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The Sequences of Human and Bovine Genes of the Phosphate Carrier from Mitochondria Contain Evidence of Alternatively Spliced Forms*

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The sequences of the human and bovine genes for the phosphate carrier from the inner membranes of mitochondria have been determined. The genes have similar structures and each is divided into nine exons. In both genes, two exons, named IIIA and IIIB, are closely related, and they appear to be alternatively spliced. The human exon IIIB sequence is found in a published human heart cDNA sequence, and bovine exon IIIA forms part of a published bovine heart cDNA sequence. By further examination of the human heart cDNA library, sequences arising from both alternatively spliced forms of the phosphate carrier have been characterized. Both forms were also found in several bovine tissues, but the ratios of expression of the two forms varied. The form containing exon IIIA was expressed most highly in bovine heart and liver, less highly in brain and kidney, and only in low amounts in lung. The opposite hierarchy was found for the form containing exon IIIB; it was most highly expressed in lung and least in heart and liver. The alternative splicing mechanism affects amino acids 4–45 of the mature phosphate carrier protein, which is believed to form one of six transmembrane segments of the phosphate carrier and to emerge into a large extramembranous loop. The alternative splicing mechanism changes 13 and 11 amino acids in the human and bovine carrier proteins, respectively. As the function of this region of the phosphate carrier is not known, the effects of the changes on carrier function are not understood at present.

The inner membranes of mitochondria contain a number of proteins that are responsible for the transport of various metabolites back and forth (Krämer and Palmieri, 1989, 1992; Walker, 1992; Walker and Runswick, 1993). An example is provided by the phosphate carrier, which catalyzes the transport of phosphate into the mitochondrial matrix, either by proton cotransport or in exchange for hydroxyl ions. The purified protein from both pig and bovine heart (Bisaccia *et al.*, 1984; Kolbe *et al.*, 1984) has been reconstituted into liposomes in an active form. The primary structure of the bovine phosphate carrier is 313 amino acids long and contains three related segments, each of about 100 amino acids arranged in tandem (Runswick *et al.*, 1987). The rat, human, *Saccharomyces cerevisiae*, and *Caenorhabditis elegans* sequences have similar features (Ferreira *et al.*, 1989; Dolce *et al.*, 1991; Phelps *et al.*, 1991; Runswick *et al.*, 1994). These repetitive elements are related to those found in the other characterized members of the mitochondrial carrier family, namely, the ADP/ATP (Saraste and Walker, 1982) oxoglutarate-malate (Runswick *et al.*, 1990) and citrate carriers (Kaplan *et al.*, 1993), and the uncoupling protein from brown fat (Aquila *et al.*, 1985). They are also found in a number of other proteins of known sequence but of unknown function, which therefore belong to the same protein superfamily (Walker, 1992; Runswick *et al.*, 1994). Among the family members, the mammalian, but not the yeast, phosphate carrier is exceptional in having a processed N-terminal sequence that helps to target the protein into mitochondria (Runswick *et al.*, 1987; Dolce *et al.*, 1991; Phelps *et al.*, 1991; Zara *et al.*, 1992; Ferreira *et al.*, 1989). By examination of the transmembrane topography of the phosphate carrier in the inner mitochondrial membrane, it has been shown that the N-terminal and the C-terminal regions of the phosphate carrier both protrude toward the cytosol. Therefore, its polypeptide chain spans the membrane an even number of times (Capobianco *et al.*, 1991; Palmieri *et al.*, 1993).

It is known that isoforms encoded by different ADP/ATP carrier genes are present in several species. Two bovine and three human genes have been detected for ADP/ATP carrier (Walker *et al.*, 1987a; Battini *et al.*, 1987; Neckelmann *et al.*, 1987; Houldsworth and Attardi, 1988; Cozens *et al.*, 1989; Powell *et al.*, 1989). Three isoforms have been characterized in *S. cerevisiae* (Gawaz *et al.*, 1990; Kolarov *et al.*, 1990). Single genes for the phosphate and oxoglutarate carriers were detected in the human and bovine genomes by Southern blotting (Runswick *et al.*, 1987, 1990), and there is a unique gene for the uncoupling protein (Ricquier *et al.*, 1991).

In this paper, the sequences of the genes of the human and bovine phosphate carriers are described. The human sequence has been determined from genomic clones obtained by screening a human genomic library with two human cDNA probes. The bovine genomic sequence has been derived from genomic fragments generated by polymerase chain reactions, using primers and probes based upon the bovine cDNA (Runswick *et al.*, 1987). The two genes are spread over 7.9 and 6.1 kb[1] of human and bovine DNA, respectively. Both contain nine exons separated by eight introns. The sequences show that there are two related exons IIIA and IIIB in each gene that appear to be alternatively spliced. The two alternatively spliced forms have

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) X77337 and X77338.

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[1] The abbreviation used is: kb, kilobase(s).

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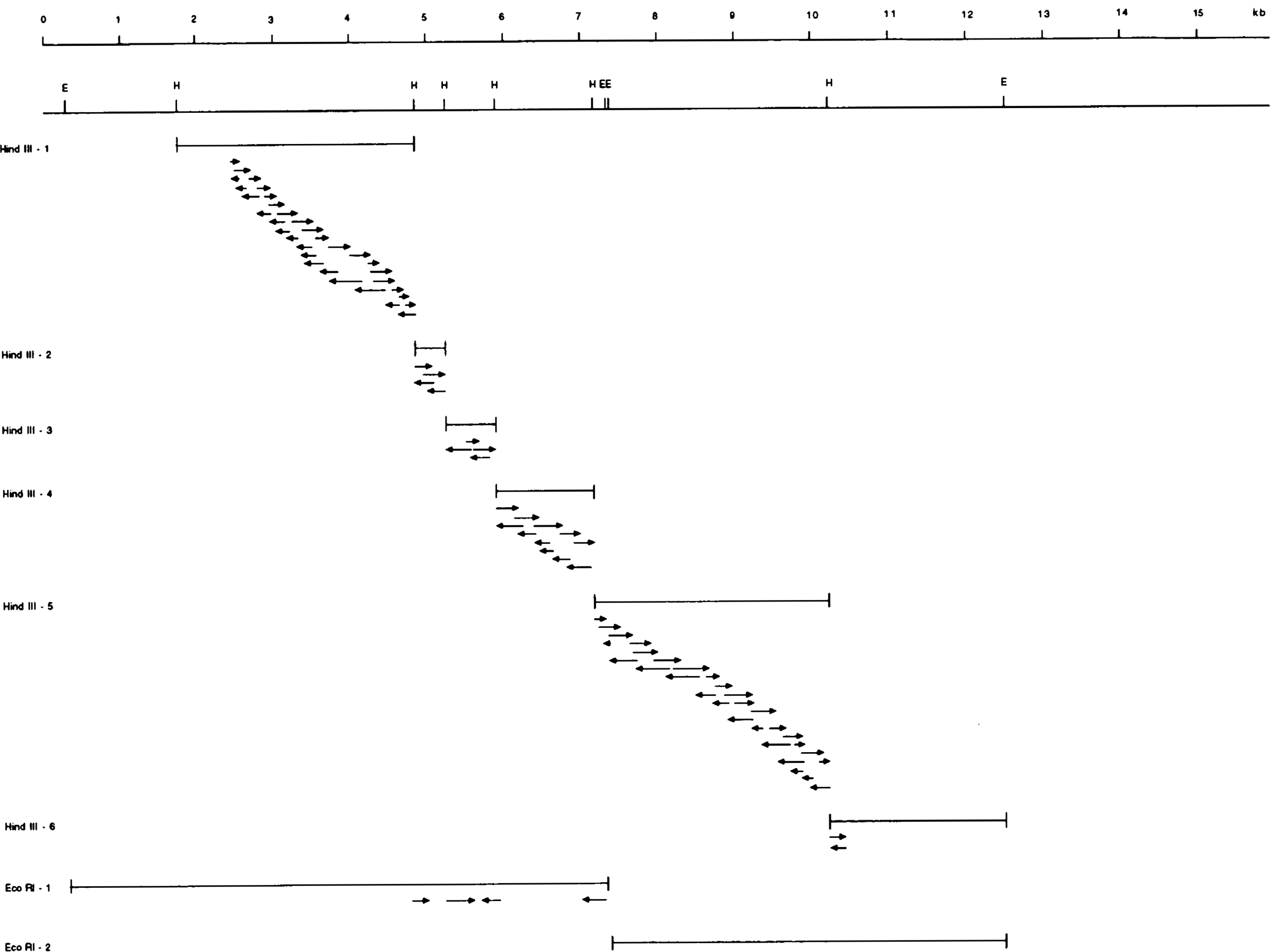

