P-type ATPases are a venerable family of ATP-dependent ion transporters. Recently, evidence was presented that a rabbit gene in the type IV subfamily of P-type ATPases was missing a transmembrane helix (transmembrane domain 4) thought to be critical for ion transport, a deletion that would place the two major catalytic loops of the enzyme on opposite sides of the membrane. It was proposed that the resulting protein was a RING finger-binding protein that targets transcription factors to specific domains within the nucleus. From analysis of human genomic sequence data, it is shown here that the region containing transmembrane domain 4, corresponding to exon 12, is present in the human homolog of the gene, ATP11B. PCR analysis indicates that the predominant ATP11B transcripts in a rabbit cDNA library and in a mouse cDNA library also contain exon 12. The results suggest that the transcript proposed to encode the RING finger-binding protein is a minor rabbit-specific splice variant. The ATP11B gene thus may not encode a protein with a function radically different from that of other P-type ATPase transporters.

Mechanisms that directly link active transport to the ATP pool have not arisen often in evolution. One of the few cases is represented by the family of P-type ATPases, in which ion movement is driven by conformational changes between phosphorylated and nonphosphorylated forms of the enzyme. Recently, the fundamental structure of one of those conformations was determined to 2.6 Å resolution (1), revealing two large cytosolic loops organized into three domains anchored to 10 transmembrane (TM) helices. A subset of these helices, including the critical TM4 and TM5 that bracket the largest of the cytosolic loops of the protein, contains the residues that compose the ion binding sites. This structure confirmed a wealth of sequence and mutational analyses that had identified amino acids, both within and without the membrane, critical to ion transport (1).

Recently, evidence was presented for the existence of an atypical member of the P-type ATPase family with a radically different structure and potential function (2, 3). Screening a Agt11 rabbit endometrial cDNA expression library with a RING motif polypeptide probe yielded a partial sequence; a more complete sequence was obtained from the library using 5' and 3' rapid amplification of cDNA ends, although the 5' end of the expressed gene was never identified. The composite sequence resembled a P-type ATPase with two putative cytosolic loops, the larger of which contained the aspartate phosphorylation motif and ATP-binding domain characteristic of the enzymes. In addition, 7 of 8 P-type ATPase diagnostic consensus sequences (3) were present, but only 9 rather than the typical 10 TM helices were present. The missing helix was TM4, which includes the critical consensus sequence (region D (4), sequence C (5)) thought to be integral to ion binding and transport (6).

The possibility was considered that the sequence might represent a splice variant of a more typical P-type ATPase gene (2). To test that possibility, sequences encoding part of TM2 on one side of the missing TM4 and the conserved aspartate phosphorylation sequence on the other side were used as PCR primers with genomic DNA as template. Because the reaction produced only a product corresponding to the cloned DNA with TM4 still missing, it was concluded that the sequences encoding TM4 are not present in the gene itself. The absence of TM4 would result in a protein with altered topology, with one of the large loops on a side of the membrane opposite its normal location. Based on this information, it was proposed that the protein encoded might be a RING finger-binding protein (RFBP) located in the inner nuclear membrane, with one loop in the periplasmic space and the other, larger loop extending into the nucleoplasm, where it could interact with euchromatin and a RING finger-containing transcription factor.

The sequence of the putative RFBP includes 13 of 14 consensus sequences diagnostic of a subfamily of P-type ATPases first identified as potential amphipath transporters (5, 7) and classified as type IV P-type ATPases (4). Present in a wide range of organisms from yeast to parasites to man, genes in this subfamily have been recognized and reported by both the human and mouse nomenclature committees as ATP (human) or Atp (mouse) 8 through 11. Although a substrate transported by type IV P-type ATPases has been identified only for the amphipath lipid transporter (ATP8A1) (8), mutation of the FIC1 gene (ATP8B1) results in cholestasis or impaired bile flow in humans (9), and the pftap gene (Atp10a) is linked to obesity in mice (10), implicating the transporters in important physiological processes whose disruption lies at the root of human diseases.

