Chacko, S. (1981) *Biochemistry 20*, 702–707.
Chalovich, J. M., Cornelius, P., & Benson, C. E. (1987) *J. Biol. Chem. 262*, 5711–5716.
Cross, R. A., Cross, K. E., & Small, J. V. (1987) *FEBS Lett. 219*, 306–310.
Dabrowska, R., Gouch, A., Galazkiewicz, B., & Osinska, H. (1985) *Biochim. Biophys. Acta 842*, 70–75.
Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82*, 70–77.
Fujita, H., Ishimura, K., Ban, T., Kurosumi, M., Sobue, K., & Kakiuchi, S. (1984) *Cell Tissue Res. 237*, 375–377.
Furst, D. O., Cross, R. A., Mey, J. D., & Small, J. V. (1986) *EMBO J. 5*, 251–257.
Graceffa, P. (1987) *FEBS Lett. 218*, 139–142.
Graceffa, P., & Lehrer, S. S. (1980) *J. Biol. Chem. 255*, 11296–11300.
Haugland, R. P. (1970) Ph.D. Thesis, Stanford University, Palo Alto, CA.
Heaslip, R. J., & Chacko, S. (1985) *Biochemistry 24*, 2731–2736.
Horiuchi, K. Y., & Chacko, S. (1988) *Biophys. J. 53*, 463a.
Horiuchi, K. Y., Miyata, H., & Chacko, S. (1986) *Biochem. Biophys. Res. Commun. 136*, 962–968.
Ishii, Y., & Lehrer, S. S. (1985) *Biochemistry 24*, 6631–6638.
Ishimura, K., Fujita, H., Ban, T., Matsuda, H., Sobue, K., & Kakiuchi, S. (1984) *Cell Tissue Res. 235*, 207–209.
Kaminski, E. A., & Chacko, S. (1984) *J. Biol. Chem. 259*, 9104–9108.
Lash, J. A., Sellers, J. R., & Hathaway, D. R. (1986) *J. Biol. Chem. 261*, 16155–16160.
Lehrer, S. S., Betteridge, D. R., Graceffa, P., Wong, S., & Seidel, J. C. (1984) *Biochemistry 23*, 1591–1595.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Lynch, W. P., Riseman, V. M., & Bretscher, A. (1987) *J. Biol. Chem. 262*, 7429–7437.
Marston, S. B., Lehman, W., Moody, C. J., & Smith, C. W. J. (1985) in *Advances in Protein Phosphatases II* (Merlevede, W., Lens, H., & DiSalvo, J., Eds.) pp 171–189, Leuven University Press, Leuven, Belgium.
Miyata, H., & Chacko, S. (1986) *Biochemistry 25*, 2725–2729.
Ngai, P. K., & Walsh, M. P. (1984) *J. Biol. Chem. 259*, 13656–13659.
Ngai, P. K., & Walsh, M. P. (1985) *Biochem. J. 230*, 695–707.
Smith, C. W. J., Pritchard, K., & Marston, S. B. (1987) *J. Biol. Chem. 262*, 116–122.
Sobue, K., Muramoto, Y., Fujita, M., & Kakiuchi, S. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 5652–5655.
Sobue, K., Morimoto, K., Kanda, K., Maruyama, K., & Kakiuchi, S. (1982) *FEBS Lett. 138*, 289–292.
Sobue, K., Takahashi, K., & Wakabayashi, I. (1985) *Biochem. Biophys. Res. Commun. 132*, 645–651.
Wang, J. L., & Edelman, G. M. (1971) *J. Biol. Chem. 246*, 1185–1191.
Weltman, J. K., Szaro, R. P., Frackelton, A. R., Jr., Dowben, R. M., Binting, J. R., & Cathou, R. E. (1973) *J. Biol. Chem. 248*, 3173.

Gene Sequence and Primary Structure of Mitochondrial Malate Dehydrogenase from *Saccharomyces cerevisiae*[†,‡]

Leslie M. Thompson, Pamela Sutherland, Joan S. Steffan, and Lee McAlister-Henn*

Department of Biological Chemistry, California College of Medicine, University of California, Irvine, California 92717

Received March 24, 1988; Revised Manuscript Received May 26, 1988

ABSTRACT: The nucleotide sequence was determined for a 1.5-kilobase genomic fragment containing the mitochondrial malate dehydrogenase gene (*MDH*1) of *Saccharomyces cerevisiae*. The open-reading frame encodes a precursor form of the mature enzyme containing an amino-terminal extension of 17 amino acid residues. In vitro translation experiments confirm that the initial translation product of *MDH*1 is larger than the mature polypeptide. Transcription of *MDH*1 initiates at several sites from 83 to 97 nucleotides 5′ of the translational start site. Alignment of the amino acid sequence for the mature yeast enzyme with those for mammalian mitochondrial and for *Escherichia coli* malate dehydrogenases reveals polypeptides of very similar sizes with identical amino acids at 54% and 48% of the residue positions, respectively. The amino acid sequences of the yeast and mammalian mitochondrial targeting sequences are similar but less related than the mature polypeptides. The yeast *MDH*1 gene is shown to reside on chromosome XI.

Two forms of malate dehydrogenase, a mitochondrial and a cytoplasmic isozyme, catalyze the NAD^+-dependent oxidation of malate in eucaryotic cells. As components of the malate/aspartate shuttle cycle, this compartmentalized isozyme system represents an important mechanism for exchange of substrates and reducing equivalents between central metabolic pathways separated by the mitochondrial membrane. The mitochondrial and cytoplasmic forms of malate dehydrogenase from many species are dimers of identical subunits with approximate molecular weights of 68 000 and 72 000, respectively (Banaszak & Bradshaw, 1975). X-ray crystallography and amino acid sequence analyses of the porcine isozymes (Birktoft et al., 1982, 1987; Roderick &

[†] Supported by USPHS Research Grant GM 33218.
[‡] The nucleic acid sequence in this paper has been submitted to GenBank under Accession Number J02841.
* To whom correspondence should be addressed.

0006-2960/88/0427-8393$01.50/0

Banaszak, 1986) have provided detailed information about the structures of the enzymes. The main chain folding of the two enzymes is remarkably similar, and they share catalytic mechanisms involving homologous histidine and aspartate residues (Birktoft & Banaszak, 1983). However, the porcine enzymes are only marginally related at the level of primary sequence with identities at approximately 20% of the residue positions in aligned amino acid sequences. A similar relationship (23% identity) has recently been reported for the compartmentalized isozymes of mouse (Joh et al., 1987a,b). In contrast, the high degree of identity between the amino acid sequences of eucaryotic mitochondrial and *Escherichia coli* (McAlister-Henn et al., 1987) malate dehydrogenases as described in this report and by others (Joh et al., 1987b) suggests highly conservative evolution of the tricarboxylic acid cycle enzyme and extensive divergence of the cytoplasmic enzyme following the presumptive duplication of an ancestral gene. That this duplication event occurred prior to the emergence of eucaryotes is suggested by the similarity between primary structures of the cytoplasmic enzymes and the enzyme from another procaryote, *Thermus flavus* (Nishiyama et al., 1986).