FIG. 1. **Sequence analysis of the human gene for the mitochondrial phosphate carrier.** The restriction map of the λ12D3 clone is shown at the *top*, where *E* and *H* represent *Eco*RI and *Hin*dIII restriction sites, respectively. The sequence was determined by sequencing fragments prepared by subdigesting two *Eco*RI fragments (6.7 and 5.0 kb) with *Hin*dIII. The *Hin*dIII sites were overlapped by priming on the intact *Eco*RI fragments with appropriate oligonucleotides. The two *Eco*RI fragments were joined by the sequence of an overlapping *Hin*dIII fragment (nucleotides 4710–7769 in Fig. 2). The *arrows* denote the directions and the extents of the individual DNA sequences from which the complete sequence was compiled.

been characterized from a human cDNA library, and their expression has been investigated in various bovine tissues.

MATERIALS AND METHODS

Synthetic Oligonucleotides—Oligonucleotide primers based on the bovine phosphate carrier cDNA were synthesized in an Applied Biosystems model 320B DNA synthesizer. Some were used as primers in polymerase chain reactions, and others as hybridization probes with the products. Forward and reverse primers 25 bases in length were made with an appropriate 5′-linker (*Eco*RI, *Hin*dIII, *Bam*HI, or *Xba*I). Oligonucleotides were also employed as primers in sequencing reactions. The purification, radiolabeling, and use as hybridization probes of synthetic oligonucleotides have been described before (Powell *et al.*, 1989; Walker *et al.*, 1989).

Isolation of Human Genomic Clones—The human genomic library consisted of partial *Sau*3A fragments cloned into λ2001 (LeFranc *et al.*, 1986). About 7 × 10^5 recombinants were screened by plaque hybridization (see Dyer and Walker (1993)). Duplicate filters were screened with two different probes; one was derived from the 5′ end of the human phosphate carrier cDNA (nucleotides 1–562), and the other from the 3′ end of the cDNA for the human phosphate carrier (nucleotides 612–1114) (Dolce *et al.*, 1991). DNA was purified by glycerol step gradient centrifugation (Garber *et al.*, 1983) from two positively hybridizing recombinant phages, λ12D3 and λ2F2. Restriction fragments from these phages were cloned into M13mp18 or M13mp19.

Amplification of Bovine Genomic DNA Sequences—Overlapping segments of bovine genomic DNA from liver (Walker *et al.*, 1987b) were amplified in four polymerase chain reactions (Walker *et al.*, 1992; Iacobazzi *et al.*, 1992), using synthetic oligonucleotide primers (with appropriate linkers) and probes based on the bovine cDNA sequence (Runswick *et al.*, 1987). These oligonucleotides are listed in Table I. The reactions were carried out for 30 cycles and, after addition of fresh portions of enzyme and primers, for an additional 30 cycles. The reaction products were analyzed on 1.4% high melting agarose gels. The DNA fragments were transferred to Hybond-N membranes (Amersham International, Amersham, United Kingdom), cross-linked to membranes by irradiation with uv light, and hybridized with radioactively labeled synthetic oligonucleotides at 5 °C below the minimum dissociation temperature. Fragments that hybridized with the probes were recovered from the gel by the Gene Clean procedure (Bio 101, La Jolla, CA), and the products were cloned into M13mp18 and M13mp19 vectors.

Characterization of Human cDNAs—A human cDNA library derived from cardiac muscle cloned in λgt11 was kindly donated by Dr. E. R. M. McCabe. This library was screened as described previously (Dolce *et al.*, 1991) with sequences derived from the bovine cDNA for the phosphate carrier (Runswick *et al.*, 1987). These sequences were an *Eco*RI-*Hin*dIII fragment and a *Pst*I fragment corresponding to nucleotides 628–1130 and 104–339 of the bovine sequence, respectively.

Polymerase Chain Reactions with Bovine mRNAs—Poly(A^+) RNA was prepared from total RNA from bovine heart, liver, brain, kidney and lung (Chirgwin *et al.*, 1979; Viñas *et al.*, 1990). Samples of poly(A^+) RNA (2 μg) from each tissue were employed as templates in polymerase chain reactions carried out as described above, except that the annealing temperature was 50 °C. The primers from exon IIIA (IIIA-F and IIIA-R) and from exon IIIB (IIIB-F and IIIB-R) were 19 bases long (see Fig. 4 for their sequences). Two sets of the amplified products from each tissue were analyzed on an agarose gel, transferred to Hybond membranes, hybridized with the 16-mer oligonucleotide probes (IIIA-P and-IIIB-P), and autoradiographed at room temperature for 1 h. The products from heart and liver were recovered from the gel, cloned, and sequenced.

DNA Sequence Analysis—DNA sequences were determined by the modified dideoxy chain termination method (Sanger *et al.*, 1977; Biggin *et al.*, 1983) with T7 DNA polymerase (Sequenase, U. S. Biochemical Corp.). Sequencing reactions were primed with either the 17-mer universal primers or the primers used in the polymerase chain reactions. Other 17-mer synthetic primers were used to extend existing sequences. Both DNA strands were sequenced completely. In order to avoid sequence errors being introduced by the polymerase chain reaction, at least three independent M13-isolated clones were sequenced from each reaction. When an ambiguity was observed, further clones were sequenced. Compressions were resolved by the use of dITP instead of dGTP in sequencing reactions. Data bases were compiled and analyzed with the computer program DBUTIL (Staden, 1982).

RESULTS AND DISCUSSION

Cloning and Sequencing the Human and Bovine Genes for the Phosphate Carrier—Two independent and overlapping genomic clones, λ12D3 and λ2F2, were isolated from a human genomic library. Together they cover about 26 kb of genomic sequence. It was shown by Southern blot analysis that λ12D3 contained the entire phosphate carrier gene, and by sequencing appropriate restriction fragments from this phage (see Fig. 1), a sequence of about 7,969 bases encompassing the human phosphate carrier gene was established (see Fig. 2).

In the case of the bovine gene, a different strategy was adopted. Four overlapping sequences about 1.0–1.8 kb in length (PCR-1 to PCR-4 in Fig. 3) were amplified by the polymerase chain reaction using bovine genomic DNA as template, and with synthetic oligonucleotide primers based on the bovine cDNA sequence (see Table I and legend to Fig. 3). The sequences of PCR-1 to PCR-4 were then determined with the use of synthetic primers. In this way, a bovine genomic sequence of 6,161 nucleotides containing the phosphate carrier gene was determined (see Fig. 4).

