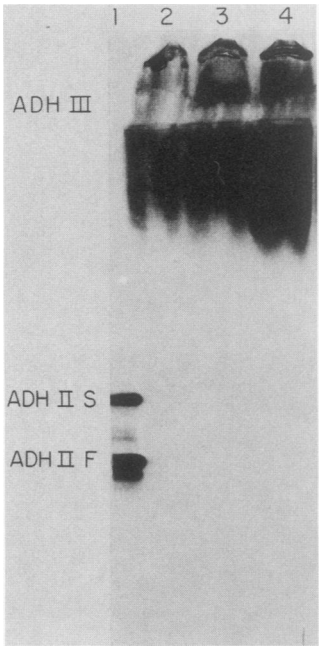


FIG. 2. Electrophoretic analysis of the ADH activity produced by the cloned *ADH3* gene. Cell extracts were prepared and analyzed as described in Materials and Methods from the *adh*-null strain 302-21(YEpADH3-9) (lanes 3 and 4, growth in YPD and YPGL, respectively) or the *ADH3* strain 301.10a (lane 2, growth in YPGL). Lane 1, Purified ADH II markers (slow [S] and fast [F] alleles).

TABLE 3. Glucose repression of cloned *ADH3*[a]

Strain	ADH genotype	Plasmid	ADH activity (mU/mg of protein)	
			Glucose	Glycerol-lactate
900-17-1A	*adh1-Δ1 adh2-601 adh3-1A*		<2	<3
301.10a	*adh1-11 adh2 ADH3*		150	450
900-17-1A	*adh1-Δ1 adh2-601 adh3-1A*	YEpADH3-9	580	1,900

[a] ADH specific activity was measured as described in Materials and Methods. Cell cultures were grown in complete medium containing either 3% glucose or 3% glycerol–3% lactate as the carbon source.

out, and the entire sequence was determined from both strands. Since the *ADH3* gene was originally cloned in two parts (*Eco*RI-*Eco*RI and *Eco*RI-*Sal*I), the sequence across the internal *Eco*RI site was confirmed independently. Using the *Eco*RI-*Xba*I fragment from *ADH3* (nucleotides 0 through 370) as a probe, we used a partial *Sau*3A genomic DNA library in YRp7 (a gift of K. Nasmyth) to isolate a 5.5-kb fragment containing *ADH3*. Restriction mapping confirmed the relative orientations of the *Eco*RI and *Eco*RI-*Sal*I fragments. A 330-base-pair *Taq*I-*Hin*fI *ADH3* fragment from this plasmid was isolated, end labeled, and sequenced by the Maxam and Gilbert technique (24). The sequence of this fragment confirmed the sequences across the internal *Eco*RI site. Predicted recognition sites for 16 different restriction enzymes were confirmed by digestion with the appropriate nucleases. These included a total of 190 base pairs, 12% of the sequenced fragment.

The correct open reading frame was found by comparing the nucleotide sequence and deduced amino acid sequence with those of *ADH1* and *ADH2* (30). The differences between the sequence shown in Fig. 4 and that of *ADH1* and *ADH2* were carefully analyzed to be sure the gels were unambiguous in those positions.

Codon usage. The codon usage for the three ADH genes is shown in Table 4. As previously reported, *ADH1* (4) and *ADH2* (30) show considerable bias in their codon usage. The two proteins are translated with a restricted set of preferred codons, as has been observed for other *S. cerevisiae* glycolytic enyzmes (4). Bennetzen and Hall (4) defined a codon bias index (CBI) based on the fraction of utilized codons that corresponds to 22 preferred codons out of the set of 61 nontermination codons. A CBI of 1.0 represents the highest bias, and a CBI of 0.0 represents completely random use of codons for a particular amino acid. Bennetzen and Hall showed a rough correlation between the CBI and mRNA levels for these and other genes. *ADH1* and *ADH2* have CBI values of 0.92 and 0.71, respectively, characteristic of genes encoding highly expressed glycolytic enzymes, whereas *CYC1*, coding for iso-1-cytochrome *c*, has a value of 0.50, characteristic of genes coding for proteins of moderate abundance.

In contrast to *ADH1* and *ADH2*, the codon usage for *ADH3* shows considerably less bias (CBI of 0.41). *ADH3* also includes some relatively rare codons for *S. cerevisiae*, namely CGU (arginine) and CUG (leucine) (4, 16). Based on its enzymatic activity, ADH III appears to constitute 0.1 to 0.2% of the total cell protein under derepressed conditions, similar to the abundance of iso-1-cytochrome *c*.

ADH III not essential to the cell. Molecular cloning of *ADH3* allowed a more definitive analysis to be made of the physiological role of ADH III in metabolism. Although *S. cerevisiae* strains that lack detectable ADH activity in vitro have been isolated and are viable (2), these mutants could have partial activity in vivo. Confirmation that ADH III is not essential for cell viability or some specific function such as sporulation or germination requires a mutant strain that produces no active gene product. Such a strain was constructed by using the technique of Rothstein (29) to disrupt the *ADH3* gene. A 1.45-kilobase-pair *TRP1-ARS1* fragment flanked by *ADH3* sequences was isolated. This fragment was introduced by transformation into haploid cells of strain 900-17 (**a** *adh1 adh2 ADH3 trp1*), and diploid cells and Trp^+ transformants were isolated. Trp^+ transformants occurred in the haploid cells at the expected frequency, indicating that the disruption of *ADH3* was not lethal. Five of these haploid transformants were screened for ADH enzyme activity, and four were found to lack detectable activity. The fifth Trp^+ isolate was apparently a revertant. When the genomic DNA of one of the $Adh3^-$ haploid transformants (strain 900-17-101a) and DNA from a Trp^+ diploid and its haploid segregant were examined by Southern analysis, a new, larger band appeared of the size predicted from integration of the disrupted gene at the *ADH3* locus (Fig. 5).

