

GENE 03627

Primary structure of bovine lactate dehydrogenase-A isozyme and its synthesis in *Escherichia coli*

(cDNA library; LDH isozyme; nucleotide sequencing; recombinant DNA)

Naotaka Ishiguro^a, Satoshi Osame^b, Ryo Kagiya^b, Shigeru Ichijo^b and Morikazu Shinagawa^a

Departments of^a Veterinary Public Health and^b Veterinary Internal Medicine, Obihiro University of Agriculture and Veterinary Medicine, Obihiro 080 (Japan)

Received by A. Nakazawa: 27 January 1990

Revised: 22 March 1990

Accepted: 9 April 1990

SUMMARY

Eleven cDNA clones encoding lactate dehydrogenase(LDH)-A isozyme were isolated from a bovine lymphocyte cDNA library, and the nucleotide sequences of three of the clones (pLDH5, pLDH9 and pLDH12) were determined. With the exception of variation in the 5' portion, two cDNA clones (pLDH9 and pLDH12) appeared to contain the full-length cDNA of 1786 bp, consisting of the protein-coding sequence (996 bp), the 5'- and the 3'-untranslated regions and the poly(dA) tail. The predicted amino acid (aa) sequence of bovine LDH-A (332 aa) showed 96.7% homology with that of pig LDH-A. The protein-coding cDNA region (1650 bp) was inserted into an *Escherichia coli* expression vector ptac11 and expressed. The protein synthesized in *E. coli* showed enzyme activity of LDH and was identified by cellogel electrophoresis as LDH-5 isozyme whose subunit M chain is the product of the *LDH-A* gene.

INTRODUCTION

LDH catalyzes the interconversion of pyruvate and lactate with nicotinamide adenine dinucleotide as a coenzyme. The enzyme is a tetramer with an M_r of 140 000 (Holbrook et al., 1975). In mammals and birds, LDH is encoded by

two different genes: *LDH-A* and *LDH-B*. Two gene products, M and H chains from the *LDH-A* and *LDH-B* genes, respectively, give rise to five different isozymes, M₄, H₄ and their three hybrids (Holbrook et al., 1975; Kiltz et al., 1977).

During the screening for the bovine Tcr-encoding gene in a bovine lymphocyte cDNA library using mouse C α -encoding DNA as a probe (Ishiguro et al., 1990), we isolated at a high frequency cDNA clones which were homologous, but not identical to bovine Tcr cDNA under low-stringency hybridization conditions. After determining their nt sequences and searching for homologous genes in a GenBank database, we thought that they were *LDH* cDNA clones, although no complete primary structure has so far been reported for bovine *LDH*.

In the present paper, we describe the entire nt sequence of the cDNA clones and the aa sequence predicted from it. The cDNA sequence proved to direct the active LDH synthesis in *E. coli*. The bacterially produced protein was the bovine M chain (the *LDH-A* gene product).

Correspondence to: Dr. N. Ishiguro, Department of Veterinary Public Health, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080 (Japan) Tel. (0155)48-5111, ext. 281; Fax (0155)48-2693.

Abbreviations: aa, amino acid(s); Ap, ampicillin; bp, base pair(s); C α , constant region of Tcr α -chain; IPTG, isopropyl- β -D-thiogalactopyranoside; kb, kilobase(s) or 1000 bp; LDH, lactate dehydrogenase; *LDH-A*, gene (DNA) encoding LDH-A; *LDH-B*, gene (DNA) encoding LDH-B; nt, nucleotide(s); ORF, open reading frame; PAGE, polyacrylamide-gel electrophoresis; R , resistance; SDS, sodium dodecyl sulfate; SSC, 0.15 M NaCl/0.015 M Na₃ citrate pH 7.6; Tc, tetracycline; Tcr, T-cell receptor; Tcr, gene (cDNA) encoding Tcr; WU, Wróblewski unit.

