

Lactate Dehydrogenase-B cDNA from the Teleost *Fundulus heteroclitus*: Evolutionary Implications¹

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A cDNA that encodes the heart-type lactate dehydrogenase (LDH-B) from the teleost fish *Fundulus heteroclitus* was cloned and sequenced. The protein encoded by the cDNA was analyzed in relation to 13 LDH proteins from a variety of taxa. One of the deductions from this analysis is that LDH-B proteins have residues in the active site that are unique and that may be important in determining the biochemistry of the heart-type isozyme. Phylogenetic analysis of the LDH sequences indicates that the branch lengths are greater in lower vertebrates, suggesting that the amino acid replacement rates vary depending on the evolutionary constraints within each taxon. Furthermore, the analysis suggests that LDH-C arose prior to the divergence of the LDH-A and LDH-B isozymes and thus that it is probably ancestral to these isozymes.

Introduction

Lactate dehydrogenase (LDH) reversibly reduces pyruvate to lactate with the concurrent oxidation of NADH. There are at least three LDH isozymes in vertebrates: LDH-A (muscle form), LDH-B (heart form), and LDH-C (Holbrook et al. 1975; Markert et al. 1975; Whitt et al. 1975). LDH-A and LDH-B tend to have specific biochemical functions and tissue distributions (Everse and Kaplan 1975; Markert et al. 1975; Whitt et al. 1975). LDH-A is best suited for pyruvate reduction in anaerobic tissues, whereas LDH-B is superior for lactate oxidation in aerobic tissues. In lower-teleost fishes, LDH-C has a generalized tissue distribution, but it is either eye or liver specific in advanced teleosts (Shaklee et al. 1973; Markert et al. 1975; Kettler and Whitt 1986). In mammals and birds, LDH-C is expressed only in mature testes (Blanco et al. 1975; Wheat and Goldberg 1983). It is surprising that LDH-C has only been found in one family of birds, Columbidae (Matson 1986).

The LDH isozymes were thought to have arisen from a single *Ldh-A*-like locus in Agnatha (lampreys have only a single LDH isozyme; Markert et al. 1975). This primordial locus was presumably duplicated to form *Ldh-A* and *Ldh-B*; then *Ldh-B* was duplicated to form *Ldh-B* and *Ldh-C* (fig. 1A; Markert et al. 1975; Whitt et al. 1975). Support for this theory is based on many types of evidence. There appear to be only two LDH isozymes in lower vertebrates (reptiles and below; Fisher et al. 1980). Advanced teleosts are an exception in that they have three LDH isozymes (Shaklee et al. 1973; Markert et al. 1975; Whitt et al. 1975; Fisher et al. 1980). The

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two isozymes that occur in most vertebrates are thought to be LDH-A and LDH-B because of their tissue-specific distribution, biochemical parameters, and immunoaffinity (Wilson et al. 1964; Holmes 1972; Shaklee et al. 1973; Holmes and Scopes 1974; Markert et al. 1975; Whitt et al. 1975). The existence of LDH-A prior to tetrapod divergence is indicated by the similarity between the primary structures of the dogfish LDH-A (class Chondrichthyes) and mammalian LDH-A (Li et al. 1983). LDH-B is thought to be the second LDH isozyme (i.e., other than LDH-A) in lower vertebrates, and its gene (*Ldh-B*) was presumably duplicated to form *Ldh-C* in advanced teleosts (Markert et al. 1975; Whitt et al. 1975). Finally, the enzyme kinetics for the mammalian LDH-C are more similar to those of the LDH-B isozyme than to those of LDH-A (Blanco et al. 1975). Both the taxonomic distribution of these three LDH isozymes and other evidence presented above suggest that LDH-C evolved independently in teleosts and mammals.

Recently, this theory of LDH evolution has been questioned (Li et al. 1983; Rehse and Davidson 1986; Baldwin and Lake 1987). It has been suggested that the primordial vertebrate LDH is structurally more similar to LDH-C. This conclusion is based on the analysis of LDH amino acid sequences from five vertebrate species (Li et al. 1983) and on the fact that the single LDH isozyme in lamprey is more similar immunologically to LDH-C in teleost than to either LDH-A or LDH-B (Baldwin and Lake 1987). The major problem with an LDH-C-like isozyme being ancestral is the current taxonomic distribution of the LDH isozymes. There are only two isozymes in amphibians and reptiles, and these have the conserved biochemical and physiological functions of LDH-A and LDH-B. This presents a dilemma because taxonomically older and younger groups contain LDH-C, and there is evidence from both groups that these isozymes are most similar to the ancestral LDH isozyme. If an LDH-C-like protein is ancestral, then the current taxonomic distribution can be explained either by LDH-B evolving independently in advanced teleost and terrestrial vertebrates or by all three isozymes being present prior to the divergence of teleost and terrestrial vertebrates. A prediction of the former hypothesis, that LDH-B has two origins (paralogous), is that the teleostean LDH-B and terrestrial vertebrate LDH-Bs will not have the greatest similarity—and thus, in a phylogenetic analysis, will not appear to have a common origin.

These hypotheses can be tested by the analysis of specific LDH sequences. In particular, the sequences of LDH-B and LDH-C need to be determined for representative lower vertebrates. As a first step in that direction, we have cloned and sequenced an *Ldh-B* cDNA from the teleost fish *Fundulus heteroclitus*. We have used both the amino acid sequence deduced from this cDNA and 13 other LDH amino acid sequences to determine the homology between isozymes in different vertebrate classes. Our findings suggest that an isozyme similar to LDH-C was ancestral to both LDH-A and LDH-B and that the teleost LDH-B is homologous to the mammalian and avian LDH-B. Finally, a different scheme of LDH isozyme evolution is presented (fig. 1B).

Material and Methods

Construction and Screening of cDNA Libraries

A cDNA library, ligated into both lambda gt-11 and lambda gt-10, was constructed from poly-A RNA isolated from livers of *Fundulus heteroclitus* that express only one of the allelic variants of LDH-B (i.e., *Ldh-B^b*; Place and Powers 1978). A modification (Agellon and Chen 1986a) of Gubler and Hoffman's (1983) RNase H method was used to synthesize the cDNA.

