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Tissue-specific isoforms of the bovine mitochondrial ATP synthase γ-subunit

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Tissue-specific isoforms of the γ-subunit of the bovine F_oF_1-ATP synthase were identified for the first time. The two isoforms, heart and liver type, were generated by alternative splicing, the liver-type RNA transcript containing a 37-nucleotide sequence as a cassette exon. Protein sequencing of the C-terminal fragments of the two isoforms of the $F_1\gamma$-subunit indicated that the liver-type isoform had an additional aspartate residue at the C terminus, not present in the heart type one.

F_1-ATPase; ATP synthase; Isoform; Alternative splicing

1. INTRODUCTION

Mitochondrial ATP synthase (F_oF_1) is the main enzyme complex for oxidative phosphorylation [1–7]. F_1 contains five different polypeptides, α, β, γ, δ, and ε with the stoichiometry $\alpha_3\beta_3\gamma\delta\,\varepsilon$ [4–7]. The catalytic sites are located on α–β subunit interfaces [8]. However, the exact role of the γ-subunit in mammals is not known. The γ-subunit of *Escherichia coli* is essential for reconstruction of an ATPase activity and for the in vitro assembly of the F_1 portion [5,9]. The protein sequence of the bovine heart $F_1\gamma$-subunit was the first mammalian sequence reported [10]. The amino acid sequence deduced from bovine cDNA [11] agrees exactly with that determined by protein sequencing of a preparation, so that isoforms of the $F_1\gamma$-subunit in other tissues have been overlooked. Among components of F_oF_1, no tissue-specific isoforms have been reported in mammals except proteolipid [12,13]. Human and bovine isoforms of proteolipid were encoded by two genes, respectively, and two cDNAs encode the same mature subunit. In this study, for the first time we showed the existence of two tissue-specific isoforms of the $F_1\gamma$-subunit in bovine heart and liver at both the mRNA and polypeptide levels.

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Abbreviations: RT-PCR, reverse transcription-polymerase chain reaction; F_1, catalytic portion of ATP synthase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography; DEAE, diethylaminoethyl; PTH, phenylthiohydantoin.

2. MATERIALS AND METHODS

2.1 *RT-PCR analysis of RNAs from tissues*

Total RNA was purified from bovine heart and liver by the acid guanidine method [14]. Reverse transcription was carried out using total RNA and oligo dT_{16} primer. Alternative splicing of the newly identified exon in the bovine liver was confirmed by DNA amplification by PCR using a pair of specific primers, the sense-strand oligonucleotide on exon 7 (5′-GTCATCACCAAGGAGCTGATA-3′) and the antisense-strand on exon 8 (5′-ATGGACCAATAAATTCTTCT-GAC-3′). These primers corresponded to nucleotides 901–921, and 999–1020, respectively, of bovine cDNA [11]. PCR products were subcloned into dideoxy T-tailed vector [15] and sequenced by the dideoxy termination method [16].

2.2. *Purification and protein sequencing of the γ-subunit of F_1-ATPase*

F_1-ATPase was isolated from bovine heart and liver by a modified chloroform extraction method [17] followed by DEAE-chromatography (DEAE-Toyopearl 650M; TOHSO, Japan). Elution was performed with a linear gradient of 50–300 mM K^+ phosphate/pH 7.8 in 20% glycerol at 25°C. F_1 was stored in 50% ammonium sulfate at 4°C. Subunits were separated by SDS-PAGE [18] and electroblotted onto a polyvinylidene difluoride (PVDF) membrane (ProBlott; Applied Biosystems). Blots were stained with 0.1% Ponceau S. The band of the γ-subunit was cut out and subjected to in situ digestion [19] with *Achromobacter* protease I (Lysyl Endopeptidase; Wako Pure Chemicals, Japan) at a molar ratio of 1:90 for 2 days at 37°C. This protease cleaves Lys-X bonds. The digest was fractionated by reverse-phase high performance liquid chromatography (HPLC) on μ Bondasphere S-5 C18–300 Å (150 × 3.9 mm; Waters Japan) with a linear gradient of acetonitrile (0–100%) in 0.1% trifluoroacetic acid. The peptide fraction corresponding to the C-terminal fragment was sequenced in a protein sequencer (model 473A; Applied Biosystems).