TABLE I

Synthetic oligonucleotide primers and probes used to generate partial overlapping clones comprosing the bovine gene for the mitochondrial phosphate carrier protein

Their sequences were based on the bovine cDNA sequence. F, R, and P denote forward and reverse primers and probes, respectively, employed in polymerase chain reactions PCR-1 to PCR-4.

Oligonucleotide	Position in bovine cDNA
1F	1–25
1R	285–310
1P	194–211
2F	250–275
2R	445–470
2P	340–357
3F	420–445
3R	847–862
3P	735–752
4F	822–847
4R	1300–1325
4P	973–990

Gene Structures—The exons of the human and bovine genes for the phosphate carrier were identified by comparison of the genomic sequences with the human and bovine cDNA sequences (Dolce *et al.*, 1991; Runswick *et al.*, 1987). Consensus rules for splice sites that predict conservation of the dinucleotides GT and AG, respectively, next to the 5′ and 3′ boundaries of the introns, were also taken into consideration (Breathnach and Chambon, 1981). In this way, it was found that the human gene contains nine exons separated by eight introns, and that the bovine gene has the same organization. The introns in the bovine gene are mostly smaller than those in the human gene (Fig. 5). The 3′ extremities of both genes were established by the cDNA sequences, but the transcriptional start sites of the human and bovine phosphate carrier genes have not been mapped. Therefore, the 5′ extremity of neither of exons I is known, and it is unlikely that the rather short 5′-noncoding sequences present in the cDNAs represent the full extent of these exons.

The eight introns in both genes interrupt the coding sequences at exactly the same positions. In other members of the carrier family, it has been noted that the introns tend to interrupt the coding sequences in or near to the extramembranous loops (Cozens *et al.*, 1989; Krämer and Palmieri, 1992). A similar tendency is found in the phosphate carrier genes with introns B, C, D, E, F, and H. However, intron G appears to be in the middle of an α-helical segment.