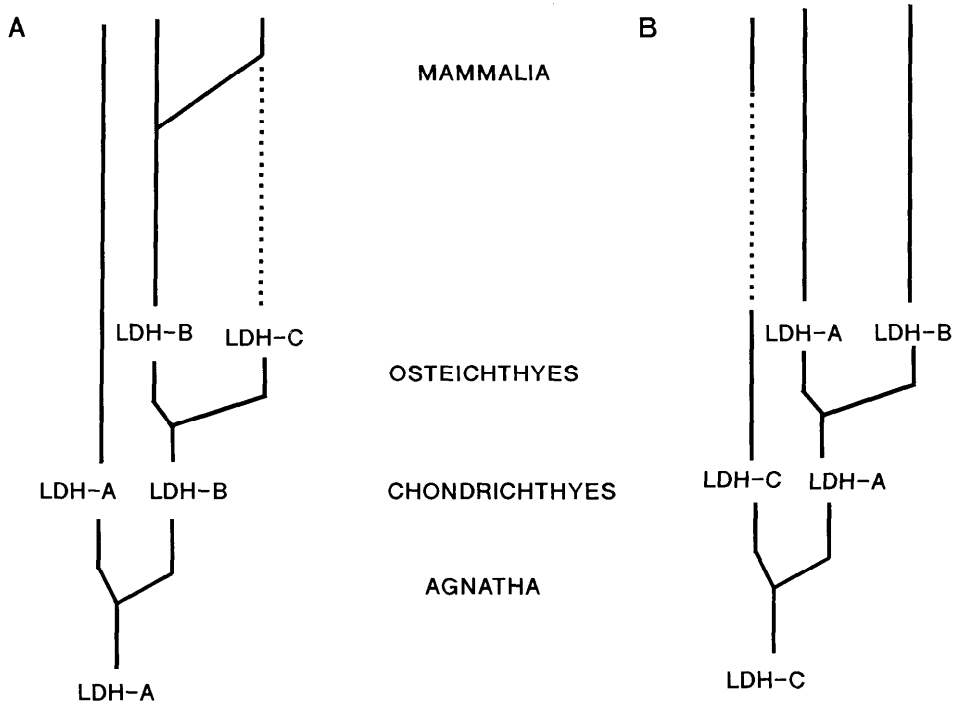


FIG. 1.—Evolution of LDH isozymes. Two alternative patterns of LDH isozyme evolution are presented: (A) the scheme of LDH evolution according Markert et al. (1975) and (B) the model of LDH evolution as the synthesis of the information presented in the present paper. Dotted lines indicate alternative or tentative evolutionary pathways.

The lambda gt-11 library was screened using an affinity-purified anti-LDH-B antibody and an alkaline-phosphatase conjugated anti-rabbit antibody. Antibodies were made by immunizing rabbits with LDH-B, which was purified according to the method of Place and Powers (1984) and then isolated from a sodium-dodecylsulfate-polyacrylamide-gel-electrophoresis gel. LDH-B bound to nitrocellulose was used as an affinity matrix to purify the antibodies (Olmsted 1981). Procedures for expression and plaque lifts of lambda gt-11 are described by Huynh et al. (1985). In brief, nitrocellulose filters were used and preincubated in 5% powdered milk (Johnson et al. 1984). Antibody complexes were detected with the use of two color reagents (0.33 mg nitroblue tetrazolium/ml and 0.16 mg 5-bromo-4-chloro-3-indolyl phosphate/ml in 100 mM Tris-HCl pH 9.6, 100 mM NaCl, 10 mM MgCl as recommended by the supplier, Promega Biotec).

The cDNA insert isolated from lambda gt-11 library was used as a probe for further screening. Nylon filters (Hybond-NTM; Amersham) were used for plaque lifts, and then these filters were UV irradiated to cross-link the DNA to the filters. Filters were hybridized at 37°C in 5 × SSPE (0.8 M NaCl, 10 mM NaH₂PO₄ pH 7.5, 1 mM ethylenediaminetetraacetic acid), 5 × Denhardt's (Maniatis et al. 1982), 50% formamide, 0.1% sodium dodecyl sulfate, and 100 µg calf-thymus DNA/ml. Hybridization probes were labeled by random priming using ³²P-dCTP (Feinberg and Vogelstein 1983, 1984). All cDNA inserts were sequenced using a combination of chemical degrada-

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-70      -60      -50      -40      -30
AGC  TGGCCTTCAC  CTGCGTCCGC  GGGCACCACCT  CAGTCTCTGTA  TCCCAGACTT

-20      -10      15      30
TTCTCCTGAT  CTCTGCAATC  ATG  TCC  TCA  GTC  CTG  CAG  AAG  CTG  ATC  ACC  CCC  CTG
Met  Ser  Ser  Val  Leu  Gln  Lys  Leu  Ile  Thr  Pro  Leu

45      60      75      90
GCC  AGC  TCC  TCC  GCT  GAG  CCT  CCC  AGG  AAC  AAG  GTG  ACC  GTG  GTG  GGT  GTG  GGC
Ala  Ser  Ser  Ser  Ala  Glu  Pro  Pro  Arg  Asn  Lys  Val  Thr  Val  Val  Glu  Val  Gly

105      120      135
CAG  GTC  GGC  ATG  GGG  TGT  GCC  GTC  AGC  ATC  CTG  CTG  CGG  GAC  CTG  TGC  GAC  GAG
Gln  Val  Gly  Met  Ala  Cys  Ala  Val  Ser  Ile  Leu  Lys  Gly  Leu  Arg  Asp  Lys  Cys  Asp  GAG

150      165      180      195
CTC  GCT  CTG  GTG  GAC  GTG  ATG  GAG  GAC  CGC  CTC  AAA  GGG  GAG  ATG  ATG  GAC  CTG
Leu  Ala  Leu  Val  Asp  Val  Met  Glu  Asp  Arg  Leu  Lys  Gly  Leu  Val  Phe  Lys  Asp  Leu

210      225      240
CAG  CAC  GGC  CTC  CTC  TTC  CTG  AAG  ACG  TCC  AAG  GTG  GTT  GCT  GAT  AAA  GAC  TAC
Gln  His  Gly  Leu  Leu  Phe  Leu  Lys  Thr  Ser  Lys  Val  Val  Ala  Asp  Lys  Asp  Tyr