The growth characteristics of strain 900-17-101a, which contains the disrupted *ADH3*, were compared with those of the isogenic parent strain 900-17. Strain 900-17-101a showed no discernible mutant phenotype other than the lack of ADH III activity. It had the same growth rate in liquid culture as the isogenic parent strain on all media tested (YEPD, YEPGL, YEPE, SD, SGL, SGL+YE). Both strains grew faster with glycerol-lactate as the carbon source than with either glucose or ethanol. Diploid strains both heterozygous and homozygous for the disrupted gene were able to sporulate normally, and the spores were viable.

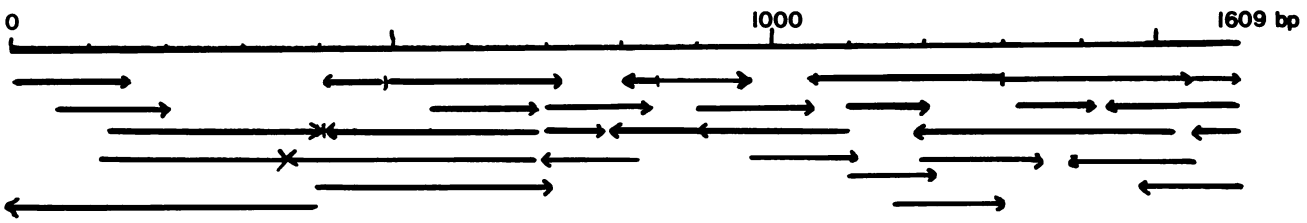
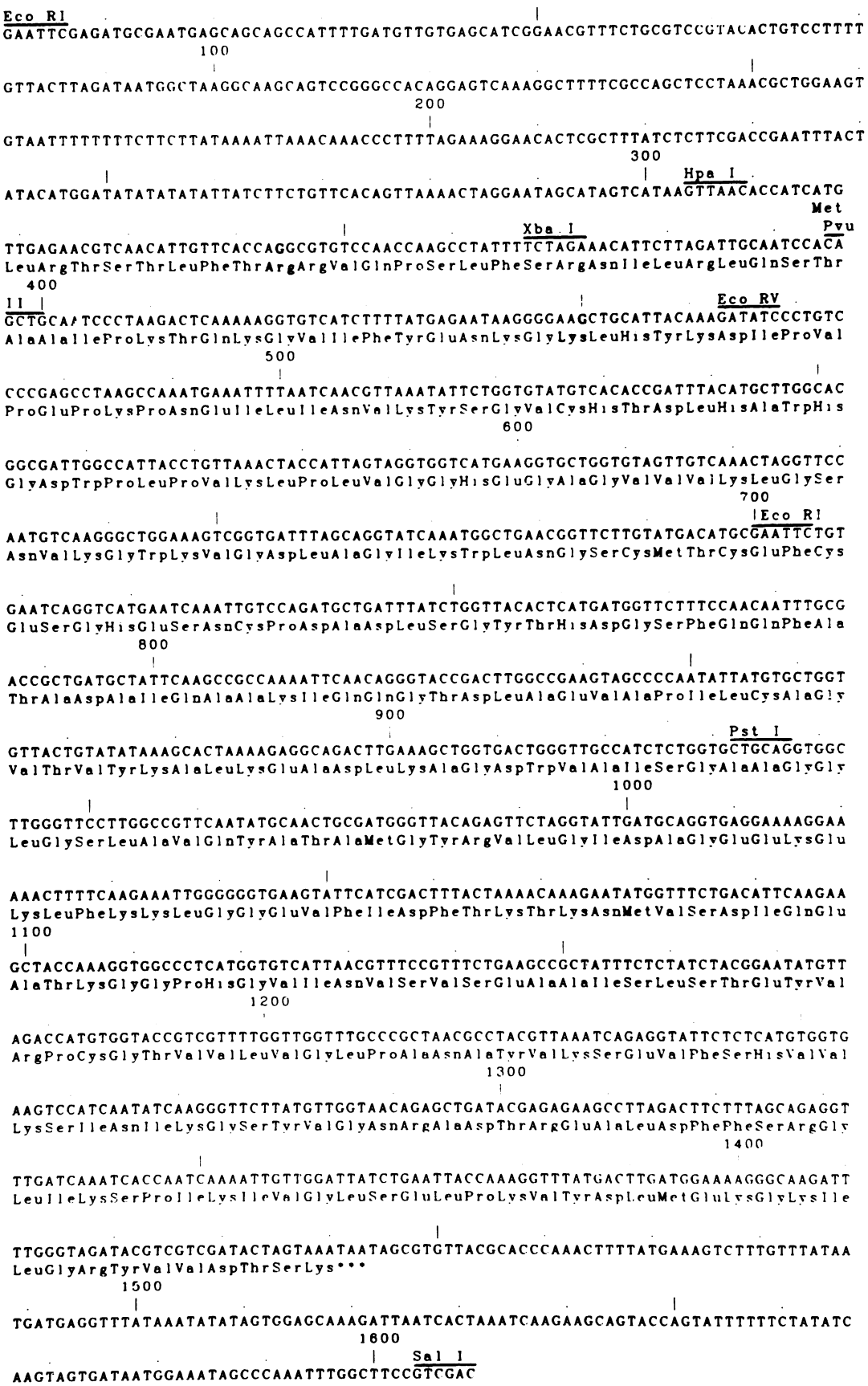


