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Nucleotide sequence for yeast dihydrolipoamide dehydrogenase

(flavoprotein/conservation of sequence)

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ABSTRACT **Rabbit antiserum to the dihydrolipoamide dehydrogenase (dihydrolipoamide:NAD$^+$ oxidoreductase, EC 1.8.1.4) component of the pyruvate dehydrogenase complex from bakers' yeast was used to screen plaques produced by a λgt11 yeast cDNA library. A 2.1-kilobase insert was isolated that also hybridized to a 17-base mixed oligonucleotide probe corresponding to the amino-terminal sequence of the yeast dihydrolipoamide dehydrogenase. The cDNA has a coding sequence of 499 amino acids that corresponds to a 21-residue signal peptide and a 478-residue mature protein (M_r = 51,558). Computer analysis shows that yeast dihydrolipoamide dehydrogenase has about 41% amino acid identity with *Escherichia coli* dihydrolipoamide dehydrogenase. Particularly striking is the conservation of sequence in the active site region of the dihydrolipoamide dehydrogenases from *E. coli*, yeast, and pig heart.**

Dihydrolipoamide dehydrogenase (dihydrolipoamide:NAD$^+$ oxidoreductase, EC 1.8.1.4) is the flavoprotein component (E_3) of the multienzyme pyruvate, α-ketoglutarate, and branched-chain α-keto acid dehydrogenase complexes (1, 2). In eukaryotic cells these multienzyme complexes are located in mitochondria, within the inner membrane–matrix compartment. Dihydrolipoamide dehydrogenase contains a disulfide bridge that undergoes reduction–oxidation as part of the catalytic mechanism (2). The amino acid sequence around this active center cystine is highly conserved in dihydrolipoamide dehydrogenases from *Escherichia coli* (3, 4), *Bacillus stearothermophilis* (5), and pig heart (2) as well as in glutathione reductases from yeast (2), *E. coli* (6, 7), and human erythrocytes (8) and in *Pseudomonas aeruginosa* transposon mercuric reductase (9).

About one-half of the amino acid sequence of pig heart dihydrolipoamide dehydrogenase has been determined (10), and the complete amino acid sequence of the *E. coli* K-12 enzyme has been deduced from the nucleotide sequence of its structural gene (11). Dihydrolipoamide dehydrogenase has been purified to homogeneity from bakers' yeast (12, 13) and partially characterized (14). In this report, we present the cloning and sequencing of cDNA for yeast dihydrolipoamide dehydrogenase§ and make comparisons with the corresponding enzymes from pig heart and *E. coli*.

MATERIALS AND METHODS

Materials. Restriction enzymes and DNA-modifying enzymes were purchased from Bethesda Research Laboratories and New England Biolabs. The sequencing reagents were purchased from United States Biochemical. Horseradish peroxidase-labeled goat anti-rabbit antibody was obtained from Kirkegaard and Perry (Gaithersburg, MD). [γ-^{32}P]ATP, [α-^{32}P]dCTP, and [α-[^{35}S]thio]dATP were purchased from New England Nuclear. The λgt11 yeast cDNA library (15) was generously provided by Hans Trachsel and Michael Altmann (Universität Bern, Switzerland). *E. coli* strains Y1090 and TB-1 were used to grow the λ library and plasmids, respectively.

Purification of Dihydrolipoamide Dehydrogenase. Pyruvate dehydrogenase complex was purified to homogeneity from bakers' yeast as described (16). Dihydrolipoamide dehydrogenase was obtained by resolution of the complex on hydroxylapatite in the presence of 8 M urea (13).

Preparation of Antiserum. Antibodies to purified dihydrolipoamide dehydrogenase were raised in female New Zealand White rabbits. An emulsion containing about 200 μg of sodium dodecyl sulfate-denatured enzyme in Freund's complete adjuvant was injected subcutaneously. Booster injections were given at 20-day intervals using Freund's incomplete adjuvant. For the second and third booster injections, 1 mg of native enzyme was used. Blood was collected 10 days after the last injection, and the serum was separated and stored at −70°C. Immunoblot analysis showed that the antiserum was specific for dihydrolipoamide dehydrogenase (17). Antibodies reacting with *E. coli* and λ phage proteins were removed from the serum by incubation with *E. coli* and phage lysates immobilized on nitrocellulose (18).

Amino-Terminal Sequence Analysis. The amino-terminal sequence of native dihydrolipoamide dehydrogenase was determined with an Applied Biosystems (Foster City, CA) model 470A gas-phase sequenator equipped with a model 120A on-line phenylthiohydantoin-amino acid analyzer.