255      270      285      300
GCC  GTG  ACG  GCC  AAC  TCC  CGC  CTG  GTG  GTG  GTG  ACG  GCC  GGC  GTG  CGG  CAG  CAG
Ala  Val  Thr  Ala  Asn  Ser  Arg  Leu  Val  Val  Val  Thr  Ala  Gly  Val  Arg  Gln  Gln

315      330      345      360
GAG  CGC  GAG  AGC  CGC  CTC  AAC  CTC  GTC  CAG  AGC  AAC  CTC  AAC  GTC  TTC  AAG  TGC
Glu  Gly  Glu  Ser  Arg  Leu  Asn  Leu  Val  Gln  Arg  Asn  Val  Asn  Val  Phe  Lys  Cys

375      390      405
ATC  ATC  CCG  CAG  ATC  ATA  AAG  TAC  AGC  CCC  AAC  TGC  ACA  ATC  CTG  GTG  GTG  TCC
Ile  Ile  Pro  Gln  Ile  Ile  Lys  Tyr  Ser  Pro  Asn  Thr  Thr  Ile  Leu  Val  Val  Ser

420      435      450      465
AAC  CCC  GTT  GAC  GTG  CTG  ACC  TAC  GTG  ACC  TGG  AAG  CTG  AGC  GGT  CTG  CCC  AAG
Asn  Pro  Val  Asp  Val  Leu  Thr  Tyr  Val  Thr  Trp  Lys  Leu  Ser  Gly  Leu  Pro  Lys

480      495      510
CAC  CGG  GTC  ATC  GGC  AGC  GGC  ACC  AAC  CTG  GAC  TCG  GCT  CGC  TTC  CGC  TAC  ATG
His  Arg  Val  Ile  Gly  Ser  Gly  Thr  Asn  Leu  Asp  Ser  Ala  Arg  Phe  Arg  Tyr  Met

525      540      555      570
ATG  GCC  GAG  CGC  CTC  GGC  ATC  CAC  GCC  AGT  TCC  TTC  AAC  GGC  TGG  GTG  CTG  GGC
Met  Ala  Glu  Arg  Leu  Gly  Ile  His  Ala  Ser  Phe  Asn  Gly  Trp  Val  Leu  Leu  Gly

585      600      615      630
GAG  CAC  GGA  GAC  ACC  AGC  GTT  CCC  GTG  TGG  AGC  GGC  GCA  AAC  GTC  GCC  GGG  GTC
Glu  His  Gly  Asp  Thr  Ser  Val  Pro  Val  Trp  Ser  Gly  Ala  Asn  Val  Ala  Gly  Val

645      660      675
AGC  CTG  CAG  AAG  CTG  AAC  CCG  GAG  ATC  GGC  ACC  GAC  GGC  GAC  AAG  GAG  CAG  TGG
Ser  Leu  Gln  Lys  Leu  Asn  Pro  Glu  Ile  Gly  Thr  Asp  Gly  Asp  Lys  Glu  Gln  Trp

690      705      720      735
AAG  GCC  ACC  CAC  AAG  GCC  GTG  GTG  GAC  AGC  GCC  TAC  GAG  GTG  ATC  AAG  CTG  AAG
Lys  Ala  Thr  His  Lys  Ala  Val  Val  Asp  Ser  Ala  Tyr  Glu  Val  Ile  Lys  Leu  Lys

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tion (Maxam and Gilbert 1980) and dideoxy termination methods (Agellon and Chen 1986*b*).

Protein Comparisons

The protein encoded by the LDH-B cDNA from *F. heteroclitus* was compared with 13 other LDH proteins. These sequences were obtained from NEWAT (Doolittle 1981), National Biomedical Research Foundation protein data bank, GenBank (NIH) DNA sequence library and from Li et al. (1983) for LDH-C sequences. Protein sequences were compared by the use of Feng and Doolittle's (1987) multiple-alignment and tree-construction programs. The advantage of these algorithms is that they progressively align the sequences, giving more weight to recently diverged sequences than to those which are more phylogenetically distant.

Terminology

Two residue numbers are given for each amino acid. The first number, in superscript, refers to the position relative to the LDH-B isozymes of human, *F. heteroclitus*, and pig. This numbering system is simpler because it is based on the longest peptide sequence and thus avoids having to use the same number twice. The second number, in brackets (< >), is the number used in older studies and is based on the

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          750          765          780
GGT TAC ACC AAC TGG GCC ATT GGC TTC AGC GTG GCT GAC CTG ACT GAG AGC ATC
Gly Tyr Thr Asn Trp Ala Ile Gly Phe Ser Val Ala Asp Leu Thr Glu Ser Ile

          795          810          825          840
GTG AAG AAC CTG AGC CGC GTC CAC CCC GTC TCC ACC ATG GTT AAG GAC ATG TTC
Val Lys Asn Leu Ser Arg Val His Pro Val Ser Thr Met Val Lys Asp Met Phe

          855          870          885          900
GGC ATC GGC GAG GAG GTC TTC CTG TCT CTG CCC TGC GCC CTG AAC GGC AGC GGC
Gly Ile Gly Glu Glu Val Phe Leu Ser Leu Pro Cys Val Leu Asn Gly Ser Gly

          915          930          945
GTC GGC AGC GTG GTG AAC ATG ACC CTG ACG GCC GCG GAG GTG GCC CAG CTG AAG
Val Gly Ser Val Val Asn Met Thr Leu Thr Ala Ala Glu Val Ala Gln Leu Lys

          960          975          990
AAG AGC CCC GAC ACC CTC TGC GGC ATC CAG AAG GAC CTC T
Lys Ser Ala Asp Thr Leu Trp Gly Ile Gln Lys Asp Leu Lys Asp Leu

1,010          1,020          1,030          1,040          1,050          1,060
GAACACG   TCCACCCCAC   CGTGCAGAAG   ACCCCCGTCG   TCTCACAGCG   TTCCTTCGTT

1,070          1,080          1,090          1,100          1,110          1,120
TTTCTATCGT   CCCGTTTCA   GACTTTAACG   CCCTTACTGT   AGGAAGCTAT   GAGTGCCTTT

1,130          1,140          1,150          1,160          1,170          1,180
TAGAGCCGAG   GCAGGGAGGA   TTTGAGAGATT   CTATCGGTAT   GTATCAGTGC   TGCAGTCTGT