Within this context, the possibility that the protein encoded by one of the genes in the subfamily might have a dramatically different function as a RFBP represents a radical alteration of our views on this group of enzymes. Accordingly, we report here a critical reanalysis of this gene.

**EXPERIMENTAL PROCEDURES**

Human ATP11B Gene Sequence—Human ESTs AL046573 and BF372979 as well as cDNA clones AB025173 and AF156548 identify...
most of the ATP11B ORF. To fill in the regions not covered by these clones, first, using the TBLASTN algorithm (11) to search the human genome, AB023173 was assigned to chromosome 3, based on identical matches to several discrete but widely separated regions of the working draft sequences AC069431 and AC036127, suggesting exons with long introns. Then, the sequence of chromosome 3 was searched using the complete ORF of the most closely related gene, ATP11A (found on chromosome 13), to identify exons not represented by ATP11B ESTs. In each case, the new exons identified were on the same contig as an exon represented by ATP11B ESTs. Because a gap in intron 10 remains, the possibility cannot be excluded that the sequences on either side of the gap belong to different genes. However, no other type IV P-type ATPases are known to exist on chromosome 3, and the homology between the amino acid sequence of the human ORF on the 5′/H11032 side of the gap and the corresponding rabbit sequence is 96% identity and 98% similarity, and the homology between the sequences on the 3′ side of the gap is 94% identity and 97% similarity, arguing that both sides of the human sequence are from the same gene. The sequence of the ATP11B

![Diagram of ATP11B ORF and subfamily relationships.](image-url)

**FIG. 1.** Type IV P-type ATPase subfamily. A, the phylogenetic relationship among all known type IV subfamily sequences from diverse eukaryotic organisms and the (type IIB) plasma membrane Ca²⁺-ATPase, expressed as a neighbor-joining tree, using p-distance, complete deletion, and Felsenstein's bootstrap test (17) (1000 replications). Large Arabic numerals designate classes. Numbered sequences forming each branch are as follows (GenBank accessions or accession number): 1, plasma membrane Ca²⁺-ATPase (PMCA2; Q01814); 2, Ab20B (AF155913); 2a, Ab9A (AF152243); 4, AAF53280; 5, Y73C5C (AF101318) and F02C9.3 (U80025); 6, F36H2 (Z81078) and VF36H2L (AL021466); 7, CA93618, SPAC6C3 (Z69731.1); 8, ATC7_YEAST, YIL048W (Z69731.1); 9, CAB45102; 10, AFO067496; 11, PFML3P6.27 (Z89551); 12, BAI10991; 13, Oryctolagus cuniculus RING finger-binding protein, AF236061; 14, 6f, ATP11B (described herein); 15, 6g, ATP11C (AF156547 + AI371849 + BE168255 + AI162137 + comparisons with AL356785, human chromosome X sequence); 16, 6h, Atp11A (AF156551); 17, AAF48606; 18, AAF48605; 19, T24H7.5 (U28940); 20, W09D10.2, CAB07859.1 (Z93785); 21, AAF52779; 22, 5a, Atp10A (AF156549); 23, ALA1_ARATH (P98204); 24, ATC5_YEAST, YM580.11c (Q12674); 25, PDF16955 (U16955); 26, ATC4_YEAST, YDR093W, YD8557.01 (Q12675); 27, ATC5_YEAST, YER166w (P32660, AAB64693); 28, SPAC4F10.16c (Z98980.1, CAB11719); 29, ATCX_SCHPO (Q09891); 30, AAF54749; 31, Y17G9 (AC006719, assembled using Genefinder and ESTs); 32, 1c, FIC1, ATP8B1 (AF038007); 33, 1b, ATP8A2 (AF156550); 34, 1a, APLT, ATP8A1 (U75321); 35, AAF58378.1, CAB11550; 36, SPBC887.12; 37, DRS2, YAL026C, ATC3_YEAST (AI033388); 38, AFO06421; 39, AAD38325; 40, BAB00597.1; 41, AAD31074; 42, AAD49973; 43, BAB00402.1; 44, AAF54747; 45, ATP211A (AF152243); 46, BAA9591.1; 47, AAD36098.2. B, the human ATP11B gene. The ORF is shown, interrupted by introns (vertical dashed lines) with intron length in base pairs at the bottom. Lightly shaded P-type ATPase consensus sequences are numbered, subfamily-specific and class-specific motifs within and outside these consensus sequences are lettered (4), and heavily shaded regions denote the 10 transmembrane helices characteristic of P-type ATPases. The PCR primers used by Mansharamani et al. (2) are shown as arrowheads. Exon 12, encoding sequence absent in RFBP, is represented by the horizontal dashed line.
gene is part of NT_022406.6 and can be viewed on the master config map found at the National Center for Biotechnology Information Human Genome Resources site (www.ncbi.nlm.nih.gov/genome/guide/human/).