We have previously described the isolation of the yeast gene encoding mitochondrial malate dehydrogenase (*MDH*1) by immunoscreening of a λ gt11 genomic library and subsequent screening of a yeast genomic library in the shuttle vector YEp13 to obtain the full-length gene (McAlister-Henn & Thompson, 1987). The one-step gene disruption technique (Rothstein, 1983) was used to produce a haploid yeast strain lacking the mitochondrial but not the cytoplasmic enzyme. This mutant strain grows at wild-type rates with glucose as a carbon source but at reduced rates if at all on various nonfermentable carbon sources, indicating that both tricarboxylic acid cycle and shuttle cycle functions are dispensable in glucose-grown cells. Expression of both *E. coli mdh* and yeast *MDH*1 genes is repressed by aerobic cultivation on glucose (McAlister-Henn & Thompson, 1987; Sutherland & McAlister-Henn, 1985).

To extend analyses of the structure and expression of malate dehydrogenase, we present here the complete nucleotide sequence of the yeast *MDH*1 gene and characterization of the gene with respect to chromosomal location and sites for initiation of transcription. The relationship between precursor and mature forms of the mitochondrial enzyme is described and the protein sequence derived for the yeast enzyme compared with those for mammalian mitochondrial and *E. coli* malate dehydrogenases.

Experimental Procedures

Strains and Growth Conditions. The haploid yeast strain S173-6B (a *leu*2-3 *leu*2-112 *his*3-Δ1 *ura*3-52 *trp*1-289; Botstein et al., 1979) transformed with the yeast/bacterial shuttle plasmid YEpM10 was used for isolation of cellular RNA and protein. As previously described (McAlister-Henn & Thompson, 1987), the YEpM10 vector carries the *MDH*1 gene as well as 2-micron sequences for multicopy replication and the yeast *LEU*2 gene for selection. Yeast were grown either in rich YP medium (1% yeast extract/2% bactopeptone) or in minimal YNB medium (0.17% yeast nitrogen base/0.5% ammonium sulfate, pH 6.0) supplemented with 20 μg/mL each of adenine, uracil, tryptophan, and histidine. The carbon sources were 2% glycerol plus 2% lactate. *E. coli* strain JM105 (Yanisch-Perron et al., 1985) was maintained on M-9 agar plates (Miller, 1972) and grown on 2 × YT medium (2% yeast extract, 3.2% bactopeptone, and 1% NaCl) for transformation with M13 vectors. Culture growth was monitored spectrophotometrically by the absorbance at 600 nm.

Nucleotide and Amino Acid Sequence Analysis. The dideoxy method (Sanger et al., 1977) was used to obtain nucleotide sequences of DNA restriction fragments subcloned into M13 vectors (Messing, 1983). The sequencing primers used for chain elongation were either 17-mer oligonucleotides complementary to M13 sequences obtained from New England Biolabs or 18–20-mer oligonucleotides complementary to *MDH*1 sequences synthesized with an Applied Biosystems DNA synthesizer (Protein and Nucleic Acid Laboratory, University of California at Irvine).

Amino-terminal sequence analysis of yeast mitochondrial malate dehydrogenase purified as previously described (McAlister-Henn & Thompson, 1987) was conducted following transfer of the protein from a polyacrylamide–sodium dodecyl sulfate (SDS)[1] gel to a poly(vinylidene difluoride) membrane (Immobilon Transfer; Matsudaira, 1987) using an Applied Biosystems 470-A gas-phase microprotein sequencer (Biotechnology Instrumentation Facility, University of California at Riverside). Protein and DNA sequence data were stored and analyzed by computer using programs developed by Schwindinger and Warner (1984).

S1 Nuclease and Primer Extension Analyses. For mRNA transcript mapping experiments, total cellular RNA was isolated from yeast transformed with YEpM10 as previously described (McAlister & Finkelstein, 1980). S1 nuclease mapping to identify the 5′ end of the *MDH*1 transcript was based on the method of Berk and Sharp (1977). A 1.1-kb *Hin*dIII restriction fragment containing 5′ noncoding sequences and 114 contiguous nucleotides of the coding region of *MDH*1 was treated with calf intestinal phosphatase (Boehringer Mannheim) and labeled at the 5′ end with [γ-^{32}P]dATP (5000 Ci/mmol) and T4 polynucleotide kinase. Total cellular RNA (100 μg) in ethanol and 3 × 10^4 cpm of the ^{32}P-labeled restriction fragment were dried together in a Savant Speed Vac. The pellet was resuspended in 30 μL of hybridization buffer (80% formamide, 50 mM PIPES, pH 6.5, and 0.4 M NaCl), denatured at 80 °C for 10 min, and incubated at 47 °C for 3 h; 300 μL of cold S1 buffer (0.3 M sodium acetate, pH 4.6, 0.5 M NaCl, 10 mM $ZnSO_4$, and 50% glycerol) and 100 units of S1 nuclease were added on ice. Following digestion at 37 °C for 30 min, the reaction was stopped by heating at 65 °C for 3 min. The protected fragments were precipitated with ethanol using 10 μg of yeast tRNA as carrier and electrophoresed on an 8% polyacrylamide–urea sequencing gel in parallel with appropriate dideoxy sequencing reactions. The optimal conditions described above were empirically determined from a range of hybridization temperatures of 42–55 °C and of S1 nuclease aliquots of 0–400 units.

For primer extension analyses (Homa et al., 1986), a 17-mer oligonucleotide (GCTACTCTTGACAACAT) complementary to the *MDH*1 sequence near the translation initiation codon was synthesized and 5′ end-labeled with [γ-^{32}P]dATP using T4 polynucleotide kinase. The primer (approximately 3 × 10^4 cpm) and 10 μg of total cellular RNA were dried together, resuspended in 12.5 μL of doubly distilled H_2O, and boiled for 6 min. An equal volume of primer extension buffer was added to give a final concentration of 75 mM KCl, 50 mM Tris-HCl, pH 7.5, and 3.0 mM $MgCl_2$, and the reactants were incubated at 65 °C for 1 h. The hybridization mix was transferred to 37 °C, 0.5 mM dNTPs and 200 units of Maloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories) were added, and extension was allowed to proceed for 1 h. Following ethanol precipitation, the primer

[1] Abbreviations: kb, kilobase(s); SDS, sodium dodecyl sulfate; PIPES, 1,4-piperazinediethanesulfonic acid.

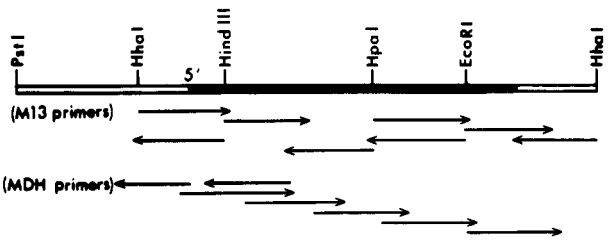

FIGURE 1: Restriction map and nucleotide sequencing strategy. Restriction endonuclease sites shown within a 1.77-kb *PstI–HhaI* yeast genomic fragment containing the *MDH*1 coding region (solid line) were used to construct M13 subclones for dideoxynucleotide sequence analysis. The arrows indicate the direction and extent of nucleotide sequences obtained from subclones using oligonucleotide primers complementary to either M13 or *MDH*1 sequences. The first nucleotides in *MDH*1-specific primers correspond to the following positions in the nucleotide sequence shown in Figure 2: 17 and 309 (leftward arrows); −24, 166, 391, 595, and 853 (rightward arrows).

extension products were resolved by electrophoresis and compared with nucleotide sequences generated with the same primer.

