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Isolation and Expression of the Gene Encoding Yeast Mitochondrial Malate Dehydrogenase

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The mitochondrial tricarboxylic acid cycle enzyme malate dehydrogenase was purified from *Saccharomyces cerevisiae*, and an antibody to the purified enzyme was obtained in rabbits. Immunoscreening of a yeast genomic DNA library cloned into a lambda gt11 expression vector with anti-malate dehydrogenase immunoglobulin G resulted in identification of a lambda recombinant encoding an immunoreactive β-galactosidase fusion protein. The yeast DNA portion of the coding region for the fusion protein translates into an amino acid sequence which is very similar to carboxy-terminal sequences of malate dehydrogenases from other organisms. In *S. cerevisiae* transformed with a multicopy plasmid carrying the complete malate dehydrogenase gene, the specific activity and immunoreactivity of the mitochondrial isozyme are increased by eightfold. Expression of both the chromosomal and plasmid-borne genes is repressed by growth on glucose. Disruption of the chromosomal malate dehydrogenase gene in haploid *S. cerevisiae* produces mutants unable to grow on acetate and impaired in growth on glycerol plus lactate as carbon sources.

The mitochondrial isozyme of malate dehydrogenase has key functions in the tricarboxylic acid (TCA) cycle and in the malate-aspartate shuttle of reducing equivalents across the organelle membrane. Due to the unfavorable equilibrium for oxaloacetate production, it has been postulated that a physical association between malate dehydrogenase and citrate synthase, the next enzyme in the TCA cycle, must exist to allow direct exchange of this molecule (40). In vitro, purified malate dehydrogenase can form a stable physical complex with citrate synthase (9) or with aspartate aminotransferase (2), an association which presumably would enhance shuttle cycle function. An additional level of regulation, or perhaps correlative to formation of alternative enzyme complexes, is the allosteric interaction of malate dehydrogenase with citrate (28).

In addition to extensive kinetic analyses of the enzyme, the structure of mitochondrial malate dehydrogenase is well defined. The enzyme from most organisms is a dimer composed of identical subunits with approximate molecular weights of 34,000 (1). The amino acid sequence of the porcine enzyme has been determined (4), and the sequence has been correlated with the crystallographic structure of the enzyme (32). Particularly instructive are comparisons of this structure with that determined for the porcine cytoplasmic enzyme, which is also a dimer with a subunit molecular weight of 36,000 and which also functions in the malate-aspartate shuttle. The primary structures of the compartmentalized isozymes show a low degree of similarity (approximately 20% [4]), yet the main chain folding of the two proteins is remarkably similar (32; J. J. Birktoft, R. A. Bradshaw, and L. J. Banaszak, submitted for publication). Structural features associated with catalysis, substrate and cofactor binding, and subunit interactions are similar. For example, the catalytic mechanisms of both enzymes involve homologous histidine and aspartate residues (3). The overall low level of sequence relatedness presumably reflects other differential functions for the compartmentalized isozymes. This idea is supported by comparison of the primary sequences for eucaryotic mitochondrial malate dehydrogenases with that for malate dehydrogenase from *Escherichia coli* (L. McAlister-Henn, M. Blaber, R. A. Bradshaw, and S. J. Nisco, submitted for publication). The amino acid sequences are 59% identical, indicative of dramatic evolutionary conservation of the TCA cycle enzyme.

We wish to develop a eucaryotic molecular genetic system that can be exploited for in vivo tests of correlates between structure and function of mitochondrial malate dehydrogenase and of the role of this enzyme in regulation of the TCA cycle. Since yeast genes can be manipulated in vitro and reintroduced into their proper chromosomal location through transformation (17), and since *Saccharomyces cerevisiae* is particularly amenable to study of mutations affecting mitochondrial functions (recently reviewed by Tzagoloff and Myers [42]), we have isolated the gene for mitochondrial malate dehydrogenase from *S. cerevisiae*. Details of the isolation of this gene through immunoscreens of a yeast genomic library in lambda gt11 are presented in this report. Initial studies of expression of the gene are also described.

MATERIALS AND METHODS

Strains and growth conditions. *E. coli* Y1090 {Δ*lacU169 proA*$^+$ Δ*lon araD139 strA supF* [*trpC222*::Tn*10*](pMC9); 43} was used for lambda gt11 transfection, immunoscreening, and preparation of lysogens. This strain was grown in LB medium containing 0.2% maltose and 50 μg of ampicillin per ml. *E. coli* HB101 was grown on M-9 minimal medium (27) containing 0.2% glucose and 50 μg of ampicillin per ml for amplification of plasmid DNAs. Strain JM105, used for propagation of M13 phage derivatives, was maintained on M-9 agar plates and grown on 2× YT medium (26) for transformations and plating.

The haploid yeast strain Sg7 (α *leu2 his3-Δ1 trp1 gcr1-1*), constructed through a cross of *S. cerevisiae* strains S173-29A (α *leu2-3 leu2-112 his3-Δ1 ade1-101 trp1-289*; 5) and DFY67 (a *leu2 lys1 trp1 gcr1-1*; 8), was used for isolation of mitochondrial malate dehydrogenase. Yeast transformations were conducted by using the lithium acetate protocol (18) with strains S173-6B (a *leu2-3 leu2-112 his3-Δ1 ura3-52 trp1-289*; 5) and S173-6B/SB, a diploid constructed by cross-

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ing S173-6B with S173-SB (α *leu2-3 leu2-112 ade1-101 ura3-52 trp1-289*; 24). Yeast cells were cultivated with YP (1% yeast extract, 2% Bacto-Peptone [Difco Laboratories, Detroit, Mich.]) or minimal YNB (0.17% yeast nitrogen base, 0.5% ammonium sulfate, pH 6.0) medium supplemented with 20 μg each of adenine, uracil, tryptophan, leucine, and histidine per ml as required for auxotrophic mutants. The carbon source utilized was 2% glucose, 2% glycerol plus 2% lactate, or 2% acetate. Cell growth for both yeast and bacterial cultures was monitored spectrophotometrically by A_{600}.

Isolation of yeast mitochondrial malate dehydrogenase. Yeast strain Sg7 was cultivated in YP medium containing 2% glycerol plus 2% lactate as carbon source. Cells were harvested at mid-log phase (A_{600}, 1.5) by centrifugation at 3,000 × *g*. The cell pellets were washed with distilled water and suspended to a concentration of 1 g of cells per 5 ml of a buffer containing 50 mM $NaPO_4$ (pH 7.4), 5 mM EDTA, 5 mM β-mercaptoethanol, and 50% glycerol. The cell suspension was frozen by dripping into liquid nitrogen and stored at −70°C.