FIG. 3. Sequencing strategy for the *ADH3* 1.6-kilobase-pair *Eco*RI-*Sal*I DNA fragment. Arrows indicate DNA strand sequence: (⟶, 5′ ⟶ 3′ "upper" strand; ⟵, 3′ ⟵ 5′ "lower" strand.


Eco RI
GAATTCGAGATGCGAATGAGCAGCAGCCATTTTGATGTTGTGAGCATCGGAACGTTTCTGCGTCCGTACACTGTCCTTTT
100
GTTACTTAGATAATGGCTAAGGCAAGCAGTCCGGGCCACAGGAGTCAAAGGCTTTTCGCCAGCTCCTAAACGCTGGAAGT
200
GTAATTTTTTTTCTTCTTATAAAATTAAACAAACCCTTTTAGAAAGGAACACTCGCTTTATCTCTTCGACCGAATTTACT
300
Hpa I
ATACATGGATATATATATATTATCTTCTGTTCACAGTTAAAACTAGGAATAGCATAGTCATAAGTTAACACCATCATG
Met
Xba I
Pvu
TTGAGAACGTCAACATTGTTCACCAGGCGTGTCCAACCAAGCCTATTTTCTAGAAACATTCTTAGATTGCAATCCACA
LeuArgThrSerThrLeuPheThrArgArgValGlnProSerLeuPheSerArgAsnIleLeuArgLeuGlnSerThr
400
Eco RV
GCTGCAATCCCTAAGACTCAAAAAGGTGTCATCTTTTATGAGAATAAGGGGAAGCTGCATTACAAAGATATCCCTGTC
AlaAlaIleProLysThrGlnLysGlyValIlePheTyrGluAsnLysGlyLysLeuHisTyrLysAspIleProVal
500
CCCGAGCCTAAGCCAAATGAAATTTTAATCAACGTTAAATATTCTGGTGTATGTCACACCGATTTACATGCTTGGCAC
ProGluProLysProAsnGluIleLeuIleAsnValLysTyrSerGlyValCysHisThrAspLeuHisAlaTrpHis
600
GGCGATTGGCCATTACCTGTTAAACTACCATTAGTAGGTGGTCATGAAGGTGCTGGTGTAGTTGTCAAACTAGGTTCC
GlyAspTrpProLeuProValLysLeuProLeuValGlyGlyHisGluGlyAlaGlyValValValLysLeuGlySer
700
Eco RI
AATGTCAAGGGCTGGAAAGTCGGTGATTTAGCAGGTATCAAATGGCTGAACGGTTCTTGTATGACATGCGAATTCTGT
AsnValLysGlyTrpLysValGlyAspLeuAlaGlyIleLysTrpLeuAsnGlySerCysMetThrCysGluPheCys
GAATCAGGTCATGAATCAAATTGTCCAGATGCTGATTTATCTGGTTACACTCATGATGGTTCTTTCCAACAATTTGCG
GluSerGlyHisGluSerAsnCysProAspAlaAspLeuSerGlyTyrThrHisAspGlySerPheGlnGlnPheAla
800
ACCGCTGATGCTATTCAAGCCGCCAAAATTCAACAGGGTACCGACTTGGCCGAAGTAGCCCCAATATTATGTGCTGGT
ThrAlaAspAlaIleGlnAlaAlaLysIleGlnGlnGlyThrAspLeuAlaGluValAlaProIleLeuCysAlaGly
900
Pst I
GTTACTGTATATAAAGCACTAAAAGAGGCAGACTTGAAAGCTGGTGACTGGGTTGCCATCTCTGGTGCTGCAGGTGGC
ValThrValTyrLysAlaLeuLysGluAlaAspLeuLysAlaGlyAspTrpValAlaIleSerGlyAlaAlaGlyGly
1000
TTGGGTTCCTTGGCCGTTCAATATGCAACTGCGATGGGTTACAGAGTTCTAGGTATTGATGCAGGTGAGGAAAAGGAA
LeuGlySerLeuAlaValGlnTyrAlaThrAlaMetGlyTyrArgValLeuGlyIleAspAlaGlyGluGluLysGlu
AAACTTTTCAAGAAATTGGGGGGTGAAGTATTCATCGACTTTACTAAAACAAAGAATATGGTTTCTGACATTCAAGAA
LysLeuPheLysLysLeuGlyGlyGluValPheIleAspPheThrLysThrLysAsnMetValSerAspIleGlnGlu
1100
GCTACCAAAGGTGGCCCTCATGGTGTCATTAACGTTTCCGTTTCTGAAGCCGCTATTTCTCTATCTACGGAATATGTT
AlaThrLysGlyGlyProHisGlyValIleAsnValSerValSerGluAlaAlaIleSerLeuSerThrGluTyrVal
1200
AGACCATGTGGTACCGTCGTTTTGGTTGGTTTGCCCGCTAACGCCTACGTTAAATCAGAGGTATTCTCTCATGTGGTG
ArgProCysGlyThrValValLeuValGlyLeuProAlaAsnAlaTyrValLysSerGluValPheSerHisValVal
1300
AAGTCCATCAATATCAAGGGTTCTTATGTTGGTAACAGAGCTGATACGAGAGAAGCCTTAGACTTCTTTAGCAGAGGT
LysSerIleAsnIleLysGlySerTyrValGlyAsnArgAlaAspThrArgGluAlaLeuAspPhePheSerArgGly
1400
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LeuIleLysSerProIleLysIleValGlyLeuSerGluLeuProLysValTyrAspLeuMetGluLysGlyLysIle
TTGGGTAGATACGTCGTCGATACTAGTAAATAATAGCGTGTTACGCACCCAAACTTTTATGAAAGTCTTTGTTTATAA
LeuGlyArgTyrValValAspThrSerLys***
1500
TGATGAGGTTTATAAATATATAGTGGAGCAAAGATTAATCACTAAATCAAGAAGCAGTACCAGTATTTTTTCTATATC
1600
Sal I
AAGTAGTGATAATGGAAATAGCCCAAATTTGGCTTCCGTCGAC