Alternative Splicing of Exons IIIA and IIIB—The most striking feature, uncovered by the comparison of the human and bovine genomic sequences with the corresponding cDNAs, was that both genes contain evidence of alternative splicing. Exon

```
Exon I
GCAACCTTTCCAAGGGAGTGGTTGTGTGATCGCCATCTTAGGGAGTGAGTGTGGCCGGGCTTCTCCTGTGGCGGGTGTGGGGAGCGGCAGCCAGAGCTCCTGTGGGGCCGCTGCTTTGGC   120

GGTGGGCCAGCCGGGAGCAGCCTCTTTCGAAGGCCGCCGTGACCTCTTCAAGGGCGTGGAGACGGAAGGAAAAGGCCGGTTGGGGTTCCAGGGCGCCGGTAACGTTAACGGCGCCTTGC   240

                                         -49   Exon II             -40                                -30
                                         M  F  S  S  V  A  H  L  A  R  A  N  P  F  N  T  P  H  L  Q  L  V  H  D  G  L  G  D
CTGTCCTCTAACCGTCGCTCCCTCCTCCCCTAGAAAGATGTTCTCGTCCGTGGCGCACCTGGCGCGGGCGAACCCCTTCAACACGCCACATCTGCAGCTGGTGCACGATGGTCTCGGGGA   360

 -20                     -10                  -1 1
 L  R  S  S  S  P  G  P  T  G  Q  P  R  R  P  R  N  L  A  A  A  A  V  E
CCTCCGCAGCAGCTCCCAGGGCCCACGGGCCAGCCCCGCCGCCCTCGCAACCTGGCAGCCGCCGCCGTGGAAGGTGAGATCAGACCCTGCCCAATACAGTCGTGTGGTCTACTTGTGGG   480

CCGCCCAGCCTTTGAGGCCTGGACCCGGCTTGGAGGACAGGGAAGGACGAGTCCAGCCCGCTGCACCGTAGACGGCGCCCAGTTGCTTGCCCTGGGGTATCGGCCTTTTCCACCATAGGC   600

GGGTGTCAGATCCTTGGCCTTCCCTCAAGGGCGTGGAAGCGTGAGGCCCTGTGTCCTTCGTGACCTCCGTGTGCCGAGGAAGAGAGGCCGTGACTAGCTCTTTCTCGCCGTGAGCTCGCT   720

TGGTCAGCAAACTAAGTACTGGGTAAACTCTCCTCCCGAAACTACTGACCACCCACAGTTCTACTAACTATATTACCTGTCCTTTGATTTGCCCTTAGTTTTCTTAAAAATAGTCCTTTC   840

CTAAGTTTATATTTGTCGACGCCAGGTCAGTCATTTTTGCACTTAATTGTATATGAGATAACTAGATGTCTGTTTTCGTGCGACTGGAGGAGGCAGCTTGCAGCCTACATAGGCAATAGA   960

AAGGAGATAATTGGTGGGCGTCCCTTTGGTGTGATTATCTCTTGGGATTCTTGGTGGTTGGGCCCAGCTTTAACTTAGTTGTGTGTGTTTTAAATCCTAGTGAAGTATGTACATACAGAG   1080

AAACCTGAAAACCGATGTAGGAGTCCTAAGAATGTTATCTACCTTGTGCCTGTAAAATCGAAAGATTTTTTTCTTTAAATCATTCATGTGCTTCTGTTGTCTTTAGTATGATAGAGTCCT   1200

TATTTACACTATCTGTCGTTCAGTTTAAAAGTTAAAGCTAACATTATTTGTGTATTAAATGTATTTGACTGCAGGAATTTGTGCGTATCCGTTTTTATGTTACAGGAGTTCTTAAGTGGG   1320

GATAGTATTGGTTAGTGGCTGGGATAAACTTTGAAGTTGTTTGCAAGCCTTTTACATGTCGAGTGATCTTTCTGAGTTTCGTTTGTACTCTGAAGTGTGCTCTTACCAGTGGTTAATAAT   1440

TTGAAGTTTGACTTTAATTGTAAAAGCCGTTAATGTAGAAAACTTTTAAAAAAATTAATATGGCAGAATACGAGCTATTAATAGTATCAGATACAGAACCCTCATAATTTTTAAATGATC   1560

TGAATTTGGCAGTGAATTTTTTTTTAACCGACTTAATTGTGGCCAAAGCTATTCAGCTGTGATAATAGTATCTCACAGGGAAACATTTTCTTTCATTCAGTGGCCTTAGTCATCTCTGAA   1680

                                        Exon IIIA   10                         20                         30
                                         E  Q  Y  S  C  D  Y  G  S  G  R  F  F  I  L  C  G  L  G  G  I  I  S  C  G  T  T  H
GAAATACTTACTTGATTTTTTTTTTTCCAATCAAACAGAGCAGTATAGCTGTGACTATGGATCTGGCAGATTCTTTATCCTTTGTGGACTTGGAGGAATTATTAGCTGTGGCACAACACA   1800

             40
 T  A  L  V  P  L  D  L  V  K  C  R  M  Q
TACAGCATTGGTTCCTCTAGATCTGGTTAAATGCAGAATGCAGGTTTGTTTTGCATGCTGGACTAGAGCATATTGAAGCATGACTGACTGTTAAGTTATAAGATATAGTCATCTAACAAG   1920

                                                                                                     Exon IIIB
                                                                                             E  Y  S  C  E  F  G  S  A  K
CTGTGTGGAAACCTAACTGTCCAGGAGGTAAGTTTATGACCAGTAACATGAGTTTTATATTAAAATGCATGGTGTGTCTTCTCTTACTACAGAGTACAGTTGTGAATTTGGCTCCGCGAA   2040

 Y  Y  A  L  C  G  F  G  G  V  L  S  C  G  L  T  H  T  A  V  V  P  L  D  L  V  K  C  R  M  Q
GTATTATGCACTGTGTGGCTTTGGTGGGGTCTTAAGTTGTGGTCTGACACACACTGCTGTGGTTCCCCTGGATTTAGTGAAATGCCGTATGCAGGTTTGTATTGAGATGACAACATTGAA   2160

AGTATCTCTCATGTGACTTAACTCTAAATAAAATCTTAAATGAGAATTTGAAGACATCACAACTGCTAGAAACTTCAGTAGGAAACCACATAGAATTTCTTTAAATTGTGGGCTTTATTT   2280

TGACTAGTGTTTTAAATCCAATAAATGCCTCTATTGTTTGAGTAAATTGTTCACTTTTAGAGTCTGGCTGTAAATGGCTCTTTGCTGCTGCTCTTCACTTCTGGCCGGAGTTCTTCAAGC   2400

TTACTAGTGCCCCAAATAGTCATTTAACAAGCACCTATGTGTTGGAGTGGGTAAACAAGTCTGTGTATTATGTGAAGTAACAATGTCCTTTATCTACTAATAAGACCAAGGCAAAGAGTA   2520

CATGTAGATTTATTTAAAGTGCTCTTAATATCTTAGTATGAAAATTTTGTCAGATTAGCCTCTTGTTTATTTACACAACATTTTAAAATAATTTGAATATATTTTAATTAAGATCATTGA   2640

AACAAAAACAAGACATCGAAAATTTATTGTTCCAAACTGGATTAGAAGTCTACTTACCTAAAAGAGAAATGAATTTGAAATATTAGGAGGGAGAGGAAGGTGAGTATACCATCCCTTCTA   2760

GTACTGGCAGAAATAGATAAGCTTTTCCTCCCCCCCCATTAGTTAATGTAATGGATATGAAATGTACCTTTGCTAAAACGTGTTATGCATGTCTCTTGAAGTGAATTTAGTTTATGTGCT   2880

TTTTTTTTTTAACTTGTGTTTAGTTTGTTTAGTGTTCACAAAACTTTATGCTATAAGCTACTTAAAGAACTAGCATATGTTAGGAATACAGTTATATCTATTATCTAGACAGTAGCTTTT   3000

AAAAAGTAGGAGGTGGGCCAAGCATGGTCGGCCCCAGACCGTGGAATGCCGAGCATTTTGGGTAGCGGAAGCAGGTGGGTCACTTGAGGTCGGAGTTCGAGATCAGAACTCAATCTAGCC   3120

AACATGGTGAAACCTCATCTCTGCTAAAATACAAAAATTAGCTGGGCATGGTGGCGTGCCCTGTAGTCGCAGCTACTCGGAGGCTGAGGCAGGAGAATCGCTTGAACCTGGGAGGTGGAG   3240

GTTGCAGTGAGCTGAGATCACACCATGGCACTCATCCTGGGCTACAGAGCAGACTCAAAAAACAAACAAACAAAAAACACAAAAAAAGTAGTAGGAGTTGGGTGGTAAGAAATCTGTTAA   3360

GAATCAGTTTTGTTACGTATTTTTGTTGAAATTTTACCCTCTTAACTATCACTCCAAGACTTAAGTTTGACACAGAAAGCTTTTGTTGAAATTTTACCCTCTTAACTATCACTCCAAGAC   3480

TTAGGTTTGACACAGAAAGCTAGACCTTAATGTACAACAGTTTCTATTTATAAAAGTAATCAAATGAACAATAATAAACAGTGATAAGCATAAACTAATAAAAGTTATATGAATGTAGTT   3600

CCTCACTCAAAATATCTTAATATTTTCAGATGTCAGTTGACCTTGGATAACTGAAGCCACAGAAAATGAACTCTTGGATAAGGGAGAGCTACTGTATTTCTTTTTTTTTTTTTGAGATGG   3720
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FIG. 2. **DNA sequence of the human phosphate carrier gene.** The nucleotide sequence is *numbered*, and the location of exons I–VIII and the protein sequences they encode are shown. Exon-intron boundaries are denoted by *small arrows*.

AGTCTTGCTCTGCTGCCCAGGCTGGAGTGTAGTGGTGCCATCGTGCTCACTGCAACCTCCACCTCCCTCAGGCTCAAGCGATTGTTCTGTCTCACCCTCACCCTCCCGAGTAGCTGGGAT 3840

