MOLECULAR AND CELLULAR BIOLOGY, Jan. 1991, p. 370–380
0270-7306/91/010370-11$02.00/0

Vol. 11, No. 1

Isolation, Nucleotide Sequence Analysis, and Disruption of the *MDH2* Gene from *Saccharomyces cerevisiae*: Evidence for Three Isozymes of Yeast Malate Dehydrogenase

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The major nonmitochondrial isozyme of malate dehydrogenase (MDH2) in *Saccharomyces cerevisiae* cells grown with acetate as a carbon source was purified and shown by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to have a subunit molecular weight of approximately 42,000. Enzyme assays and an antiserum prepared against the purified protein were used to screen a collection of acetate-nonutilizing (acetate$^-$) yeast mutants, resulting in identification of mutants in one complementation group that lack active or immunoreactive MDH2. Transformation and complementation of the acetate$^-$ growth phenotype was used to isolate a plasmid carrying the *MDH2* gene from a yeast genomic DNA library. The amino acid sequence derived from complete nucleotide sequence analysis of the isolated gene was found to be extremely similar (49% residue identity) to that of yeast mitochondrial malate dehydrogenase (molecular weight, 33,500) despite the difference in sizes of the two proteins. Disruption of the *MDH2* gene in a haploid yeast strain produced a mutant unable to grow on minimal medium with acetate or ethanol as a carbon source. Disruption of the *MDH2* gene in a haploid strain also containing a disruption in the chromosomal *MDH1* gene encoding the mitochondrial isozyme produced a strain unable to grow with acetate but capable of growth on rich medium with glycerol as a carbon source. The detection of residual malate dehydrogenase activity in the latter strain confirmed the existence of at least three isozymes in yeast cells.

Two isozymes of malate dehydrogenase in mammalian cells catalyze the interconversion of malate plus NAD^+ and oxaloacetate plus NADH. The mitochondrial isozyme functions in the tricarboxylic acid cycle, and the cytosolic isozyme catalyzes a step in gluconeogenesis from pyruvate. Also, as components of the malate/aspartate shuttle cycle, the malate dehydrogenase isozymes control the exchange of metabolic intermediates and reducing equivalents across the mitochondrial membrane. Analyses of amino acid sequences for the isozymes from porcine heart (4, 5) and of nucleotide sequences for the porcine and murine cDNAs (25, 26) have shown that the two mammalian mitochondrial enzymes share greater than 90% residue identity, as do the two cytosolic enzymes. The compartmentalized isozymes within each species, however, show only limited relatedness, with approximately 20% residue identity of aligned sequences. Despite the disparity in primary sequences, crystallographic analyses have shown that the porcine mitochondrial and cytosolic isozymes have very similar three-dimensional structures (6, 41).

Saccharomyces cerevisiae cells also contain genetically distinct isozymes of malate dehydrogenase. The yeast *MDH1* gene encoding the mitochondrial isozyme has been cloned and disrupted, producing a strain that retains substantial malate dehydrogenase activity and is able to grow on both fermentable and nonfermentable carbon sources with the exception of acetate (34). The yeast MDH1 enzyme shares a 54% residue identity with the mammalian mitochondrial enzymes (48).

In addition to mitochondrial and cytosolic enzymes, a third compartmentalized isozyme of malate dehydrogenase in plant cells is localized in organelles called glyoxysomes and functions in the glyoxylate pathway (9). This pathway allows bypass of the two decarboxylation steps in the tricarboxylic acid cycle and subsequent biosynthesis of C_4 and C_6 metabolites from C_2 precursors. *S. cerevisiae* can utilize acetate and ethanol as carbon sources by inducing synthesis of isocitrate lyase and malate synthase (30), key glyoxylate cycle enzymes not expressed in mammalian cells, but whether the requirement for malate dehydrogenase activity in this cycle is fulfilled by the cytosolic isozyme or by a unique glyoxysomal enzyme has not been established. Various analyses of the isozymes of malate dehydrogenase in *S. cerevisiae* have been reported, with contradictory conclusions that this yeast contains two (21, 30) or three (1) biochemically distinct enzymes. To resolve this question and to compare the structures and expression of the yeast enzymes, we have purified the major nonmitochondrial isozyme from yeast cells grown on acetate and have cloned the corresponding gene. By disrupting this gene in a haploid strain also containing a chromosomal disruption of the *MDH1* gene encoding the mitochondrial isozyme (34), we have obtained evidence for three genetically distinct isozymes of malate dehydrogenase in *S. cerevisiae*. Evidence that MDH2 is the glyoxylate cycle enzyme is discussed.

MATERIALS AND METHODS

Strains and growth conditions. The haploid yeast strain Sg7 (*MATα leu2 his3-Δ1 trp1 gcrl-1-1*; 34) was used for isolation of the nonmitochondrial isozyme of malate dehydrogenase. Yeast strains S173-6B (*MATα leu2-3,112 his3-Δ1 ura3-57 trp1-289*; 7) and SΔMDH1, a derivative of S173-6B containing a chromosomal disruption of the MDH1 gene (47), were used for transformation, growth studies, and cell fractionation. The parental *S. cerevisiae* strain MMY011 (*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100*) and

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various acetate-nonutilizing (acetate$^-$) mutants derived from this strain by UV mutagenesis were provided by Mark T. McCammon (University of Texas Southwestern Medical Center, Dallas). Yeast strains were cultivated on rich YP medium (1% yeast extract, 2% Bacto-Peptone [Difco]) or on minimal YNB medium (0.17% yeast nitrogen base, 0.5% ammonium sulfate, pH 6.0) containing supplements of 20 μg/ml each to satisfy auxotrophic requirements for growth. Carbon sources were glucose, glycerol, lactate, or acetate added to 2%. Cell growth was monitored spectrophotometrically and cultures were routinely harvested at an A_{600} of 1.0 to 1.5. *Escherichia coli* DH5αF′ (39) was used for amplification of plasmid DNAs.