For enzyme isolation, 90 g of frozen cells was thawed in 100 ml of buffer A (10 mM $NaPO_4$ [pH 7.4], 1 mM EDTA, 1 mM β-mercaptoethanol) plus 10 mM phenylmethylsulfonyl fluoride and broken with glass beads, using a Bead Beater (Biospec Products, Bartlesville, Okla.). During breaking, the cells were maintained at 4 to 7°C in an ice-chilled chamber by alternating eight cycles of 1-min rotor bursts with 1-min intervals. The lysate was cleared by centrifugation at 10,000 × *g* for 10 min. The glass bead break was repeated, using the lysate pellet, and the supernatants from both breaks were combined. Nucleic acids were removed from the crude supernatant by precipitation with 0.25% protamine sulfate and centrifugation. The resulting supernatant was loaded slowly onto a CL-6B Blue Sepharose column (70-ml column bed; Pharmacia, Inc., Piscataway, N.J.) previously equilibrated with buffer A. The column was washed with 600 ml of buffer A, and bound proteins were eluted with a 500-ml 0.0 to 2.0 M KCl gradient. Malate dehydrogenase, assayed in 10-ml fractions collected during the gradient elution, was resolved into two distinct forms. The fractions containing the mitochondrial isozyme, initially identified on the basis of elution profiles reported by others (16; E. R. Dickman, Ph.D. thesis, Albert Einstein College of Medicine, New York, N.Y., 1976) and later by size and cellular localization of the purified protein as described in the text, were pooled and dialyzed for 24 h at 4°C with three changes of 3 liters each of buffer A. The dialysate was loaded onto a DEAE-cellulose column preequilibrated with buffer A. Proteins were eluted with a 0.0 to 0.5 M KCl gradient, and fractions containing malate dehydrogenase activity were pooled and precipitated with 60% ammonium sulfate. The precipitate was suspended in 3 ml of buffer B (50 mM $NaPO_4$, pH 7.4), dialyzed, and loaded onto a Sephacryl S-200 gel filtration column (1.5 by 80 cm; Pharmacia) equilibrated with the same buffer. Following elution with buffer B, fractions containing malate dehydrogenase activity were pooled and loaded onto a 2-ml CL-6B/Blue Sepharose column preequilibrated with buffer B. Malate dehydrogenase was eluted with buffer B containing 12 mM NADH, dialyzed, and stored in buffer B containing 50% glycerol. These steps resulted in an overall increase in specific activity of malate dehydrogenase from 3.63 U/mg of protein in the crude extract to 308.7 U/mg in the purified sample. Protein concentrations were measured by the method of Lowry et al. (20). Malate dehydrogenase activity was measured as the oxaloacetate-dependent rate of NADH oxidation (ΔA_{340}) in assay mixes containing 45 mM KPO_4 (pH 6.5), 0.12 mM NADH, and 0.33 mM oxaloacetate. A unit represents 1 μmol of NAD^+ produced per min.

Immunochemical methods. Antiserum specific for yeast malate dehydrogenase was obtained through a commercial source (Bethyl Laboratories, Montgomery, Tex.) by injecting rabbits with the purified native protein. Immunoglobulin G (IgG) was prepared from the serum by ammonium sulfate precipitation followed by DEAE- and carboxymethyl cellulose chromatography as described by Palacios et al. (30).

For Western blot (immunoblot) analyses, protein samples were electrophoresed on 8 or 10% polyacrylamide-sodium dodecyl sulfate (SDS) gels (11) and electrotransferred to nitrocellulose filters. The filters were blocked and washed as described by Burnette (6) and then incubated with anti-malate dehydrogenase IgG diluted 1:100 in a solution containing 50 mM Tris-chloride (pH 8.1), 0.15 M NaCl, and 1% bovine serum albumin (radioimmunoassay grade; Sigma Chemical Co., St. Louis, Mo.). Bound IgG was detected by autoradiography following a 1-h incubation of the washed filters with ^{125}I-labeled protein A.

Immunoscreening of lambda gt11 plaques was conducted as described by Snyder and Davis (37). The recombinant yeast library was plated at a density of 10^5 bacteriophage per 20-cm petri plate. Following incubation and lysis at 42°C for 3 h, the plaques were overlaid with nitrocellulose filters which had been presoaked in 10 mM isopropyl-β-D-thiogalactopyranoside and air dried. Overlays with duplicate sets of filters were conducted at 37°C overnight and then at 37°C for 4 h. Incubation of the filters with IgG and ^{125}I-labeled protein A were as described previously (37). The IgG used to screen the library was a 1:100 dilution of the anti-malate dehydrogenase IgG which had been preincubated with nitrocellulose filters lifted from mock-infected plaques, a procedure which substantially reduces IgG interactions with bacterial antigens.

Lysogens were prepared by infecting *E. coli* Y1090 cells and isolating colonies from the center of phage plaques which were capable of growth at 30°C but not at 42°C. The latter temperature inactivates the temperature-sensitive *c*I857 repressor encoded by the phage leading to prophage induction and lysis in this strain (43). To examine fusion proteins, lysogens were grown in 15-ml cultures of LB containing 10 mM MgCl and 0.2% maltose. When the cultures reached A_{600} of 0.5, isopropyl-β-D-thiogalactopyranoside was added to a concentration of 10 mM to induce the *lacZ* promoter and the cultures were shifted to 42°C for 20 min and then to 37°C for 2 h. A 300-μl portion of chloroform to ensure complete lysis and 300 μl of a 10-mg/ml concentration of phenylmethylsulfonyl fluoride were added, and the incubations were continued for 30 min at 37°C. Cell debris was removed by centrifugation at 10,000 × *g* for 10 min. Soluble proteins in the supernatant were recovered by precipitation with 70% ammonium sulfate. The precipitates were suspended in 200 μl of phosphate-buffered saline (10 mM $NaPO_4$ [pH 7.0], 15 mM NaCl) and dialyzed with three changes of the same buffer at 4°C for 24 h. The dialysates were diluted with an equal volume of SDS sample buffer (0.1 M Tris-chloride [pH 6.8], 6% SDS, 4 mM EDTA, 5% β-mercaptoethanol, 20% glycerol, 0.1% bromophenol blue) and heated in a boiling-water bath for 3 min prior to electrophoresis.

Total cellular protein samples from yeast cells were obtained by vortexing cell pellets with glass beads as previously described (25). The pellets were broken in buffer A (described above) with 10 mM phenylmethylsulfonyl fluo-

ride, and the lysates were cleared by centrifuging for 10 min in an Eppendorf centrifuge. The supernatants were used directly for enzyme assays or diluted with an equal volume of SDS sample buffer for electrophoresis.