TACAGGTGCACGCCACATGCCCGTCCAATTTTTTATATTTTAGTAGAGATGGGGTTTCACTGTGTTGCCCAGGCTGGTCTTGAACTCCTGAGCTCAGGTAATCCACCTGCCTCAGCCTCC 3960

CAAAGCGATGGGATTACAGATGTGAGCCACCGTGACCTGGCAGGAATTACTGTAGTTACCTTTTGTGTAGTTGCTGAAATTTATTATGGAACCCAAACAAAAATAATCATGAGATTAAAT 4080

50 Exon IV 60

V D P Q K Y K G I F N G F S V T L K E D G V

TTTTATGTTAGCTGTTTGGTGCATTTAATTTTTTTTTTCTTGTTTTAAATAAAGGTGGACCCCCAAAAGTACAAGGGCATATTTAACGGATTCTCAGTTACACTTAAAGAGGATGGTGTT 4200

70 80 90 100

R G L A K G W A P T F L G Y S M Q G L C K F G F Y E V F K V L Y S N M L G E

CGTGGTTTGGCTAAAGGATGGGCTCCGACTTTCCTTGGCTACTCCATGCAGGGACTCTGCAAGTTTGGCTTTTATGAAGTCTTTAAAGTCTTGTATAGCAATATGCTTGGAGAGGTATGT 4320

AATTAACTTTAAAATTGAATGTTCCGAGTGTTTAAGACTTTCCGAGTGTTCTTAGATTTTTGTCTGTCTTGCCTATTTTAGCACTGAAGAAAATGGTCCTACAAAGTGAGCAACTGTTTT 4440

AATCGTATGCCTGTGTCAGCAGAGACAGGTTGATAAATGTATTCATTAAATTACAATTCTCCTTTTAATATACTATCTTGTTTTCTAAGTAAGTTGGATTTTGAGTTTTTTAGAGAAGGG 4560

CTCTGGGACTCCTTTGCGTAGTTCCGGGGCATGGCATCTAGATATTTTTTGAATATTCATGTTGTTCACATAGTTACGTGTTTTGTTAATGAGGTTATGAGACCACATATATATTCCATA 4680

E

TCATCGTGTGTTACTTTATAAAATGTCTGAAGCTTAGTTGTTTTCTAGAACTGTCACTTCAATTGAAAACCAACACAACAACAATTCACATCCCTTCCTTGTGTTTTGGATTTTAGGAGA 4800

110 Exon V 120 130 140

N T Y L W R T S L Y L A A S A S A E F F A D I A L A P M E A A K V R I Q T Q P G

ATACTTATCTCTGGCGCACATCACTATATTTGGCTGCCTCTGCCAGTGCTGAATTCTTTGCTGACATTGCCCTGGCTCCTATGGAAGCTGCTAAGGTTCGAATTCAAACCCAGCCAGGTT 4920

150 160

Y A N T L R D A A P K M Y K E E G L K A

ATGCCAACACTTTGAGGGATGCAGCTCCCAAAATGTATAAGGAAGAAGGCCTAAAAGCGTAAGTAAACACTTAAAAATTTATACTATGAAAGTACTTATTTTAAGTGAACTTCATTTTTT 5040

TTTGTTTTTTTTTTTGTTTTGTTTTTTTGACGAAAAGTCTCGCTCTGTCACCCAGGCTGGAGTGCAGTGGCGTGATCTCGGCTCACTGCAAGCTCCGCCTCCCGGTTCACGCCATTCTCC 5160

TGCCTCAATCCCAAAGAGCTAGGACTACAGGCGCCTGCACTGCCCAGCTAATTTTTTGTATTTTTCAGTATAGACGGAGTTTCACCGTCTCCATCTCCTGACCTCATGATCCGCCCACCT 5280

TGGCCTCCCAAAATGCTGGGATTACAGGTGTGAGCCACCAGCACCCGGCTTCTTTTTTTTTTTTTCTTTGAGACGGAGTTTTGCTCTTGTTGTCCAGGCTGGAGTGCAATGGCATATCTCA 5400

GCTCACTGCAACCTCCGCCTCCCAGGTTCAAGTGATTGTCTGCTCAGCCTGCTGAATAGCTGGGATTACAGGCATCACCACCACGCCTGGCTAATTTTGTATTTTTAGTAGAGACTCCAT 5520

GTTGGTCAGGCTGGTCTCGAACTCCTGACCTCAGTGATCCACCGCCTCGCCTCCCAAAGTGCTGGGATTACAGGCGTGAGACACCGCGTCTGGCTGTGAACTTCATTTTTTAGTATTAAG 5640

CCTACCAGACTTTTTAAAAAGACTATTATAACATTTAATGTCTATTTACCTGTATGTAGAACATAAACTTGAGAGGTAAAGATAAGTTAATACATGGTTTGAGCCCTGGACTGAGTTTTT 5760

TTTTTTTCCCTAGAGTAAGGACCATTTGTCGGTCTACAAATGACTTGATGTTTGGCCTAAACATTTACTATTAACTTTTAGGGAGGGCTTGTTAGTATCCTTGTGATCTATTCACAGTTA 5880

AGAATTTCCATGACCACAACATGCTTCCTTAGATCCACCTTTGTGGATGAATCTTGAACTGAGTTCCACTTGTAAACTTCTTGTTTCTTGTGGTTCCAGTAGTCAAAGAAACATCCAGCA 6000

ACTTTTTTGGTTGTATAGTCAAAGGTGCTTGAGTCATTGGCATGTAAGAGAAATATACCTGCATGTTAGTCTAACGTATCTGATAGAAATGACATGCATTTACTGGCCATTTGTTACTAT 6120

170

F Y K G V A P

CAGGACTCGACTCGTGTGCGGACATTTCTGTTAATAATGACAGTCTCTGATAGATGAGTGTATGCAACTGTGTAAAACAAGTCTCTTGATTTCCTAGATTCTACAAGGGGGTTGCTCCTC 6240

Exon VI 180 190 200 210

L W M R Q I P Y T M M K F A C F E R T V E A L Y K F V V P K P R S E C S K P E Q

TCTGGATGAGACAGATACCATACACCATGATGAAGTTCGCCTGCTTTGAACGTACTGTTGAAGCACTGTACAAGTTTGTGGTTCCTAAGCCCCGCAGTGAATGTTCAAAGCCAGAGCAGC 6360

220

L V V T F V A G Y I

TGGTTGTAACATTTGTAGCAGGTTACATAGGTACGAATTACTTAGAACACACTTGTCTGAAATTATGAACAAATAATCATTTCCATTATTGGCGTTTTTTGAGAGGGAAAAATACTCCAA 6480

AGGTTATAAAGCAGTGTTTTTGTTTTTTTGGTTTCCTGAGGCATAGTCTCGCTCTGTTGCCCAGGCTGGAGTGCAGTGGCATGATAATAGCTCACTGCAACCTCCTCCTCCTGGGTTCAA 6600

GTGATTCTTCTGCCGCAGCCTCCCAAGTAGCTGGGACAGGCATGCACCGCCATGCCGCGCTGATTTTTGTTTTTGTTTTTTGTTTTTTTTTGAGATGGAGTTTTGCCCTTGTTGCCCAGG 6720

CTGGAGTGCAGTGGTGCAATCTTGGCTCACTGCAGCCTCCGCCTCCTGGGTTCAAGCGATTCTCCTGTCTCAGCCTCCTGAGTAGCTGGGATTGCAGGCGTCTGCACTACGCCCGGCTAA 6840

TTTTTAGTATTTTTAGTAGAGACGGTTTCACTATGTTGGCCAGGCTGGTCTCAAACTCCTGGGGCAGGTGATCTGCCTGCCTCGCGCTCCCAAAGTGCTGGGATTACAGGCGTGAGCCAC 6960

CGCCGACCTGGCCTGATTTTTGTAAAGATGGGGTTTCACCATGTTGATGAGTCTGGTCTCAAACTCCTGACCTCAGGTGATGTGCCTGGCCTATAAAGCAGTTTTATCTTCCCATAACAT 7080

TTTATATGCTTTTTAGGATTCATAAATACTTGCTAACTAGTTCCTAAAAGGGCTGTTTCATGGGATGTCATTATTAGGCTTGGGAATGTATTGATCTTACTGTTGCACCATTTTTTTGTT 7200

GTTGTTTATTAAGAATTTTATACAGAAAGTGTTGAATAAAATCTGAAAAACTTTAAAAGATTGTTCCGTTTTACATAGATAGACAGCTTGATAACTGTACCCGTGTCACCCTGAGTCTGA 7320

Fig. 2—*continued*

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                                                                                                                   A  G  V
TTTTTATTTTAGAACTAGAAAAATATTATTTTAAAATCATAAAAATGATTATTGGCTCTGTGAAGTTTTGTTTTTAACTGGCACTGTATGGGATCCTTTATCTTTTTTCAGCTGGAGTCT   7440

    230 Exon VII                    240                                 250                                 260
F  C  A  I  V  S  H  P  A  D  S  V  V  S  V  L  N  K  E  K  G  S  S  A  S  L  V  L  K  R  L  G  F  K
TTTGTGCAATTGTTTCTCACCCTGCTGATTCTGTGGTATCTGTGTTGAATAAAGAAAAAGGTAGCAGTGCTTCTCTGGTCCTCAAGAGACTTGGATTTAAAGGTAGGATGATGTTTTTTT   7560

                                                                          Exon VIII                   270
                                                                      G  V  W  K  G  L  F  A  R  I  I  M  I  G  T  L  T  A
CTTGAAAGAAAGAACAACAGTTTTGGATATGTTGCATTTTTTTCATTTGCTTTTCCTGTTTGAACCAGGTGTATGGAAGGGACTGTTTGCCCGTATCATCATGATTGGTACCCTGACTGC   7680

  280                           290                           300                           310
 L  Q  W  F  I  Y  D  S  V  K  V  Y  F  R  L  P  R  P  P  P  P  E  M  P  E  S  L  K  K  K  L  G  L  T  Q  *
ACTACAGTGGTTTATCTATGACTCCGTGAAGGTCTACTTCAGACTTCCTCGCCCTCCTCCACCCGAGATGCCAGAGTCTCTGAAGAAGAAGCTTGGGTTAACTCAGTAGTTAGATCAAAG   7800

CAAATGTGGACTGAATCTGCTTGTTGATCAGTGTTTGAAGAAAGTGCAAAAGGAACTTTTATATATTTGACAGTGTAGGAAATTGTCTATTCCTGATATAATTACTGTAGTACTCTTGCT   7920

TAAGGCAAGAGTTTCAGATTTACTGTTGAAATAAACCCAACTGTTCATG   7969
```