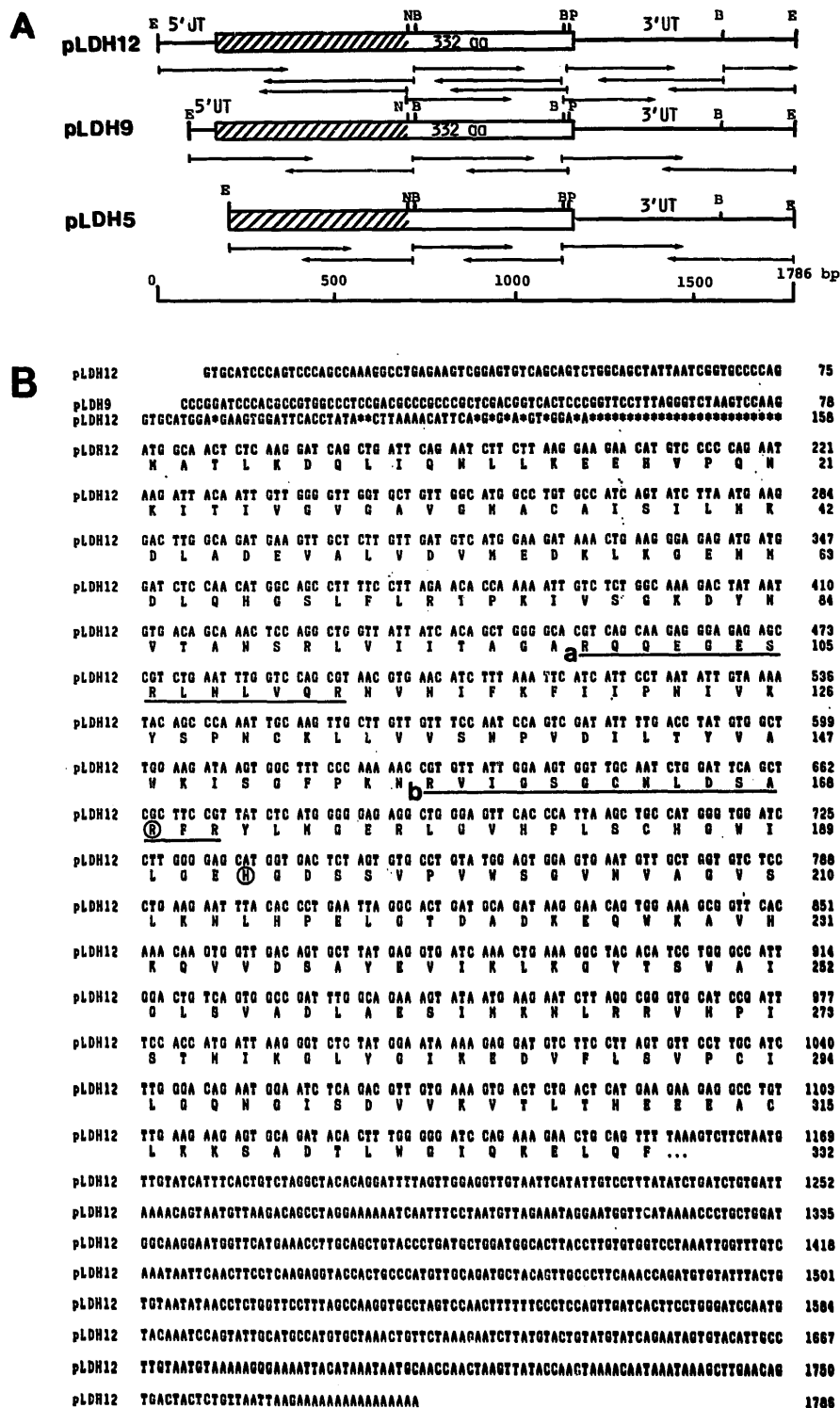


Fig. 1. Restriction map and nucleotide sequence of bovine *LDH* cDNA. (A) Partial restriction map and strategy for nt sequencing of three cDNA clones. The open boxes indicate the protein (332 aa)-coding as well as the 5'- and the 3'-untranslated regions. The hatched portions of the boxes indicate regions hybridized with the mouse *Tcr* α probe in Southern hybridization (Maniatis et al., 1982). Filters were washed extensively in $2 \times$ SSC and 0.1% SDS at room temperature (low stringency) before autoradiography. General procedures for cloning and DNA manipulations were as described in Maniatis et al. (1982). DNA from cDNA clones was subcloned in Bluescript M13+ and M13-, and sequenced by the chain-termination method (Sanger et al., 1977). Arrows indicate the directions and the extent of sequence determination. Sites of cleavage by restriction enzymes are: B, *Bam*HI; E, *Eco*RI; N, *Nco*I; P, *Pst*I. (B) The nt sequences of three LDH cDNAs, pLDH12 (1786 bp), pLDH9 (1706 bp) and pLDH5 (1602 bp), and predicted aa sequences. The numbers on the righthand side of the upper and lower lines indicate those for nt and aa sequences, respectively. Asterisks indicate the sequences identical to that of clone pLDH9. Two solid lines (a and b) under the aa sequence show homologies to the loop region and the substrate-binding region of LDH, respectively. Two circles indicate the particularly important residues of the substrate-binding region. The nt sequence data reported in this paper have been submitted to the GenBank nt sequence database under accession numbers D90141–D90143.