TABLE 4. Codon usage in *ADH3*

Amino acid	Codon	No. of uses			Amino acid	Codon	No.of uses		
		ADH1	*ADH2*	*ADH3*			*ADH1*	*ADH2*	*ADH3*
Phe	UUU	0	2	5	Tyr	UAU	0	1	7
	UUC	8	6	7		UAC	13	12	5
Leu	UUA	2	7	10	His	CAU	1	2	7
	UUG	19	15	13		CAC	10	9	2
Leu	CUU	0	0	2	Gln	CAA	9	6	9
	CUC	0	1	0		CAG	0	0	1
	CUA	3	2	6					
	CUG	0	0	2	Asn	AAU	0	1	6
						AAC	11	12	6
Ile	AUU	9	9	10					
	AUC	12	12	11	Lys	AAA	4	4	19
	AUA	0	1	1		AAG	20	20	13
Met	AUG	7	5	5	Asp	GAU	2	6	11
						GAC	14	9	7
Val	GUU	19	16	17					
	GUC	17	12	10	Glu	GAA	20	18	15
	GUA	0	3	7		GAG	0	3	5
	GUG	0	1	2					
					Cys	UGU	8	6	6
Ser	UCU	14	13	13		UGC	0	2	1
	UCC	7	7	5					
	UCA	0	1	5	Trp	UGG	5	5	5
	UCG	0	1	0					
					Arg	CGU	0	0	1
Pro	CCU	2	2	5		CGC	0	0	0
	CCC	1	1	2		CGA	0	0	0
	CCA	10	12	9		CGG	0	0	0
	CCG	0	0	0					
					Ser	AGU	0	1	1
Thr	ACU	5	5	6		AGC	0	1	2
	ACC	9	9	6					
	ACA	0	0	4	Arg	AGA	8	7	9
	ACG	0	0	3		AGG	0	1	1
Ala	GCU	19	18	13	Gly	GGU	41	32	31
	GCC	16	12	9		GGC	3	8	5
	GCA	0	3	7		GGA	0	1	1
	GCG	0	4	2		GGG	0	1	2

Integration of the cloned *ADH3* gene at the *ADH3* locus. Integration of yeast genes either as plasmids or as linear fragments of DNA occurs by homologous recombination, allowing genetic mapping of cloned yeast genes (29). Ciriacy (7) identified a locus required for ADH III function (formerly called *ADM*) (36). Integration of the *TRP1*-containing fragment of *ADH3* at this locus would indicate that the gene cloned was from the *ADH3* locus and that this was the structural gene for ADH III rather than a regulatory gene.

Strain 900-17-101a (*adh3*::*TRP1*) was crossed with a strain containing the original *adm* mutation of Ciriacy, SF4-1B (*adh3 trp1*), and the Adh3 and Trp phenotypes were scored after tetrad analysis. All spores resulting from the cross were Adh3$^-$, ruling out the possibility that the previously isolated *adh3* mutation was in a regulatory gene. If Ciriacy's *adm* mutation was in an unlinked gene required for expression of *ADH3*, 25% of all spores resulting from the cross would have had normal ADH III levels. This evidence indicates that the *ADH3* locus defined genetically by Ciriacy (7) is the structural gene for ADH III.