In Vitro Translation. For in vitro translation, a 20-μg sample of cellular RNA was translated with [^{35}S]methionine (1100 Ci/mmol) in a 15-μL reaction using the rabbit reticulocyte lysate system (Bethesda Research Laboratories). Following the addition of 0.6 μL of 10 mg/mL phenylmethanesulfonyl fluoride, the translation mix was diluted into 1.0 mL of a buffer containing 0.5% Tween 20, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1 mM EDTA. ^{35}S-Labeled translation products were immunoprecipitated as described by Bedwell et al. (1987) using a 1:50 dilution of antimitochondrial malate dehydrogenase IgG (McAlister-Henn & Thompson, 1987) following preliminary precipitation with preimmune antiserum.

Chromosomal Mapping. Filters containing yeast chromosomes were generously provided by J. Wasmuth and B. Smith. Briefly, intact chromosomes isolated in agarose beads (Overhauser & Radic, 1987) from the yeast strain A364A were electrophoresed on a transverse alternating field gel (Gardiner et al., 1986) and blotted onto Genetran filters (Plasco, Inc.). Hybridization probes were total yeast DNA or specific restriction fragments labeled with [α-^{32}P]dCTP (6000 μCi/mmol) using the random primer method (Feinberg & Vogelstein, 1984). Hybridizations were conducted at 65 °C as previously described (Sutherland & McAlister-Henn, 1985). Chromosome assignment was based on the yeast karyotype described by Carle and Olsen (1985).

RESULTS

Nucleotide Sequence Analysis of MDH1. The *Saccharomyces cerevisiae* gene (*MDH*1) encoding mitochondrial malate dehydrogenase was previously isolated from a YEp13 plasmid library, and sequences required for expression of the gene were localized to a 2.8-kb *Sph*I restriction fragment of yeast DNA (McAlister-Henn & Thompson, 1987). Partial nucleotide sequence analysis extending from an *Eco*RI restriction site near the 3′ end of the coding region established the extent and orientation of the coding region. A partial restriction map and the strategy for complete nucleotide sequence analysis of the *MDH*1 gene are illustrated in Figure 1. Restriction fragments were subcloned into M13 vectors for dideoxy nucleotide sequence analysis using as primers for chain elongation either commercially available M13 primers or *MDH*1-specific oligonucleotides synthesized on the basis of accumulated sequence data. Ninety-eight percent of the sequence of 1450 nucleotides containing the *MDH*1 gene was determined from both DNA strands.

```
-200        -190        -180        -170        -160        -150
GCCATAATAC  AATGGCGGAT  ATTACAATTA  ATTAATTAAT  TCGTGCGCTT  TAAAAAGAAG

-140        -130        -120        -110        -100        -90
TGTTAGCACG  CATTGGGTGT  TTGTACTGTA  TTCCTCCACT  TAAGATAACG  AATACACATA
                                                * *  **        **
-80         -70         -60         -50         -40         -30
TAGATATACA  ATATATTATA  CGTGGACATC  TAGGGAAAGG  AAGAAAAAAA  ACAAAAGGAA
                *

-20         -10                              15                  30
AAGGAAGGAT  ACCATATACA  ATG TTG TCA AGA GTA GCT AAA CGT GCG TTT TCC TCT
                        Met Leu Ser Arg Val Ala Lys Arg Ala Phe Ser Ser

          45                  60                  75                  90
ACA GTT GCC AAC CCT TAT AAA GTG ACT GTT TTG GGT GCA GGC GGT GGT ATT GGA
Thr Val Ala Asn Pro|Tyr|Lys Val Thr Val Leu Gly Ala Gly Gly Gly Ile Gly

                105                 HindIII 120                 135
CAA CCA TTG TCT TTG CTT CTA AAG CTT AAC CAT AAA GTC ACG GAC TTA AGA CTG
Gln Pro Leu Ser Leu Leu Leu Lys Leu Asn His Lys Val Thr Asp Leu Arg Leu

    150                 165                 180                 195
TAC GAC CTA AAG GGC GCA AAA GGT GTT GCC ACC GAT TTG TCT CAT ATT CCA ACA
Tyr Asp Leu Lys Gly Ala Lys Gly Val Ala Thr Asp Leu Ser His Ile Pro Thr

            210                 225                 240
AAC TCC GTG GTC AAG GGG TTT ACT CCA GAA GAG CCA GAC GGA TTG AAC AAC GCT
Asn Ser Val Val Lys Gly Phe Thr Pro Glu Glu Pro Asp Gly Leu Asn Asn Ala

255                 270                 285                 300
TTA AAG GAC ACA GAC ATG GTT TTA ATT CCT GCT GGT GTG CCC AGA AAG CCT GGT
Leu Lys Asp Thr Asp Met Val Leu Ile Pro Ala Gly Val Pro Arg Lys Pro Gly

        315                 330                 345                 360
ATG ACA CGT GAT GAC TTG TTC GCC ATC AAC GCA AGC ATC GTT CGC GAT TTG GCA
Met Thr Arg Asp Asp Leu Phe Ala Ile Asn Ala Ser Ile Val Arg Asp Leu Ala

                375                 390                 405
GCA GCA ACC GCC GAA TCC GCT CCC AAT GCT GCC ATT CTG GTC ATT TCC AAC CCA
Ala Ala Thr Ala Glu Ser Ala Pro Asn Ala Ala Ile Leu Val Ile Ser Asn Pro

    420                 435                 450                 465
GTC AAT TCT ACC GTT CCA ATT GTC GCC CAA GTC TTG AAA AAC AAG GGT GTT TAC
Val Asn Ser Thr Val Pro Ile Val Ala Gln Val Leu Lys Asn Lys Gly Val Tyr

            480                 495                 510
AAC CCA AAG AAA TTG TTC GGT GTG ACT ACC TTG GAC TCT ATT AGA GCC GCC AGA
Asn Pro Lys Lys Leu Phe Gly Val Thr Thr Leu Asp Ser Ile Arg Ala Ala Arg

525                 540                 555          HpaI    570
TTC ATC TCA GAA GTC GAG AAC ACC GAT CCA ACT CAG GAA AGG GTT AAC GTC ATC
Phe Ile Ser Glu Val Glu Asn Thr Asp Pro Thr Gln Glu Arg Val Asn Val Ile

        585                 600                 615                 630
GGT GGA CAT TCT GGT ATT ACC ATC ATC CCA TTG ATT TCG CAA ACA AAC CAT AAG
Gly Gly His Ser Gly Ile Thr Ile Ile Pro Leu Ile Ser Gln Thr Asn His Lys

                645                 660                 675
TTG ATG TCT GAT GAC AAG AGA CAC GAA TTG ATT CAC AGA ATA CAG TTT GGT GGT
Leu Met Ser Asp Asp Lys Arg His Glu Leu Ile His Arg Ile Gln Phe Gly Gly

    690                 705                 720                 735
GAC GAA GTC GTC AAA GCA AAG AAT GGT GCT GGC TCT GCT ACG TTG TCA ATG GCC
Asp Glu Val Val Lys Ala Lys Asn Gly Ala Gly Ser Ala Thr Leu Ser Met Ala

            750                 765                 780
CAT GCT GGT GCT AAA TTC GCT AAC GCT GTT TTG TCC GGT TTC AAA GGC GAA AGA
His Ala Gly Ala Lys Phe Ala Asn Ala Val Leu Ser Gly Phe Lys Gly Glu Arg

795                 810                 825                 840
GAC GTC ATC GAG CCT TCC TTC GTG GAC TCT CGC TTG TTC AAA TCC GAA GGC ATC
Asp Val Ile Glu Pro Ser Phe Val Asp Ser Pro Leu Phe Lys Ser Glu Gly Ile

 EcoRI  855                 870                 885                 900
GAA TTC TTT GCA TCT CCG GTC ACT TTG GGC CCA GAT GGT ATT GAA AAG ATC CAT
Glu Phe Phe Ala Ser Pro Val Thr Leu Gly Pro Asp Gly Ile Glu Lys Ile His

                915                 930                 945
CCA ATA GGT GAG TTA TCT TCA GAA GAA GAA GAA ATG CTA CAA AAA TGT AAA GAA
Pro Ile Gly Glu Leu Ser Ser Glu Glu Glu Glu Met Leu Gln Lys Cys Lys Glu

    960                 975                 990
ACC TTG AAG AAG AAT ATC GAA AAG GGT GTC AAC TTT GTT GCT AGT AAA
Thr Leu Lys Lys Asn Ile Glu Lys Gly Val Asn Phe Val Ala Ser Lys

  1,010       1,020       1,030       1,040       1,050       1,060
TAGAGCAT  TGTTCAAGAT  CAGAAATAGA  GTGAAAAATA  GGGCAAAAAA  AAAAAAAATT

    1,070       1,080       1,090       1,100       1,110       1,120
CTACTAATAA  GAACGGAAGA  GTACTCGCCA  TCATGAGATT  ACGACATCTT  TTTTATTTAA

    1,130       1,140       1,150       1,160       1,170       1,180
TTCTTTGTTA  TATTTAGCCA  ATTGAAAGGA  AGGAAACTGC  CAAAAATCAA  CTAGAGAAAA

    1,190       1,200       1,210       1,220       1,230       1,240
ATTCATGCTA  TTCGTTGCTC  CTACACCTTT  TTTTCTTCCG  TAGACGAGGA  AAAACAAAAA

    1,250   1,257
TGATTAAATA  GGCGCGC
```