Fig. 2—*continued*

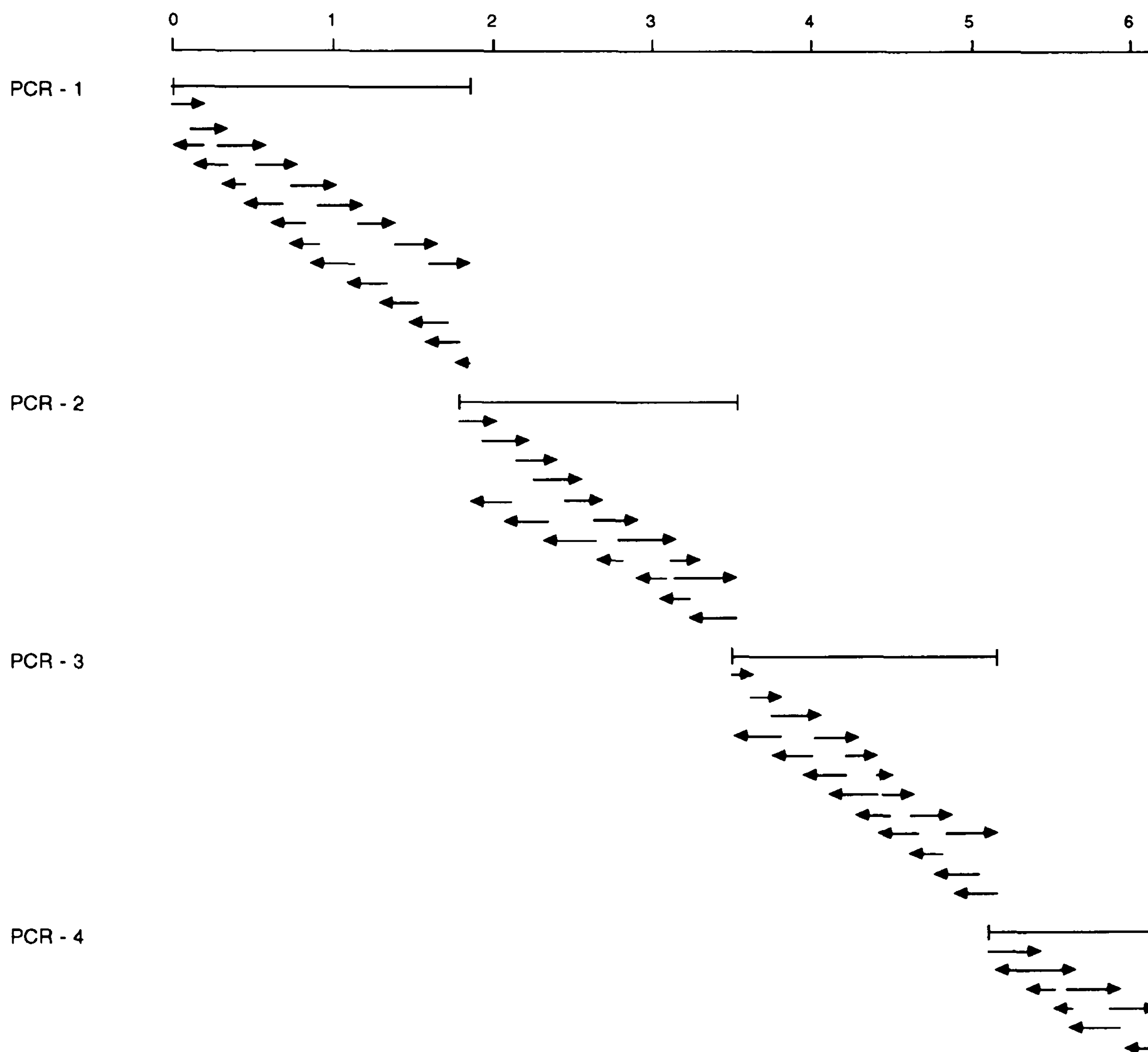

FIG. 3. **Generation by polymerase chain reactions and sequence analysis of genomic clones of the bovine phosphate carrier gene.** The *heavy horizontal lines* are proportional to the lengths of these DNA segments, and the *arrows* represent the directions and the extents of the determined DNA sequences. The primers are listed in Table I.

IIIA in the bovine gene encodes amino acids 4–45 of the known sequence of the bovine phosphate carrier (Runswick *et al.*, 1987), whereas human exon IIIB encodes amino acids 4–45 of the published human phosphate carrier (Dolce *et al.*, 1991). The nucleotide sequence of bovine exon IIIA is identical to nucleotides 220–344 in the published bovine cDNA sequence, and human exon IIIB is identical to nucleotides 206–327 of the cDNA sequence. The sequences of amino acids 4–44 of the rat phosphate carrier encoded in a cDNA derived from liver (Ferreira *et al.*, 1989) and of the *C. elegans* phosphate carrier are both more closely related to the protein sequence encoded in exon IIIB than to the sequence encoded in exon IIIA. The N-