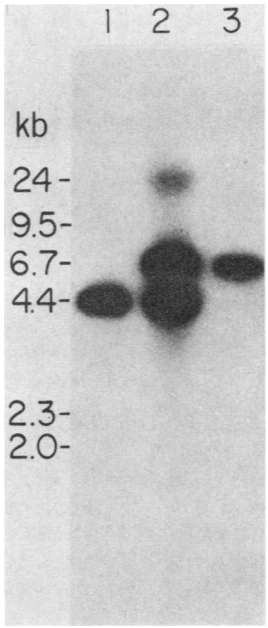

FIG. 5. Southern blot of *ADH3* gene disruption. Genomic DNA was digested with *Sal*I and blotted as described in Materials and Methods. The probe used was the *ADH3*-specific *Eco*RI-*Xba*I DNA fragment (nucleotides 1 through 367). Lanes: 1, strain 701.1; 2, Trp$^+$ transformant 701.1-1-b (strain 701.1 transformed with linear disruption fragment); 3, Trp$^+$ spore from tetrad dissection of strain 701.1-1-b. Genomic DNA isolated from the integrated *ADH3* gene disrupted by *TRP1* in haploid strain 900-17 is not shown here, but the pattern was identical to that in lane 3. Trp$^-$ spores from dissection of strain 701.1-1-b showed a pattern identical to that in lane 1. Trp$^+$:Trp$^-$ spores segregated 2:2 in all tetrads dissected.

DISCUSSION

As expected from the hybridization results, the nucleotide sequence of *ADH3* is very closely related to those of *ADH1* and *ADH2* (Fig. 6). In the region that is most highly conserved between the three genes (nucleotides 402 through 1445 in *ADH3*), the nucleotide homology is: *ADH1* and *ADH2*, 88%; *ADH1* and *ADH3*, 74%; and *ADH2* and *ADH3*, 73%. This region includes all but two codons of *ADH1* and *ADH2*, but does not contain the first 29 codons of *ADH3*, suggesting that ADH III is synthesized with an amino-terminal extension relative to ADH I and ADH II, as discussed in detail below. The nucleotides coding for the ADH III amino-terminal extension have homology to the 5′ noncoding regions of *ADH1* and *ADH2* (Fig. 7); the implications of this homology are discussed below. *ADH1* and *ADH2* have homology to each other further 5′ as well (30),

FIG. 4. DNA sequence for *ADH3* and predicted amino acid sequences for ADH III. Only the coding strand is shown. Restriction enzyme sites having 6-base recognition sequences are included. All sites have been confirmed by digestion. The open reading frame for ADH III was assigned by homology to ADH I and ADH II. Numbering begins with the distal *Eco*RI site. No distinction is made in the figure between sequences derived by the methods of Sanger (32) and those of Maxam and Gilbert (24; Christensen, Ph.D. thesis, 1982).