FIGURE 2: Nucleotide sequence of *MDH*1. The nucleotide sequence of a 1.45-kb genomic DNA fragment containing the *MDH*1 gene and the inferred amino acid sequence for yeast mitochondrial malate dehydrogenase are shown. Positions of transcription initiation sites determined by both S1 nuclease and primer extension analyses (see text) are indicated by asterisks. The presumptive "TATA" box is underlined. The tyrosine residue corresponding to the amino-terminal residue of the mature protein is boxed. Positions of restriction sites within the coding region used to generate M13 subclones are indicated.

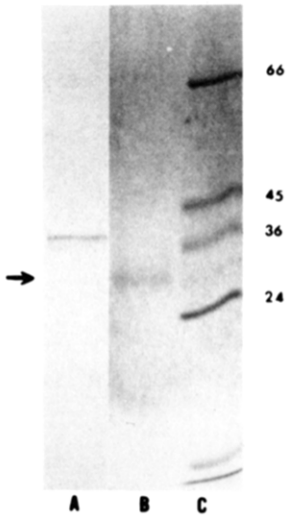

FIGURE 3: In vitro translation and immunoprecipitation of the malate dehydrogenase precursor protein. ^{35}S-Labeled polypeptides from an in vitro translation reaction programmed with total yeast RNA were immunoprecipitated with antimitochondrial malate dehydrogenase IgG as described under Experimental Procedures. Immunoprecipitated proteins were detected by autoradiography (lane A) following resolution on a 10% polyacrylamide–SDS gel. Parallel lanes from the same gel loaded with 10 μg of purified yeast malate dehydrogenase (lane B) and molecular weight markers (lane C) were stained with Coomassie brilliant blue. The arrow indicates the position of the purified protein.

Mature and Precursor Forms of Mitochondrial Malate Dehydrogenase. The DNA sequence shown in Figure 2 contains an open-reading frame of 1002 nucleotides extending from the AUG codon at position 1 that could encode a polypeptide of 334 amino acid residues with a predicted molecular weight of 35 600. The mature mitochondrial enzyme purified from yeast has an apparent molecular weight of approximately 33 500 as determined by SDS–polyacrylamide gel electrophoresis. To establish the amino terminus of the mature protein, the purified enzyme was subjected to stepwise Edman degradation for gas-phase sequence analysis. The amino acid sequence determined for residues 1–16 begins with the tyrosine residue indicated in Figure 2 and correlates exactly with that derived from nucleotide sequence data. This suggests that the initial translation product for the mitochondrial enzyme is a precursor with an amino-terminal extension of 17 amino acids.

To examine the mitochondrial malate dehydrogenase precursor, total cellular RNA was isolated from yeast cells transformed with YEpM10, a multicopy 2-μm plasmid carrying the *MDH*1 gene. As previously described, levels of mitochondrial malate dehydrogenase and of hybridizable messenger RNA are 5–10-fold higher in YEpM10 transformants than in cells transformed with the vector alone (McAlister-Henn & Thompson, 1987). Samples of isolated RNA were translated in vitro by using a rabbit reticulocyte lysate system and translation products immunoprecipiated with anti-malate dehydrogenase IgG as described under Experimental Procedures. As shown in Figure 3 (lane A), the primary translation product recovered by immunoprecipitation has an apparent molecular weight of 37 000–38 000 as determined by SDS–polyacrylamide gel electrophoresis, a value somewhat larger than that predicted from the gene sequence for the precursor polypeptide. In light of RNA transcript mapping studies described below, this discrepancy is probably due to aberrant electrophoretic migration of the precursor on SDS gels.

Transcription Initiation of MDH1. To identify the site(s) of transcription initiation, the 5′ termini of the *MDH*1 transcript were mapped (Figure 4). As determined by S1 nuclease mapping and the relative abundance of protected DNA fragments, there appear to be three predominant sites of initiation at nucleotide positions −97 and −91 and a cluster at position −64 relative to the AUG codon of the open-reading frame. There is also a minor start site at position −84. Primer extension mapping experiments reveal the same pattern with discrepancies of one to two nucleotides. Major initiation sites are apparent at positions −95 and −92 with a minor start site at position −83. The nucleotide sequences TAAG and GATAA around the start site at position −95 conform to the general sequence motifs PyAAPu and PuPuPyPuPu, respectively, often found at major transcription initiation sites in yeast genes (Dobson et al., 1982; Healy et al., 1987; Hahn et al., 1985). Likewise, the sequence AACGA near position −92 fits the latter pattern. The minor initiation sites do not conform to reported consensus start sites. The absence of other AUG codons in the 5′ flanking sequence and the mapped positions for *MDH*1 transcription initiation sites support the conclusion that the methionine indicated in Figure 2 is the first translated amino acid of the mitochondrial malate dehydrogenase protein.