Nucleic acid hybridizations. Lambda DNA was isolated from 250-ml cultures of lysogens and purified by cesium chloride gradient centrifugation as described by Maniatis et al. (21). Plasmid DNA was prepared from chloramphenicol-treated bacterial cultures and purified by hydroxyapatite column chromatography (36). Yeast genomic DNA was isolated as described previously (23). Hybridization probes labeled with [α-^{32}P]dATP were prepared by the random primer method (12) with DNA fragments isolated from 0.8% low-melt agarose gels.

To screen the Naysmyth-Tatchell yeast genomic recombinant plasmid library (29), *E. coli* HB101 was transformed and plated at a density of 600 transformant colonies per plate on 12 large (20-cm) petri plates containing LB agar plus 50 μg of ampicillin per ml. The colonies were transferred to Whatman 540 filter papers for lysis and hybridization (13). Hybridization with ^{32}P-labeled DNA fragments was conducted at 65°C, using 3× SSC (1× SSC = 0.15 M NaCl plus 15 mM sodium citrate, pH 7.0) containing 0.02% each bovine serum albumin, Ficoll, and polyvinylpyrrolidone plus 10 μg of depurinated salmon sperm DNA per ml. Positive transformants obtained in the initial screen were streaked onto LB-ampicillin plates to obtain single colonies for secondary hybridization.

For Southern hybridizations, DNA restriction fragments were electrophoresed on 0.8% agarose gels, transferred to nitrocellulose as described by Southern (39), and hybridized with ^{32}P-labeled DNA probes as described above for colony hybridizations.

For Northern (RNA) blot hybridizations, total cellular RNA was prepared from yeast cell pellets as described previously (22). RNA samples of 15 μg were electrophoresed on 1% agarose-formaldehyde gels and transferred to nitrocellulose filters. The filters were hybridized with ^{32}P-labeled DNA fragments for 24 h at 42°C in 5× SSC containing 50% formamide, 10 μg of salmon sperm DNA per ml, and 0.2% each bovine serum albumin, Ficoll, and polyvinylpyrrolidone and then washed with three changes of 5× SSC at 42°C for 30 min prior to autoradiography.

Nucleotide sequence analysis. The dideoxy primer extension protocol (35) was used for nucleotide sequence analysis of DNA fragments subcloned into single-stranded M13 plasmids (26). The primers were 17-mer sequencing oligonucleotides obtained from New England BioLabs, Inc., Beverly, Mass.

Cell fractionation. Mitochondria were prepared as described by Daum et al. (10) from S173-6B and S173-6B transformed with YEpM10. For enzyme and protein assays, the mitochondrial fractions were diluted 1:10 or 1:20 in the mannitol-Tris buffer containing 1% Triton.

RESULTS

Purification of yeast mitochondrial malate dehydrogenase. *S. cerevisiae* Sg7 was used for isolation of mitochondrial malate dehydrogenase. This strain contains a mutation (*gcr1*) which results in substantial reduction in the levels of otherwise very abundant glycolytic enzymes (8), including glyceraldehyde-3-phosphate dehydrogenase, which we have found to copurify with malate dehydrogenase on Blue Sepharose columns. Also, presumably because of a combination of low levels of glycolytic enzymes and to the requirement for mitochondrial functions for growth of this strain, the specific activity of malate dehydrogenase is two- to threefold higher in crude extracts prepared from Sg7 than in extracts from other yeast strains which lack the *gcr* mutation. Approximately 5 mg of the enzyme was purified from 90 g (wet weight) of cells harvested from cultures grown to early log phase on YP medium with 2% glycerol plus 2% lactate as carbon sources. Details of the purification protocol are described in Materials and Methods. The mitochondrial and cytoplasmic isozymes were resolved in an initial chromatography step, using a 0.0 to 2.0 M KCl gradient to obtain differential elution from a CL-6B Blue Sepharose column. In subsequent purification steps, malate dehydrogenase activity correlated with the presence of a single mitochondrial isozyme. The profile of proteins at each stage of purification is illustrated in Fig. 1. Malate dehydrogenase was purified to apparent homogeneity as judged by the presence of a single polypeptide band with an approximate molecular weight of 33,000 following SDS-polyacrylamide gel electrophoresis. This value is in close agreement with previous estimates of the subunit molecular weight of the yeast mitochondrial isozyme (16). The cellular localization of the purified enzyme is further substantiated by cell fractionation and immunoblot analyses described below.

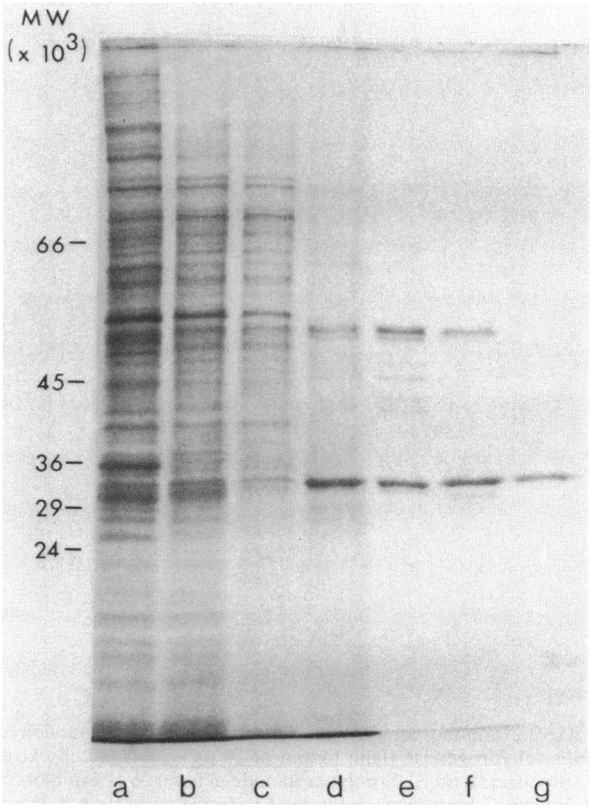

FIG. 1. Purification of yeast mitochondrial malate dehydrogenase. Protein samples (10 to 20 μg) obtained at each stage in the purification of the mitochondrial isozyme of malate dehydrogenase from yeasts were electrophoresed on SDS-polyacrylamide gels and stained with Coomassie blue. Lane a, Whole-cell protein extract from S173-6B; lane b, cellular protein extract from Sg7; and extracts from Sg7 following protamine sulfate precipitation (lane c), KCl gradient elution from CL-6B Blue Sepharose (lane d), DEAE-cellulose column chromatography (lane e), Sephacryl S-200 gel filtration (lane f), and elution with NADH from CL-6B Blue Sepharose (lane g). MW, Molecular weight.