Preparation of Oligonucleotides. A 17-base mixed probe 5′ GC$^{C}_{T}$AT$^{C}_{T}$AACAA$^{G}_{A}$TC$^{T}_{C}$CA 3′ based on the amino-terminal sequence Ala-Ile-Asn-Lys-Ser-His of yeast dihydrolipoamide dehydrogenase and the preferred codon usage of yeast (19) was synthesized on an Applied Biosystems model 381A DNA synthesizer and purified on a DuPont Zorbax Bioseries oligo column according to the manufacturers' instructions. Oligonucleotide primers for sequencing were prepared similarly. The oligonucleotide probe was 5′ end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (20).

Screening of cDNA Library. Plaques produced by the λgt11 yeast cDNA library were screened with rabbit antisera to yeast dihydrolipoamide dehydrogenase essentially as described by Huynh *et al.* (18). Positive clones were rescreened with antibody and with the oligonucleotide probe (20). The approximate size of the inserts was determined, and one clone corresponding to an insert of 2.1 kilobases was designated E3-3. The λ phage used to prepare DNA for subcloning was grown in liquid culture and purified by poly(ethylene glycol) precipitation and centrifugation in a CsCl gradient as described by Maniatis *et al.* (20). The λ DNA containing the insert was digested with *Eco*RI for 12 hr

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§The sequence reported in this paper is being deposited in the EMBL/GenBank data base (Bolt, Beranek, and Newman Laboratories, Cambridge, MA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J03645).

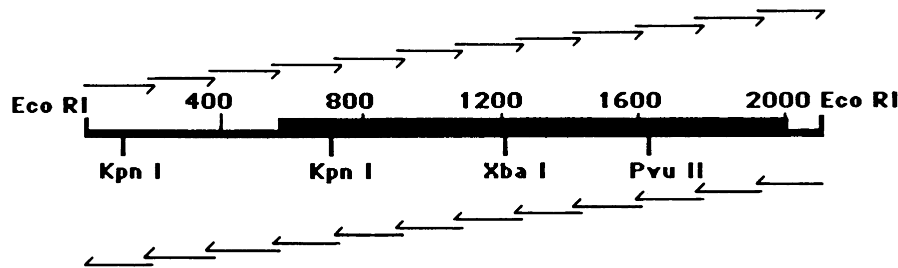

FIG. 1. Restriction endonuclease map and sequencing strategy for dihydrolipoamide dehydrogenase cDNA. The thick bar represents the coding region for the enzyme. The arrows show the direction and extent of the nucleotide sequencing.

and the fragments were resolved on a 0.5% agarose gel. The 2.1-kilobase DNA fragment was electroeluted by using an Elutrap (Schleicher & Schuell) and precipitated with ethanol. The fragment was ligated into the plasmid vector Bluescript (Stratagene Cloning Systems, San Diego, CA), which had been previously digested with *Eco*RI and treated with bacterial alkaline phosphatase. The resulting plasmid containing the 2.1-kilobase insert was designated pE3-3.

DNA Sequencing. Two sets of deletion clones of pE3-3, corresponding to each strand of DNA, were prepared by using Erase-A-Base (Promega Biotec, Madison, WI). Double-stranded DNA was prepared from mini-plasmid preparations and sequenced with Sequenase (United States Biochemical). The mini-plasmid preparations were prepared as described by Maniatis *et al.* (20) with the following changes. The cell pellet was resuspended in 200 μl of the lysis buffer and 10 μl of a 10 mg/ml solution of lysozyme was added. To the supernatant fluid obtained after boiling and centrifugation was added 200 μl of isopropanol, and the mixture was centrifuged immediately. The pellet was resuspended in 100 μl of 10 mM Tris·HCl, pH 7.5/1 mM EDTA and incubated with DNase-free RNase for 30 min at 37°C. The mixture was extracted with phenol/chloroform until the white interface was no longer apparent (usually three or four times). Approximately 20 μl of 10 mM Tris·HCl, pH 7.5/1 mM EDTA was added as needed to keep the volume at 100 μl during the extraction procedure. The supernatant fluid was extracted with chloroform, and 1 vol of 7.5 M ammonium acetate and 2 vol of ethanol were added. The precipitate was collected by centrifugation, washed with 70% ethanol, and then dissolved in 20 μl of 10 mM Tris·HCl, pH 7.5/1 mM EDTA. A 7-μl portion of these mini-plasmid preparations was denatured with 4 μl of 1 M NaOH/1 mM EDTA in a final volume of 20 μl. The denatured DNA was precipitated with 2 μl of 2 M ammonium acetate (pH 5) and 55 μl of ethanol. The mixture was placed on dry ice for 5 min, and the DNA was pelleted by centrifugation. The pellet was washed with 70% ethanol and taken up in 7 μl of 10 mM Tris·HCl, pH 7.5/1