Purification and analysis of MDH2. For purification of MDH2, yeast strain Sg7 was cultivated at 30°C on YP medium with 2% acetate as a carbon source. The cells were harvested by centrifugation and stored at −70°C. Cells (200 g) were thawed in 100 ml of buffer A (10 mM $NaPO_4$ [pH 7.4], 1 mM EDTA, 1 mM β-mercaptoethanol) containing 2 mM phenylmethylsulfonyl fluoride and broken with glass beads in a Bead Beater (Biospec Products, Bartlesville, Okla.). This and all subsequent steps were conducted on ice or at 4°C. The lysate was cleared by centrifugation at 12,000 × *g* for 10 min, the resulting pellet was rebroken and centrifuged, and the supernatants were combined. Nucleic acids were precipitated from the crude supernatant by adding protamine sulfate to a final concentration of 0.25% and centrifugation. The supernatant was loaded onto a CL-6B Blue Sepharose column (110-ml column bed; Pharmacia) preequilibrated with buffer A. The column was washed with 10 volumes of buffer A, and proteins were eluted with an 800-ml 0 to 2.0 M KCl gradient. Fractions containing MDH2 activity were pooled and dialyzed against buffer B (10 mM $NaPO_4$ [pH 6.8], 1 mM EDTA, 1 mM β-mercaptoethanol). The dialysate was loaded onto a 150-ml DEAE-cellulose column preequilibrated with buffer B. The column was washed with 10 volumes of buffer B, and the proteins were eluted with an 800-ml 0 to 0.5 M KCl gradient. Fractions containing MDH2 activity were pooled, dialyzed against buffer B, and loaded onto a second 35-ml CL-6B Blue Sepharose column preequilibrated with buffer B. The column was washed with buffer B, and proteins were eluted with a 400-ml 0 to 25 mM NADH gradient. The fractions containing MDH2 activity were pooled, dialyzed against buffer B containing 50% glycerol, concentrated in an Amicon filtration unit, and stored at −20°C. These steps resulted in an overall increase in specific activity of malate dehydrogenase from 9.2 U/mg in the crude supernatant to 379.4 U/mg in the purified sample. Malate dehydrogenase activity was measured as the oxaloacetate-dependent rate of NADH oxidation (ΔA_{340}) in assay mixes containing 45 mM KPO_4 (pH 7.5), 0.12 mM NADH, and 0.33 mM oxaloacetate. A unit represents 1 μmol of NAD^+ produced per min. Protein concentrations were determined by the method of Bradford (8).

The size of MDH2 was examined by gel filtration analysis, using a column (43 by 1 cm) of Sephadex G200 superfine with β-amylase, bovine serum albumin, and carbonic anhydrase as molecular weight standards. For amino-terminal sequence analysis, 100-pmol samples of MDH2 were electrophoresed in each of two lanes on a 10% sodium dodecyl sulfate-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Immobilon), using a graphite semidry electroblotter. The peptide band, identified by Coomassie blue staining of the membrane, was used directly for amino-terminal sequence analysis with an Applied Biosystems 470-A gas-phase microprotein sequencer (Biotechnology Instrumentation Facility, University of California at Riverside).

A polyclonal antiserum was obtained from a commercial source (Bethyl Laboratories, Montgomery, Tex.) by injection of rabbits with purified native MDH2. For immunoblot analyses, protein samples were electrophoresed and transferred to membranes as described above. The membranes were blocked and washed as described by Burnette (10) prior to incubation with the anti-MDH2 antiserum (diluted 1:100 in 50 mM Tris hydrochloride [pH 8.0]–0.15 M NaCl–1.0% bovine serum albumin) and ^{125}I-labeled protein A. Protein samples were obtained from whole cells by breakage with glass beads as previously described (34) and from mitochondrial and postmitochondrial supernatant cell fractions prepared as described by Daum et al. (12). The latter corresponded to supernatant fractions obtained after centrifugation of cellular homogenates to obtain mitochondrial pellets.

Subcloning strategies and nucleotide sequence analysis. To localize the amino-terminal coding region of the *MDH2* gene, a redundant oligonucleotide [(C/T)TGNCC(A/G/T)AT NCCNCCNGC] complementary to the possible coding sequences for amino acids 21 to 26 (29) was obtained (Operon, San Pablo, Calif.) and 5′ end labeled with [γ-^{32}P]ATP by using polynucleotide kinase. The ^{32}P-labeled oligonucleotide was used for Southern blot analysis (46) of restriction fragments from plasmids isolated by complementation of acetate$^-$ yeast mutants.

*Pst*I, *Hin*cII, and *Eco*RI restriction sites within the *MDH2* gene were used to construct subclones in Bluescribe vectors for nucleotide sequence analysis. Oligonucleotide primers corresponding to plasmid sequences near the multicloning sites were used for dideoxynucleotide sequence analysis (43). In addition, primers complementary to *MDH2* sequences were synthesized to extend sequence information and to overlap various restriction sites. Both single-stranded and double-stranded DNA sequencing strategies were used with Klenow (Boehringer Mannheim) and Sequenase (United States Biochemical Corp.) polymerases to produce sequences nearer or more distant from the primers.

For gene disruption, the 2.35-kbp *Bam*HI-*Eco*RI fragment from plasmid YEpMDH2 was subcloned into a Bluescribe vector lacking the *Hin*dIII restriction site in the polylinker. The internal 300- and 50-bp *Pst*I fragments of *MDH2* were removed, and the remaining *Pst*I site was converted to a *Hin*dIII site by using oligonucleotide linkers. The yeast *URA3* gene contained a 1.1-kbp *Hin*dIII fragment was cloned into this site. The *Bam*HI-*Eco*RI fragment from *MDH2* containing the *URA3* insertion was used for one-step gene disruption (42), using the lithium acetate method for yeast transformation (24). Gene disruptions were verified by Southern blot analyses, using genomic DNA isolated as previously described (32) from wild-type and mutant yeast strains. ^{32}P-labeled DNA probes were prepared by the random-primer method (15).

Nucleotide sequence accession number. The sequence reported has been assigned GenBank accession number M37632.

RESULTS

Purification and expression of yeast MDH2. The levels of malate dehydrogenase activity in yeast cells grown on various carbon sources have been shown to be highly variable. Levels of the mitochondrial enzyme are low in cells grown

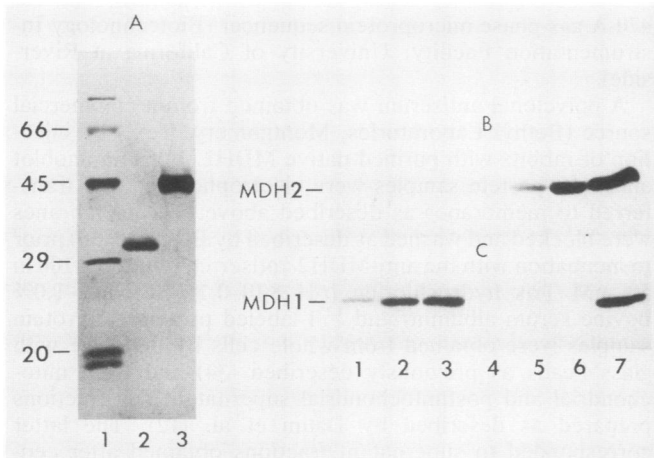

FIG. 1. Comparison of the sizes and expression of yeast MDH2 and MDH1. (A) Analysis in which 1 μg of MDH1 (lane 2) purified as previously described (34) and 2 μg of MDH2 (lane 3) purified as described in Materials and Methods were electrophoresed on a 10% sodium dodecyl sulfate-polyacrylamide gel and stained by using a Bio-Rad silver staining kit. The values represent sizes in kilodaltons of standard proteins (lane 1) coelectrophoresed on the same gel. (B and C) Immunoblot analysis conducted by using anti-MDH2 (B) and anti-MDH1 (C) antiserum as described in Materials and Methods with 25-μg extracts from mitochondrial fractions (lanes 1 to 3) or with 100-μg protein extracts from postmitochondrial supernatant fractions (lanes 4 to 6). The cellular fractions were prepared as described by Daum et al. (12) from S173-6B cells grown with 2% glucose (lanes 1 and 4), with 2% glycerol plus 2% lactate (lanes 2 and 5), or with 2% acetate (lanes 3 and 6) as a carbon source. Lanes 7 contained 0.5 μg of purified MDH2 (B) or MDH1 (C).