PCR Amplification and Sequencing—Phage from the murine teratocarcinoma PC4 xZAPII cDNA library (Stratagene) were mass excised to phagemids according to the manufacturer's instructions. Briefly, host cells (XLI-Blue; Stratagene) were infected with library phage at a multiplicity of infection of 10 and with R408 helper phage at a multiplicity of infection of 100. After incubation at 37 °C for 2–3 h with vigorous shaking, the host cells were heat-killed at 70 °C and centrifuged at 2500 × g. Single-stranded DNA was purified from the phagemids in the supernatant using the Prep-A-Gene DNA Purification System (Bio-Rad) according to the manufacturer's instructions. The sequence of the forward primer (nucleotides 487–512 of mouse EST AW230681) was AGCTGAAGAAAAATGGGATGAACCT, and the sequence of the reverse primer (nucleotides 717–737 from mouse EST BE283895) was TGGAGTCTGGGCTTGGTCCTT. A rabbit cDNA library was prepared from leukocytes using the E.Z.N.A. Blood RNA Midiprep kit (Omega Bio-tek), according to the manufacturer's instructions. Briefly, 10 ml of blood was collected from the central ear vein of each of two New Zealand White rabbits, erythrocytes were lysed, and leukocytes were collected by centrifugation. Total RNA was isolated from the leukocytes and purified on a silica-based spin column. An immortalized rabbit cDNA library was prepared from the RNA using the SOLIDscript Solid Phase cDNA Synthesis kit (CPG Inc.), according to the manufacturer's instructions. Briefly, mRNA was immunoisolated on biotinylated oligo(dT)25 complexed to streptavidin-linked magnetic particles, and reverse transcriptase was then used to generate single-stranded DNA. Two different sets of PCR primers derived from the GenBank™ RF sequence (AP236061) were used: for set 1, the primers were CAGGGGTACGAAGATTGGTTG (forward) and TTCTCTACCTTGCTCTGGAATCC (reverse); and for set 2, the primers were ATGGGATGAGCCTTGGTATAA (forward) and AAGAATCTGGTTGCTATGCCTT (reverse). PCR was carried out using the Taq-FOUR amplification system (CPG Inc.). Reaction mixtures contained 500 μM deoxynucleotide triphosphates, 2.5 units of Taq-FOUR DNA polymerase, each primer at 1 μM, and either 500 ng of single-stranded phagemid DNA (mouse) or 2.5 μl (about 12 μg) of the immobilized rabbit cDNA library. The resulting 400-bp (mouse), 388-bp (rabbit; primer set 1), and 400-bp (rabbit; primer set 2) products were purified from low melt agarose by phenol/chloroform extraction and cloned for sequencing using the pGem-T Easy Vector System (Promega) according to the manufacturer's instructions. Plasmid DNA from white colonies containing the PCR product insert was prepared for sequencing using the Quantum Prep Plasmid Miniprep kit (Bio-Rad) or Wizard Miniprep kit (Promega) according to each manufacturer's instructions.