1,190          1,200          1,210          1,220          1,230          1,240
CGCTCATAGG   TTACCGTCGC   TCTAAATCCA   CTGATGTGAT   TCCTAAAGTA   AGATGTCGGC

1,250          1,260          1,270          1,280          1,290          1,300
TTCCAAACCG   CTCGTGGCT   TCTTCTTCTC   CCGTGTTTG   TGTGCCGAC   TCCTACTAGC

1,310          1,320          1,330          1,340          1,350          1,360
CTTCTCAGGA   AGGGTTCTTA   CGTGTGGCTT   TGACGATCAA   ACACCTTCTA   AAGGCTCTTT

1,370          1,380          1,390          1,400          1,410          1,420
ATCTGTCTGT   TCTACTGATC   TCACTTGACT   CCTCTTAGCT   GCAGGGATTT   TCCTACTAAG

1,430          1,440          1,450          1,460          1,470          1,480
AGCTCACAAAC   TGCCCTGAAA   GACCCTTAAA   GTCCAACTGC   GGTGTTCTCT   TGGCCTCATA

1,490          1,500          1,510          1,520          1,530          1,540
CTCGGCTGTA   TGTTGACAGA   GCAGCACAGT   GAACCAGTCG   CATGGAATG   TTGTCAAAGT

1,550          1,560          1,570          1,580          1,590          1,600
GAGGGGAAAC   CAGTTTGTCA   TAAGATGTGT   TGAATAATA   AATATGGTCA   CAAAGGAAA

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FIG. 2.—LDH-B^b cDNA sequence. The first nucleotide of the start codon is number 1. The conserved polyadenylation (AATAAA) signal is underlined. Only three A's, added by polyadenylation, are shown.

dogfish LDH-A residue positions. The species and abbreviations used in this study are given in figure 3.

Results

The nucleotide and predicted amino acid sequence for LDH-B from *Fundulus heteroclitus* is shown in figure 2. Three overlapping inserts were sequenced so that every base pair was read at least twice in both directions. This yielded a structure that is 1,670 bp long and contains the complete protein coding region, 73 bases in the 5' nontranslated region and 595 bases in the 3' nontranslated region not including the poly-A tail. Eighteen base pairs upstream from the poly-A site is the 5' end of the conserved poly-A signal (AATAAA; Proudfoot and Brownlee 1976). Only one of the five consensus nucleotides surrounding the translation start codon (Kozak 1984) is present (-3A, relative to the first codon).

The protein predicted on the basis of the cDNA sequences (fig. 2) is 36 kDa (not including the first methionine) and contains five cysteines, 33 acidic amino acids, and 40 basic amino acids. Its amino acid composition is as follows: 23 Ala (6.9%), 12 Arg (3.6%), 15 Asn (4.5%), 18 Asp (5.4%), 5 Cys (1.5%), 11 Gln (3.3%), 15 Glu (4.5%), 26 Gly (7.8%), 6 His (1.8%), 15 Ile (4.5%), 36 Leu (10.8%), 22 Lys (6.6%), 10 Met (3%), 7 Phe (2.1%), 11 Pro (2.2%), 28 Ser (8.4%), 18 Thr (5.4%), 6 Trp (1.8%), 6 Tyr (1.8%), and 44 Val (13.2%).

There is an extreme bias in codon usage, favoring G or C: 91% of the amino acids that have more than 1 codon have a G or C in the wobble position. In addition,

ATLKDQILVILLKKEEQ P QNKITVVGVGAVGMACAISILMKDLADELALVDVMDKLGEMMDLQHGSL
 -A-----N-----V-----I-----
 -----YN-----T-----I-----
 -----HN-----HV -H-----E-----I-----
 S--H--HNVH--H-HAH--S-----T-----V-----
 -K--GH-ATSQEP RSY-----V-----
 ---EK--APVAE--ATV-N-----Q-----G-S-----L-----
 ---EK--APVAQO--TTI-N-----Q-----G-S-T-----L-----
 ---EK--TPVA AGSTV-S-----Q-----G-G-C-----L-----
 SSVLQK--TP-ASSAEP-R-V-----Q-----V--LR-C-----R-----L-----
 S-V-E-----QN-VP-DKL SRC-----N-----L-G-----ADT--R--AL--L-----
 S-V-EE--QN-AP DKQ SRC-----D-----L-G-----AD-----AL--L-----
 -SIT-K-----DHQ-VIL--D-----SSY-FAMVLQGI-Q-IGI--IFK--T--DAT--SNALP
 -NN-----GGARVV-I-A-F--ASYVFALMNQGI--IV-I-AN-S-AI-DA--FN--KV

MU-A
 RA-A
 HU-A
 PI-A
 CH-A
 DF-A
 HU-B
 PI-B
 CH-B
 FU-B
 MU-C
 RA-C
 LB
 BS

FLKTPKIVSSKDYCVTANSKLVIIITAGARQQEGESRLNLVQRNVNIFKFIIPNIVKYSPhCKLLIVSNPV
 -----S-----V-----Q-----
 ---R-----G-N-----V-----N-----
 ---R-----G-N-----R-V-----V-----N-----V-----
 ---T-G-S-H-----V-----V-----D-----
 ---H-A-----G-S-S-G-----V-----H--D-II-V-----
 ---Q-----AD-S-----I-VV-V-----V-----Q-----D-IIIV-----
 ---Q-----AN-S-----I-VV-V-----V-----Q-----N-IIIV-----
 ---Q-H--AD-A-----I-VV-V-----V-----Q-----N-VI-V-----
 ---S-V-AD--A-----R-VV-V-----V-C-Q-I-----N-TI-V-----
 ---S-----FG-N-S-----MVS--T--D-L--A-M-A-V-GVIQN--D-II--T-----
 ---S-----FG-S-S-----MVS-QS--A-L--T-M-A-V-GVIQN--D-IM--T-----
 ---TS--K-IY-AE-SDAKDAD--V-----PK-P-T--D--NK-IL-K-L-S-VDP--DSGFNLI-F-VAA-----
 ---APK-VDIWHG--DDCRDAD--V-C-----N-KP--T--D--DK-IA--RS-VESVMASGFQGLF-VAT---