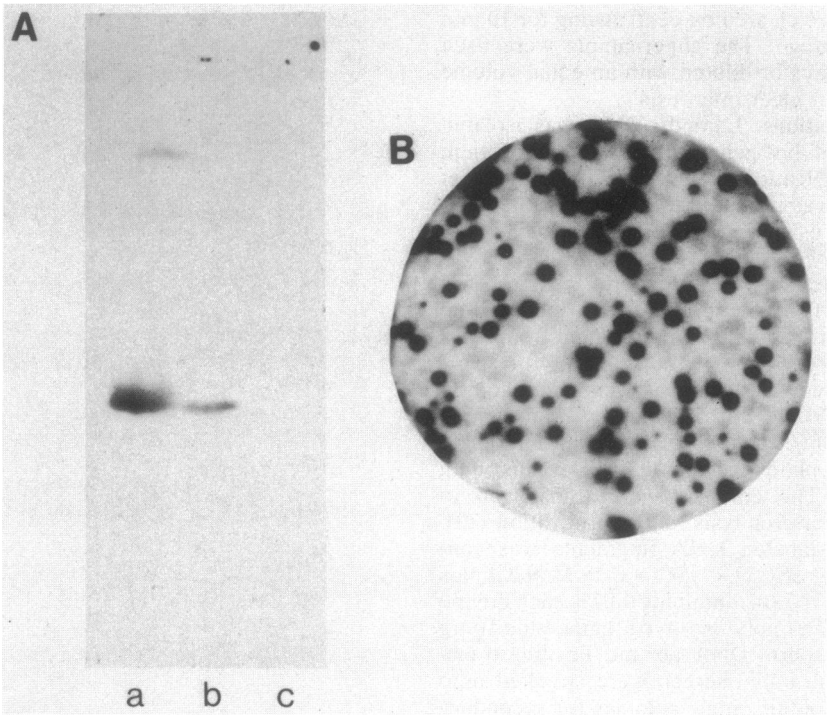

FIG. 2. Immunoreactivity of anti-mitochondrial malate dehydrogenase IgG. (A) Protein samples of 25 ng of purified yeast mitochondrial malate dehydrogenase (lane a) and of 10 μg of whole-cell extracts from yeast strain Sg7 (lane b) and bacterial strain Y1090 (lane c) were electrophoresed on SDS-polyacrylamide gels for Western blot analysis, using anti-malate dehydrogenase IgG and ^{125}I-labeled protein A. (B) Anti-malate dehydrogenase IgG and ^{125}I-labeled protein A were used to screen a library of lambda gt11 plaques containing yeast genomic DNA. Immunoreactivity during a secondary stage of plaque purification is shown for plaques from a positive isolate, lambda M8, relative to nonreactive plaques on the same plate.

Antiserum against yeast mitochondrial malate dehydrogenase was prepared by injection of rabbits with the native form of the purified enzyme. The IgG fraction obtained from the rabbit serum as described in Materials and Methods reacts well against immunoblots of the purified enzyme and with a polypeptide with the appropriate molecular weight in total yeast cellular extracts (Fig. 2A). The antiserum strongly inhibits the activity of the purified mitochondrial enzyme, but does not affect the activity of a partially purified cytoplasmic isozyme or react with immunoblots of the latter (data not shown).

Isolation of the mitochondrial malate dehydrogenase gene. The anti-malate dehydrogenase IgG was used to screen the lambda gt11 yeast genomic DNA library constructed by Snyder and Davis (37), using protocols described in Materials and Methods. This library contains 2- to 4-kilobase-pair (kbp) fragments of randomly sheared yeast chromosomal DNA cloned into the *Eco*RI restriction site near the 3′ end of the *lacZ* gene of *E. coli*. Yeast genes in the proper orientation and reading frame may be expressed as fusion proteins under control of the *lacZ* promoter. One million recombinant phage were screened and eight positive phage were identified with the anti-malate dehydrogenase IgG. The eight phage were purified through two or three cycles of plating to obtain single plaques. Only three of the original eight phage maintained strong immunoreactivity after replating, and plaques of one of these phage, lambda M8, were particularly reactive (Fig. 2B).

Lysogens were prepared by infecting *E. coli* Y1090 with the three purified recombinant phage recovered from the immunoscreen. Synthesis of putative β-galactosidase fusion proteins in the lysogens was induced by addition of isopropyl-β-D-thiogalactopyranoside, and lysis was induced by shifting lysogen cultures to 42°C. Soluble proteins were recovered by ammonium sulfate precipitation as described in Materials and Methods and examined by SDS-polyacrylamide gel electrophoresis and immunoblotting. Only one of the lysogen extracts, that from cells infected with phage lambda M8, was found to contain a protein larger than β-galactosidase which reacted with anti-malate dehydrogenase IgG (Fig. 3). Extracts from the other two lysogens contained reduced levels of β-galactosidase, but no immunoreactive proteins were detected (data not shown).

Since fusion proteins may exhibit differential stabilities, purified lambda DNA was obtained from the three Y1090 lysogens to examine relatedness through cross-hybridization. Yeast DNA fragments of 2.5 kbp from lambda M8 and 3.0 and 3.5 kbp from the other lambda DNAs were recovered by *Eco*RI restriction digestion. These fragments failed to cross-hybridize in dot blot or Southern blot analyses and therefore appear to be nonoverlapping fragments of yeast genomic DNA.