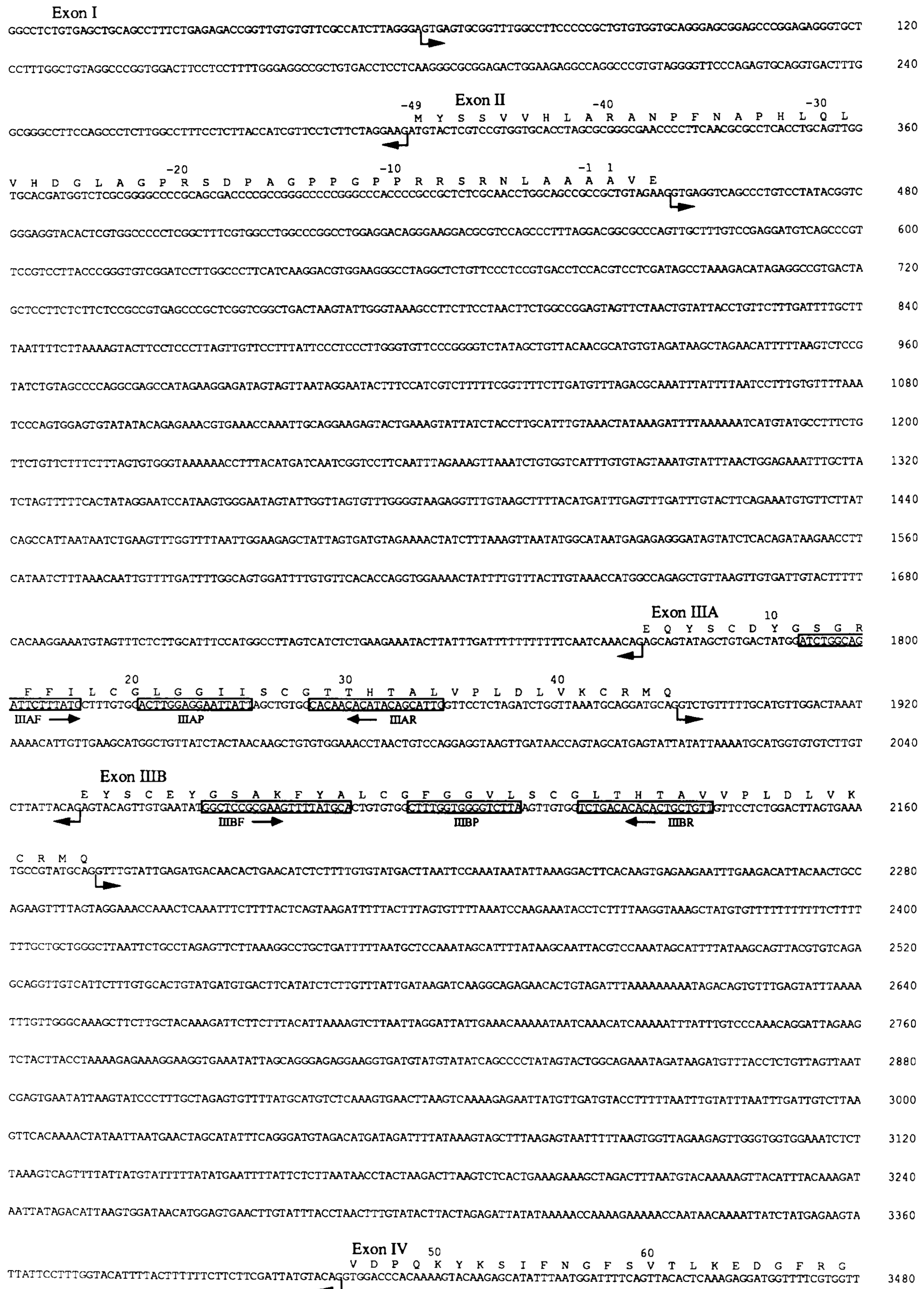

FIG. 4. **DNA sequence of the bovine phosphate carrier gene.** In exons IIIA and IIIB are shown the positions of primers (IIIA-F, IIIA-R, IIIB-F, and IIIB-R) and probes (IIIA-P and IIIB-P) employed in the investigation of alternatively spliced forms in various bovine tissues. For the meaning of the other symbols and further information, see legend to Fig. 2.

```
70                         80                         90                         100
L  A  K  G  W  A  P  T  F  I  G  Y  S  L  Q  G  L  C  K  F  G  F  Y  E  V  F  K  V  L  Y  S  N  M  L  G  E
TGGCCAAAGGATGGGCTCCGACTTTCATTGGCTACTCCCTGCAGGGACTCTGCAAGTTTGGCTTTTATGAAGTCTTCAAAGTTTTGTACAGCAACATGCTTGGAGAGGTATGTAATTTAA   3600

CTTTGAAATGGAAAGTCCTAAGCTTTTAACTTAAAGGGTACGACCAAAAAGCATCTTCTTTGTTTGATCCACTTTATTTTAGAGCTGAGGAAATAGTCTCAGAAAGAGTGAACGTGTTTC   3720

AAATCATAAGGCTATTTTAATAGAGACAGGATAAGATAAACTGCATTTGTTAGATTTCACTGCTTGTTTTTTATGCTGTGCTGCTTTCCAAGGAAGTTGGATTTGAGTTTAAAAATATTC   3840

AGTGTACTATGGTTGATGTTCAGTTCAGTTGCTCAGTGGTGTCCAGCTCTTTGCCACCCCACGGACGCCAGGCTTCCCTGTCCAATCACCAACTCCTGGAGCTTGCTCAAACTCATGTCC   3960

ATCGAGTTGGTGATGCCATCCCAACCATCTCATCCTCTATCGTCGCTTTCTCCTCCTATCTTCAATCTTTCCCAGCATCAGGGTCTTTTCAAATTAGTCAGTTCTTTGCATCAGGTGGCC   4080

AAAGTACTGAAGTTTCAGCTTCAGCATTAGTCCTTCCAATGAACATTCAGGATTGATTTCCCTTAGGATGGACTGGTTGGATCTCCTTGGTGTCCAAAGTAAACACTAAATACTGAGAAG   4200

TATTCAGTGTTTTGTGTTATCTGTGGACTGTGTGATAGAGAAACACTTAAAATACCTTGAAATTTGGCCATTTTCCAGAACTGTCTCTTACTGAGAAGAGCCCATGCAACACGAATTCAT   4320

                         Exon V 110                        120                        130
                         E  N  A  Y  L  W  R  T  S  L  Y  L  A  A  S  A  S  A  E  F  F  A  D  I  A  L  A  P  M  E  A
GTTCCTCCTTGTGTTTCTTCATTTAGGAGAATGCCTATCTGTGGCGCACATCACTGTATTTGGCTGCCTCTGCCAGTGCTGAATTCTTTGCTGACATTGCTCTGGCTCCTATGGAAGCTG   4440

   140                        150                        160
A  K  V  R  I  Q  T  Q  P  G  Y  A  N  T  L  R  D  A  A  P  K  M  Y  K  E  E  G  L  K  A
CTAAGGTTCGAATTCAAACCCAGCCAGGTTATGCTAACACTCTGAGGGATGCAGCTCCCAAAATGTATAAGGAAGAAGGCTTAAAGGCGTAAGTAAACATTTGCCTAAATAGTAGTATAA   4560

AATACTCATTTGAGAAAACTGTATTTGTTAGCACATTAACTCTCAACTTGGGGGTTGGAAGTACCCTTGTTAGAATTCCTGTCATCTGTGTTCACAGTTAACACTTGGATAGGCAGGCCA   4680

CACATGCTCCTAAGATCACCTTTGTGGATGCCCGTCTTGAGCTGAGTCTACTTGTAAACTCTGGTTTCTTGTAGTCAGTCAAAGAAACATCCAGCAACTTTTTGGTTGATAGTCAAAGGT   4800

GCTTGAGTCATTGGCATGTAAGATAAATATACCTGCATGTTAGTCTTAGGTTCTGATAGAAATGACATGCAATTATGCTGCCATTTTTTACTATCAGGACTCGACTGGTGTGCGGACACT   4920

                                                                                       170 Exon VI             180
                                                                                    F  Y  K  G  V  A  P  L  W  M  R  Q  I  P  Y  T
TCCGTTAGTAATGAAGAACTCTGCTAGATGAATATGTGCAACTGAGTGAAATAAGTCTTCTGATTTCCTAGGTTCTACAAGGGGGTTGCCCCTCTCTGGATGAGGCAGATACCATACACC   5040

                        190   .                    200                        210                        220
M  M  K  F  A  C  F  E  R  T  V  E  A  L  Y  K  F  V  V  P  K  P  R  S  E  C  S  K  P  E  Q  L  V  V  T  F  V  A  G  Y
ATGATGAAGTTTGCCTGCTTTGAACGTACTGTTGAAGCATTGTACAAGTTTGTGGTTCCCAAGCCCCGAAGTGAATGTTCAAAGCCAGAGCAGCTGGTTGTCACATTTGTGGCAGGTTAC   5160

I
ATAGGTATGAACTACTTAGAACATGTTGTGAAATTAAGAACAAAGAACATTTCCATTCTTGACTTCTTTTGTGAGGGAAAAATATTCCAAAGAAGTTTTACCTTTCCATTGCATTTGATA   5280

TACTCATAAAATACTTGCTAATTAGTTCCTAAAATAATTGTTTTGTGGTTTGCATGTCATAATTAAACTTGGGAGTGTATTGACTTCACTGCTATACCTTTTTTTGTTAGTTTTTCCAAGA   5400

ACAAATTTTTAATACAAGAGAAAGTGTTGGTTAAAATCTGAATATAACTTGCGGGATGGTTCTGTTTTACATAGGGTAGACTGCTTTATAATTAATTTGTCAGTCTCACTCTAAAATACG   5520

                                                                                                                ExonVII
                                                                                                                A  G  V  F
GTTTTTTAAAAAACAAAATTTTACTACTCATGGAAAGGTTGTCGGCTATCGTGTGAAAAAAGTTTTTTAATTGACTCCATAATGGGTCATTTGTCTTGACTTTTTCAGCTGGAGTCTTC   5640

   230                        240                        250                        260
C  A  I  V  S  H  P  A  D  S  V  V  S  V  L  N  K  E  K  G  S  S  A  S  E  V  L  K  R  L  G  F  R
TGTGCCATTGTTTCTCACCCTGCTGATTCCGTGGTGTCTGTGTTGAATAAAGAGAAGGGTAGCAGTGCCTCTGAGGTCCTGAAGAGGCTTGGATTTAGAGGTAAGATTTGTTTCCTCCCC   5760

                                                              Exon VIII         270                        280
                                                              G  V  W  K  G  L  F  A  R  I  I  M  I  G  T  L  T  A  L  Q
TCTAAAGAAAGAGTAACACTTGGGAGTATATTTTTCACTTACACTTCATATTTTATCCAGGTGTATGGAAGGGACTCTTTGCCCGTATCATCATGATCGGCACTCTGACTGCACTACAGT   5880

                           290                        300                        310
W  F  I  Y  D  S  V  K  V  Y  F  R  L  P  R  P  P  P  P  E  M  P  E  S  L  K  K  K  L  G  Y  T  Q  *
GGTTTATCTATGACTCTGTGAAGGTCTACTTCAGGCTCCCTCGCCCTCCTCCACCTGAGATGCCAGAATCTCTGAAGAAAAAGCTTGGGTACACTCAATAGATAAATGAAAGCAAATATG   6000

GACTGAATCTGCGTGTTGATCAGTGTTTGAGGAAAATGCAGAAGGAACTTTTATATATTTGACAGTGTAGGAAGTTGTCTATTCCTAATATAATTACTGTAGTACTCTTGTCAAAAGCAA   6120

GAGTTTCAAACTTAATGTGGAAATAAACCCAACTGTACATG   6161
```