The presumptive "TATA" sequence for *MDH*1, TAATTAATTAATT (positions −172 to −160, Figure 2), is located about 65 nucleotides 5′ of the most upstream initiation site. This distance falls within the 60–120-nucleotide spacing characteristic of promoters of yeast genes (Healy et al., 1987; Nagawa & Fink, 1985).

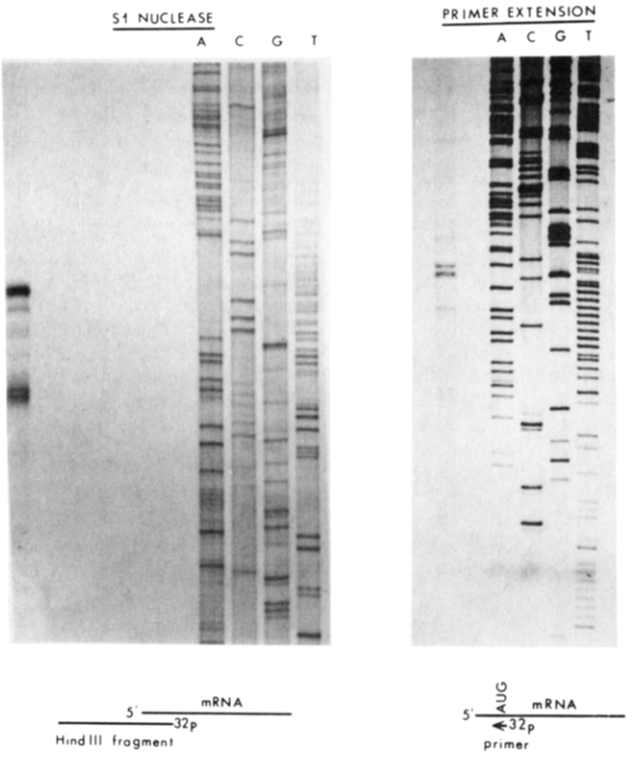

FIGURE 4: Determination of transcriptional initiation sites. Electrophoresis on an 8% polyacrylamide–urea sequencing gel and autoradiography were used to examine S1 nuclease resistant DNA fragments obtained by hybridization of yeast cellular RNA and a ^{32}P-labeled *Hin*dIII restriction fragment containing the 5′ end of *MDH*1 (left lane, S1 nuclease panel). Lanes labeled A, C, G, and T represent size standards from dideoxy sequencing reactions obtained by using a primer complementary to the coding region adjacent to the *Hin*dIII site in *MDH*1. A ^{32}P-labeled oligonucleotide primer complementary to *MDH*1 coding sequences near the translational initiation site (see Experimental Procedures) was hybridized with yeast RNA and extended with reverse transcriptase (left lane, primer extension panel). The adjacent sequencing ladder was obtained from dideoxy sequencing reactions by using the same oligonucleotide primer with an M13 subclone containing the *MDH*1 gene.

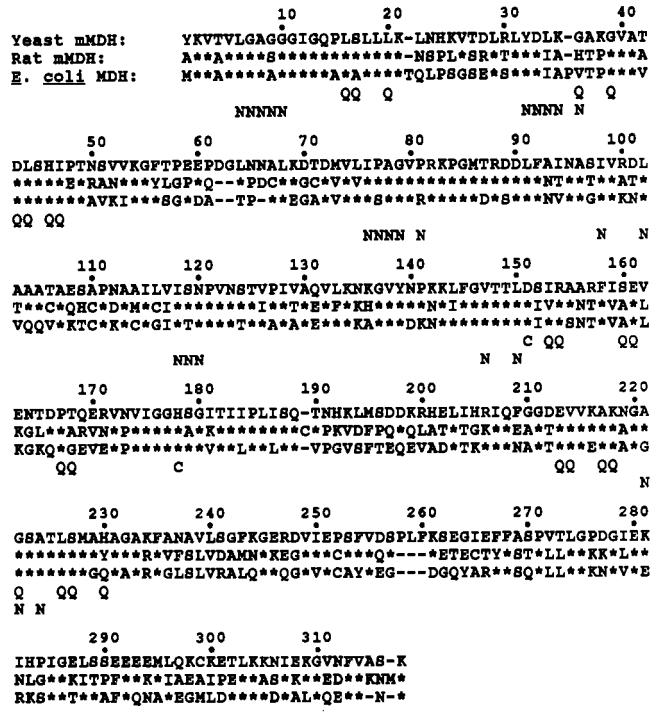

FIGURE 5: Comparison of malate dehydrogenase protein sequences. Amino acid sequences for the mature proteins derived from nucleotide sequences for the yeast *MDH*1 gene, for the rat cDNA (Grant et al., 1986), and for the *E. coli mdh* gene (McAlister-Henn et al., 1987) were aligned and gaps (indicated by dashes) introduced to optimize similarities. Numbers correspond to residue positions in the yeast protein, and asterisks indicate residues identical with those in the yeast sequence. The functions attributed to specific residues on the basis of X-ray crystallographic analyses of the porcine malate dehydrogenases (Birktoft et al., 1982; Roderick & Banaszak, 1986) are indicated by subscripts: N, NAD(H) binding; Q, subunit interactions; C, catalysis.

Comparison of Eucaryotic Mitochondrial and E. coli Malate Dehydrogenases. Amino acid sequences for malate dehydrogenases derived from the nucleotide sequences of yeast *MDH*1, of the cDNA for rat mitochondrial malate dehydrogenase (Grant et al., 1986), and of the *E. coli mdh* gene (McAlister-Henn et al., 1987) are compared in Figure 5. (Only one mammalian protein is included in this comparison since the sequences of the rat, mouse, and pig enzymes are greater than 90% identical.) The proteins are similar in size, containing respectively 317, 314, and 312 amino acid residues. Only six gaps each involving one to three residues are required for optimal alignment of the three sequences. The yeast protein is more similar to the rat mitochondrial enzyme with identities at 54% of the residue positions than to the *E. coli* protein with identities at 48% of the residue positions. The rat and *E. coli* enzymes are even more closely related with identical residues at 58% of the positions in the aligned sequences. A comparison of aligned nucleotide sequences (data not shown), however, shows a closer relationship between the eucaryotic genes (59% identity) than between either the yeast or the rat and the procaryotic genes with respective identities of 53% and 57%.