The yeast DNA *Eco*RI fragment from lambda M8 was subcloned into M13mp18 for DNA sequence analysis, since the coding region for the immunoreactive yeast protein should abut the *Eco*RI site in the β-galactosidase gene. Nucleotide sequence information was obtained from both ends of the *Eco*RI fragment. The sequence derived from the orientation shown in Fig. 4 encodes an open reading frame which can be translated into 51 amino acid residues between the *Eco*RI boundary and a UAG stop codon. This partial protein sequence is similar to aligned amino acid sequences from the carboxy termini of porcine mitochondrial and *E. coli* malate dehydrogenases (Fig. 5). The yeast sequence is

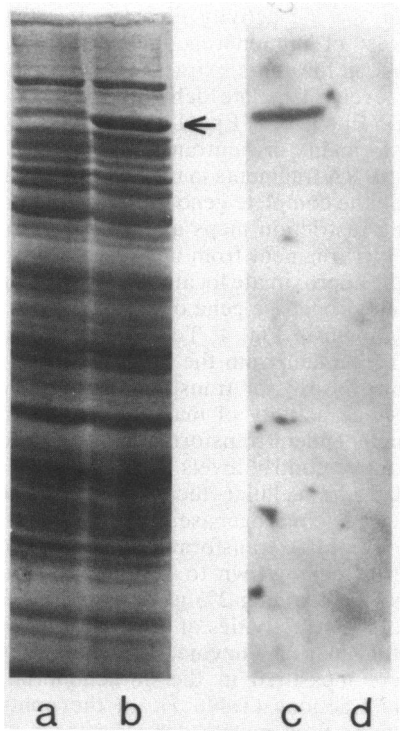

FIG. 3. Identification of an immunoreactive β-galactosidase fusion protein in lambda lysogens. Protein extracts prepared from bacterial lysogens infected with lambda gt11 (lanes b and d) and from the recombinant lambda M8 (lanes a and c) were analyzed by electrophoresis followed by staining with Coomassie blue (lanes a and b) or immunoblotting, using anti-yeast malate dehydrogenase IgG and ^{125}I-labeled protein A (lanes c and d). The position of β-galactosidase in Coomassie blue-stained electrophoretic protein standards is indicated by the arrow.

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yeast mMDH.....F F A S P V T L G P D G I E K I H P I G E L
porcine mMDH....Y * S T * L L * * K K * * * * N L G * * K I
E. coli MDH.....* * S Q * L L * * K N * V * E R K S * * T L
                                        275

S S E E E E M L Q K C K E T L K K N I E K G V N F V A S - K
* P F * * K * I A E A I P E * * A S * K * * E E * * K N M *
* A F * Q N A * E G M L D * * * * D * A L * Q E * * - N - *
                                    300
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FIG. 5. Comparison of carboxy-terminal amino acid sequences of yeast and pig mitochondrial and *E. coli* malate dehydrogenases. The yeast amino acid sequence derived from a partial nucleotide sequence of the mitochondrial malate dehydrogenase (mMDH) gene is aligned with carboxy-terminal sequences for the mitochondrial isozyme from pigs (4) and for enzyme from *E. coli* (McAlister-Henn et al., submitted). Asterisks indicate residues identical to those of the yeast enzyme. The numbers denote positions of amino acids within the porcine and *E. coli* sequences. Gaps introduced to maximize homology are indicated by dashes.

identical at 22 positions with each of the other amino acid sequences.

Expression of the yeast mitochondrial malate dehydrogenase gene. To obtain the complete gene for mitochondrial malate dehydrogenase, the yeast DNA *Eco*RI fragment from lambda M8 was used as a hybridization probe to screen the Naysmyth-Tatchell yeast DNA library (29). This library contains large (10- to 15-kbp) genomic DNA fragments cloned into the shuttle vector YEp13, which carries a yeast 2μ origin of replication and a selectable *LEU2* gene. Three independent *E. coli* transformants were identified by colony hybridization as described in Materials and Methods. The restriction maps presented in Fig. 4 show that the DNA

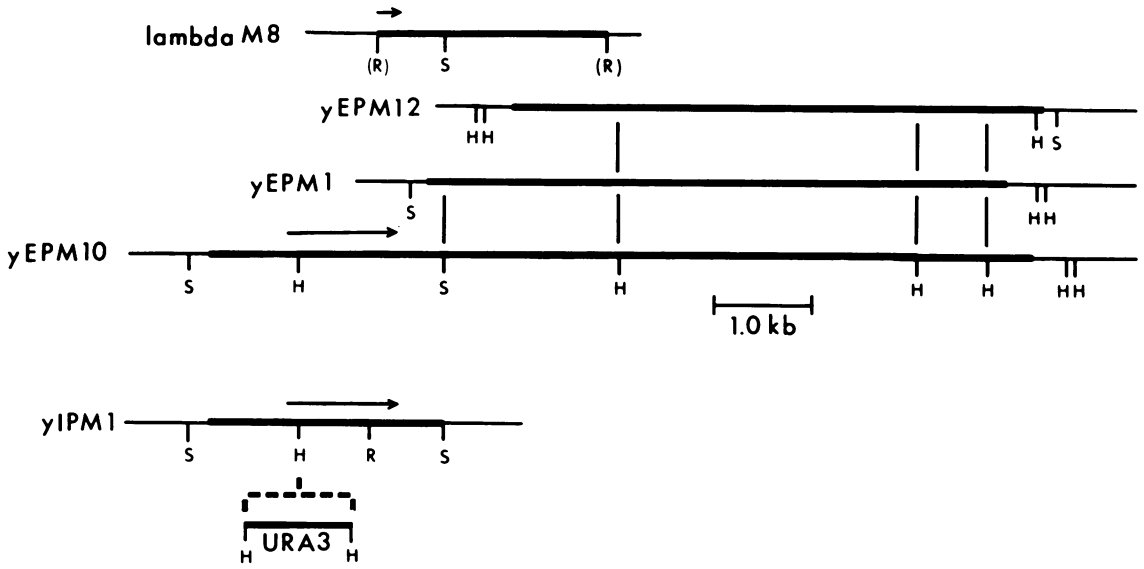

FIG. 4. Restriction endonuclease cleavage maps for subclones of the yeast mitochondrial malate dehydrogenase gene. Overlapping restriction maps are shown for the yeast DNA inserts (heavy lines) from lambda M8 and from three recombinant plasmids isolated from a yeast genomic library in plasmid YEp13. The arrow above the lambda M8 insert indicates the direction and extent of nucleotide sequence information. The arrow above the YEpM10 insert indicates the approximate location and orientation of the gene encoding mitochondrial malate dehydrogenase based on analysis of expression and nucleotide sequence data as explained in the text. YIpM1 was constructed by subcloning the *Sph*I fragment from YEpM10 into pUC19. Restriction site abbreviations: H, *Hin*dIII; S, *Sph*I; (R), artificial *Eco*RI. kb, Kilobase.