with glucose as a carbon source and three- to fivefold higher in acetate-grown cells (27). Levels of nonmitochondrial malate dehydrogenase activity measured in postmitochondrial supernatant fractions prepared as described by Daum et al. (12) are 30- to 50-fold higher in cells grown with acetate than in cells grown with glucose as a carbon source (27). Therefore, purification of the nonmitochondrial isozyme (designated MDH2) was initiated with yeast cells grown with acetate as a carbon source. Theoretically, this purification would have been facilitated by use of a yeast strain carrying a chromosomal disruption of the *MDH1* gene encoding the mitochondrial enzyme (MDH1). Unfortunately, this mutant is unable to utilize acetate as a carbon source (34). Therefore, a strain containing both enzymes was used. The purification scheme summarized in Materials and Methods was empirically developed. It utilizes an initial affinity chromatography step (CL-6B Blue Sepharose) to separate the cellular isozymes. Two major peaks of malate dehydrogenase activity were eluted with a KCl gradient from this column, the earlier corresponding to MDH1 (as determined by trial chromatography using extracts from the *MDH1* disruption strain and by immunoblot analysis; data not shown) and the later corresponding to MDH2. Subsequent chromatography steps, including DEAE-cellulose and CL-6B Blue Sepharose with an NADH gradient elution, resulted in purification of 8.8 mg of MDH2 from 200 g of cells.

Purified yeast MDH2 was examined by denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 1A, lane 3) and shown to be composed of a polypeptide with an approximate molecular weight of 42,000. Gel filtration chromatography (see Materials and Methods) produced an estimated size of 70,000 for the native enzyme, suggesting that MDH2 is a homodimer, as are most other malate dehydrogenases (2). The MDH2 subunit was significantly larger than the 33,500-molecular-weight subunit of yeast mitochondrial MDH1 (Fig. 1A, lane 2; 34) and larger than expected on the basis of previous reports of subunit molecular weights of 35,000 to 37,000 for mammalian cytosolic isozymes (4, 25). Amino-terminal amino acid sequence analysis was conducted following electrotransfer of purified MDH2 to a polyvinylidene difluoride filter. The resulting 16-residue sequence, Pro His Ser Val Thr Pro Ser Ile Glu Gln Asp Ser Leu Lys Ile Ala, corresponds exactly with that for the initial residues in a 34-residue sequence previously reported for the yeast cytosolic enzyme by Kopetzki et al. (29).

Purified MDH2 was used to prepare a polyclonal antiserum in rabbits. To confirm the cellular localization and to examine expression patterns, mitochondrial and postmitochondrial supernatant fractions were prepared for immunoblot analysis from yeast cells cultivated with glucose, glycerol plus lactate, or acetate as a carbon source. Highest levels of the 40,000-molecular-weight polypeptide recognized by the anti-MDH2 antiserum were present in postmitochondrial supernatant fraction from acetate-grown cells (Fig. 1B, lane 6). Levels in the same cellular fraction from glycerol-lactate-grown cells (lane 5) were twofold lower than acetate levels, as estimated by densitometry. No MDH2 was observed in the postmitochondrial supernatant fraction from glucose-grown cells (lane 4) or in any of the mitochondrial fractions (lanes 1 to 3) under these conditions. As a control, immunoblots of the same cellular extracts were incubated with an anti-MDH1 antiserum (34). MDH1 was detected only in mitochondrial extracts (Fig. 1C, lanes 1 to 3), and the immunochemical levels, while varying less dramatically than those of MDH2, were lowest in cells cultivated with glucose. No cross-reactivity was observed between MDH1 and MDH2 in these or in other immunoblots of the purified enzymes (data not shown).

Isolation of the *MDH2* gene. On the assumption that high levels of MDH2 could indicate an important function for this isozyme for grown of yeast cells on acetate as a carbon source and given the acetate$^-$ growth phenotype of yeast *MDH1* mutants (34), activity and immunoblot analyses for MDH2 were used to examine a collection of acetate$^-$ yeast mutants isolated by Mark T. McCammon and Joel M. Goodman (University of Texas Southwestern Medical Center). This collection consists of mutants in 14 complementation groups selected for an inability to grow on semisynthetic medium (YNB supplemented with 0.05% yeast extract) with acetate or oleate as a carbon source (34a).

In initial screens for mutants with defects in MDH2, cultures of strains representing each of the 14 complementation groups and of the parental strain MMY011 were grown to early log phase in rich YP medium with glucose as a carbon source. The cells were pelleted, washed, and shifted to rich medium with acetate for 5 h prior to harvesting, a period of time that we had previously determined to be sufficient to obtain the high levels of cellular malate dehydrogenase activity characteristic of cells grown on acetate. Total cellular protein extracts were prepared for assays as described in Materials and Methods. Extracts from one mutant strain, G38-5, representing complementation group E were found to consistently exhibit values for whole-cell malate dehydrogenase activity that were two- to threefold lower than those measured for extracts from the wild-type strain or other mutant strains. Extracts from the total of 11 strains comprising complementation group E were therefore

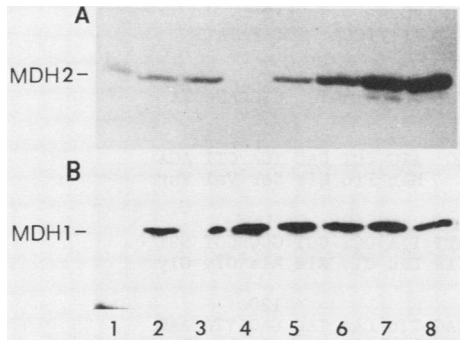

FIG. 2. Expression of MDH2 in acetate$^-$ yeast mutants and transformants. Immunoblot analysis was conducted by using anti-MDH2 (A) or anti-MDH1 (B) antiserum as described in Materials and Methods with 100-μg samples of whole-cell protein extracts prepared from yeast strains grown on YP medium with glucose as a carbon source to an A_{600} of 0.5 and then shifted to YP medium with acetate as a carbon source for 5 h. The strains were MMY011 (lanes 2 and 5), mutants G38-5 and G37-2 representing complementation group E (lanes 3 and 4, respectively), and three transformants of G37-2 carrying plasmid YEpMDH2 (lanes 6 to 8). Lanes 1 contained 0.5 μg of purified MDH2.