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GGGAATTCGCTGGTATCCAAATTGTTGCTGATGACGTGACGGTCACCAACCCAGCTAGAA
TTGCTACCGCCATCGAAGAGAAGGCTGCTGACGCTTTGTTGTTGAAGGTTAACCAAATCG
GTACCTTGTCTGAATCCATCAAGGCTGCTCAAGACTCTTTCGCTGCCAACTGGGGTGTTA
TGGTTTCCCACAGATCTGGTGAAACTGAAGACACTTTCATTGCTGACTTGGTTGTCGGTT
TGAGAACTGGTCAAATCAAGACTGGTGCTCCAGCTAGATCCGAAAGATTGGCTAAGTTGA
ACCAATTGTTGAGAATCGAAGAAGAATTGGGTGACAAGGCTGTCTACGCCGGTGAAAACT
TCCACCACGGTGACAAGTTGTAAAGTGCTTTTAACTAAGAATTATTAGTCTTTTCTGCTT
ATTTTTTCATCATAGTTTAGAACACTTTATATTAACGAATAGTTTATGAATCTATTTAGG
TTTAAAAATTGATACAGTTTTATAAAAAAAAAAAAAAAAAAAAAAATTCCACAATGTTAAGA
                                                   M  L  R
                                                 -21
ATCAGATCACTCCTAAATAATAAGCGTGCCTTTTCGTCCACAGTCAGGACATTGACCATT
 I  R  S  L  L  N  N  K  R  A  F  S  S  T  V  R  T  L  T  I
                                                       1
AACAAGTCACATGATGTAGTCATCATCGGTGGTGGCCCTGCTGGTTACGTGGCTGCTATC
 N  K  S  H  D  V  V  I  I  G  G  G  P  A  G  Y  V  A  A  I
                                                    20
AAAGCTGCTCAATTGGGATTTAACACTGCATGTGTAGAAAAAAGAGGCAAATTAGGCGGT
 K  A  A  Q  L  G  F  N  T  A  C  V  E  K  R  G  K  L  G  G
                                                 *  *  *
ACCTGTCTTAACGTTGGATGTATCCCCTCCAAAGCACTTCTAAATAATTCTCATTTATTC
 T  C  L  N  V  G  C  I  P  S  K  A  L  L  N  N  S  H  L  F
 *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *
CACCAAATGCATACGGAAGCGCAAAAGAGAGGAATTGACGTCAACGGTGATATCAAAATT
 H  Q  M  H  T  E  A  Q  K  R  G  I  D  V  N  G  D  I  K  I
                                                    80
AACGTAGCAAACTTCCAAAAGGCTAAGGATGACGCTGTTAAGCAATTAACTGGAGGTATT
 N  V  A  N  F  Q  K  A  K  D  D  A  V  K  Q  L  T  G  G  I
                                                   100
GAGCTTCTGTTCAAGAAAAATAAGGTCACCTATTATAAAGGTAATGGTTCATTCGAAGAC
 E  L  L  F  K  K  N  K  V  T  Y  Y  K  G  N  G  S  F  E  D
                                                   120
GAAACGAAGATCAGAGTAACTCCCGTTGATGGGTTGGAAGGCACTGTCAAGGAAGACCAC
 E  T  K  I  R  V  T  P  V  D  G  L  E  G  T  V  K  E  D  H
                                                   140
ATACTAGATGTTAAGAACATCATAGTCGCCACGGGCTCTGAAGTTACACCCTTCCCCGGT
 I  L  D  V  K  N  I  I  V  A  T  G  S  E  V  T  P  F  P  G
                                                   160
ATTGAAATAGATGAGGAAAAAATTGTCTCTTCAACAGGTGCTCTTTCGTTAAAGGAAATT
 I  E  I  D  E  E  K  I  V  S  S  T  G  A  L  S  L  K  E  I
                                                   180
CCCAAAAGATTAACCATCATTGGTGGAGGAATCATCGGATTGGAAATGGGTTCAGTTTAC