The catalytically active form of malate dehydrogenase is a dimer of identical subunits (Banaszak & Bradshaw, 1975). The amino acid residues participating in associations at the subunit interface (*Q* axis) as defined by X-ray crystallographic studies (Birktoft et al., 1982) are indicated in Figure 5. These amino acids have been highly conserved among the three tricarboxylic acid cycle enzymes with identities at 74% of the *Q*-axis residues. Most of these invariant residues are also conserved in several lactate dehydrogenases (Birktoft et al., 1982). Also indicated are residues involved in NAD(H) binding; 68% are identical in the yeast, rat, and *E. coli* enzymes. Almost completely conserved are amino acids at positions 6, 32–34, 76, 78, 98, and 102 that form a hydrophobic crevice for the binding of the adenine ring of NAD(H), and the remainder represent conservative amino acid replacements. Other invariant residues include Asp-151 and His-178 that are hydrogen bonded in the catalytic center of the porcine enzyme and may act as part of a proton relay system (Birktoft & Banaszak, 1983) and residues Leu-150 and Arg-154 that shield the catalytic site.

Table I: Comparison of Amino Acid Compositions for Procaryotic and Eucaryotic Malate Dehydrogenases

amino acid	no. of residues		
	yeast mMDH[a]	rat mMDH[b]	*E. coli* MDH[c]
alanine	27	32	34
arginine	10	7	9
asparagine	17	13	11
aspartic acid	17	12	12
half-cystine	1	8	3
glutamine	6	8	15
glutamic acid	20	17	19
glycine	28	28	36
histidine	8	5	2
isoleucine	23	23	17
leucine	29	28	33
lysine	26	26	21
methionine	5	6	4
phenylalanine	12	11	10
proline	18	20	13
serine	22	15	17
threonine	18	23	18
tryptophan	0	0	0
tyrosine	3	5	4
valine	27	27	34
total	317	314	312

[a] Amino acid composition of yeast mitochondrial malate dehydrogenase derived from the nucleotide sequence presented in Figure 2. [b] Composition of rat mitochondrial malate dehydrogenase from the cDNA sequence (Grant et al., 1986). [c] Composition of *E. coli* malate dehydrogenase from the gene sequence (McAlister-Henn, et al., 1987).

The most striking differences in the three malate dehydrogenase sequences are reflected in a comparison of the amino acid compositions (Table I). Whereas the overall content of most residues is comparable, the yeast enzyme contains only one cysteine residue as compared with eight in the rat enzyme and eight histidine residues as compared with two in the *E. coli* enzyme. None of the proteins contains tryptophan.

The codon utilization within the yeast *MDH*1 coding region is presented in Table II. On the basis of the method described by Bennetzen and Hall (1982), a codon bias of 0.47 is obtained for this gene, reflecting a relatively high frequency of preferred codon usage for a nuclear yeast gene encoding a mitochondrial protein. The codon bias calculated for the *E. coli mdh* gene by the method of Ikemura (1981) is also relatively high: 80% of the codons are amoing 22 of 61 possible coding triplets (data not shown).

Chromosomal Localization of MDH1. As an initial step toward genetic mapping of the *MDH*1 locus, the chromosomal location of the yeast gene was determined. Intact yeast chromosomal DNA electrophoresed on a pulsed field gel was transferred to a nylon filter and hybridized with a ^{32}P-labeled restriction fragment containing the coding region of the *MDH*1 gene. The hybridization pattern was compared with the electrophoretic pattern of chromosome markers as shown in Figure 6 (panel A), and *MDH*1 was found to reside on chomosome XI (panel B). As a control, a ^{32}P-labeled restriction

Table II: Codon Utilization in the *MDH*1 Gene

amino acid	codon	no.	amino acid	codon	no.	amino acid	codon	no.
Ala	GCA	8	Gly	GGA	3	Pro	CCA	11
	C	9		C	6		C	3
	G	1		G	1		G	1
	U	12		U	18		U	4
Arg	CGA	0	His	CAC	2	Ser	AGC	1
	C	1		U	6		U	1
	G	0					UCA	4
	U	2	Ile	AUA	2		C	7
	AGA	8		C	10		G	1
	G	1		U	11		U	11
Asn	AAC	14	Leu	CUA	3	Thr	ACA	5
	U	4		C	0		C	7
				G	2		G	2
Asp	GAC	11		U	2		U	5
	U	6		UUA	4			
				G	19	Trp	UGG	0
Cys	UGC	0						
	U	1	Lys	AAA	13	Tyr	UAC	2
				G	14		U	1
Gln	CAA	4						
	G	2	Met	AUG	6	Val	GUA	1
							C	13
Glu	GAA	16	Phe	UUC	8		G	5
	G	4		U	5		U	10

Chart I

					5							
yeast:	Met	Leu	Ser	Arg	Val	–	–	–	–	–	–	–
rat/mouse:	Met	Leu	Ser	Ala	Leu	Ala	Arg	Pro	Val	Gly	Ala	Ala
					5					10		
					10					15		
	Ala	Lys	Arg	Ala	Phe	Ser	Ser	Thr	Val	Ala	Asn	Pro
	Leu	Arg	Arg	Ser	Phe	Ser	Thr	Ser	Ala	Gln	Asn	Asn
			15					20				

fragment containing the *URA*1 gene known by genetic mapping studies to reside on chomosome XI (Loison et al., 1981) was hybridized with a parallel filter. The latter produced the same hybridization pattern (panel C) as *MDH*1.

Discussion

We have determined the nucleotide sequence for the gene encoding yeast mitochondrial malate dehydrogenase and compared the derived amino acid sequence with those for homologous mammalian mitochondrial and *E. coli* enzymes. A striking primary sequence conservation is observed ranging from 48% identity between *E. coli* and yeast enzymes to 58% identity between rat and *E. coli* malate dehydrogenases. If conservative amino acid changes are considered, the relationships among these proteins exceed 70%. Many of the differences in the nucleotide sequences of the coding regions for these proteins are due to third-position codon changes. While only a small percentage (approximately 5%) of these result in amino acid residue differences, they limit the extent of regions of nucleotide identity. Consequently, we can detect only very low levels of cross-hybridization among the malate dehydrogenase coding regions at a variety of stringency conditions (data not shown).

Clearly, by comparison of the structures of the mammalian mitochondrial enzymes with their functionally homologous cytoplasmic counterparts (Roderick & Banaszak, 1986; Birktoft et al., 1987), this high degree of structural conservation of the tricarboxylic acid cycle enzyme is not essential for preservation of basic catalytic function. Even more convincing in this regard is a recent report (Clarke et al., 1987) that changes in only three amino acid residues at the active site of a lactate dehydrogenase are required to convert the

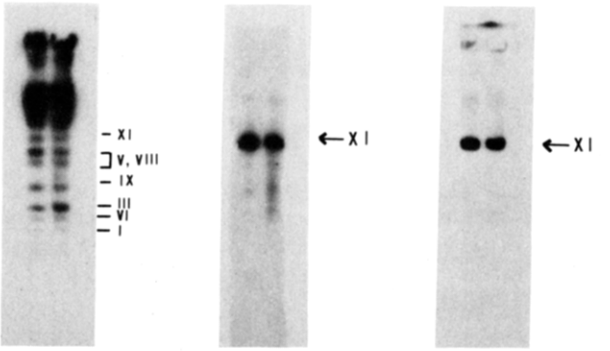

FIGURE 6: Chromosomal location of *MDH*1. Intact yeast chromosomes isolated and electrophoretically resolved as described under Experimental Procedures were transferred to a nylon filter. Duplicate lanes from the filter were hybridized with various ^{32}P-labeled DNA fragments including sheared yeast genomic DNA (panel A), a 2.8-kb *Sph*I restriction fragment containing the *MDH*1 gene (panel B), and a 2.5-kb *Hin*dIII fragment containing the *URA*1 gene (kindly provided by F. Lacroute, panel C). Chromosome assignments following the yeast karyotype described by Carle and Olsen (1985) are indicated.