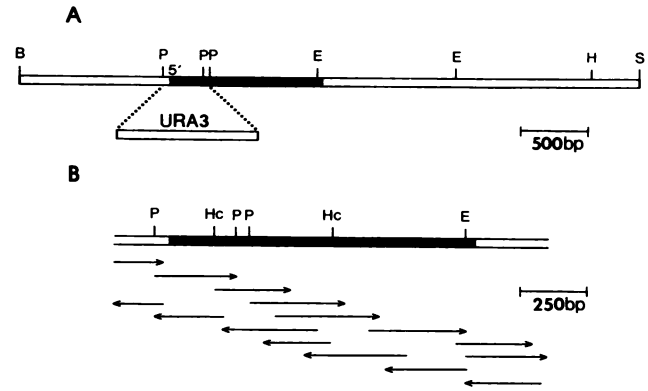

FIG. 3. Restriction map of *MDH2* subclones and strategies for nucleotide sequence analysis and gene disruption. (A) Partial map of restriction endonuclease sites within the genomic DNA insert from plasmid YEpMDH2. The location and orientation of the *MDH2* coding region (solid line) were determined by DNA sequence analysis as described in the text. The location of DNA fragments removed and replaced by the yeast *URA3* gene for disruption experiments is shown. (B) Restriction endonuclease sites used to construct subclones for dideoxynucleotide sequence analysis. Arrows indicate the direction and extent of nucleotide sequences obtained from subclones by using oligonucleotide primers complementary to either plasmid or *MDH2* sequences. Abbreviations: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; Hc, *Hin*cII; P, *Pst*I; S, *Sau*3A.

examined for malate dehydrogenase activity and MDH2 immunoreactivity following similar shifts to acetate medium. Under these conditions, all of the mutants in this group were found to exhibit reduced levels of total cellular malate dehydrogenase activity ranging from 20 to 50% of the wild-type level. Immunoblot analysis demonstrated the absence of detectable MDH2 in extracts from 5 of the 11 mutants. For example, extracts from mutant G38-5 (Fig. 2A, lane 3) contained immunoreactive but apparently inactive MDH2, whereas extracts from another mutant, G37-2 (lane 4), in the same complementation group lacked immunoreactive MDH2. Extracts from G38-5 and G37-2 (Fig. 2B) and from all other mutants in complementation group E (data not shown) were found to contain immunoreactive levels of MDH1 indistinguishable from those in wild-type cell extracts.

Mutant G37-2 was chosen for complementation to isolate the gene (designated *MDH2*) encoding MDH2. The mutant strain was transformed with the Naysmyth-Tatchell yeast DNA library (37), which contains 5- to 15-kbp fragments of genomic DNA obtained by partial restriction with *Sau*3A and cloned into the shuttle vector YEp13. This vector contains a multicopy 2μm origin of replication and a yeast *LEU2* gene for selection. To ensure an adequate representation of the library, approximately 10,000 Leu$^+$ transformants were obtained on plates containing minimal YNB medium with glucose. The transformants were then replated maintaining selection for the *LEU2* gene onto YNB plates with acetate as the carbon source. Plasmid DNAs were isolated from eight resulting Leu$^+$ acetate$^+$ yeast transformants by using the miniprep procedure of Hoffman and Winston (22) and amplified in *E. coli*. Partial restriction map analysis indicated that the isolates were apparently identical representatives of the same plasmid. The isolated plasmid, designated YEpMDH2, contains a 4.5-kbp yeast DNA insert (Fig. 3).

Plasmid YEpMDH2 was used for retransformation of mutant G37-2. The plasmid was found to consistently confer the acetate$^+$ growth phenotype to all resulting Leu$^+$ transformants. Immunoblot analysis conducted with cellular extracts from representative transformants (Fig. 2A, lanes 6 to 8) demonstrated that these extracts contained MDH2 and that levels of the protein were three- to fivefold higher than wild-type levels (lanes 2 and 5), consistent with expression of the corresponding gene on the 2μm plasmid. Immunochemical levels of MDH1 are unchanged in extracts from these transformants.

Nucleotide sequence analysis of the *MDH2* gene. To localize the *MDH2* coding region within the 4.5-kbp yeast DNA insert on plasmid YEpMDH2, a redundant oligonucleotide synthesized on the basis of the amino-terminal sequence of the purified protein as described in Materials and Methods was radiolabeled and used for Southern blot analysis of the plasmid DNA. The oligonucleotide was found to hybridize preferentially (data not shown) with the 300-bp *Pst*I fragment illustrated in Fig. 3. Nucleotide sequence analysis of this fragment confirmed the presence of a sequence encoding the amino terminus of MDH2 and established the orientation of the coding region shown.

Complete nucleotide sequence analysis of the *MDH2* gene was conducted, using the dideoxy method with subclones and nucleotide primers specific for plasmid sequences or for internal *MDH2* sequences on the basis of accumulated sequence data (see Materials and Methods and Fig. 3). The resulting nucleotide sequence with a 1,131-bp open reading frame is presented in Fig. 4. The coding region for the amino-terminal residues of the isolated MDH2 protein determined as described above is preceded by a Met residue which is presumed to be the translation initiation site because of the absence of similar residues upstream and the match of nucleotides surrounding the Met codon (AAC AUGCCU) to the initiation sequence context (ANNAU GNNU) of other yeast genes (13). The coding region is followed by two adjacent in-frame stop codons. The *MDH2* gene encodes a polypeptide with 376 amino acids (excluding the initiator methionine) and a calculated molecular weight of 40,684.