MU-A
 RA-A
 HU-A
 PI-A
 CH-A
 DF-A
 HU-B
 PI-B
 CH-B
 FU-B
 MU-C
 RA-C
 LB
 BS

DILTYVANKISGFPKNRVI GSGCNLDSARFRYLMGERLGVHALSCHGWVLGEHGDSSVPVWVG VNVAGVS
 -----P-----
 -----P-----M-----
 -----H-----H-----I-P-----IV-Q-----
 -V-----L-L-MH-I-----SC-----I-----VPS-----MWN-----
 ---T-L-L-H-----A-K-I-PS-----I-----A-----
 ---T-L-L-H-----A-K-PS-----I-----A-----
 ---T-L-L-H-----T-----A-I-PT-----I-----A-----
 -V---T-L-L-H-----T-----M-A--I-S-FN-----T-----A-----
 ---V-----V-G-----I-K-NPT-----I-----T-----
 ---V-----L-VSS-----I-K-NPT-----T-----T-----
 ---AT--L--L--V--TS--T--QSI A-MVN-D-R-V-AYIM--TEF--HA-IG--T-----
 ---AT--F--L-HE-----TI--T--F-L--YFS-APQNV-AYII--TEL--QAYIGVMP-----

MU-A
 RA-A
 HU-A
 PI-A
 CH-A
 DF-A
 HU-B
 PI-B
 CH-B
 FU-B
 MU-C
 RA-C
 LB
 BS

LKSLNPELGTADKEQWKEVHKQVVD S AYEVIKLRGYT S W A I G L S V A D L A E S I M K N L R R V H P I S T M I K G L
 -----Q-----D-----
 -T-H-D--K-----E-----V-----
 -N-H-----H-A--E-----
 -A-H-DM-----H-----T-----AV--M-----
 -E-H-----NK-QD-KL-D-----T-----C-----V--V-DF-----
 -QE--M--N-S-N-----M-E-----N-----I-ML--S-I-V--V-M-----
 -QQ--M--N-S-N-----M-E-----N-----I-ML--S-I-V--VQ-M-----
 -QQ-D-AM--K-S-N-----E-----R-----N-----E-C-TML--Y--SV--LV--T-----
 -QK--I--G-----AT--A-----F-----T--V--S--V--V-DM-----
 ---AI--S--H--N-----EGG--LNM-----T--R--L--K--VT-LV--F-----
 ---AI-S-N-QE-T--GG--LD-----T--R--L--K--AVT-LV-----
 IAENVKHAHPEIKED KLVMFED-R-A--I-----A-FYG-ATAL-RISKA-LNDENA-L-LVYMD-Q-----
 IRK-VESK-EE-Q- DLERIFVN-R-A--QI-EK--A-YYG-AMGL-RVTRA-LH-ENAILTV-AYLD--

MU-A
 RA-A
 HU-A
 PI-A
 CH-A
 DF-A
 HU-B
 PI-B
 CH-B
 FU-B
 MU-C
 RA-C
 LB
 BS

YGINEDVFLSVP CILGQNGISDVVKVTLTPEEEARLKSADTLWGIQKEL QF
 -K-----D-----
 ---KD-----L-----S-----
 ---K-N-----H-----
 H--KD-----V--SS--T--MI-K-D-EKI-----
 ---KDN--L--V-NDH--NI--MK-K-D--QQ-Q--T--D--D--K-----
 ---ENE--L--NAR-LTS-INQK-KDD-V-Q-----D--D-KDL-----
 ---ENE--L--V-NAR-LTS-INQK-KDD-V-Q-N-----D-KDL-----
 ---EN--L--V-SAS-LTS-INQK-KDD-V-Q-----S--D-KDL-----
 F--G-E--L--V-NGS-VGS--NM--AA-V-Q-----D-KDL-----
 H--K-E--I--V--S--T-F--NM-A--GL-----NM--D--EL-----
 ---K-EI--I--V--ES--T-L-S-B-NT--LF--C-I--NI--N--EL-----
 ---LYIG-AVINR--QNILEIP--DH--ESMQ--SQ-KKVLTDFAKNDIETRO
 ---ER--YIG-AVINR--RE-IEIE-NDD-KN-FHH--A--KSVLARAFTR-----

MU-A
 RA-A
 HU-A
 PI-A
 CH-A
 DF-A
 HU-B
 PI-B
 CH-B
 FU-B
 MU-C
 RA-C
 LB
 BS

96% of the Arg and Leu codons have a C occurring at the first position (vs. an A or a T, respectively), whereas random usage would predict 66%. This bias in G/C preference has been observed in other aquatic vertebrates (Bohonus et al. 1986), including several fish species (D. L. Crawford and L. B. Agellon, unpublished observation).

The progressive alignment of the 14 LDH amino acid sequences is shown in figure 3. In the present study, gaps were aligned within a group of closely related sequences, and, in turn, these subgroups were globally aligned. This approach creates maximum similarities within subgroups rather than between distantly related pairs. One result of this is that the chicken LDH-B N-terminal gap does not align with the N-terminal gaps in LDH-A sequences.

The similarity between either bacterial sequence and any single vertebrate sequence is 32%–39%. The sequence similarity among all 14 LDH proteins is only 18%; 27% of these 14 interact with the substrate or cofactor. The greatest sequence similarity is in the active site; 14 (48%) of the 29 amino acids that interact with either the substrate or coenzyme are conserved in all 13 LDH sequences (table 1). The conserved nature of these residues suggests that the bacterial and vertebrate LDH proteins were derived from a common ancestor.

The unvaried residues within the active site are probably critical for enzyme activity. However, the nonconserved amino acids may also be important in determining the biochemical parameters and physiological role of each isozyme. Among the nonconserved active-site residues in the vertebrate isozymes, three have replacements specific for LDH-B: Ile¹¹⁶ <119>, Ala³⁰ <31>, and Ala⁹⁸ <100> in LDH-As are a Val, a Glu, and a Val, respectively, in and only in all LDH-Bs. It has been suggested that Glu³⁰ <31> in LDH-B increases the affinity for NAD (Eventoff et al. 1977). Although there is some debate about the importance of this residue (Grau et al. 1981), the fact that there is a conserved and unique replacement within each isozyme suggests that this position is important in determining enzymatic function.