EXPERIMENTAL AND DISCUSSION

(a) Construction and screening of bovine cDNA library

A λ gt10 library was obtained from poly(A)⁺ mRNA isolated from bovine peripheral blood lymphocytes (Ishiguro et al., 1990). Of 6×10^4 plaques screened, 19 positive clones were detected using the mouse *Tcr C α* probe (450 bp) under low-stringency conditions (washing in $2 \times$ SSC, 0.1% SDS at room temperature). Eight out of 19 clones were still strongly hybridized with the *Tcr C α* probe under the high stringency conditions (washing in $2 \times$ SSC, 0.1% SDS at 68°C), and subsequently they were found to contain the bovine *Tcr α* cDNA (Ishiguro et al., 1990). The remaining eleven clones had partially homologous DNA regions to the mouse *Tcr C α* probe. Therefore, cDNA fragments in the representative three clones (pLDH5, pLDH9 and pLDH12) were subcloned into the single-strand generating pBluescript plasmid vector and analyzed.

(b) Nucleotide sequence of bovine LDH-A cDNA

The three cDNA clones had the same restriction map despite the different lengths of cDNA inserts (Fig. 1A). The homologous DNA sequence to the mouse *Tcr C α* probe resided on the region from the 5' terminus to the *Nco*I site in all three clones as analyzed by Southern hybridization analysis (indicated by hatched regions in Fig. 1A). The nt sequences of three cDNAs were identical except for the variation of the 5' termini between pLDH9 and pLDH12 (Fig. 1B). However, both pLDH9 and pLDH12 contained a common ORF of a 332-aa protein with an M_r of 36 597 (Fig. 1B). A search in a GenBank database (Genetyx program, Tokyo; GenBank Release 56.0) revealed that the predicted aa sequence is 96.7%, 93.7% and 84.6% homologous to those of pig, human and chicken *LDH-A* gene products (M chain), respectively (NBRF-PDB, Release 16.0). The sequence was also homologous to those of the *LDH-B* gene products (H chain) from pig (74.6%) and chicken (73.9%) (Li et al., 1983). The sequence homology at the nt level was greatest in the *LDH-A* gene from human (92.0%), followed by the *LDH-A* pseudogene from human (86.2%) and the *LDH-A* gene from rat (86.0%). Neither the complete aa sequences of the bovine *LDH-A* and *LDH-B* gene products nor their nt sequences has been reported. However, by comparison of the aa and nt sequences with those of mammalian LDH genes, the cDNA isolated here may well be that for bovine LDH M chain. The 5' variation between pLDH9 and pLDH12 may result from alternative splicing of mRNA, because an intron is present at 24 nt upstream from the translation start site in other mammalian *LDH-A* genes (Chung et al., 1985; Fukasawa et al., 1987). When the 3'-untranslated region was used as a probe for hybridization, all eleven cDNA clones were hybridized with

the probe under the high stringency conditions, suggesting that eleven cDNA clones obtained here contain an identical LDH cDNA.

When compared the nt sequences between pLDH12 and the mouse *Tcr C α* region of clone TT11 (Chien et al., 1984), sequence homology was about 31% in the entire nt sequence. However, on examination of homology of consecutive 50 bp along the sequence, 50–60% homology was found in the limited area, which may account for the cross-hybridization between the *LDH-A* cDNA clones and the *Tcr C α* probe.