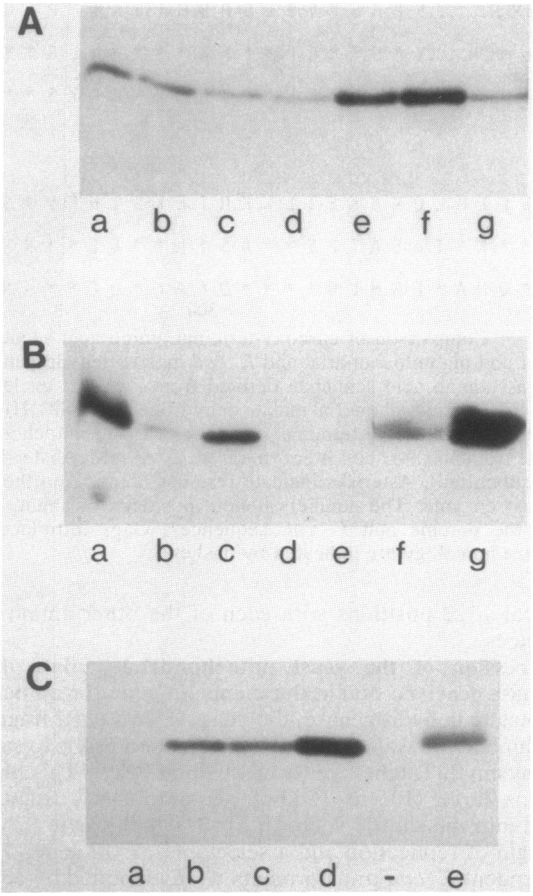

FIG. 6. Immunoblot analyses of expression of mitochondrial malate dehydrogenase in yeast cell. (A) Protein extracts were prepared from yeast S173-6B transformants containing the recombinant plasmid YEpM1 (lanes a and b), YEpM12 (lanes c and d), or YEpM10 (lanes e and f) or a control YEp13 plasmid (lane g). Equivalent concentrations of total cellular proteins were electrophoresed for Western blot analysis, using anti-mitochondrial malate dehydrogenase IgG and ^{125}I-labeled protein A. (B) Mitochondria were isolated from S173-6B and S173-6B transformed with YEpM10, and samples containing equivalent concentrations of protein were examined by SDS-polyacrylamide gel electrophoresis and immunoblotting. Lane a, 50 ng of purified yeast mitochondrial malate dehydrogenase. Samples were from nontransformed and transformed cells, respectively, from whole-cell extracts (lanes b and c), from postmitochondrial supernatants (lanes d and e), and from isolated mitochondria (lanes f and g). (C) Whole-cell protein extracts for electrophoresis and immunoblotting were obtained from S173-6B grown on 2% glucose (lane a) or 2% glycerol plus 2% lactate (lane b) and from S173-6B transformed with YEpM10 grown on glucose (lane c) or glycerol plus lactate (lane d). Lane e, 25 ng of yeast mitochondrial malate dehydrogenase.

inserts from plasmids (designated YEpM1, YEpM10, and YEpM12) isolated from these transformants contain nonidentical but overlapping fragments of yeast genomic DNA.

Yeast strain S173-6B was transformed with each of the YEp13 recombinant plasmids, and transformants were isolated as Leu$^+$ colonies. Protein extracts were prepared from the transformants and examined for expression of malate dehydrogenase by enzyme assays and immunoblotting. The total specific activity of malate dehydrogenase measured for extracts from cells transformed with YEpM10 was five- to sevenfold higher than levels measured for cells transformed with YEpM1, YEpM12, or a YEp13 control plasmid. These differences in specific activity correlated well with differences in levels of immunoreactive mitochondrial malate dehydrogenase in the same extracts as shown in Fig. 6A.

Since the levels of malate dehydrogenase in cells transformed with YEpM1 or YEpM12 were similar to those in control transformants or nontransformed cells, we assume that the yeast DNA fragments in these recombinant plasmids do not contain the complete gene for malate dehydrogenase. By comparing restriction maps and hybridization patterns, using the *Eco*RI fragment from lambda M8 as a probe, we determined the approximate location of the coding region for the malate dehydrogenase gene on the yeast DNA fragment in YEpM10 shown in Fig. 4. To test this localization, the 3.0-kbp *Sph*I fragment from the YEpM10 DNA insert was subcloned into YEp13 for transformation into yeast cells. The total specific activity of malate dehydrogenase in extracts from independent transformants was found to be six- to eightfold above control levels.

To examine the cellular localization of the plasmid-encoded malate dehydrogenase, mitochondria were prepared from YEpM10 transformants and from nontransformed S173-6B cells grown to mid-log phase on minimal medium (YNB) containing 2% glycerol plus 2% lactate as carbon sources. The activities of malate dehydrogenase and a mitochondrial marker enzyme, isocitrate dehydrogenase (NAD$^+$), were measured in the mitochondrial and postmitochondrial fractions (Table 1). In the nontransformed cells, 13% of the total malate dehydrogenase activity was found to be associated with the mitochondria, whereas in YEpM10 transformants the mitochondrial fraction contained 49% of the total activity. The absolute levels of malate dehydrogenase activity in YEpM10 transformants were more than eightfold higher than those in the mitochondrial fraction but only slightly elevated over levels in the postmitochondrial fraction from nontransformed cells. Judging from the distribution of isocitrate dehydrogenase activity in the subcellular fractions, the mitochondrial fractions are estimated to be 85 to 90% pure. The levels of the mitochondrial enzyme in the different cell fractions were examined by immunoblotting (Fig. 6B) and estimated by densitometric measurements. The increase in malate dehydrogenase activ-

TABLE 1. Subcellular distribution of malate and isocitrate dehydrogenases

Enzyme[a]	Strain[b]	Mitochondrial fraction		Postmitochondrial supernatant	
		Sp act (U/mg)	% Total activity	Sp act (U/mg)	% Total activity
Malate dehydrogenase	S173-6B	8.04	13.1	1.07	86.9
	S173-6B (YEpM10)	66.70	48.9	1.39	51.1
Isocitrate dehydrogenase	S173-6B	2.54	85.0	0.009	15.0
	S173-6B (YEpM10)	2.39	90.0	0.005	10.0

[a] Malate dehydrogenase activity was determined as described in Materials and Methods. Isocitrate dehydrogenase activity was measured as micromoles of NAD$^+$ reduced per minute per milligram of protein in assays containing 50 mM Tris-chloride (pH 7.6), 4 mM $MgCl_2$, 2 mM isocitrate, and 0.5 mM NAD$^+$. The values shown represent averages of two independent determinations.

[b] Nontransformed cells and cells transformed with YEpM10 were grown on YNB medium with 2% glycerol and 2% lactate as carbon sources. Mitochondrial and postmitochondrial cellular fractions were prepared as described in Materials and Methods.

ity in YEpM10 transformants was due to an eightfold increase in the level of the isozyme associated with the mitochondria in these cells.