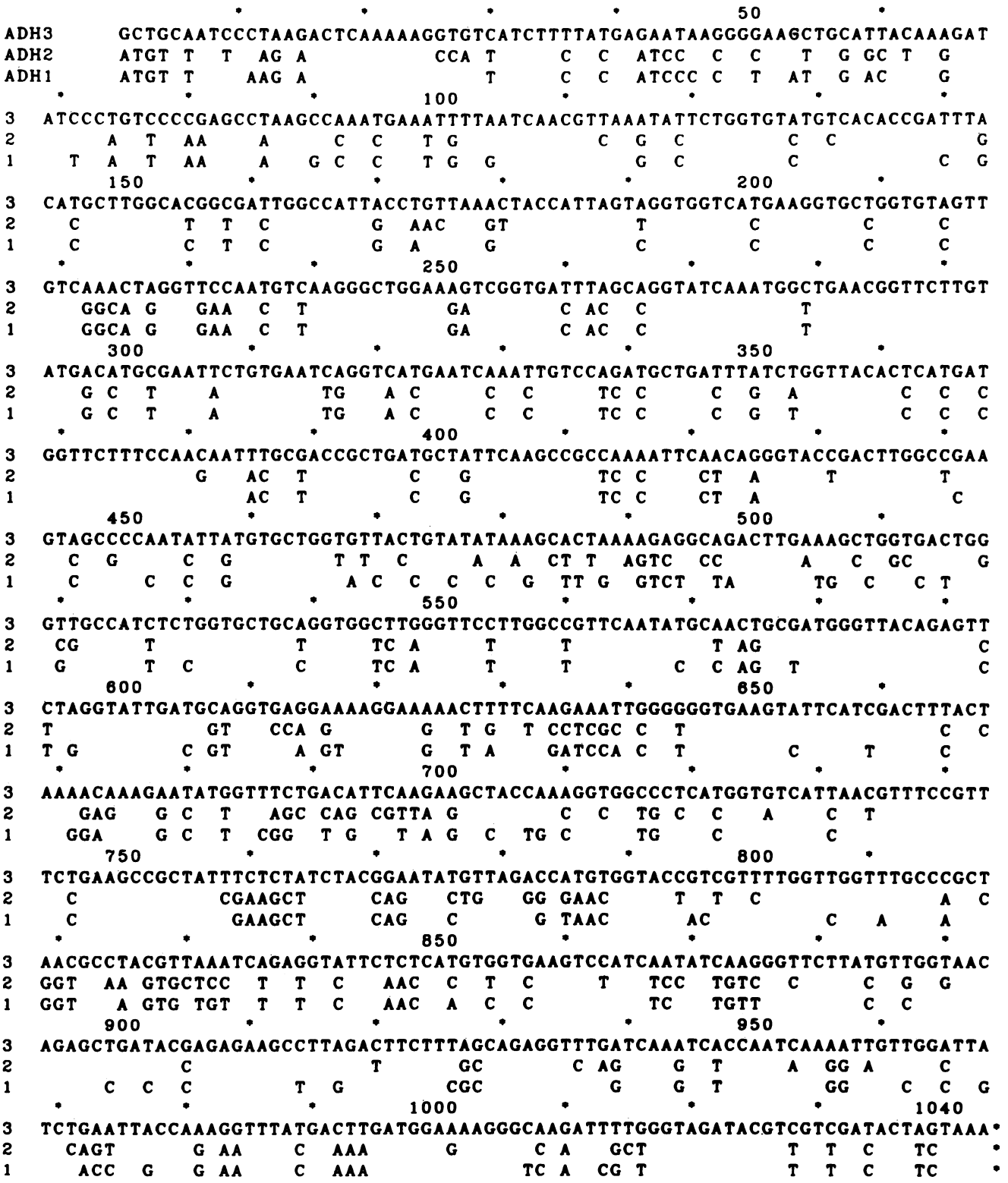

FIG. 6. Comparison of *ADH3* with *ADH1* and *ADH2* DNA coding sequences. The coding regions of *ADH2* and *ADH1* are indicated beneath DNA sequences 397 through 1440 of *ADH3*. When a base in either *ADH2* or *ADH1* is different from the corresponding base in *ADH3*, it is indicated. The numbering begins with the A of the initiator codons of *ADH1* and *ADH2*.

```
          -90          -80          -70          -60          -50
ADH3  ATGTTGAGA--ACGTCAACATTGTTCACCAGGCGTGTCCAA--CCA
ADH2  tTGTTtctt t--tg  AaATaG  tAtCAaGC------tA a  A
ADH1  tTGTTtctt t CtgCA aATa---t tCAaGC------tA aCCA

               -40          -30          -20          -10          +1
ADH3    AGCCTATTTTCTAGAAACA----TT--CTTAGATTGCAATCCACA
ADH2    AGCaTAcaaTCaActAtCA    TT  C-TA AT GtAATaCACA
ADH1    AGCaTAcaaTCaActAtC-----------------t ATa ACA
```

FIG. 7. Comparison of *ADH3* translated leader sequence to the 5′ flanking regions of *ADH2* and *ADH1*. Dashes indicate where sequences have been inserted to maximize identity. Uppercase letters in the sequences of *ADH1* and *ADH2* indicate identity to *ADH3*. Lowercase letters indicate identity only between *ADH1* and *ADH2*. Blanks indicate bases unique to *ADH1* or *ADH2*. Numbering begins at the ATGs of *ADH1* and *ADH2*. mRNA start sites for *ADH1* are at −45 and −61 and for *ADH2* are at −61 and −65 in this figure.

but these regions have no significant homology to *ADH3*. Comparing the protein-coding portions of *ADH2* and *ADH3*, there are 154 transition and 125 transversion substitutions. These changes are distributed randomly throughout the gene, including the region corresponding to nucleotides 193 to 431 in *ADH1* and *ADH2*. Sequences between these positions are highly conserved in *ADH1* and *ADH2*, even at the third position of the codons, suggesting a recent nonreciprocal exchange or conversion between these two genes. This presumed conversion event must have occurred after the divergence of *ADH3* from *ADH1* and *ADH2* since *ADH3* contains 51 substitutions within this region.

The predicted amino acid sequence of ADH III is even more closely related to those of ADH I and ADH II than is the nucleotide sequence of *ADH3* to those of *ADH1* and *ADH2*, indicating a prevalence of silent mutations in the codons and thus preservation of amino acid identities, presumably for functional reasons. Comparing *ADH2* and *ADH3*, 63% of the substitutions are in the third position of the codons. The amino acid homology is: ADH I and ADH

```
ADH III AMINO                                  -20       -10
TERMINAL EXTENSION                          MLRTSTLFTRRVQPSLFSRNILRLQST

                      *           *           *           *          50          *
ADH III   AAIPKTQKGVIFYENKGKLHYKDIPVPEPKPNEILINVKYSGVCHTDLHAWHGDWPLPVK
ADH II    MS  E   AI    SN   EH      K     L                              T
ADH I     MS QE         H   EH      K  A  L
                      *           *           *          100          *           *
ADH III   LPLVGGHEGAGVVVKLGSNVKGWKVGDLAGIKWLNGSCMTCEFCESGHESNCPDADLSGY
ADH II                GM E      I  Y           A  Y  L N     H
ADH I                 GM E      I  Y           A  Y  L N     H
                      *           *          150          *           *           *
ADH III   THDGSFQQFATADAIQAAKIQQGTDLAEVAPILCAGVTVYKALKEADLKAGDWVAISGAA
ADH II          EY     V   H P                I       S N R  H A
ADH I            Y     V   H P      Q         I       S N R  H G
                      *           200          *           *           *           *
ADH III   GGLGSLAVQYATAMGYRVLGIDAGEEKEKLFKKLGGEVFIDFTKTKNMVSDIQEATKGGP
ADH II     K          G PG  E  TS            F DI  AVVK  N  A
ADH I      K          G  G  E  RSI           F DI GVVLK  D  A
                   250          *           *           *           *          300
ADH III   HGVINVSVSEAAISLSTEYVRPCGTVVLVGLPANAYVKSEVFSHVVKSINIKGSYVGNRA
ADH II      I          EA  R C AN           G KCS D  N      S VC
ADH I                  EA  R   AN         M  G KCC D  NQ     S VC
                      *           *           *          340
ADH III   DTREALDFFSRGLIKSPIKIVGLSELPKVYDLMEKGKILGRYVVDTSK*
ADH II              A   V     V    S  EI EK    Q A          *
ADH I               A   V     V    T  EI EK    Q V          *
```