enzyme to a specific and catalytically active malate dehydrogenase. Thus, the conservative evolution of the eucaryotic mitochondrial enzyme may reflect structural requirements for functions other than catalysis, for example specific interactions within the tricarboxylic acid cycle and the malate/aspartate shuttle. Because the equilibrium for the reaction in the direction of oxaloacetate production is unfavorable, specific protein interactions involving malate dehydrogenase have been proposed to ensure direct channeling of this metabolite (Srere, 1972). Studies showing in vitro interactions

of the mitochondrial enzyme with aspartate aminotransferase and with citrate synthase (Srere et al., 1978; Beeckmans & Kanarek, 1981) provide a theoretical mechanism for alternative interactions within the tricarboxylic acid or shuttle cycles.

The apparent pattern of evolution of the malate dehydrogenase is that predicted by the endosymbiotic theory of mitochondrial origin, i.e., that the eucaryotic mitochondrial enzyme is more closely related than the cytoplasmic enzyme to its procaryotic (*E. coli*) counterpart (McAlister-Henn, 1988). Interestingly, this pattern is not shared with other tricarboxylic acid cycle enzymes that have cytoplasmic counterparts. For example, yeast mitochondrial and cytoplasmic citrate synthases are encoded by two nuclear genes, *CIT*1 and *CIT*2 (Rosenkrantz et al., 1986), respectively. In this case, the compartmentalized isozymes are closely related (75% identity) whereas the mitochondrial and *E. coli* enzymes share identities at only 20% of the residues in aligned amino acid sequences. Fumarase is also present in both compartments in yeast, but the two isozymes are encoded by a single nuclear gene, *FUM*1 (Wu & Tzagoloff, 1987). Some transcripts apparently lack coding sequences necessary for mitochondrial targeting and are translated to produce the cytoplasmic form of fumarase.

We have presented evidence that the mitochondrial targeting sequence for yeast malate dehydrogenase is a 17 amino acid extension of the amino terminus of the mature polypeptide. The yeast presequence is compositionally similar to the 24-residue identical presequence described for rat and mouse mitochondrial malate dehydrogenase (Joh et al., 1987b; Grant et al., 1986) (see Chart I). Both presequences begin with Met-Leu-Ser and contain a very similar internal region (underlined) at the same position relative to the mature protein sequences. Common features shared with many mitochondrial targeting sequences (Douglas et al., 1986) are a high content of amino acid residues with basic and hydroxylated side chains and none with acidic side chains. Extensive analyses of the function of the rat targeting sequence in mitochondrial import of malate dehydrogenase in vitro (Chu et al., 1987a,b) suggest critical roles for several residues including Arg-14 for efficient import and an uncharged residue at the Leu-13 position for binding to mitochondria. Analogous residues in the yeast presequence at exactly the same positions relative to the cleavage site are, respectively, Lys-7 and Ala-6. The yeast presequence may be fit into a secondary structural pattern to form the amphipathic α-helical element thought to be characteristic of many targeting sequences (Roise et al., 1986; von Heinje, 1986). Although it is not entirely clear that an α-helical structure is necessary for import, a conformation providing a sidedness of charge distribution may play a role in association with the mitochondrial membrane (Douglas et al., 1986). Experiments in progress will test the role of the *MDH*1 presequence in directing localization of the enzyme to mitochondria and the ability of heterologous malate dehydrogenase to function in yeast mitochondria.

Registry No. *MDH*1, 116669-01-3; mature malate dehydrogenase, 116669-06-8; precursor malate dehydrogenase, 116669-07-9; malate dehydrogenase, 9001-64-3; premalate dehydrogenase, 99332-64-6.

REFERENCES

Banaszak, L. J., & Bradshaw, R. A. (1975) *Enzymes (3rd Ed.) 11A* 369–396.

Bedwell, D. M., Klionsky, D. J., & Emr, S. D. (1987) *Mol. Cell. Biol. 7*, 4038–4047.

Beeckmans, S., & Kanarek, L. (1981) *Eur. J. Biochem. 117*, 527–535.

Bennetzen, J. L., & Hall, B. D. (1982) *J. Biol. Chem. 257*, 3026–3031.

Berk, A. J., & Sharp, P. A. (1977) *Cell (Cambridge, Mass.) 12*, 721–732.

Birktoft, J. J., & Banaszak, L. J. (1983) *J. Biol. Chem. 258*, 472–482.

Birktoft, J. J., Fernley, R. T., Bradshaw, R. A., & Banaszak, L. J. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 6166–6170.

Birktoft, J. J., Bradshaw, R. A., & Banaszak, L. J. (1987) *Biochemistry 26*, 2722–2734.

Botstein, D., Falco, S. D., Stewart, S. E., Brennan, M., Scherer, S., Stinchcomb, D. T., Struhl, K., & Davis, R. W. (1979) *Gene 8*, 12–24.

Carle, G. F., & Olson, M. V. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 3756–3760.

Chu, T. W., Grant, P. M., & Strauss, A. W. (1987a) *J. Biol. Chem. 262*, 12806–12811.

Chu, T. W., Grant, P. M., & Strauss, A. W. (1987b) *J. Biol. Chem. 262*, 15759–15764.

Clarke, A. R., Smith, C. J., Hart, K. W., Wilks, H. M., Chia, W. N., Lee, T. V., Birktoft, J. J., Banaszak, L. J., Barstow, D. A., Atkinson, T., & Holbrook, J. J. (1987) *Biochem. Biophys. Res. Commun. 148*, 15–33.

Dobson, M. J., Tuite, M. F., Roberts, N. A., Kingsman, A. J., & Kingsman, S. M. (1982) *Nucleic Acids Res. 10*, 2625–2637.

Douglas, M. G., McCammon, M. T., & Vassarotti, A. (1986) *Microbiol. Rev. 50*, 166–178.

Feinberg, A. P., & Vogelstein, B. (1984) *Anal. Biochem. 137*, 266–267.

Gardiner, K., Laas, W., & Patterson, D. (1986) *Somatic Cell Mol. Genet. 12*, 185–195.

Grant, P. M., Tellam, J., May, V. L., & Strauss, A. W. (1986) *Nucleic Acids Res. 14*, 6053–6066.

Hahn, S., Hoar, E. T., & Guarente, L. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 8562–8566.

Healy, A. M., Helser, T. L., & Zitomer, R. S. (1987) *Mol. Cell. Biol. 7*, 3785–3791.

Homa, F. L., Otal, T. M., Glorioso, J. C., & Levine, M. (1986) *Mol. Cell. Biol. 6*, 3652–3666.

Ikemura, T. (1981) *J. Mol. Biol. 151*, 389–409.