Expression of yeast mitochondrial malate dehydrogenase was also examined in YEpM10 transformants and nontransformed cells grown with either 2% glucose or 2% glycerol plus 2% lactate as carbon sources, conditions which respectively result in repression and derepression of many mitochondrial functions in yeast cells. Cells were harvested from cultures grown in YNB medium with different carbon sources, and the pellets were divided for isolation of total cellular RNA and for preparation of protein extracts as described in Materials and Methods. The protein extracts were assayed for malate dehydrogenase activity and immunoreactivity. Total cellular malate dehydrogenase activity in nontransformed cells was sevenfold higher with growth on glycerol plus lactate as compared with growth on glucose. A similar sixfold differential with these carbon sources was observed for cells transformed with YEpM10. However, the absolute levels of malate dehydrogenase activity in transformants were approximately fivefold higher than in nontransformed cells with both types of carbon sources, reflecting increased levels of the plasmid-encoded enzyme. The specific derepression of the mitochondrial isozyme, examined by immunoblotting (Fig. 6C), was approximately sixfold in both transformed and nontransformed cells.

To determine whether the increased levels of the mitochondrial enzyme in glycerol/lactate-grown cells are due to an increase in levels of mRNA, Northern blot analysis was conducted, using total cellular RNA isolated from the YEpM10 transformant and nontransformed cells. The ^{32}P-labeled *Sph*I fragment from YEpM10 which contains the malate dehydrogenase gene hybridized with a single RNA species of approximately 1.7 kilobases in RNA samples from transformed and control cells (Fig. 7). The level of the hybridizing transcript was elevated in RNA samples from both the transformant (lane b) and control cells (lane d) grown on glycerol plus lactate as compared with growth on glucose (lanes a and c, respectively). The relative level of the transcript estimated from dot blot titrations was eight- to tenfold higher in transformed cells than in controls under both growth conditions.

Disruption of the chromosomal malate dehydrogenase gene. To assess the phenotype of a yeast mutant deficient in expression of mitochondrial malate dehydrogenase, the isolated gene was used to disrupt (34) the chromosomal gene. For this disruption, a 1.1-kbp *Hin*dIII fragment containing a selectable yeast *URA3* gene was inserted into the *Hin*dIII restriction site in the malate dehydrogenase gene on plasmid YIpM1 (Fig. 4). This insertion interrupted the coding region within sequences encoding the amino terminus of the protein, as determined by partial nucleotide sequencing (data not shown). The *Sph*I fragment containing the *URA3* insertion was transformed into haploid and diploid yeast strains. The disruption of the chromosomal gene in Ura$^+$ transformants was confirmed by Southern blot analyses (Fig. 8). Insertion of the 1.1-kbp *URA3* gene increased the size of a 2.2-kbp *Eco*RI genomic DNA fragment containing a portion of the malate dehydrogenase gene to approximately 3.3 kbp. This fragment in DNA from haploid and diploid transformants hybridized with ^{32}P-labeled DNA probes from both the malate dehydrogenase (Fig. 8A) and the *URA3* (Fig. 8B) genes. The absence of the mitochondrial isozyme in extracts from haploid strains containing the gene disruption was confirmed by Western immunoblot analyses (data not shown).

The gene disruption mutants and control strains were

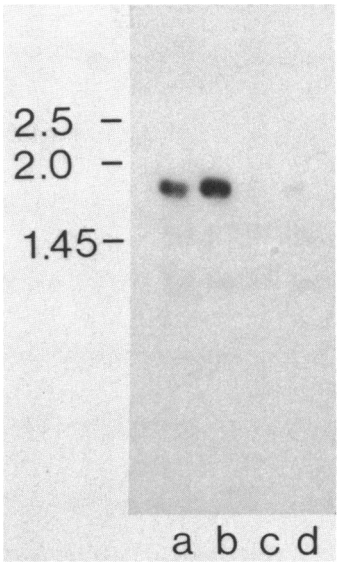

FIG. 7. Northern blot analysis of malate dehydrogenase transcript. Total cellular RNA was isolated from S173-6B transformed with YEpM10 (lanes a and b) and from nontransformed cells (lanes c and d) grown on glucose (lanes a and c) or glycerol plus lactate (lanes b and d). RNA samples of 15 μg each were electrophoresed, transferred to nitrocellulose, and hybridized with a 3.0-kbp ^{32}P-labeled *Sph*I fragment containing the yeast mitochondrial malate dehydrogenase gene. Numbers indicate sizes (in kilobases) of DNA fragments electrophoresed as standards.

plated to test a variety of carbon source and auxotrophic growth requirements. On plates containing minimal medium (YNB), the phenotypes of the haploid malate dehydrogenase mutants were indistinguishable from those of respiratory-deficient ([*rho*$^-$]) strains. No growth was observed with glycerol plus lactate or with acetate as carbon sources, conditions permissive for growth of the wild-type strain, nor did addition of aspartate or glutamate result in growth of the mutants under these conditions (data not shown). On plates containing rich medium (YP), haploid strains containing the gene disruption also exhibited no growth with acetate and reduced growth relative to the parental haploid with glycerol plus lactate. The latter phenotype does distinguish the malate dehydrogenase mutants from [*rho*$^-$] strains which do not grow on glycerol plus lactate; the haploid gene disruption mutants have also been confirmed to be respiratory competent by genetic crosses with a [*rho*$^-$] strain (data not shown). A possible explanation for the observed growth of the haploid gene disruption strains on YP but not on YNB containing glycerol plus lactate is the presence of low but sufficient levels of glucose or of some untested growth supplement in yeast extract. The diploid strain containing a single allele disruption exhibited no mutant growth phenotypes in these tests.

Levels of malate dehydrogenase activity were measured with extracts from the haploid control strain and from disruption mutants grown on YP-glucose and on YP-glycerol/lactate (Table 2). The approximate twofold increase in total cellular activity in control cells grown on glycerol/lactate as compared with those grown on glucose is not as dramatic as the sixfold increase observed with minimal medium as described above. However, disruption of the mitochondrial malate dehydrogenase gene resulted in an approximate fourfold decrease in total cellular malate dehydrogenase in cells grown on glycerol/lactate compared