Two of the many replacements within the vertebrate sequences are unique to *F. heteroclitus*. At position 163 <165>, all vertebrates (including bullfrog; Holbrook et al. 1975) have a cysteine, whereas *F. heteroclitus* and the bacterial sequences have a threonine. This residue has no obvious function and occurs in the interior of the protein. The second replacement is at position 57 <58>, where Arg replaces a Lys which is found in all the other LDH sequences. This amino acid interacts with the O-3' of the adenine ribose (Holbrook et al. 1975). Experiments with analogues of NAD, in which the active O-3' group was replaced with H, show that the interaction between this group and residue 57 is not necessary for binding (K_m remains the same), but it affects V_{max} (Suhadolnik 1977). Thus, in *F. heteroclitus*, the K_m may not be

FIG. 3.—Alignment of 13 LDHs. The first sequence is mouse LDH-A; all the sequences below this show a dash (–) where an amino acid is identical relative to mouse LDH-A; a blank represents a gap. Thirteen different LDH sequences are shown: Mu-A = mouse LDH-A (*Mus musculus*; Li et al. 1985); Ra-A = rat LDH-A (*Rattus norvegicus*; Matrisian et al. 1985); Hu-A = human LDH-A (*Homo sapiens*; Tsujibo et al. 1985); Pi-A = pig LDH-A (*Sus scrofa domestica*; Kiltz et al. 1977; Grau et al. 1981); Ch-A = chicken LDH-A (*Gallus gallus*; Torff et al. 1977); DF-A = dogfish LDH-A (*Squalus acanthias*; Eventoff et al. 1977; Taylor 1977); Hu-B = human LDH-B (Sakai et al. 1987b); Pi-B = pig LDH-B (Kiltz et al. 1977); Ch-B = chicken LDH-B (Torff et al. 1977); Fu-B = the teleost *Fundulus heteroclitus* LDH-B; Ra-C = rat LDH-C; Mu-C = mouse LDH-C (Li et al. 1983; Sakai et al. 1987a); LB = the bacterial LDH from *Lactobacillus casei* (Hensel et al. 1983); BS = the bacterial LDH from *Bacillus stearothermophilus* (Wirz et al. 1983; Barstow et al. 1986).

Table 1
Comparison of Active Site in LDH

	LDH				
	A	B	C	LB	BS
Adenine:					
Val 26 <27>	*	*	*	*	I
Val 51 <52>	*	*	*	*	I
Asp 52 <53>	*	*	*	*	*
Val 53 <54>	*	*	A	I	*
Tyr 83 <85>	*	*	*	*	*
Ile 94 <96>	* ^a	V	*	*	*
Ile 116 <119>	*	V	*	*	*
Ile 120 <123>	*	*	*	*	*
Adenine Ribose:					
Gly 27 <28>	*	*	*	*	*
Gly 29 <30>	*	*	*	*	*
Asp 52 <53>	*	*	*	*	*
Lys 57 <58>	*	* ^b	*	*	*
Gly 97 <99>	*	*	*	*	*
Pyrophosphate:					
Ala 30 <31>	*	Q	B	*	F
Val 31 <32>	*	*	*	*	*
Lys 57 <58>	*	* ^b	*	*	*
Gly 97 <99>	*	*	*	*	*
Arg 99 <101>	*	*	*	P	N
Tyr 247 <245>	*	*	*	A	A
Nicotinamide Ribose:					
Val 31 <32>	*	*	*	*	*
Thr 95 <97>	*	*	*	*	C
Ala 98 <100>	*	V	*	*	*
Arg 99 <101>	*	*	*	P	N
Glu 100 <102>	*	*	M	K	Q
Arg 106 <109>	*	*	*	*	*
Val 136 <138>	*	*	*	A	A
Ser 137 <139>	*	*	T	A	T
Asn 138 <140>	*	*	*	*	*
Nicotinamide:					
Val 31 <32>	*	*	*	*	*
Glu 100 <102>	*	*	M	K	Q
Val 135 <137> ^c	I	*	*	*	*
Ser 137 <139>	*	*	T	*	T
Asn 138 <140>	*	*	*	*	*
Leu 165 <167>	*	*	*	*	*
Thr 247 <246>	*	*	*	A	A
Ile 252 <250>	*	*	*	*	*
Substrate:					
Arg 106 <109>	*	*	*	*	*
Arg 169 <171>	*	*	*	*	*
Asp 166 ^d <168>	*	*	*	*	*

NOTE.—The five LDH columns represent the three vertebrate isozymes—A = LDH-A; B = LDH-B; C = LDH-C—and the two bacterial Ldh sequences—LB = *Lactobacillus casei*; BS = *Bacillus stearothermophilus*. The active-site residues are listed under the subunit of the cofactor with which they interact. Amino acids involved in the active site were deduced by X-ray crystallography for shark LDH-A and pig LDH-B (Holbrook et al. 1975; Eventoff et al. 1977; Grau et al. 1981). An asterisk (*) denotes identity with the active-site amino acids in column 1 in all members of the group designated by the column headings. Residues that vary and are pervasive within an isozyme are indicated by the appropriate amino acid. Exceptions to the above are indicated by the following footnotes:

^a Ile 94 is replaced by a Val in chicken LDH-A.

^b Lys 57 is replaced by an Arg in *Fundulus heteroclitus* LDH-B.

^c Val 136 is conserved in pig and dogfish LDH-A and in mouse LDH-C.

^d Proposed aspartic acid involved in histidine-aspartic acid proton relay (29).

affected by the replacement of Arg⁵⁷, but this replacement may affect V_{\max} . This can be experimentally tested with site-directed mutagenesis.

The progressive alignment presented above was used for the construction of the phylogenetic tree (fig. 4). This analysis used the two bacterial sequences as an outgroup to root the tree. In addition, the tree was rooted with each bacterial sequence separately to substantiate the branching order. The distribution of taxa within each isozyme cluster is in agreement with the known evolutionary relationships, and the branching order for the isozyme clusters is the same as that determined by Li et al. (1983). The branch lengths and positions were insensitive when rooted to each bacterial polypeptide separately, with the exception of the dogfish LDH-A sequence: dogfish LDH-A had a negative branch length when rooted with *Lactobacillus casei* separately. Attempts to remedy this increased the standard deviation or moved the DF-A node to the base of the LDH-B isozyme branch.

The two major points of interest suggested by the protein phylogenetic tree are the origin of LDH-C and the evolution rate between taxa. The branching order implies that the mammalian LDH-C diverged prior to the gene duplication creating LDH-A and LDH-B. Branch lengths appear to be longer in lower vertebrates. For DF-A, the branch length is outside the 99% confidence limit of the other LDH-A isozymes. In addition, Fu-B is 20% longer than the next longest LDH-B, when measured from the closest common branch point.