 P  K  R  L  T  I  I  G  G  G  I  I  G  L  E  M  G  S  V  Y
                                                   200
TCTAGATTAGGCTCCAAGGTTACTGTAGTAGAATTTCAACCTCAAATTGGTGCATCTATG
 S  R  L  G  S  K  V  T  V  V  E  F  Q  P  Q  I  G  A  S  M
                                                   220
GACGGCGAGGTTGCCAAAGCCACCCAAAAGTTCTTGAAAAAGCAAGGTTTGGACTTCAAA
 D  G  E  V  A  K  A  T  Q  K  F  L  K  K  Q  G  L  D  F  K
                                                   240
TTAAGCACCAAAGTTATTTCTGCAAAGAGAAACGACGACAAGAACGTCGTCGAAATTGTT
 L  S  T  K  V  I  S  A  K  R  N  D  D  K  N  V  V  E  I  V
                                                   260
GTAGAAGATACTAAAACGAATAAGCAAGAAAATTTGGAAGCTGAAGTTTTGCTGGTTGCT
 V  E  D  T  K  T  N  K  Q  E  N  L  E  A  E  V  L  L  V  A
                                                   280
GTTGGTAGAAGACCTTACATTGCTGGCTTAGGGGCTGAAAAGATTGGATTAGAAGTAGAC
 V  G  R  R  P  Y  I  A  G  L  G  A  E  K  I  G  L  E  V  D
                                                   300
AAAAGGGGACGCCTAGTCATTGATGACCAATTTAATTCCAAGTTCCCACACATTAAAGTG
 K  R  G  R  L  V  I  D  D  Q  F  N  S  K  F  P  H  I  K  V
                                                   320
GTAGGAGATGTTACATTTGGTCCAATGCTGGCTCACAAAGCCGAAGAGGAAGGTATTGCA
 V  G  D  V  T  F  G  P  M  L  A  H  K  A  E  E  E  G  I  A
                                                   340
GCTGTCGAAATGTTGAAAACTGGTCACGGTCATGTCAACTATAACAACATTCCTTCGGTC
 A  V  E  M  L  K  T  G  H  G  H  V  N  Y  N  N  I  P  S  V
                                                   360
ATGTATTCTCACCCAGAAGTAGCATGGGTTGGTAAAACCGAAGAGCAATTGAAAGAAGCC
 M  Y  S  H  P  E  V  A  W  V  G  K  T  E  E  Q  L  K  E  A
                                                   380
GGCATTGACTATAAAATTGGTAAGTTCCCCTTTGCGGCCAATTCAAGAGCCAAGACCAAC
 G  I  D  Y  K  I  G  K  F  P  F  A  A  N  S  R  A  K  T  N
                                                   400
CAAGACACTGAAGGTTTCGTGAAGATTTTGATCGATTCCAAGACCGAGCGTATTTTGGGG
 Q  D  T  E  G  F  V  K  I  L  I  D  S  K  T  E  R  I  L  G
                                                   420
GCTCACATTATCGGTCCAAATGCCGGTGAAATGATTGCTGAAGCTGGCTTAGCCTTAGAA
 A  H  I  I  G  P  N  A  G  E  M  I  A  E  A  G  L  A  L  E
                                                   440
TATGGCGCTTCCGCAGAAGATGTTGCTAGGGTCTGCCATGCTCATCCTACTTTGTCCGAA
 Y  G  A  S  A  E  D  V  A  R  V  C  H  A  H  P  T  L  S  E
                                                   460
GCATTTAAGGAAGCTAACATGGCTGCCTATGATAAAGCTATTCATTGTTGAAAACAGGAA
 A  F  K  E  A  N  M  A  A  Y  D  K  A  I  H  C