Joh, T., Takeshima, H., Tsuzuki, T., Setoyama, C., Shimada, K., Tanase, S., Kuramitsu, S., Kagamiyama, H., & Morino, Y. (1987a) *J. Biol. Chem. 262*, 15127–15131.

Joh, T., Takeshima, H., Tsuzuki, T., Shimada, K., Tanase, S., & Morino, Y. (1987b) *Biochemistry 26*, 2515–2520.

Loison, G., Jund, R., Nguyen-Juilleret, M., & Lacroute, F. (1981) *Curr. Genet. 3*, 119–123.

Matsudaira, P. (1987) *J. Biol. Chem. 262*, 10035–10038.

McAlister, L., & Finkelstein, D. B. (1980) *J. Bacteriol. 143*, 603–612.

McAlister-Henn, L. (1988) *Trends Biochem. Sci. (Pers. Ed.) 13*, 178–181.

McAlister-Henn, L., & Thompson, L. M. (1987) *J. Bacteriol. 169*, 5157–5166.

McAlister-Henn, L., Blaber, M., Bradshaw, R. A., & Nisco, S. J. (1987) *Nucleic Acids Res. 15*, 4993.

Messing, J. (1983) *Methods Enzymol. 101*, 20–78.

Miller, J. H. (1972) *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Nagawa, F., & Fink, G. R. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 8557–8561.

Nishiyama, M., Matsubara, N., Yamamoto, K., Iijima, S., Uozumi, T., & Beppu, T. (1986) *J. Biol. Chem. 261*, 14178–14183.

Overhauser, J., & Radic, M. Z. (1987) *Focus MHL 9*, 8-9.
Roderick, S. L., & Banaszak, L. J. (1986) *J. Biol. Chem. 261*, 9461-9464.
Roise, D., Horvath, S. J., Tomich, J. M., Richards, J. H., & Schatz, G. (1986) *EMBO J. 5*, 1327-1334.
Rosenkrantz, M., Alam, T., Kim, K., Clark, B. J., Srere, P. A., & Guarente, L. P. (1986) *Mol. Cell. Biol. 6*, 4509-4515.
Rothstein, R. J. (1983) *Methods Enzymol. 101*, 202-211.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463-5467.
Schwindinger, W. F., & Warner, J. R. (1984) *Nucleic Acids Res. 12*, 601-604.
Srere, P. A. (1972) in *Energy Metabolism and the Regulation of Metabolic Processes* (Mehlman, M. A., & Hanson, R. E., Eds.) pp 79-91, Academic, New York.
Srere, P. A., Halper, L. A., & Finkelstein, M. B. (1978) in *Microenvironments and Metabolic Compartmentation* (Srere, P. A., & Estabrook, R. W., Eds.) Academic, New York.
Sutherland, P., & McAlister-Henn, L. (1985) *J. Bacteriol. 163*, 1074-1079.
von Heijne, G. (1986) *EMBO J. 5*, 1335-1342.
Wu, M., & Tzagoloff, A. (1987) *J. Biol. Chem. 262*, 12275-12282.
Yanisch-Perron, C., Vieira, J., & Messing, J. (1985) *Gene 33*, 103-119.

Structure–Function Relationships in the Na,K-ATPase α Subunit: Site-Directed Mutagenesis of Glutamine-111 to Arginine and Asparagine-122 to Aspartic Acid Generates a Ouabain-Resistant Enzyme†

Elmer M. Price and Jerry B. Lingrel*

Department of Microbiology and Molecular Genetics, University of Cincinnati College of Medicine, 231 Bethesda Avenue, Cincinnati, Ohio 45267-0524

Received May 3, 1988; Revised Manuscript Received June 21, 1988

ABSTRACT: Na,K-ATPases from various species differ greatly in their sensitivity to cardiac glycosides such as ouabain. The sheep and human enzymes are a thousand times more sensitive than the corresponding ones from rat and mouse. To define the region of the α1 subunit responsible for this differential sensitivity, chimeric cDNAs of sheep and rat were constructed and expressed in ouabain-sensitive HeLa cells. The construct containing the amino-terminal half of the rat α1 subunit coding region and carboxyl-terminal half of the sheep conferred the ouabain-resistant phenotype to HeLa cells while the reverse construct did not. This indicates that the determinants involved in ouabain sensitivity are located in the amino-terminal half of the Na,K-ATPase α subunit. By use of site-directed mutagenesis, the amino acid sequence of the first extracellular domain (H1–H2) of the sheep α1 subunit, Gln-Ala-Ala-Thr-Glu-Glu-Glu-Pro-Gln-Asn-Asp-Asn, was changed to that of the rat, Arg-Ser-Ala-Thr-Glu-Glu-Glu-Pro-Pro-Asn-Asp-Asp. When expressed in HeLa cells, this mutated sheep α1 construct, like the rat/sheep chimera, was able to confer ouabain resistance to these cells. Furthermore, similar results were observed when HeLa cells were transfected with a sheep α1 cDNA containing only two amino acid substitutions. This double mutation was a Gln-111 → Arg and Asn-122 → Asp change at the amino terminus and carboxyl terminus, respectively, of the H1–H2 extracellular region. The resistant cells, whether transfected with the rat α1 cDNA, the rat/sheep chimera, or the mutant sheep α1 cDNAs, exhibited identical biochemical characteristics including ouabain-inhibitable cell growth, $^{86}Rb^+$ uptake, and Na,K-ATPase activity. These results demonstrate that the presence of arginine and aspartic acid on the amino end and carboxyl end, respectively, of the H1–H2 extracellular domain of the Na,K-ATPase α subunit together is responsible for the ouabain-resistant character of the rat enzyme and the corresponding residues in the sheep α1 subunit (glutamine and asparagine) are somehow involved in ouabain binding.

The plasma membrane derived Na,K-ATPase is an ubiquitous enzyme which establishes and maintains the Na^+ and K^+ electrochemical gradient across the plasma membrane of animal cells (Jorgensen, 1982). This gradient serves as the energy source for numerous cellular activities such as active transport of certain solutes (Ullrich, 1979), regulation of cell volume (Macknight & Leaf, 1977), and restoration of the membrane potential in electrically excitable membranes (Thomas, 1972). The enzyme exists as a heterodimer consisting of a large catalytic α subunit and a smaller glycoslated β subunit whose function has not yet been determined. In addition to its role as a ion transporter, Na,K-ATPase is the target enzyme for a pharmacologically important class of drugs known as cardiac glycosides, such as digitalis and ouabain (Schwartz et al., 1975; Hansen, 1984). It is known that ouabain binds avidly to and subsequently inhibits the enzyme from a variety of sources including sheep and human whereas the corresponding rodent enzyme is virtually resistant to the drug (Repke et al., 1965; Wallick et al., 1980; Gupta et al., 1986; Schonfeld et al., 1986), apparently due to an increase in the dissociation rate of the drug from the binding site on the enzyme (Tobin & Brody, 1972; Wallick et al., 1980). Immunological (Ball & Lane, 1986) and biochemical (Peri-

† Supported by NIH Grants RO1 HL28573 and PO1 HL22619.
* To whom correspondence should be addressed.

0006-2960/88/0427-8400$01.50/0