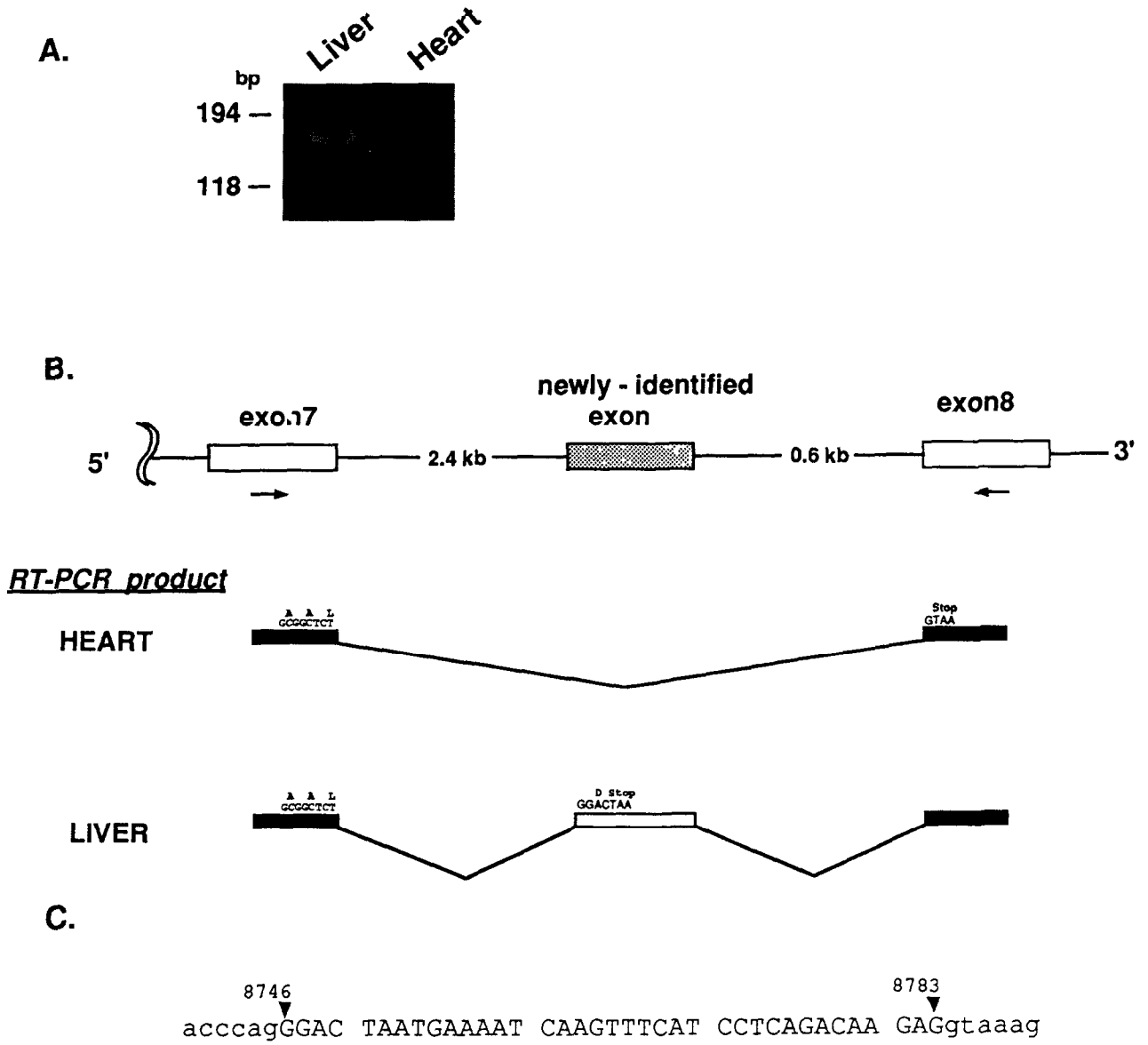

Fig. 1. Alternative splicing of the newly identified exon in bovine heart and liver. (A) Electrophoresis of PCR products amplified from heart and liver cDNA. The DNA size marker was an *Hae*III digest of φX174 phage DNA. (B) Schematic diagram of alternative splicing The upper line represents the 3'-side of the $F_1\gamma$-subunit gene. The arrows represent a pair of specific primers used for RT-PCR. Boxes represent exons, and thin lines represent introns. The splicing patterns used to generate two different transcripts detected by RT-PCR are represent under the gene. For each transcript, the exons utilized are connected by thin lines. The partial sequence of the nucleotides and amino acids encoded by exons are shown. (C) Nucleotide sequence of the gene around the newly-identified exon in bovine cDNAs. Uppercase letters represent exon sequences numbered according to Dyer et al. [11].

3. RESULTS AND DISCUSSION