ATAATAAACAGTATAGTATATATATTTATGAAGAACCGCTTAGTATTGAGTAAAAAAAAA
AGGAATTC                                                        2108
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FIG. 2. Nucleotide sequence of pE3-3 and deduced amino acid sequence of dihydrolipoamide dehydrogenase. The amino acids are numbered at 20-residue intervals. The experimentally determined amino-terminal sequence of the enzyme is underlined. Asterisks represent the putative sequence around the active center cystine. A putative poly(A) signal is indicated by the overbar. The complete nucleotide sequence was determined for both strands.

FIG. 3. Transfer blot analysis of yeast poly(A)$^+$ RNA with pE3-3. See text for blot preparation and hybridization. The positions of yeast 18S (1.65 kilobases) and 25S (3.36 kilobases) rRNA are indicated.

mM EDTA. The manufacturer's instructions for Sequenase were followed for the DNA sequencing reactions except that the denatured DNA and oligonucleotide primer were hybridized for 15 min at 37°C.

RNA Transfer Blot Analysis. A 10-μg sample of poly(A)$^+$ RNA from *Saccharomyces cerevisiae* strain 20B-12 was denatured with glyoxal, separated on a 1.2% agarose gel, and blotted onto nitrocellulose as described (21). pE3-3 was labeled by nick-translation with [α-^{32}P]dCTP by using a nick-translation kit (Bethesda Research Laboratories) according to the manufacturer's instructions. Hybridization conditions were as described (21).

Computer Analysis. All computer analyses were performed with Beckman Microgenie, version 4.

RESULTS AND DISCUSSION

Amino-Terminal Sequence of Dihydrolipoamide Dehydrogenase. The amino-terminal sequence of the native dihydrolipoamide dehydrogenase was determined to be Xaa-Ile-Asn-Lys-Ser-His-Asp-Val-Val-Ile-Ile-Gly-Gly-Gly-Pro-Ala-Gly-Tyr-Val-Ala-Ala-Ile-Lys-Ala-Ala. The first residue was uncertain, but alanine was thought to be a likely possibility. However, the cDNA sequence (see Fig. 2) shows that the first residue is threonine. This change would cause a one-base mismatch between the 5′ end of the oligonucleotide probe and the cDNA. An additional mismatch occurs at position 15 of the oligonucleotide; the serine codon has adenine in the wobble position rather than the preferred cytosine or thymine. These two mismatches did not prevent hybridization of the probe with the cDNA for dihydrolipoamide dehydrogenase.

Isolation of cDNA for Dihydrolipoamide Dehydrogenase. Plaques produced from a yeast cDNA λgt11 library were screened with antibody to the dihydrolipoamide dehydrogenase component of the pyruvate dehydrogenase complex from bakers' yeast. Three positive clones were obtained. One of these clones also hybridized to a 17-base mixed oligonucleotide probe corresponding to the amino terminus of the enzyme. This clone was found to contain an insert of about 2 kilobases (Fig. 1). This insert was subcloned into the plasmid vector Bluescript for DNA sequencing.

Sequencing of Dihydrolipoamide Dehydrogenase cDNA. The nucleotide sequence of clone pE3-3 and the deduced amino acid sequence of the protein are shown in Fig. 2. This precursor of dihydrolipoamide dehydrogenase contains 499 amino acid residues and has a calculated M_r = 54,017. It contains a putative 21-residue signal sequence that is rich in basic and hydroxylated amino acid residues and is devoid of