(c) Structure–function relationship of LDH

The complete aa sequence of a bovine LDH M chain made it possible to compare the structure of LDH of various species. Taylor and Oxley (1976) isolated a peptide segment from bovine LDH-1 (H4 subunits) and assigned two functional regions of the substrate-binding site and the loop region from aa sequence analysis. In the substrate-binding site, two residues, His¹⁹³ and Arg¹⁶⁹ are of particular importance (Taylor, 1977), which are also predicted to exist in bovine LDH-A (indicated by circles in Fig. 1B). His¹⁹³ is thought to act as a proton donor in the dehydrogenase reaction (Holbrook et al., 1975). Another important region of LDH molecule is a 'loop' of polypeptide chain which extends from aa 99–112. In the apoenzyme, this loop is accessible to the solvent, whereas in the holoenzyme the loop is folded down from the surface of the enzyme molecule, the coenzyme being bound on the surface (Taylor, 1977). The aa sequence of the loop region of bovine LDH M chain (indicated by the solid line in Fig. 1B) is identical to the corresponding segments of all known mammalian LDH-M and LDH-H chains, but is remarkably different from those of testicular *LDH-C* gene products of mouse and rat (Pan et al., 1983).

(d) Expression of bovine LDH cDNA in *Escherichia coli*

To detect a 332-aa polypeptide encoded by cDNA clones, the cDNA fragment was expressed in *E. coli*, and the gene product was analyzed by SDS–PAGE. The 5'-noncoding region preceding the ORF in pLDH12 was digested with nuclease BAL 31, ligated with the *Eco*RI linker at both ends, and was cloned into the *Eco*RI site located downstream from the *tac* promoter in p $\text{tac}11$ (Amann et al., 1983). Two kinds of recombinant plasmids (pLDH17 and pLDH18), which carried the cDNA fragment at different orientations, were obtained (Fig. 2). *E. coli* RB791 carrying each plasmid was incubated with or without IPTG, and the lysates were subjected to SDS–PAGE (Fig. 3). A 34-kDa band corresponding to LDH was observed in *E. coli* RB791 carrying pLDH17 but not in that carrying pLDH18. The protein band synthesized in *E. coli* migrated closely with M4 rather than with H4 in

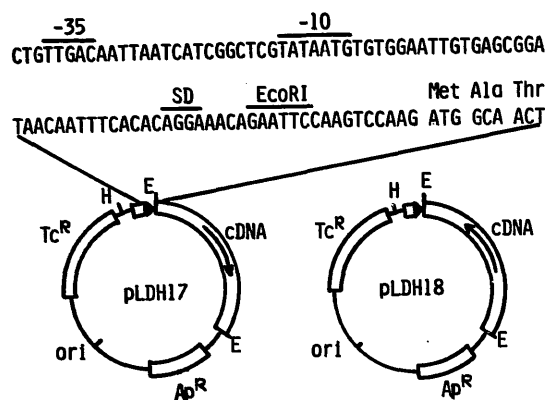


Fig. 2. Structures of plasmids pLDH17 and pLDH18. Both plasmids carry the 1650-bp *EcoRI* fragment inserted downstream from the *tac* promoter in the inverted orientation (Amann et al., 1983). The nt sequence of the 97 bp above the plasmid map shows the promoter region combined with the 5'-terminal portion of the cDNA insert in pLDH17. The open boxes show the ORFs. The arrows indicate the translation directions of the ORFs. The small boxes with triangular tips show the *tac* promoter region. SD, ribosome-binding site; ori, DNA replication origin; E, *EcoRI*; H, *HindIII*.

SDS-PAGE (Fig. 3). However, no significant difference in the amounts of protein was noted between the induced and uninduced cells.