FIG. 8. Comparison of protein sequences of ADH III, ADH II, and ADH I. The sequences of ADH II and ADH I are indicated below the predicted ADH III sequence. Where an amino acid in ADH II or ADH I differs from one in ADH III, it is indicated. Numbering begins with the Met of ADH II and ADH I.

TABLE 5. Nonconservative amino acid changes in ADH III[a]

Amino acid position in ADH I and II	Amino acid in ADH:			Amino acid position in ADH III
	I	II	III	
1	Met	Met	Ala	28
75	Gly	Gly	Lys	102
88	Tyr	Tyr	Leu	115
106	Leu	Leu	Ser	133
174	Gly	Ala	Val	201
231	Val	Val	Asp	258
255	Ala	Ala	Leu	282
263	Asn	Asn	Cys	290
276	Lys	Lys	Tyr	303
277	Cys	Cys	Val	304
292	Val	Val	Lys	319

[a] Based on data from reference 9.

II, 94% ADH 1 and ADH III, 79%; and ADH II and ADH III, 80%. Of the 67 amino acids in ADH III that are not present in identical positions in either ADH II or ADH I (Fig. 8), 56 are conservative amino acid changes as calculated by Dayhoff et al. (9). All of the active-site, cofactor-binding, and noncatalytic zinc-binding residues identified for *S. cerevisiae* ADH I by Jörnvall (19) are conserved in both ADH II and ADH III. The 11 nonconservative amino acid changes are listed in Table 5.

When the amino acid compositions of the three proteins are compared, the most striking change is in the number of lysine residues (from 24 in ADH I and ADH II to 31 in the conserved region of ADH III). The ratio of lysine-plus-arginine to aspartic acid-plus-glutamic acid is 0.88 for ADH I and ADH II and 0.98 for ADH III. The ratio increases to 1.1 if the amino-terminal extension of ADH III is included. Thus, the basic character of both the mature and presumed precursor polypeptides is increased relative to that of the cytoplasmic isozymes. The increased basicity explains the slower electrophoretic mobility, but not the heterogeneity, of ADH III relative to ADH I and ADH II.

Since *S. cerevisiae* ADH I and ADH II, as well as horse liver ADH, have acylated serine as the amino-terminal amino acid, cleavage of the ADH III precursor might occur between residues 25 and 26, to leave Ser-26 at the amino terminus. The cleavage site for at least six other imported mitochondrial precursor polypeptides in different systems (cytochrome *c* peroxidase [21], ATP synthase proteolipid subunit [38], EF-Tu [26], cytochrome oxidase subunit VI [46], OTCase [37], and cytochrome P-450 [25]) all lie within one amino acid of a serine residue.

The most interesting feature of the predicted amino acid sequence of ADH III is the presence of a 27-amino-acid amino-terminal extension. Several lines of evidence indicate that these 27 amino acids constitute or are included in a leader sequence essential for targeting the ADH III isozyme to the mitochondria and that they are cleaved from a precursor to yield the mature ADH III polypeptide (unpublished data). Most mitochondrial proteins encoded by nuclear genes are synthesized as precursors with amino-terminal extensions that are removed proteolytically within the mitochondrion (15, 33). No consensus amino acid sequence within this leader region has been found for either mitochondrial targeting or intramitochondrial localization. The presumed leader of ADH III follows the general rule of being highly basic and lacking acidic amino acids (15).

The origin of presequences on either secreted proteins or proteins targeted to organelles such as mitochondria is unknown. One hypothesis is that these sequences evolved from the amino terminus of a nonsecreted protein. A comparison of the nucleotide sequences of the presumed *ADH3* leader and the 5′ transcribed but untranslated portion of the mRNAs of *ADH1* and *ADH2* showed extensive homology (Fig. 7). This identity is maximized by postulating several short deletions in the region. Since these regions in *ADH1* and *ADH2* perform a very different function from the homologous region in *ADH3*, they cannot have arisen by convergent evolution. The homology suggests that the progenitor of the three ADH isozymes contained this or a closely related sequence. ADH I and ADH II are the more closely related of the three isozymes (94% amino acid homology) and presumably represent genes that diverged from a common ancestor more recently in evolution than *ADH3* diverged from *ADH1* and *ADH2*. On the other hand, ADH I and ADH II are equally distant in terms of amino acid identity from ADH III (ADH I and ADH III, 79% homology; ADH II and ADH III, 80% homology), suggesting that they are equally divergent from ADH III. These observations make it unlikely that *ADH3* arose by integration of an RNA copy of either *ADH1* or *ADH2*, since in that case ADH III would be more closely related to either ADH I or ADH II. By similar reasoning, it is unlikely that genomic rearrangement of a duplicated copy of either *ADH1* or *ADH2* was the progenitor of *ADH3*.