Discussion

Whitt's (1969, 1970) conclusion that *Ldh-B* is expressed in *Fundulus heteroclitus* livers is strongly supported by our data. Both the amino acids in the active site and the C-terminus are typical of LDH-B proteins. Moreover, the amino acid sequence of *F. heteroclitus* LDH-B is more similar to those of the LDH-B isozymes of other species than it is to that of either LDH-A or LDH-C.

In figure 4, the branch lengths of the two lower vertebrates are longer than those of higher vertebrates, suggesting that the rate of evolution is different between vertebrate taxa. Since rapid evolution of LDH in lower vertebrates is contrary to the accepted paradigm that lower vertebrates have a slower rate of evolution (Bush et al. 1977), perhaps this paradigm deserves closer scrutiny in the future.

The greater sequence divergence in lower-vertebrate LDHs may be due to reduced interactions between the subunits of different multilocus isozymes. For example, LDH-A and LDH-B in lower vertebrates have restricted association in vivo (Markert et al. 1975). In fact, heterotetramers of LDH-A and LDH-B from *F. heteroclitus* are not formed in vitro or in vivo (data not shown). Perhaps the amino acid replacement rate, which varies among vertebrate classes, depends on the evolutionary constraints within each taxon.

The evolutionary scheme of Markert et al. (1975) and Whitt et al. (1975) suggests that the three vertebrate LDH isozymes arose by duplication from a single primordial locus (fig. 1A). The data that support this hypothesis suggest that only one of the duplicated isozymes will be structurally and functionally similar to the enzyme from which it was derived. For example, LDH-A appears to maintain greater structural and functional similarity to the primordial vertebrate isozyme than does LDH-B. This process was repeated when the primitive *Ldh-B* locus was duplicated, yielding *Ldh-B* and *Ldh-C* (fig. 1A). Since the mammalian and teleostean LDH-Cs are not functionally similar to phylogenetically older isozymes, they appear to have evolved by recent and independent duplications. A recent and independent evolution of the teleostean and

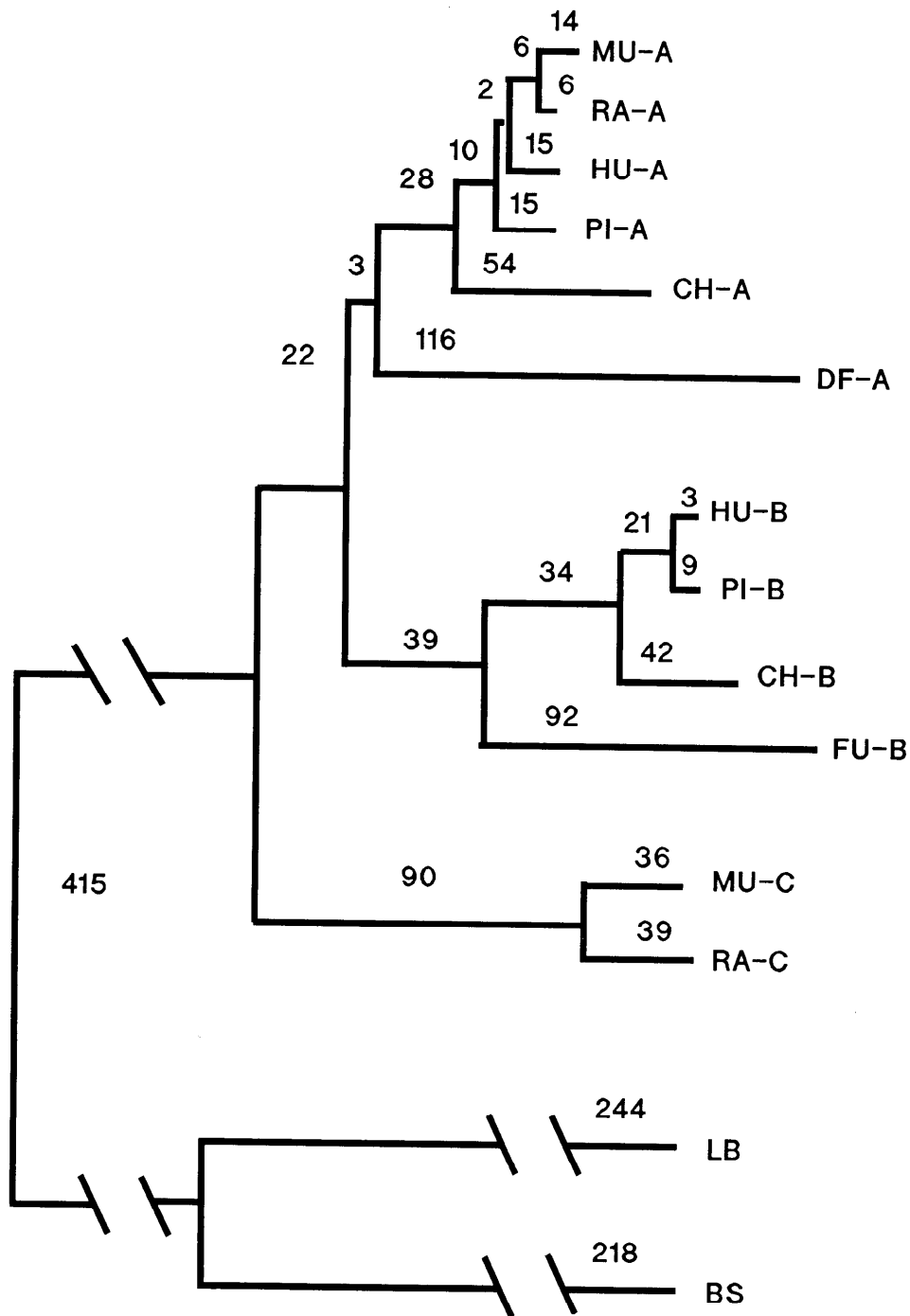


FIG. 4.—Phylogenetic tree. The phylogenetic relationship between the 14 LDH isozymes is shown. Abbreviations are the same as in fig. 3. Except for the bacterial sequences, phylogenetic distances are proportional to branch lengths (vertical lines) and are typed above the branch. Distances are in arbitrary units

mammalian LDH-Cs is also suggested by its limited taxonomic distribution, specialized tissue distributions, and differential developmental regimes (Shaklee et al. 1973; Champion et al. 1975; Millan et al. 1987).