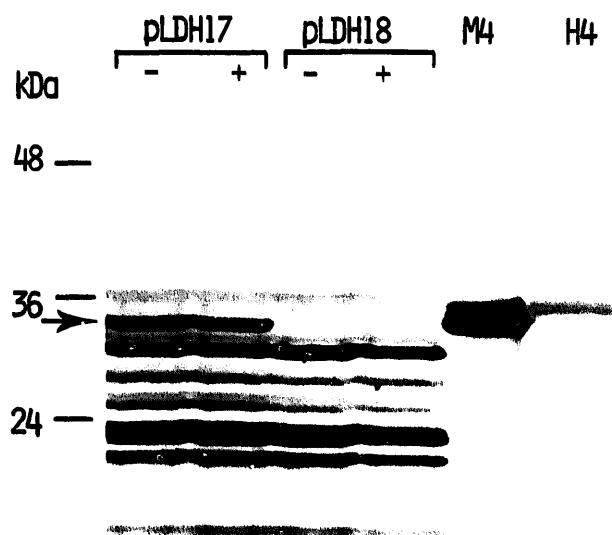


Fig. 3. SDS-PAGE of LDH protein synthesized in *E. coli*. *E. coli* RB791 (W3110 *lacI*^Q L8) carrying pLDH17 or pLDH18 were grown in L broth in the presence of 50 µg Ap/ml. At $A_{600} = 0.7$, IPTG was added, and the culture was incubated for another 2 h. An aliquot (1 ml) was withdrawn, treated as described by Amann et al. (1983), and the samples were analyzed on a 0.1% SDS-15% polyacrylamide gel (Laemmli, 1970). The arrow indicates the bovine LDH protein band (34 kDa). Purified LDH-1 (H4) and LDH-5 (M4) proteins were run as a control. Symbols - and + indicate uninduced and induced cultures, respectively. M_r standards were the cytochrome *c* monomer (12 kDa) and its covalently linked dimer, trimer, and tetramer (Oriental Yeast Co., Tokyo).

(e) Enzymatic activity of LDH and isozyme assay

To know if the protein synthesized in *E. coli* cells is functional, LDH activity was examined with an LDH Detection Kit (Chugai Pharmacy, Tokyo). The enzyme activity in the lysate of *E. coli* RB791 carrying pLDH17 was 6854 WU/mg protein, about 30–50 times higher than those in *E. coli* RB791 (133 WU/mg protein) or in *E. coli* RB791 carrying pLDH18 (190 WU/mg protein) (Tarmy and Kaplan, 1968). These results indicate that the protein expressed from pLDH17 in *E. coli* form a tetramer possessing LDH activity.

To confirm the LDH isozyme type of the bovine cDNA product, the isozyme pattern of LDH in lymphocyte extract and extracts from *E. coli* cells carrying pLDH17 or pLDH18 were analyzed by cellogel electrophoresis (Fig. 4). In lymphocytes freshly isolated from peripheral blood, LDH-2, LDH-3 and LDH-4 were predominant, and LDH-1 and LDH-5 levels were low (Kato et al., 1989). The enzyme from cells carrying pLDH17 migrated slightly faster than M4 (Fig. 4). However, when the lysate from pLDH17-carrying cells was mixed with M4 and applied on cellogel electrophoresis (pLDH17 + M4), the enzyme migrated at the same speed as M4 (Fig. 4). The unusual migration of pLDH17 band can be accounted for by difference in the

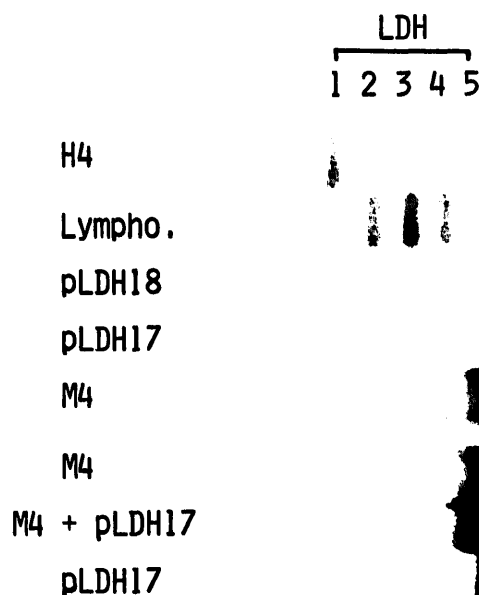


Fig. 4. Isozyme pattern of LDH protein synthesized in *E. coli*. Peripheral blood lymphocytes were obtained from a healthy Holstein cow using the Ficoll-Paque method (Pharmacia, Sweden). The lymphocytes were lysed by the freezing and thawing procedure, and the resulting lysate was used in the isozyme assay. *E. coli* RB791 cells carrying plasmids pLDH17 or pLDH18 were disrupted by sonication for 3 min, and the extracts were subjected to LDH isozyme analysis. The samples (1–4 µl) were loaded on a cellogel (Chemtron, Milan, Italy), and the LDH zymograms were made visible by tetrazolium staining (Kato et al., 1989). LDH-1 (H4) and LDH-5 (M4) proteins were used as controls. Lympho., lymphocyte extract. Migration of protein bands is leftward.

ionic composition of sample solution or by difference in the N-terminal modification. It is not clear if LDH isozyme synthesized in *E. coli* is N-acetylated or not. From the isozyme pattern, it is concluded that the 332-aa protein forms the tetrameric form of bovine LDH-5 and the subunit is LDH-M chain.