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-158       -150        -140        -130        -120        -110
ATATGAGA  TAAAGATTGC  TGCATGATTC  TCCTTCTGAT  TCTTTTTCCC  TGTATATATT

-100        -90         -80         -70         -60         -50
TTCTCCCCTT  CTGTATAAAC  GTACAGTCAG  AAGTAGTCCA  GAATATAGTG  CTGCAGACTA

-40         -30         -20         -10                         15
TTACAAAAGT  TCAATACAAT  ATCATAAAAG  TTATAGTAAC  ATG CCT CAC TCA GTT ACA
                                                Met Pro His Ser Val Thr

             30                  45                  60
CCA TCC ATA GAA CAA GAT TCG TTA AAA ATT GCC ATT TTA GGT GCT GCC GGT GGT
Pro Ser Ile Glu Gln Asp Ser Leu Lys Ile Ala Ile Leu Gly Ala Ala Gly Gly

 75                  90                 105                 120
ATC GGG CAG TCG TTA TCG CTG CTT TTG AAA GCT CAG TTG CAA TAC CAG TTA AAG
Ile Gly Gln Ser Leu Ser Leu Leu Leu Lys Ala Gln Leu Gln Tyr Gln Leu Lys

        135                 150                 165                 180
GAG AGC AAC CGG AGC GTT ACC CAC ATT CAT CTG GCT CTT TAC GAT GTC AAC CAA
Glu Ser Asn Arg Ser Val Thr His Ile His Leu Ala Leu Tyr Asp Val Asn Gln

                195                 210                 225
GAA GCC ATC AAC GGT GTT ACC GCC GAC TTG TCT CAT ATA GAC ACC CCC ATT TCC
Glu Ala Ile Asn Gly Val Thr Ala Asp Leu Ser His Ile Asp Thr Pro Ile Ser

    240                 255                 270                 285
GTG TCG AGC CAC TCT CCT GCA GGT GGC ATT GAG AAC TGT TTG CAT AAC GCT TCT
Val Ser Ser His Ser Pro Ala Gly Gly Ile Glu Asn Cys Leu His Asn Ala Ser

             300                 315                 330
ATT GTT GTC ATT CCT GCA GGT GTT CCA AGA AAA CCT GGC ATG ACT CGT GAT GAC
Ile Val Val Ile Pro Ala Gly Val Pro Arg Lys Pro Gly Met Thr Arg Asp Asp

345                 360                 375                 390
TTA TTT AAC GTG AAT GCT GGT ATC ATT AGC CAG CTC GGT GAT TCT ATT GCA GAA
Leu Phe Asn Val Asn Ala Gly Ile Ile Ser Gln Leu Gly Asp Ser Ile Ala Glu

        405                 420                 435                 450
TGT TGT GAT CTT TCC AAG GTC TTC GTT CTT GTC ATT TCC AAC CCT GTT AAT TCT
Cys Cys Asp Leu Ser Lys Val Phe Val Leu Val Ile Ser Asn Pro Val Asn Ser

                465                 480                 495
TTA GTC CCA GTG ATG GTT TCT AAC ATT CTT AAG AAC CAT CCT CAG TCT AGA AAT
Leu Val Pro Val Met Val Ser Asn Ile Leu Lys Asn His Pro Gln Ser Arg Asn

    510                 525                 540                 555
TCC GGC ATT GAA AGA AGG ATC ATG GGT GTC ACC AAG CTC GAC ATT GTC AGA GCG
Ser Gly Ile Glu Arg Arg Ile Met Gly Val Thr Lys Leu Asp Ile Val Arg Ala

             570                 585                 600
TCC ACT TTT CTA CGT GAG ATA AAC ATT GAG TCA GGG CTA ACT CCT CGT GTT AAC
Ser Thr Phe Leu Arg Glu Ile Asn Ile Glu Ser Gly Leu Thr Pro Arg Val Asn

615                 630                 645                 660
TCC ATG CCT GAC GTC CCT GTA ATT GGC GGG CAT TCT GGC GAG ACT ATT ATT CCG
Ser Met Pro Asp Val Pro Val Ile Gly Gly His Ser Gly Glu Thr Ile Ile Pro

        675                 690                 705                 720
TTG TTT TCA CAG TCA AAC TTC CTA TCG AGA TTA AAT GAG GAT CAA TTG AAA TAT
Leu Phe Ser Gln Ser Asn Phe Leu Ser Arg Leu Asn Glu Asp Gln Leu Lys Tyr
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FIG. 4. Nucleotide sequence of a 1.5-kbp genomic DNA fragment containing the *MDH2* gene and inferred amino acid sequence for MDH2.

Homology between partial amino acid sequences determined for the amino termini of purified yeast mitochondrial and cytosolic malate dehydrogenases was previously noted by Kopetzki et al. (29). Alignment of the complete amino acid sequences derived from the *MDH2* and *MDH1* gene sequences (Fig. 5) demonstrates that the full-length proteins are remarkably similar despite their disparate sizes (MDH1 has 317 amino acids). Numerous gaps in the MDH1 sequence and one two-residue gap in the MDH2 sequence were required for optimal alignment. With this alignment, long regions of near identity are obvious, for example, residues 97 to 121 in MDH2 with residues 75 to 98 in MDH1 and residues 240 to 271 in MDH2 with residues 203 to 234 in MDH1. The latter region contains numerous residues located within the Q axis of twofold symmetry believed to form the subunit interface in malate dehydrogenase dimers (41). Other identities include Asp-181 in MDH2 (Asp-151 in MDH1) and His-214 in MDH2 (His-177 in MDH1), amino acids believed to participate in a proton relay catalytic mechanism (3), and many other residues located primarily within the homologous amino-terminal regions with predicted functions in pyrimidine nucleotide binding (5). Overall, 49% of the residues in MDH1 are identical with aligned residues in MDH2. MDH1 shares a similar 48% residue identity with *E. coli* malate dehydrogenase (33), compared with 40% for MDH2.

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                735                 750                 765
TTA ATA CAT AGA GTC CAA TAC GGT GGT GAT GAA GTG GTC AAG GCC AAG AAC GGT
Leu Ile His Arg Val Gln Tyr Gly Gly Asp Glu Val Val Lys Ala Lys Asn Gly

    780                 795                 810                 825
AAA GGT AGT GCT ACC TTA TCG ATG GCC CAT GCC GGT TAT AAG TGT GTT GTC CAA
Lys Gly Ser Ala Thr Leu Ser Met Ala His Ala Gly Tyr Lys Cys Val Val Gln

            840                 855                 870
TTT GTT TCT TTG TTA TTG GGT AAC ATT GAG CAG ATC CAT GGA ACC TAC TAT GTG
Phe Val Ser Leu Leu Leu Gly Asn Ile Glu Gln Ile His Gly Thr Tyr Tyr Val

885                 900                 915                 930
CCA TTA AAA GAT GCG AAC AAC TTC CCC ATT GCT CCT GGG GCA GAT CAA TTA TTG
Pro Leu Lys Asp Ala Asn Asn Phe Pro Ile Ala Pro Gly Ala Asp Gln Leu Leu

        945                 960                 975                 990
CCT CTG GTG GAC GGT GCA GAC TAC TTT GCC ATA CCA TTA ACT ATT ACT ACA AAG
Pro Leu Val Asp Gly Ala Asp Tyr Phe Ala Ile Pro Leu Thr Ile Thr Thr Lys

                1005                1020                1035
GGT GTT TCC TAT GTG GAT TAT GAC ATC GTT AAT AGG ATG AAC GAC ATG GAA CGC
Gly Val Ser Tyr Val Asp Tyr Asp Ile Val Asn Arg Met Asn Asp Met Glu Arg