Sequence Comparisons and Analysis—Sequence similarities were calculated using BLASTP. Alignments were performed using CLUSTALW, and the neighbor-joining tree was generated from alignments using MEGA (12).

RESULTS AND DISCUSSION

The type IV subfamily of putative amphipath-transporting P-type ATPases was originally subdivided into five classes (classes I–V) based on sequence similarity and the presence of class-specific diagnostic sequences (5, 13). Unfortunately, in the literature, Roman numerals have been used to designate both types (subfamilies) of P-type ATPases (4) and classes of transporters within the type IV subfamily (5, 13). To reduce confusion, the Roman numerals formerly used to designate classes of amphipath transporters have been changed to Arabic numerals; thus, classes I–VI are now designated classes 1–6.) Phylogenetic analysis of all sequences now available (Fig. 1A), including, in particular, those from Drosophila, now indicates that there are in fact six classes of type IV ATPases; three genes previously thought to form a subclass of class 1 (ATP8) genes are actually a separate class (class 6; ATP11). Mammals, Arabidopsis, Drosophila, Caenorhabditis elegans, and yeasts express class genes and 9 genes (ATP8 and 9); C. elegans and Arabidopsis express class 5 genes as well; mammals and Drosophila also express class 5 (ATP10) genes and class 6 (ATP11) genes in addition, whereas only yeasts express class 3 and 4 genes. As was recognized by Manesharamani et al. (2), a human homolog of rabbit RFBP is present in the EST data bases (fragments If and Ir in Fig. 1 of Ref. 13). More recently available sequences (ESTs and genomic sequences) consolidate EST fragments If and Ir and provide the complete ORF of this human homolog (gene ATP11B), as described under “Experimental Procedures,” this gene is 95% identical and 97% similar to the rabbit homolog over the region for which rabbit sequence is available. Sequence similarity places the gene in class 6 as ATP11B, joining ATP11A (consolidation of Ib and Is in Ref. 13) and ATP11C (consolidation of Ig and Iq in Ref. 13).

The complete genomic sequence of the ATP11B gene was derived from the human genome data base as described under “Experimental Procedures.” Identifying intron/exon boundaries by splice donor/acceptor sequences produced the intron/exon structure shown in Fig. 1B, where introns are represented by vertical dashed lines. The gene consists of 30 exons and 29

FIG. 2. Expression of ATP11B in rabbit and mouse. A, gel of PCR products. Lane 1, murine PC4 teratocarcinoma cDNA as template and sequences in exons 11 and 13 as primers; lane 2, rabbit leukocyte cDNA library as template and sequences in exons 5 and 13 as primers (primers set 2 also see the arrowheads in Fig. 1B); lane 3, rabbit leukocyte cDNA library as template and sequences in exons 11 and 13 as primers; lane M, marker DNA. B, nucleotide sequence of the cloned 400-bp product shown in A, lane 1. Primers have solid underlining, and exon 12 is shaded. C, nucleotide sequence of the cloned 393- and 388-bp products shown in A, lanes 2 and 3. Primer set 1 has solid underlining, primer set 2 has dashed underlining, and exon 12 is shaded.
introns, typical of many P-type ATPase genes (14–16), including
the type IV subfamily (5), and sequences encoding TM4 are
present in the gene. Moreover, the region containing TM4
that is missing in RFBP exactly matches the sequence of exon 12
(horizontal dashed line in Fig. 1B), suggesting that the RFBP
EST of Mansharamani et al. (2) (boxed below C) contains eight introns
totaling a minimum of 24 kb of sequence as well as all or part
of nine exons (totaling almost 1 kb). It is unlikely that a
sequence of this overall size (>25 kb) would be amplified by
PCR using genomic DNA as template, explaining the failure of
Mansharamani et al. (2) to observe it.