Although we have tried to isolate the *LDH-B* cDNA clone from the same cDNA library, all cDNA clones obtained were not bovine *LDH-B* but *LDH-A* gene. It remains to be determined why *LDH-B* cDNA (H chain) could not be isolated from this cDNA library. It has been demonstrated by in vitro cultures that changes in the LDH isozyme pattern can be induced by alterations in oxygen tension; hypoxia increased the relative amount of M subunits (Hellung-Larsen and Andersen, 1968). In addition, the proportion of M subunit increases when LDH of lymphocytes were cultured with phytohaemagglutinin. In the present study, the prolonged lymphocyte culture with mitogen concanavalin A may have resulted in a shift towards the message of the *LDH-A* gene (M chain).

REFERENCES

- Amann, E., Brosius, J. and Ptashne, M.: Vectors bearing a hybrid *trp-lac* promoter useful for regulated expression of cloned genes in *Escherichia coli*. *Gene* 25 (1983) 167–178.
- Chien, Y., Becker, D.M., Lindsten, T., Okamura, M., Cohen, D.I. and Davis, M.M.: A third type of murine T-cell receptor gene. *Nature* 312 (1984) 31–35.
- Chung, F.-Z., Tsujibo, H., Bhattacharyya, U., Sharief, F. and Li, S.S.-L.: Genomic organization of human lactate dehydrogenase-A gene. *Biochem. J.* 231 (1985) 537–541.
- Fukasawa, K.M. and Li, S.S.-L.: Complete nucleotide sequence of the mouse lactate dehydrogenase-A functional gene: comparison of the exon-intron organization of dehydrogenase genes. *Genetics* 116 (1987) 99–105.
- Hellung-Larsen, P. and Andersen, V.: Lactate dehydrogenase isoenzymes of human lymphocytes cultured with phytohaemagglutinin at different oxygen tensions. *Exp. Cell Res.* 50 (1968) 286–292.
- Holbrook, J.J., Lilijas, A., Steindel, S.J. and Rossmann, M.G.: Lactate dehydrogenase. In Boyer, P.D. (Ed.), *The Enzymes*, Vol. 11. Academic Press, New York, 1975, pp. 191–292.
- Ishiguro, N., Tanaka, A. and Shinagawa, M.: Sequence analysis of bovine T-cell receptor α -chain. *Immunogenetics* 31 (1990) 57–60.
- Kato, K., Mori, K. and Katoh, N.: Contribution of leucocytes to the origin of lactate dehydrogenase isozymes in milk of bovine mastitis. *Jpn. J. Vet. Sci.* 50 (1989) 530–539.
- Kiltz, H.-H., Keil, W., Griesbach, M., Petry, K. and Meyer, H.: The primary structure of porcine lactate dehydrogenase: isoenzymes M4 and H4. *Hoppe-Seyler's Z. Physiol. Chem.* 358 (1977) 123–127.
- Laemmli, U.K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227 (1970) 680–685.
- Li, S.S.-L., Fitch, W.M., Pan, Y.-C.E. and Sharief, F.S.: Evolutionary relationship of vertebrate lactate dehydrogenase isozymes A4 (muscle), B4 (heart), and C4 (testis). *J. Biol. Chem.* 258 (1983) 7029–7032.
- Maniatis, T., Fritsch, E.F. and Sambrook, J.: *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982.
- Pan, Y.-C.E., Sharief, F.S., Okabe, M., Huang, S. and Li, S.S.-L.: Amino acid sequence studies on lactate dehydrogenase C4 isozymes from mouse and rat tests. *J. Biol. Chem.* 258 (1983) 7005–7016.
- Sanger, F., Nicklen, S. and Coulson, A.R.: DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* 74 (1977) 5463–5467.
- Tarmy, E.M. and Kaplan, N.O.: Chemical characterization of D-lactate dehydrogenase from *Escherichia coli* B. *J. Biol. Chem.* 243 (1968) 2579–2586.
- Taylor, S.S.: Amino acid sequence of dogfish muscle lactate dehydrogenase. *J. Biol. Chem.* 252 (1977) 1799–1806.
- Taylor, S.S. and Oxley, S.: Homologies in the active site regions of lactate dehydrogenases. *Arch. Biochem. Biophys.* 175 (1976) 373–383.