Fig. 4—*continued*

terminal region of the yeast phosphate carrier is not strongly related to the mammalian sequences, and appears to be equidistant from the sequences encoded in mammalian exons IIIA and IIIB.

The continuations of the cDNA sequences after nucleotides 344 (bovine) and 327 (human) are found in exons IV. The sequences encoded in exons IIIA are closely related to those encoded in exons IIIB; 29 amino acids out of 42 in the human exons and 31 out of 42 amino acids in the bovine exons are identical. The amino acid sequence encoded in both exons IIIB lacks an equivalent of the first amino acid of exons IIIA. The human exons IIIA and IIIB differ in 44 out of 125 nucleotides, and the bovine ones in 41 out of 125. Bovine and human exons IIIA have identical nucleotide sequences. The nucleotide sequences of exons IIIB are 93% identical, and the encoded sequences differ in 5% of amino acids.

Evidence of the existence of alternatively spliced forms of the human phosphate carrier gene was found by characterization of cDNA clones from a human heart cDNA library. Using sequences of the bovine cDNA as hybridization probes, several

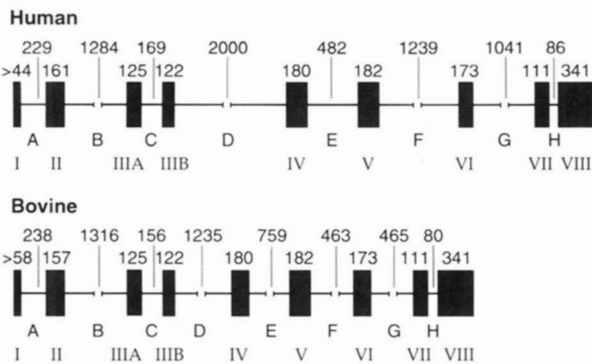

FIG. 5. **Structures of the human and bovine genes encoding the mitochondrial phosphate carrier.** The exons and introns are shown as *filled boxes* and *continuous lines*, respectively, and their sizes are given in base pairs.

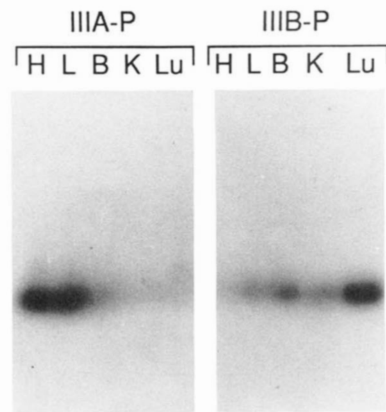

FIG. 6. **Hybridization of the products of polymerase chain reactions on mRNAs from various bovine tissues using primers from bovine exons IIIA and IIIB.** The products from heart (*H*), liver (*L*), brain (*B*), kidney (*K*), and lung (*Lu*) were separated by electrophoresis and were detected by hybridization with radiolabeled oligonucleotide probes, IIIA-P (*left-hand side*) and IIIB-P (*right-hand side*; see Fig. 4 for details of primers and probes).

positively hybridizing clones were identified. Three of them, λ11D, λ9M and λ4D, were analyzed, but only λ11D contained the full-length cDNA. This is the human cDNA sequence of the phosphate carrier reported previously (Dolce *et al.*, 1991). The λ9M and λ4D clones are partial and extend from the *Xba*I site at nucleotide 1804 in the genomic sequence. Sequences (960 and 826 bases, respectively) were determined from the 3′ ends of λ9M and λ4D. It is clear that they contain the sequence found in human exon IIIA, whereas the reported sequence of λ11D (Dolce *et al.*, 1991) contains exon IIIB.

Alternative Splicing of Exons IIIA and IIIB in Bovine Tissues—The distribution of the two different transcripts arising from alternative splicing of exons IIIA and IIIB were studied by polymerase chain reactions conducted on mRNAs from bovine heart, liver, brain, kidney, and lung. The primers (shown in Fig. 4) were chosen from the regions of greatest nucleotide sequence divergence between bovine exons IIIA and IIIB. The amplified products from these polymerase chain reactions were hybridized with specific probes from exons IIIA and IIIB, respectively. A hybridizing band was detected in all of the tissues that were examined (Fig. 6), and therefore both of the possible alternative transcripts were present in all instances. In order to confirm that the specific product was being amplified by each pair of primers, the products from the heart and liver polymerase chain reactions were cloned and sequenced. The sequences of the products obtained with the primers from exon IIIA corresponded to exon IIIA, and those obtained with the primers from exon IIIB corresponded to exon IIIB.

Different intensities of hybridization with the two probes were observed with the various tissues. Since the same amount of mRNA was used in each experiment, and the conditions of reaction were the same, the amounts of transcripts containing exon IIIA and exon IIIB seem to differ from tissue to tissue. The highest expression of exon IIIA was found in heart and liver, much less was found in brain and kidney, and relatively little was present in lung. In contrast, the expression of exon IIIB was greater in lung than in brain and kidney, and it was weakest in heart and liver.

The biological reasons underlying these observations are not understood at present. Exons IIIA and IIIB encode the N-terminal region of the phosphate carrier protein, which encompasses its first transmembrane segment and part of the first hydrophilic loop. The functions of these regions have not been established. The effects of the alternative splicing on the activity of the phosphate carrier require further study, probably involving their overexpression and reconstitution, as described recently for the related oxoglutarate-malate carrier (Fiermonte *et al.*, 1993). Alternative splicing has been shown to operate in one other protein associated with the inner membranes of mammalian mitochondria, namely the γ-subunit of the ATP synthase complex (Matsuda *et al.*, 1993), but again the biological significance is not understood.

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