The existence of two tissue-specific mRNAs in bovine tissues was confirmed by RT-PCR with cDNA as a template. The results of 5% agarose gel electrophoresis showed that the PCR product amplified from liver cDNA was approximately 40 bp longer than that from the heart (Fig. 1A). DNA sequencing of subcloned PCR products revealed that 37 bp nucleotides were inserted between exon 7 and exon 8 in the liver cDNA (Fig. 1B). This newly identified exon corresponded to nucleotides

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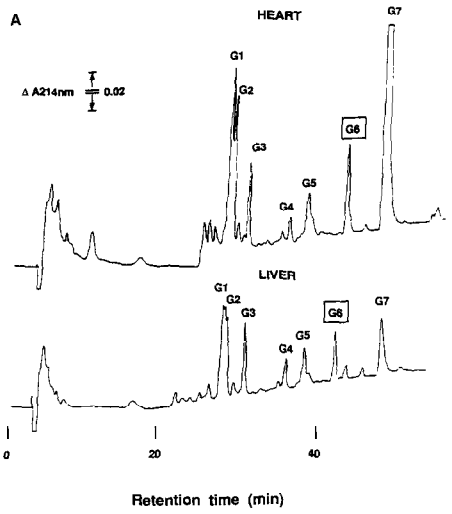
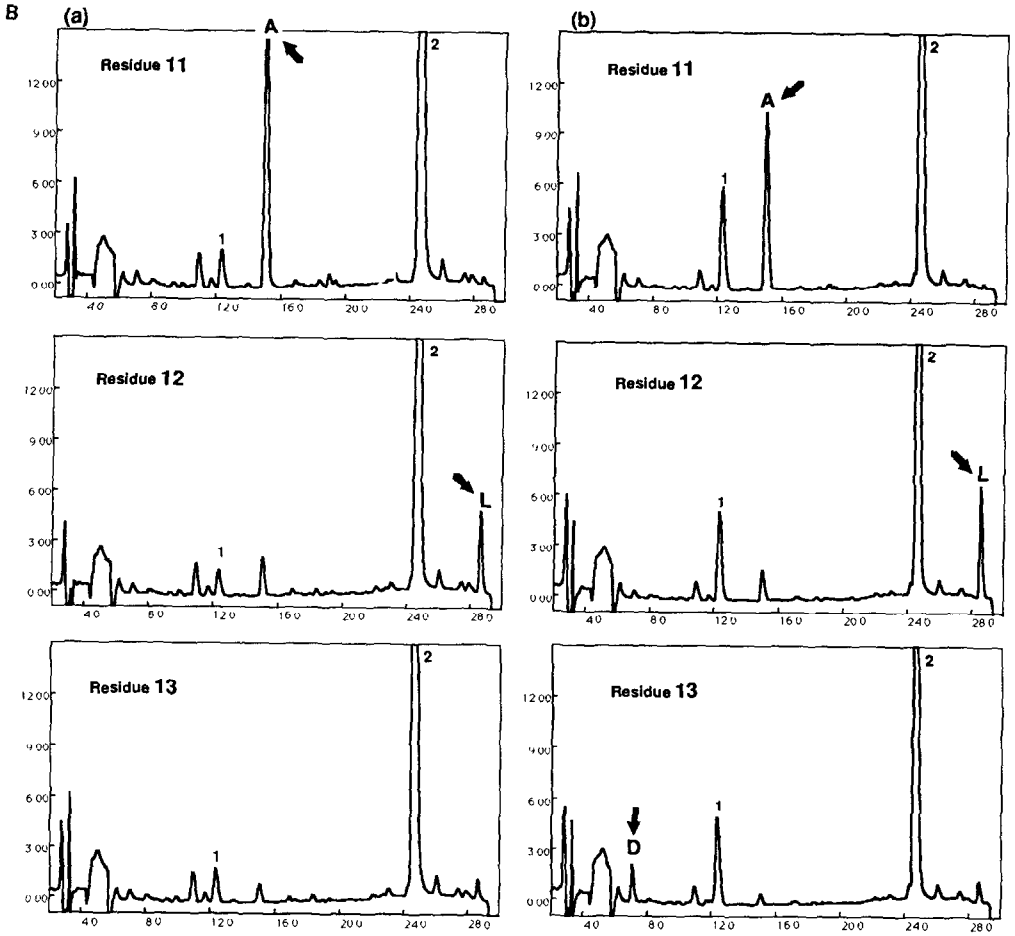
Fig. 2 (A) Reverse-phase HPLC of peptides in digests of the heart (upper) and liver (lower) $F_1\gamma$-subunits with lysyl-endopeptidase. The flow rate was 0.2 ml/min. (B) PTH-amino acid analyses of the heart (a) and liver (b) $F_1\gamma$-subunits. Three residues in the C-terminal are shown by the one-letter code. Peak 1 is *N'N*-dimethyl-*N'*-phenylthiourea, and peak 2 is *N'N*-diphenylthiourea. (C) Amino acid sequences of the C-terminal peptide fragments generated by digestion with lysyl-endopeptidase.