    1050                1065                1080                1095
AAC CAA ATG TTG CCA ATT TGC GTC TCC CAG TTA AAG AAA AAT ATC GAT AAG GGC
Asn Gln Met Leu Pro Ile Cys Val Ser Gln Leu Lys Lys Asn Ile Asp Lys Gly

            1110                1125            1,140       1,150
TTG GAA TTC GTT GCA TCG AGA TCT GCA TCA TCT TAA TGAGCA  TCGGACCGAA
Leu Glu Phe Val Ala Ser Arg Ser Ala Ser Ser  *

     1,160       1,170       1,180       1,190       1,200       1,210
GCATAAGAAT  GCAGCAAATT  GATAATATTC  CCGTTAAGCC  AGTCGTAGGA  AAAGGAAAAG

     1,220       1,230       1,240       1,250       1,260       1,270
AATGAGTAGC  AAATGTCACA  CAACAAACGC  GCACCATCCC  TACATACACA  AACACACACA

     1,280       1,290       1,300       1,310       1,320       1,330
CACACACACA  TATATATATA  TTCTACCCCA  TATAAGCGAG  TGATTCATCA  TGAAAAGAAT

     1,340       1,350
TTATCACTTC  GAATTTAAAT  AAT
```

Despite long regions of amino acid residue identity, comparison of the aligned nucleotide sequences of *MDH1* and *MDH2* (data not shown) showed no identities extending beyond 13 nucleotides in length. Also, no cross-hybridization between *MDH1* and *MDH2* sequences was observed under high-stringency conditions. The codon utilization and predicted residue contents of *MDH2* and *MDH1* are presented in Table 1. Calculation of the codon adaptation index for assessing codon usage bias in yeast genes as described by Sharp and Li (44) produced a value of 0.21 for *MDH2*, compared with 0.33 for *MDH1*. Interestingly, MDH2 lacks tryptophan residues, as do *E. coli* and all mitochondrial malate dehydrogenases for which sequences are available (20, 26, 33, 48) and unlike porcine and murine cytosolic isozymes, which each contain four tryptophan residues (25).

Among the striking differences between MDH1 and MDH2 (Fig. 5) is the 12-residue extension at the amino terminus of MDH2 not present on the mature form of the mitochondrial MDH1 polypeptide. Except for a high content of hydroxylated residues, the extension of MDH2 which contains 2 residues with acidic side chains shows no similarity to the 17-residue amino-terminal sequence present on the precursor form of MDH1, which contains 3 residues with basic side chains (48; data not shown). Also, large portions of the carboxy-terminal regions of the proteins between residues 272 and 345 of MDH2 corresponding to residues 235 and 293 of MDH1 are very dissimilar. Significant homology is restored only in the remaining 25 to 30 residues of the carboxy-terminal regions. Many of the shorter internal regions of nonhomology correspond with areas of nonrelatedness previously noted in comparisons of MDH1 with *E. coli* or mammalian mitochondrial malate dehydrogenases (48; data not shown).

Disruption of the *MDH2* gene. To disrupt the *MDH2* coding region, the 300- and 50-bp *Pst*I fragments containing 5′ noncoding and amino-terminal coding regions were removed and replaced with a 1.1-kbp DNA fragment containing the yeast *URA3* gene (Fig. 3) as described in Materials and Methods. The resulting 3.1-kbp *Bam*HI-*Eco*RI yeast DNA fragment containing the disrupted *MDH2* sequence was used for transformation of the haploid yeast strain S173-6B and also for transformation of strain SΔMDH1, a derivative of S173-6B which contains a deletion-insertion disruption of the chromosomal *MDH1* locus with the yeast *HIS3* gene (47). Ura^+ transformants of both strains were selected on minimal medium plates containing glucose as a carbon source. The chromosomal disruptions in representative transformants, designated SΔMDH2 and SΔMDH1/ΔMDH2, were confirmed by Southern blot analyses. Disruption of the *MDH2* gene resulted in replacement of a 2.35-kbp genomic *Bam*HI-*Eco*RI DNA fragment with the 3.1-kbp fragment that hybridized with both *MDH2* and *URA3* probes (Fig. 6). Disruption of the *MDH1* gene as previously described (47) resulted in replacement of a 6.1-kbp genomic *Sph*I fragment with a 7.0-kbp fragment that hybridized with both *MDH1* and *HIS3* probes. The absence of MDH2 and MDH1 polypeptides in

FIG. 5. Comparison of the amino acid sequences of MDH1 and MDH2. The amino acid sequence of MDH2 (top line) derived from the nucleotide sequence of the *MDH2* gene (Fig. 4) was aligned with the amino acid sequence of MDH1 (bottom line) previously derived from the corresponding gene sequence (48). The sequences shown start with amino-terminal sequences for the mature forms of both proteins as defined by partial amino acid sequence analysis of the purified yeast enzymes. Gaps (-) were introduced to optimize similarities. *, Identical residues.

these strains was also confirmed by immunoblot analyses (data not shown).

Initial analyses of growth phenotypes associated with *MDH2* gene disruption were conducted by examining the growth of wild-type and mutant strains on plates containing various carbon sources (data not shown). Mutant SΔMDH2 exhibited no growth on minimal YNB plates with either acetate or ethanol as a carbon source. Mutant SΔMDH1/ΔMDH2 was also unable to grow on rich YP plates with acetate as a carbon source, a growth phenotype previously reported for mutant SΔMDH1 (47). Importantly, the double mutant, in contrast with a [*rho*$^-$] derivative of S173-6B, exhibited some growth on YP plates with glycerol as a carbon source.

Culture doubling times and cellular levels of malate dehydrogenase activity were determined for wild-type and mutant strains grown on rich medium with various carbon sources. Disruption of either or both *MDH1* and *MDH2* genes did not substantially alter culture growth rates with glucose as a carbon source (Table 2). Levels of residual malate dehydrogenase activity measured in extracts from the glucose-grown cells were approximately 13% of wild-type

TABLE 1. Comparison of codon utilization in *MDH1* and *MDH2* genes

Amino acid	Codon	No.		Amino acid	Codon	No.		Amino acid	Codon	No.	
		MDH2	*MDH1*			*MDH2*	*MDH1*			*MDH2*	*MDH1*
Ala	GCA	7	8	Gly	GGA	1	3	Pro	CCA	6	11
	C	8	8		C	6	6		C	2	3
	G	2	0		G	4	1		G	1	1
	U	7	11		U	17	18		U	11	3
Arg	CGA	0	0	His	CAC	3	2	Ser	AGC	4	1
	C	1	1		U	8	6		U	1	1
	G	1	0	Ile	AUA	5	2		UCA	5	3