The presence of the TM4 sequence in the human ATP11B
gene does not speak to the question of the predominant expres-
sion of the form of the gene in rabbits, which might well be missing
the TM4 sequence either in the gene or in the final mRNA.
Accordingly, a cDNA library was prepared from rabbit leuko-
cytes and used as template for PCR with primers derived from
the rabbit sequence immediately upstream and downstream of
exon 12 containing TM4 (Fig. 2C, dashed underline). These
primers would yield a 388-bp PCR product from templates
containing exon 12 and a 190-bp product from templates lack-
ing it. The longer (388-bp) product was observed (Fig. 2A, lane
3). To rule out the possibility that the shorter (190-bp) sequence
was actually generated by PCR but obscured on the gel by the
presence of excess primer dimers, PCR was carried out using
the same rabbit cDNA library with a second set of primers
nearly identical to those used by Mansharamani et al. (2) (Fig.
1B, arrowheads and Fig. 2C, solid underline). These primers
would yield a 939-bp PCR product from templates containing
exon 12 and a 741-bp product from templates lacking it. The
predominant product observed was the longer (939-bp) one
(Fig. 2A, lane 2), although the lower boundary of the band was
indistinct, suggesting the possible presence of shorter PCR
products. The entire band was excised from the gel, and its
DNA was purified and cloned. Restriction analysis revealed

inserts of two different lengths, 741 and 939 bp. The majority
(>80%) of clones contained the 939-bp sequence expected for a
transcript containing the exon 12 sequence (Fig. 2C, shaded).
The shorter sequence was that expected for a transcript of the
reported sequence of rabbit RFBP, lacking the region encoded
by exon 12.

To investigate whether this minor variant was anomalous or
characteristic of transcripts of the gene, a murine PCC4 ter-
atocarcinoma cDNA library was examined for its presence.
PCR primers derived from either side of exon 12 of murine
ATP11b (Fig. 2B, underlined) would yield a 400-bp product from
templates containing exon 12 and a 202-bp product from tem-
plates lacking it. Only a single 400-bp product was observed
(Fig. 2A, lane 1). To eliminate the possibility that the 202-bp
product might be obscured by primer dimers in 1% agarose
gels, PCR products were also analyzed in 2% agarose gels.
Again, shorter PCR products were not observed (data not
shown). Nevertheless, the band was excised, and its DNA was
purified and cloned. Only a single-sized insert was observed
among the clones, which, when sequenced, was found to con-
tain the 400-bp sequence expected, including exon 12.

These results demonstrate that exon 12 is present not only in
the human ATP11B gene, but also in the major mRNA species
expressed from it in both rabbits and mice. Fig. 3 presents a
comparison of rabbit, mouse, and human ATP11B protein
sequences over the region in question compared with the type IV
P-type ATPase to which they are most closely related (Atp11a)
and the type IIB sarco/endoplasmic reticulum Ca2+-transporter
(SERCA1). Together, these results suggest that the ATP11B
gene does not encode a protein of radically different structure
and function from the rest of the P-type ATPase family of
enzymes but encodes a P-type ATPase transporter of normal
structure. The nature of the substrate transported by this
enzyme, like those of most of its cousins in the type IV subfam-
ily, remains unknown and an interesting object of further
investigation.

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Fig. 3. Sequence alignments of several class 6 proteins and the sarco/endoplasmic reticulum Ca2+-ATPase (SERCA1). CLUSTALW
was used to align the rabbit RFBP of Mansharamani et al. (2) (dashed line, missing exon 12), the predominant rabbit ATP11b sequence reported
here, murine Atp11b, human ATP11B, mouse Atp11a, and mouse SERCA1. Only the relevant region, including TM3 and TM4 (shaded), type IV
subfamily-specific sequence C (boxed below C) (5, 7, 13), class 6-specific sequence D (boxed below D) (5, 13), and P-type ATPase phosphorylation
consensus sequence 2 (boxed below #2), is shown.
Reanalysis of ATP11B, a Type IV P-type ATPase

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