C

Residue	1	2	3	4	5	6	7	8	9	10	11	12	13
HEART	E	L	I	E	I	I	S	G	A	A	A	L	
LIVER	E	L	I	E	I	I	S	G	A	A	A	L	D

8746–8783 (numbered according to Dyer et al. [11]) of the bovine genome, the sequence of which was reported as part of intron 7. The sequence of the PCR product amplified from heart cDNA was exactly consistent with that of bovine cDNA reported previously [11]. Dyer et al. [11] reported isolation of the cDNA clones from a cDNA library derived from heart and liver RNAs. According to our data, the sequence could have been derived from bovine heart mRNA. The sequence of the alternatively spliced exon is shown in Fig. 1C and the nucleotide sequence of intron/exon boundaries is compatible with the AG–GT rule. There are stop codons on the 5′-side of both the new exon and the next one in the bovine gene. From the amino acid sequences deduced from the bovine cDNAs of the two types, the L-type isoform differed by having an additional aspartate residue at its C terminus.

The aspartate residue in the C terminus of the L-type protein was identified by sequencing the C-terminal peptide fragments of the liver and heart enzymes. On reverse-phase HPLC of digests with lysyl endopeptidase, seven major peaks of fragment peptides of both were separated and named G1, G2, G3, G4, G5, G6 and G7 in order of their elutions (Fig. 2A). The elution times of all the peaks except G6 from liver and heart were identical, but the elution time of G6 of the heart $F_1\gamma$-subunit was slower than that of the liver one (Fig. 2A). The elution time of the peak would be increased by the addition of an aspartate residue at the C terminus of the liver-type isoform, in spite of the increase in molecular weight, since an aspartate residue is hydrophilic. Analyses of the primary structures of the G6 peaks from the heart and liver $F_1\gamma$-subunits by peptide sequencing revealed that G6 corresponded to the C-terminal peptide fragment, and that the sequence of the liver type protein differed from that of the heart type by having one additional aspartate at the C-terminus (Fig. 2B,C).