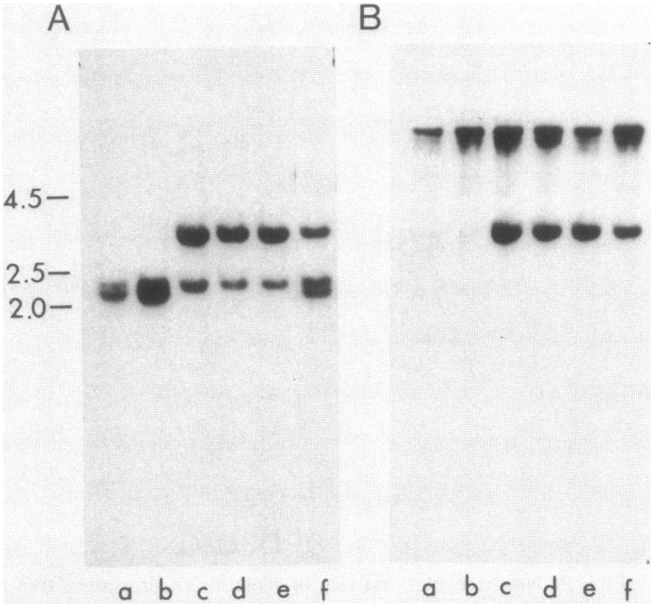

FIG. 8. Southern hybridization of chromosomal DNA from control cells and strains containing gene disruptions. Yeast genomic DNA was isolated from haploid strain S173-6B (lane a), from three independent Ura$^+$ colonies obtained by transformation of S173-6B with the malate dehydrogenase gene disrupted by a *URA3* gene insertion (lanes c, d, and e), from the diploid strain S173-6B/SB (lane b), and from a diploid strain containing the gene disruption (lane f). DNA samples of 10 μg each were digested with *Eco*RI, electrophoresed, transferred to nitrocellulose, and hybridized with the ^{32}P-labeled *Sph*I fragment containing the malate dehydrogenase gene from YEpM10 (A). Because of an internal *Eco*RI site, this DNA probe hybridized with two fragments of 2.2 and 2.3 kbp in genomic DNA from wild-type cells. In DNA from cells carrying the gene disruption, the 2.2-kbp *Eco*RI fragment was replaced by a 3.3-kbp fragment that also hybridized with a 1.1-kbp ^{32}P-labeled *URA3* gene (B). The large (>6-kbp) *Eco*RI fragment in panel B contained the resident genomic copy of the *URA3* gene.

with a twofold decrease in cells grown on glucose. The remaining cellular activity attributed to the cytoplasmic isozyme was similar for both carbon sources.

DISCUSSION

Although several collections of yeast mutants with defects in mitochondrial functions and oxidative energy metabolism have been isolated (7, 31, 41), mutants with specific defects in mitochondrial malate dehydrogenase have not been described. Thus, since the direct approach for obtaining the gene for this enzyme through complementation was not available, we instead identified a portion of the coding region by using anti-malate dehydrogenase IgG to screen the lambda expression library for *S. cerevisiae* described by Snyder and Davis (37). The identity of the gene was confirmed by partial nucleotide sequence information, by mitochondrial localization of the gene product, and by gene disruption. Haploid yeast cells containing a genomic disruption of the mitochondrial malate dehydrogenase gene did not exhibit aspartate or glutamate auxotrophy, suggesting compensatory function of the cytoplasmic isozyme or aspartate aminotransferase or both. This result was not unexpected, since disruption of yeast genes encoding both cytoplasmic and mitochondrial isozymes of citrate synthase has been shown to be necessary to produce glutamate auxotrophs (19). The malate dehydrogenase disruption mutants were impaired in growth on glycerol plus lactate and were unable to grow on acetate, suggesting key roles for the mitochondrial isozyme in energy production under these conditions.

The specific activity of mitochondrial malate dehydrogenase was elevated approximately eightfold in yeast cells transformed with a multicopy plasmid containing the entire coding region for the corresponding gene. Expression of the cytoplasmic isozyme in the same cells, measured by assays of postmitochondrial cellular fractions, was essentially unchanged from control levels. In addition to appropriate localization, the level of the mitochondrial enzyme was elevated to a similar extent above control levels in transformants grown under repressing or derepressing conditions for mitochondrial functions, indicating that the plasmid-encoded protein was regulated in the same manner as the chromosomal gene product. The extent of derepression of malate dehydrogenase expression at the level of both RNA and protein in cells grown on nonfermentable carbon sources as compared with glucose was similar to that reported for other yeast mitochondrial proteins including iso-1-cytochrome *c* (15) and citrate synthase (19).

The partial amino acid sequence derived from the yeast DNA fragment encoding an immunoreactive β-galactosidase fusion protein shares extensive identity with carboxy-terminal sequences reported for porcine and murine mitochondrial (4, 14) and *E. coli* (McAlister-Henn et al., submitted) malate dehydrogenases. The corresponding nucleotide sequences are also highly homologous but contain no identities exceeding six consecutive nucleotides in length. Furthermore, we have found no evidence for cross-hybridization of the rat, *E. coli*, and yeast genes in dot blot and Southern hybridization analyses conducted under a wide range of stringency conditions (data not presented). Despite an overall identity of 59% between the aligned nucleotide sequences for the *E. coli* gene and the rat cDNA, numerous third-position codon differences provide a likely explanation for the lack of hybridization between these genes, which may also be the case for the yeast gene. Because of the high degree of evolutionary conservation of this TCA cycle enzyme and because of differences between the rat and *E. coli* genes involving specific codon insertions and deletions which are speculated to represent intron boundaries in an ancestral gene, the complete nucleotide sequence of the yeast gene will be of obvious interest.

The gene encoding the mitochondrial isozyme of malate dehydrogenase is present in a single copy in genomic DNA

TABLE 2. Malate dehydrogenase activity in strains containing a gene disruption

Strain[a]	Sp act (U/mg)[b]	
	Glucose	Glycerol plus lactate
S173-6B	0.83	2.01
S173-6B (*mdh:URA3-1*)	0.34	0.46
S173-6B (*mdh:URA3-2*)	0.38	0.41
S173-6B (*mdh:URA3-3*)	0.40	0.43

[a] The haploid strain S173-6B and three independent Ura$^+$ strains containing the malate dehydrogenase gene (*mdh*) disruption were cultivated with YP medium with either 2% glucose or 2% glycerol plus 2% lactate as carbon sources.

[b] Malate dehydrogenase activity was determined as described in Materials and Methods. Each value represents an average of two independent measurements.

from haploid *S. cerevisiae* (Fig. 8; data not shown). We have found no evidence for structurally related sequences in DNA or RNA hybridizations or any evidence for cross-reacting proteins in immunochemical tests. This suggests that the yeast cytoplasmic enzyme is structurally distinct from the mitochondrial isozyme, as is the case for the porcine cellular isozymes which exhibit minimal relatedness at the level of amino acid sequence (4). It is interesting at this point to note the very different patterns for evolution of the yeast citrate synthase isozymes. The mitochondrial and cytoplasmic enzymes are very similar (75% homologous), whereas they share only 20% homology with citrate synthase from *E. coli* (33).

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