The present data rule out the possibility that ADH III represents an altered form of ADH I or ADH II, perhaps arising from a different transcript as in the case of secreted invertase (6, 12). The genes are unlinked (unpublished data), and the nucleotide sequences are different.

One additional clue to the evolution of the *S. cerevisiae* ADH isozymes is provided by a comparison with the well-characterized horse liver ADH enzyme (18). The alignment of the amino termini of ADHs from *S. cerevisiae* and other species is shown in Fig. 9. Alignments are calculated from the data of Jörnvall (18). The three amino-terminal amino acids of horse liver ADH do not have counterparts in the ADH I and ADH II proteins, but correspond exactly to three amino acids in the amino-terminal extension of ADH III. If these amino acids are conserved for functional regions and if they are present in the mature form of ADH III, the functions of the horse liver ADH and ADH III may be more closely related than the functions of the mitochondrial and cytoplasmic ADH isozymes, even though the vertebrate ADH enzymes are located in the cytoplasm. In any case this comparison suggests that the leader region of *ADH3* was present in some ancestral gene that preceded the separation of fungal and vertebrate evolution. Whether the ancestral enzyme was cytoplasmic or mitochondrial cannot be determined from this evidence. Mitochondrial compartmentalization of ADH III suggests a respiratory function, whereas the ancestral enzyme presumably had a fermentative role before O_2 was present in the atmosphere. In fact, no function has been identified for ADH III: yeast cells lacking a functional *ADH3* gene can still grow by respiration. On the other hand, mitochondrially located ADH III cannot fulfill a fermentative role (45), but the ADH III protein lacking the presumed leader sequence can function fermentatively if it is located in the cytoplasm of yeast cells (unpublished data).

The model we favor for the evolution of the *S. cerevisiae* ADH isozymes is as follows. The progenitor gene encoded a 374-amino-acid polypeptide that was cytoplasmic and functioned in fermentation. This gene then duplicated itself (including at least part of the noncoding region). After the initial duplication event, the amino-terminal 27 amino acids

```
ADH I   S. cerevisiae                                    aSER ILE gln GLU THR GLN LYS GLY
ADH II    "                                              aSER ILE PRO GLU THR GLN LYS ALA
ADH III   "       (met)leu/ /leu gln SER THR ALA ala ILE PRO lys THR GLN LYS GLY
Schizosaccharomyces pombe                             (met)thr ILE PRO asp LYS GLN leu ALA
Mammalian liver                          aSER THR ALA GLY LYS VAL ILE LYS CYS LYS ALA
Maize                                 (met)ala THR ALA GLY LYS VAL ILE LYS CYS LYS ---
Bacillus stearothermophilus                                                fmet LYS ALA
Drosophila melanogaster                               (met)SER phe thr leu THR asn LYS asn
Aspergillus nidulans                                  (met)SER val PRO GLU val GLN tyr ALA
```

FIG. 9. Amino termini of ADH proteins from various organisms aligned for comparison. Amino acid abbreviations are uppercase where there is identity between two or more proteins, otherwise they are lowercase. aSER, Acylated serine; fmet, formylmethionine. Where protein sequencing has not been done, the sequence was predicted from the DNA. Sequences of ADH I (16), ADH II (29), *Schizosaccharomyces pombe* (31), mammalian liver (20), maize (5), *Bacillus stearothermophilus* (20), and *Drosophila melanogaster* (22) have been previously reported. The *Aspergillus nidulans* sequence is from Gary McKnight (24a).

of the predecessor of *ADH1* and *ADH2* were lost from one copy, but one or both copies of the gene could still fulfill a fermentative function. Subsequent mutations in the gene that had lost its original amino terminus gave rise to a functional gene that could duplicate itself to give, ultimately, two genes with sophisticated regulatory properties: one (*ADH1*) that was highly expressed during fermentation and whose enzyme product was specialized for fermentation; the other (*ADH2*) that was glucose repressed and whose enzyme product was more adapted to ethanol oxidation during gluconeogenesis. The cytoplasmic isozymes specialized in glucose and ethanol metabolism that evolved may have usurped the role of the ancestral, presumably cytoplasmic, ADH III, allowing it to acquire a more specialized role in respiratory metabolism within the mitochondrion. The mitochondrial targeting of ADH III could have evolved by genomic rearrangement 5′ to the region conserved between the three isozymes or by the accumulation of single mutations.

The cloning of *ADH3* has not resolved the question raised by the heterogeneous migration of ADH III on nondenaturing gels (45). The fact that the cloned gene gives rise to the same complex pattern is strong evidence that the pattern does not arise from association of subunits derived from two different genes. Association of modified and unmodified ADH III subunits seems a more likely explanation. The modification does not involve the presence or absence of a presequence, since all of the detectable ADH III polypeptide is uniform in size and is identical in size to ADH I and ADH II, indicating complete cleavage (A. P. G. M. van Loon and E. T. Young, submitted for publication). Thus, some other form of modification that does not alter the apparent molecular weight is presumably involved. Whether this is required for mitochondrial compartmentalization or a concomitant of such localization or is involved in some specific ADH III function is unknown.

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