Recently we cloned and sequenced a human gene encoding the $F_1\gamma$-subunit. We detected two tissue-specific RNA transcripts that were generated by alternative splicing by the reverse transcription-polymerase chain reaction (RT-PCR) (Matsuda, C., Endo, H., Ohta, S. and Kagawa, Y., manuscript in preparation). Judging from the sequences of human cDNAs, the difference between the two isoforms could be an additional amino acid (Asp) at the C-terminus of the L-type protein. In this paper, we report direct evidence both at the mRNA and protein level for the existence of two different isoforms of the bovine $F_1\gamma$-subunit. Like the human isoforms, the bovine isoforms of the $F_1\gamma$-subunit are generated by alternative splicing of a newly identified exon from a single gene. The C-terminal region is highly conserved in several species [10], and the C-terminal region is reported to be important in regulation of F_1-ATPase activity in *E. coli* [20]. This fact and findings of tissue-specific distributions of the two isoforms in human and bovine tissues suggest the importance of the $F_1\gamma$-subunit in tissue-specific energy supply.

REFERENCES

[1] Mitchell, P. (1979) Science 206, 1148–1159.
[2] Penefsky, H.S. and Cross, R.L. (1991) Adv. Enzymol. 64, 173–214.
[3] Kagawa, Y. (1984) in: Bioenergetics, New Comprehensive Biochemistry (L. Ernster, Ed.) pp. 149–186, Elsevier, Amsterdam.
[4] Walker, J.E., Fearnley, I.M., Lutter, R., Todd, R.J. and Runswick, M.J. (1990) Phil. Trans R Soc. Lond. 326, 367–378.
[5] Futai, M., Noumi, T. and Maeda, M. (1989) Annu Rev. Biochem. 58, 111–136.
[6] Yoshida, M., Sone, N., Hirata, H., Kagawa, Y. and Ui, N. (1979) J Biol. Chem. 254, 9525–9533.
[7] Lutter, R., Abrahams, J.P., van Raaij, M.J., Todd, R.J., Lundqvist, T., Buchanan, S.K., Leslie, A.G.W. and Walker, J.E. (1993) J Mol. Biol. 229, 787–790.
[8] Harada, M., Ito, Y., Sato, M., Aono, O., Ohta, S. and Kagawa, Y. (1991) J Biol. Chem. 266,11455–11460.
[9] Dunn, S D. and Futai, M. (1980) J. Biol. Chem. 255,113–118.
[10] Walker, J.E., Fearnley, I.M., Gay, N.J., Gibson, B.W., Northrop, F.D., Powell, S.J., Runswick, M.J., Saraste, M. and Tybulewicz, V L.J (1985) J. Mol Biol. 184, 677–701.
[11] Dyer, M.R., Gay, N.J., Powell, S.J. and Walker, J.E. (1989) Biochemistry 28, 3670–3680
[12] Gay, N.J. and Walker, J.E. (1985) EMBO J. 4, 3519–3524.
[13] Farrell, L.B. and Nagley, P. (1987) Biochem. Biophys. Res. Commun 144, 1257–1264.
[14] Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem 162,156–159.
[15] Holton, T.A. and Graham, M.W. (1991) Nucleic Acids Res. 19, 1156.
[16] Sanger, F., Nicklen, S. and Coulson, A.R (1977) Proc Natl Acad. Sci. USA 74, 5463–5467
[17] Williams, N., Amzel, L.M and Pedersen, P L. (1984) Anal. Biochem. 140, 581–588.
[18] Laemmli, U.K. (1970) Nature 227, 680–685.
[19] Matsudaira, P. (1987) J. Biol. Chem. 262, 10035–10038.
[20] Iwamoto, A., Miki, J., Maeda, M. and Futai, M. (1990) J. Biol. Chem. 265, 5043–5048.