	U	3	1		C	7	10		C	9	6
	AGA	7	7		U	21	11		G	7	1
	G	2	1	Leu	CUA	3	3		U	11	10
Asn	AAC	18	13		C	2	0	Thr	ACA	2	4
	U	6	4		G	3	2		C	6	7
Asp	GAC	9	11		U	5	2		G	0	2
	U	11	6		UUA	14	4		U	6	5
Cys	UGC	1	0		G	11	18	Trp	UGG	0	0
	U	4	1	Lys	AAA	7	12	Tyr	UAC	5	2
Gln	CAA	8	4		G	10	14		U	5	1
	G	8	2	Met	AUG	9	5	Val	GUA	1	0
Glu	GAA	7	16	Phe	UUC	4	8		C	12	13
	G	7	4		U	5	4		G	7	5
									U	14	9

levels for SΔMDH1 and 84% of wild-type levels for SΔMDH2. These data are consistent with previous studies showing that the mitochondrial isozyme is the predominant activity in wild-type cells under this condition (27). Importantly, low levels of malate dehydrogenase activity representing approximately 3% of the activity in wild-type cell extracts were reproducibly measured in extracts from the double mutant SΔMDH1/ΔMDH2 grown on glucose.

On glycerol, growth rates of mutant SΔMDH2 were indistinguishable from those of the wild-type strain, whereas growth rates were reduced approximately twofold for mutants SΔMDH1 and SΔMDH1/ΔMDH2 (Table 2). The total cellular malate dehydrogenase activities, however, measured for the wild-type strain and each of the mutant strains grown on glycerol were 14- to 17-fold higher than glucose cellular levels. This result suggests that the residual activity in the double mutant may represent a third isozyme of malate dehydrogenase, the levels of which are derepressed

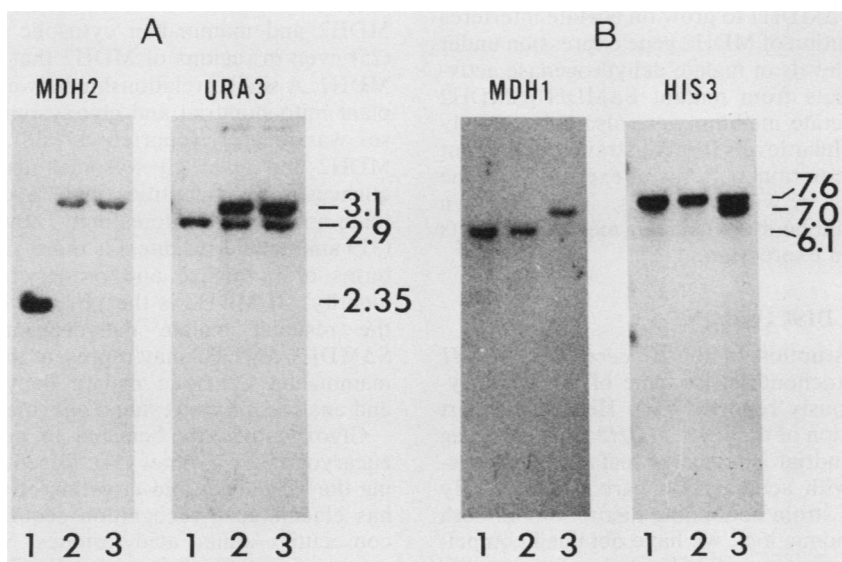

FIG. 6. Southern blot analysis of genomic DNA from yeast strains containing disruptions of the *MDH2* gene. Genomic DNA was isolated from haploid yeast strains S173-6B (lanes 1), SΔMDH2 (lanes 2), and SΔMDH1/ΔMDH2 (lanes 3). Aliquots of 10 μg were digested with *Bam*HI and *Eco*RI (A) or with *Sph*I (B), electrophoresed, and transferred to nitrocellulose filters. The yeast DNA fragments used as ^{32}P-labeled probes as indicated for each panel were a 2.35-kbp *Bam*HI-*Eco*RI fragment containing the *MDH2* gene, a 1.1-kbp *Hin*dIII fragment containing the *URA3* gene, a 0.64-kbp *Pst*I-*Hin*dIII fragment from the *MDH1* gene (48), and a 1.7-kbp *Bam*HI fragment containing the *HIS3* gene. The sizes in kilobase pairs of genomic DNA fragments that hybridize with these probes are indicted on the right. These fragments include a 2.9-kbp fragment in the *URA3* lanes and a 7.6-kbp fragment in the *HIS3* lanes which contain the resident genomic copies of those genes.

TABLE 2. Growth rates and cellular malate dehydrogenase activities of yeast *MDH2* and *MDH1* mutants

Strain	Carbon source					
	Glucose		Glycerol		Acetate	
	Doubling time[a] (h)	Malate dehydrogenase activity[b]	Doubling time (h)	Malate dehydrogenase activity	Doubling time (h)	Malate dehydrogenase activity
S173-6B	1.81	0.61	3.67	9.70	4.85	19.75
SΔMDH1	1.76	0.08	6.56	1.15	—[c]	0.52
SΔMDH2	1.79	0.51	3.65	8.35	13.20	9.05
SΔMDH1/ΔMDH2	2.06	0.02	7.38	0.35	—	0.06

[a] Yeast strains were grown at 30°C on YP medium containing carbon sources added to 2%. Doubling times were measured spectrophotometrically during logarithmic growth. Values represent averages of three experimental measurements.

[b] Expressed as micromoles of NAD^+ produced per minute per milligram of protein; determined by using extracts prepared from cells harvested at an A_{600} of 0.4 to 0.7. Values represent averages of assays from three independent cultures.

[c] —, Strains unable to grow on acetate were shifted from YP glucose medium to YP acetate medium for 5 h prior to preparation of cellular extracts.

with glycerol as a carbon source to a similar extent as those of MDH1 and MDH2 isozymes in strains SΔMDH2 and SΔMDH1, respectively. The relatively low levels of the putative third isozyme in cells grown on either a fermentable or a nonfermentable carbon source may explain our failure to detect this activity during purification of MDH2.

Mutant SΔMDH2 grew at a significantly reduced but measurable rate relative to the wild-type strain on rich medium with acetate and retained substantial cellular levels of malate dehydrogenase activity (Table 2). In contrast, no growth was measurable for mutants SΔMDH1 and SΔMDH1/ΔMDH2 with acetate as a carbon source. To try to obtain an estimate of residual malate dehydrogenase activity, cultures of these strains were shifted from glucose- to acetate-containing medium for 5 h prior to preparation of cellular extracts for assays. Exceptionally low levels of cellular malate dehydrogenase activity (3% of wild-type cellular acetate levels) were measured for mutant SΔMDH1 (Table 2). Since levels of MDH2 are normally elevated in wild-type cells grown on acetate (Fig. 1B), it is possible that the inability of strain SΔMDH1 to grow on acetate interferes with induction or execution of MDH2 gene expression under these conditions. The levels of malate dehydrogenase activity measured in extracts from mutant SΔMDH1/ΔMDH2 following a shift to acetate medium were also substantially lower than glycerol cellular levels for that strain, but without some knowledge of the normal patterns of expression of the putative third isozyme in wild-type cells, it is unknown whether this effect could be due to similar metabolic effects or to real differences in expression.

DISCUSSION

The cloning and disruption of the *S. cerevisiae MDH1* gene encoding the mitochondrial isozyme of malate dehydrogenase were previously reported (34). Here, we report the cloning and disruption of the yeast *MDH2* gene encoding the major nonmitochondrial isozyme of malate dehydrogenase in cells grown with acetate as a carbon source. By constructing a haploid strain containing disruptions in both *MDH1* and *MDH2* genomic loci, we have obtained compelling evidence for the existence of at least three genetically distinct malate dehydrogenase isozymes in *S. cerevisiae*. First, the strain lacking MDH1 and MDH2 was found to be capable of growth with glycerol as a carbon source. This finding is significant because the results obtained with yeast mutants lacking both mitochondrial and cytosolic citrate synthases (28) or aconitase (16) indicate that the expected growth phenotype of a yeast strain lacking all malate dehydrogenase activity is the inability to grow on this carbon source. Second, residual malate dehydrogenase activity representing 3 to 4% of the total cellular activity in wild-type cells grown on glucose or glycerol is retained in the strain containing the *MDH1* and *MDH2* gene disruptions.