Portions of this evolutionary scheme (fig. 1A) are supported by our analysis, but other parts are not. For example, the conclusion that LDH-A and LDH-B in lower vertebrates are homologous to the avian/mammalian LDH-A and LDH-B, respectively, is supported by our analysis (fig. 4). However, a recent and independent origin of LDH-C is not supported by our analysis. If LDH-C was recently derived, as suggested by Markert et al. (1975) and Whitt et al. (1975), then it should have the greatest similarity to the focus from which it originated, i.e., either *Ldh-A* or *Ldh-B*. Instead, the mammalian LDH-C is the least similar, suggesting either that it is closer to the ancestral isozyme or that it has evolved so rapidly that the expected similarity is obscure. Consistent with the hypothesis that LDH-C is ancestral, Baldwin and Lake (1987) showed that, relative to the other teleostean LDH enzymes, both the single isozyme in lampreys and the heart-type isozyme in hagfish have the greatest immunoaffinity to teleostean LDH-C. In addition, Baldwin et al. (1988) showed that the amino acid composition and immunocharacteristics of the only LDH in a lower chordate (subphylum Urochordata, *Pyura stoloifera*) is most similar to the teleostean LDH-C. Li et al. (1983) found that the mammalian LDH-C had the least similarity to either LDH-A or LDH-B, suggesting that it is more similar to the ancestral LDH. This is consistent with our analysis employing a larger data set and a different computer algorithm. The concept that LDH-C is similar to the primordial LDH is a major departure from the theory advanced by Markert et al. (1975), in two fundamental ways: (1) the evolutionary order from which the isozymes arose must be different; and (2) the primordial isozyme (i.e., LDH-C) has maintained its structural but not its functional similarity. We suggest that LDH-C, while maintaining its structural similarity to the primordial LDH, has undergone a canalization of function such that it is only expressed in limited amounts and in specialized tissue.

An alternative evolutionary scheme for the LDH isozymes is presented in figure 1B. As seen in the figure, LDH-C is suggested to be most like the ancestral LDH isozyme. The ancestral *Ldh* locus was duplicated to form *Ldh-C* and *Ldh-A*. Then *Ldh-A* in turn duplicated to yield *Ldh-B*. Within this scheme, another pathway would be LDH-C as a precursor of LDH-B. Although our analysis does not support this alternative pathway, several studies do support it. Others investigators have shown that teleost LDH-B is immunologically more similar to teleost LDH-C than is LDH-A (Whitt 1969; Shaklee et al. 1973; Whitt et al. 1975). The major problem with this evolutionary scheme is the lack of an observable third isozyme in amphibians, reptiles, and most birds. If it is assumed that our evolutionary scheme is correct (fig. 1B), then there are two alternatives that could resolve the missing-third-isozyme problem.

First, if LDH-C is similar to the primordial isozyme and LDH-A occurs in primitive vertebrates, then the two isozymes in primitive fishes and lower tetrapods should be LDH-A and LDH-C, not LDH-A and LDH-B. This implies that LDH-B evolved independently in advanced teleosts and mammals. As indicated above, the independent

(see Feng and Doolittle 1987). These units are based on Feng and Doolittle's distance matrix and are equal to $-\ln[S_{\text{real}} - S_{\text{rand}}]/(S_{\text{idnet}} - S_{\text{rand}})$, where S_{real} is the alignment score, S_{rand} is the score obtained with random sequences of the same length and composition, and S_{idnet} is the average score of the two sequences being compared when aligned with itself. Horizontal lines are not important in defining phylogenetic distances.

evolution of LDH-B is not supported by our analysis: LDH-Bs from all the classes analyzed appear to have a common origin. This explanation is not compelling.

Alternatively, if the three isozymes in teleosts have homologues in mammals, then all three isozymes should occur in lower-tetrapod vertebrates and in the Sarcopterygii (the bony fish from which tetrapods were derived). However, to date, a third isozyme has not been found in sarcopterygian fish. While this may be due to the scarcity of tissue samples from these fish [as suggested by Markert et al. (1975)], the absence of a third LDH isozyme in amphibians, reptiles, and most birds (Fisher et al. 1980; Matson 1986) cannot be explained by such reasoning. Since it is unlikely that a silent locus could maintain its functional and structural similarity over the 330 million years during which the tetrapods evolved, perhaps the third LDH isozyme has been overlooked or misidentified in lower tetrapods.

The lack of an observable third LDH isozyme in lower terrestrial vertebrates could be explained by the technical difficulty in detecting it. LDH-C in teleosts has the greatest variability in both tissue distribution and electrophoretic mobility, relative to other LDH isozymes (Shaklee et al. 1973; Champion et al. 1975; Whitt et al. 1975). In addition, in mammals, LDH-C is expressed only in breeding males (Millan et al. 1987). This variation and the temporal expression may explain why it has not been found in some lower tetrapods.

Finally, the potential homology between the mammalian and lower-vertebrate LDH-C is suggested by the ancestral origin of this locus, as presented in figure 3. Although the immunochemical and amino acid characterization of the single LDH in lampreys and tunicates indicate that it is most similar to the teleostian LDH-C, there is no corroborating evidence that these LDHs are orthologous to the mammalian LDH-C. Further research is needed to determine the similarity between the single LDH in lower vertebrates and the LDH-C.

Conclusion

The evidence presented in the present paper supports the concept that LDH-B isozymes are orthologous and that the LDH-C isozyme arose prior to the duplication creating LDH-A and LDH-B. In addition, LDH-C appears to have undergone a canalization of function while maintaining structural similarity to the primordial LDH. LDH-C may only be expressed in limited amounts in specialized tissue, making it difficult to detect.

These alternative evolutionary schemes (fig. 1A, 1B) can be resolved by DNA sequence analyses of specific LDHs, including the various *Ldh* loci from Agnatha, Chondrichthyes, Amphibia, and Reptilia, as well an *Ldh-C* from a representative teleost. The additional information provided by cDNA sequences, as opposed to protein sequences, should allow one to discriminate between the convergent and divergent evolutionary strategies described above.

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