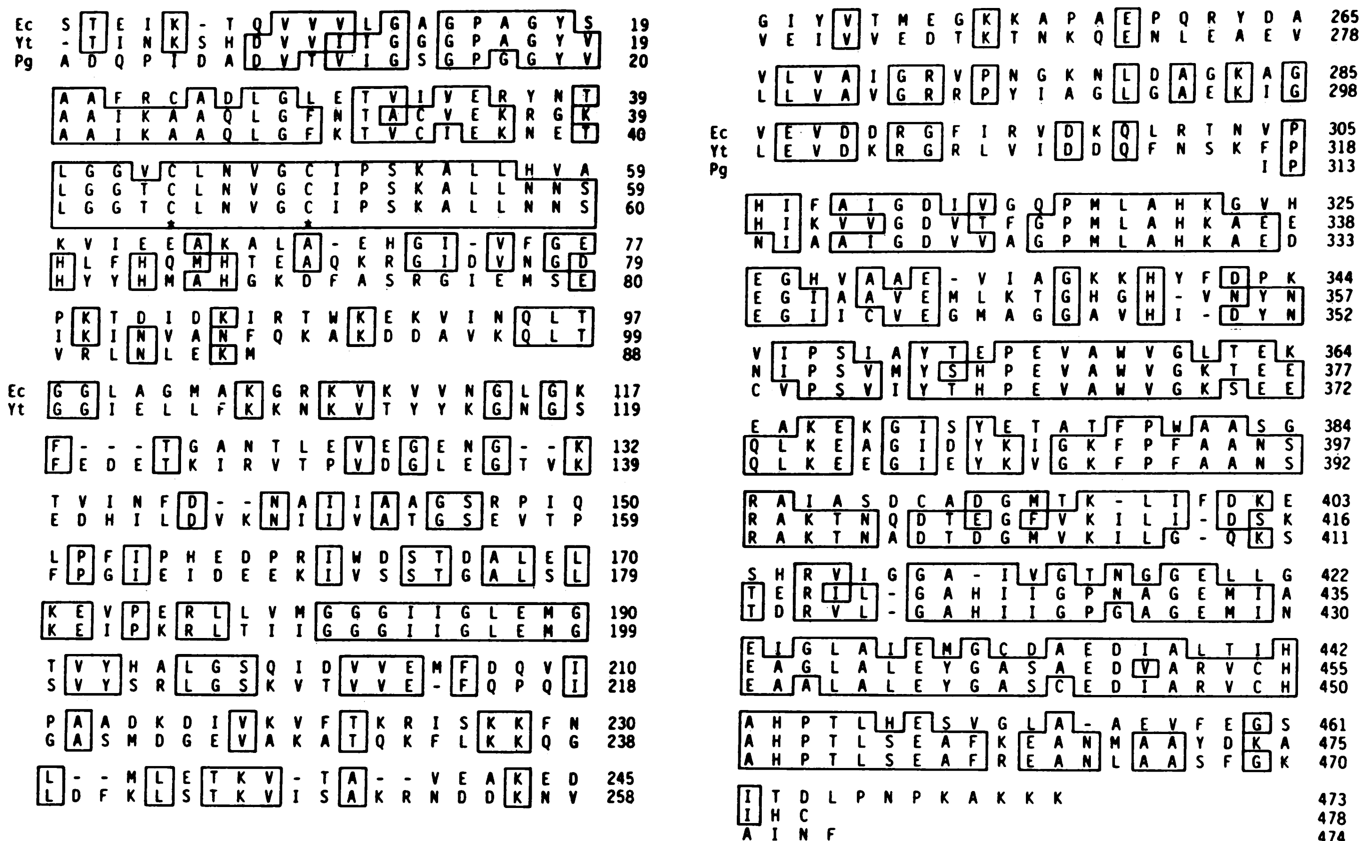

FIG. 4. Comparison of amino acid sequences of dihydrolipoamide dehydrogenases. The deduced primary structures of the *E. coli* (Ec) and yeast (Yt) enzymes and the partial sequence of the pig heart (Pg) enzyme are aligned for maximal homology. The boxed amino acid residues are those that are identical. Asterisks represent the active center cystine.

acidic amino acids. This feature is characteristic of presequences of mitochondrial matrix/inner membrane proteins (22). The presumed mature protein consists of 478 amino acids and has a calculated M_r = 51,558. Its predicted amino-terminal sequence is virtually identical with the 25-residue amino-terminal sequence of the purified protein (underlined). The only difference is the first residue, which was uncertain in the experimentally determined amino acid sequence. It is noteworthy that the amino acid sequence surrounding the putative active site cystine (asterisks), is identical with the sequence reported for the purified protein from pig heart (6).

The putative 5′ untranslated region of this cDNA is rather unusual. It is quite long (≥530 nucleotides) and contains a region of 21 adenine nucleotides immediately adjacent to the initiator ATG. Transfer blot analysis of yeast poly(A)$^+$ RNA indicates that the size of the mRNA is ≈1.6 kilobases (Fig. 3). The discrepancy between the size of the mRNA and the size of the cDNA (2.1 kilobases) indicates that another cDNA was probably ligated to the dihydrolipoamide dehydrogenase cDNA during the cloning procedures. Further work will be necessary to determine the true size and sequence of the 5′ untranslated region of cDNA for yeast dihydrolipoamide dehydrogenase.

Comparison of Dihydrolipoamide Dehydrogenases. The amino acid sequences of dihydrolipoamide dehydrogenases from *E. coli*, yeast, and pig heart are compared in Fig. 4. The amino acid sequence of the pig heart enzyme is incomplete. The partial sequence presented in Fig. 4 corresponds to the amino-terminal 88 residues and the carboxyl-terminal 163 residues (10). It is obvious that the primary structures of the three proteins are highly homologous. The yeast and *E. coli* enzymes exhibit 41% sequence identity. Particularly striking is the conservation of sequence around the active center cystine in the three enzymes. The cloning and sequence analysis of the cDNA for yeast dihydrolipoamide dehydrogenase should make it possible to test, by site-directed mutagenesis, some of the predictions made about the reaction mechanism of this flavoprotein.

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