Regarding the identity of the *MDH2* gene product, we suggest that this isozyme may function primarily in the glyoxylate cycle and that MDH2 is not analogous to the cytosolic enzymes of mammalian cells. In support of this idea, the anti-MDH2 antiserum described in this report was recently used to show that MDH2 is associated, although inefficiently, with peroxisomal fractions isolated from yeast cells grown on oleate and that malate dehydrogenase activity is significantly reduced in peroxisomal fractions from strain G37-2, which lacks MDH2 (35). More speculative evidence for this suggestion is based on the sequence analysis of *MDH2*. The primary sequences of MDH2 and MDH1, as well as of the mammalian mitochondrial malate dehydrogenases (26), are clearly very closely related, whereas there is very little similarity between the primary sequences of MDH2 and mammalian cytosolic malate dehydrogenases (25) even in regions of MDH2 that show no relatedness to MDH1. A similar relationship between primary sequences of plant mitochondrial and glyoxysomal malate dehydrogenases was recently reported (17, 18). An alignment of yeast MDH2 and plant glyoxysomal malate dehydrogenase sequences shows identities in 43% of the residue positions (data not shown). Interestingly, however, the plant enzyme (319 amino acid residues) is more similar to yeast MDH1 in terms of both size and primary sequence (46% residue identity). If MDH2 is the yeast glyoxysomal enzyme, then the residual malate dehydrogenase activity in strain SΔMDH1/ΔMDH2 may represent the enzymatic homolog of mammalian cytosolic malate dehydrogenase. Purification and analysis of the residual enzyme are in progress.

Glyoxysomes are believed to be a specialized form of eucaryotic peroxisomes (31). Recent progress in understanding the biogenesis and targeting of proteins to peroxisomes has elucidated a recognition sequence consisting of three consecutive amino acid residues, Ser-Lys-Leu, which can result in posttranslational localization to peroxisomes (19). The Ser residue can be functionally substituted with Ala or Cys, and the Lys residue can be substituted with Arg or His. This targeting sequence is frequently found at the extreme carboxy terminus of peroxisomal or glyoxysomal proteins, which include yeast cytosolic citrate synthase (45), but in some cases is apparently located internally (19). In contrast, plant glyoxysomal malate dehydrogenase contains an Ala-

His-Leu tripeptide within a 37-residue amino-terminal transit peptide which is removed upon organellar import (17). Examination of the MDH2 primary sequence shows no such consensus sequence at the carboxy terminus, and the gene sequence shows no evidence for an amino-terminal transit peptide. However, located internally there is a Ser-Arg-Leu sequence corresponding to residues 230 to 232. Assuming a similarity of three-dimensional structure, as predicted by the primary sequences of MDH2 and mitochondrial malate dehydrogenases, this tripeptide would be located within a hairpin loop between two beta sheets on the surface of the protein (41). The potential role of this tripeptide sequence in glyoxysomal localization of MDH2 will be tested by using site-directed mutagenesis.

In addition to the primary sequence of MDH2, the patterns of expression of the protein support the idea that this isozyme functions in the glyoxylate cycle. Highest levels of MDH2 are observed in cells grown on C_2 carbon sources, compared with negligible levels in cells grown on glucose. These patterns of expression are similar to those reported for other yeast glyoxylate cycle enzymes, including isocitrate lyase and malate synthase (14, 38, 49). The growth phenotypes of the yeast SΔMDH2 mutant are also compatible with glyoxylate cycle function, since this mutant is defective for growth on minimal medium with acetate or ethanol as a carbon source. The ability of the mutant to grow on rich medium with acetate suggests an auxotrophic requirement, perhaps for the C_4 products generated by the glyoxylate cycle. We have found that this is not a simple auxotrophy, however, because supplementation with succinate, malate, aspartate, or glutamate does not restore growth of the *MDH2* mutant on minimal medium with acetate as the carbon source.

Cytosolic malate dehydrogenase is among other yeast enzymes with functions in gluconeogenesis or the glyoxylate cycle that have been reported to be subject to catabolite inactivation (12a, 50). This phenomenon involves a rapid and specific proteolytic removal of these enzymes when glucose is added to an actively respiring culture, ensuring a faster metabolic response than that provided by simultaneous catabolite repression of gene expression (11, 23). We have found that MDH2 levels are rapidly depleted following the addition of glucose to cells growing with acetate as a carbon source (data not shown). The molecular mechanisms of catabolite inactivation of another yeast enzyme, fructose bisphosphatase, have been examined in some detail. Upon addition of glucose, this enzyme becomes inactivated by phosphorylation and is subsequently degraded (36). A serine at residue position 11 in the enzyme has been shown to be the target for cyclic AMP-dependent phosphorylation (40). Kopetzki et al. (29) have suggested that the amino terminus of MDH2, which contains residues with hydroxylated side chains at positions 3, 5, 7, and 12, may be similarly involved in the process of inactivation and degradation. With the *MDH2* gene and the anti-MDH2 antiserum, it should now be possible to determine the contribution of these and other controls in regulating cellular levels of MDH2.

ACKNOWLEDGMENT

This work was supported by Public Health Service grant GM-33218 from the National Institutes of Health.

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