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Genetics

Structural organization and transcription of the mouse gastric H^+,K^+-ATPase β subunit gene

(Na^+,K^+-ATPase $\beta 2$ subunit/gene family/evolution/5′ flanking sequence)

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ABSTRACT **We have cloned and characterized the mouse gene encoding the β subunit of H^+,K^+-ATPase (EC 3.6.1.36). The entire 10.5-kilobase transcription unit of the H^+,K^+-ATPase β subunit gene was cloned in three overlapping cosmids encompassing ≈46 kilobases of genomic DNA. A tight cluster of transcription initiation sites has been localized 24–25 nucleotides upstream of the translation start site and 28–29 nucleotides downstream of a TATA-like sequence. The H^+,K^+-ATPase β subunit gene is split into seven exons encoding predicted structural domains of the β subunit protein. The intracellular amino-terminal and putative transmembrane domains are encoded by individual exons, and the extracellular carboxyl-terminal domain is encoded by five exons. The exon/intron organization of the mouse H^+,K^+-ATPase β subunit gene is identical to that of the mouse Na^+,K^+-ATPase $\beta 2$ subunit gene. The conservation of genomic organization, together with the high sequence homology, indicates that the mouse H^+,K^+-ATPase β and Na^+,K^+-ATPase $\beta 2$ subunit genes originated from a common ancestral gene.**

The H,K-ATPase is a membrane-associated enzyme that provides the driving force for HCl secretion into the stomach (1). H,K-ATPase couples the electroneutral exchange of extracellular K^+ and intracellular H^+ to the hydrolysis of ATP (2), thereby generating an extremely high transmembrane pH gradient (3). The enzyme consists of two subunits. The α subunit contains the catalytic site for ATP hydrolysis (4). The β subunit is a glycosylated polypeptide of $M_r \approx$ 60,000–80,000 (5) whose function has not yet been established.

Analysis of cDNAs for the catalytic α subunit (6, 7) reveals that the encoded polypeptide is highly homologous to the Na,K-ATPase α subunit. The organizations of the H,K-ATPase (8) and Na,K-ATPase (9) α subunit genes are also strikingly similar. Recently, we and others have isolated cDNA clones encoding the H,K-ATPase β subunit (10–13). The amino acid sequence deduced from β subunit cDNA reveals a polypeptide that shares ≈41% and ≈35% amino acid sequence identity with Na,K-ATPase $\beta 2$ (14) and $\beta 1$ subunit isoforms (15), respectively. The high degree of primary sequence and secondary structural similarity between H,K-ATPase and Na,K-ATPase β subunits suggests that these polypeptides are members of a family of related cation-transport protein subunits.

Several lines of evidence indicate that H,K-ATPase exhibits a restricted tissue-specific pattern of expression. Northern and Western blot analysis has shown that H,K-ATPase α and β subunits are expressed in the stomach of a variety of mammalian species, including rat, rabbit, hog, cow, and mouse (10, 11). Histochemical studies indicate that within the stomach, H,K-ATPase appears to be specifically localized to the parietal cell, the gastric epithelial cell that secretes HCl (reviewed in ref. 16). Further, immunofluorescence and immunogold electron microscopic analyses indicate that both enzyme subunits colocalize to the parietal cell tubulovesicular network (13, 17).

As an initial step towards elucidating the control mechanisms underlying the parietal cell-specific expression of H,K-ATPase genes, we have cloned and characterized a complete mammalian H,K-ATPase β subunit gene (*Atp4b*).†‡ Two mouse H,K-ATPase β subunit cosmid clones, with inserts of ≈40 kilobases (kb), were found to span the entire coding region of the β subunit gene. The 10.5-kb transcription unit is split into seven exons and contains ≈9.2 kb of intervening sequences and ≈0.9 kb of coding sequences. Our data show that the organization of the H,K-ATPase β subunit gene is remarkably similar to that of the Na,K-ATPase $\beta 2$ subunit gene and suggest that these two genes evolved from a common ancestral gene.

MATERIALS AND METHODS

Isolation and Characterization of Genomic Clones. A 1.5-kb cDNA fragment containing the entire coding region of rat H,K-ATPase β subunit (10) was used to screen a mouse BALB/c 3T3 genomic DNA library constructed in the cosmid vector pWE15 (19). Approximately 300,000 independent colonies were plated onto LB agar plates, and replica nitrocellulose filters were prepared and screened by colony hybridization (ref. 20, pp. 1.96–1.104). Filters were hybridized and washed as previously described (10). Exon-containing restriction fragments were identified by Southern blotting using radiolabeled fragments of rat H,K-ATPase cDNA as probes. Positively hybridizing restriction fragments were subcloned and sequenced by using a Sequenase sequencing kit (United States Biochemical).

S1 Nuclease Mapping and Primer Extension Analysis. For S1 nuclease mapping, a 541-base-pair (bp) *Eco*RI–*Nsi* I genomic restriction fragment was end-labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase (Boehringer Mannheim), denatured in annealing buffer [80% (vol/vol) formamide/0.4 M NaCl/1 mM EDTA/40 mM Pipes, pH 6.4], and hybridized for 16 hr at 50°C to 100 μg of total RNA prepared from mouse (BALB/c) stomach. The DNA·RNA hybrids were digested with 300 units of S1 nuclease (Boehringer Mannheim) and the products were analyzed on a 6% polyacrylamide gel containing 7 M urea.

Abbreviations: H,K-ATPase, potassium-activated proton-transport adenosine triphosphatase (EC 3.6.1.36); Na,K-ATPase, Na^+,K^+-activated adenosine triphosphatase (EC 3.6.1.37).

†The mouse gene encoding the H,K-ATPase β subunit has been named according to the rules for mouse gene nomenclature (18).

‡The sequence of the mouse H,K-ATPase β subunit gene has been deposited in the GenBank data base (accession nos. M64685–M64688).

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A 25-base oligonucleotide (5'-GCTGGCTGCATGACT-TCTTCTCCTG-3') complementary to DNA sequences located between nucleotides +13 and +37 of the first exon served as a primer for recombinant Moloney murine leukemia virus reverse transcriptase (Boehringer Mannheim). The primer was end labeled with [γ-^{32}P]ATP and an aliquot (10^5 cpm) was hybridized to 2 μg of poly(A)$^+$ RNA prepared from mouse stomach. Primer extension was carried out essentially as described by Sambrook *et al.* (ref. 20, pp. 7.79–7.83). Primer extension products were analyzed by electrophoresis through a 6% polyacrylamide gel containing 7 M urea.

RESULTS AND DISCUSSION

Isolation and Characterization of Cosmid Clones. A mouse genomic library constructed in the cosmid vector pWE15 was probed with a full-length rat H,K-ATPase β subunit cDNA (10). Three independent clones, MG4, MG15A, and MG15B, 35 to 42 kb long, were analyzed by restriction mapping and found to encompass 46 kb of unique genomic DNA sequences (Fig. 1*A*). Southern blot analysis of the cosmid clones with rat H,K-ATPase β subunit cDNA (data not shown) revealed that the 5' end of the β subunit gene was contained on a 1.5-kb *Eco*RI fragment and that the remainder of the gene was contained on an adjacent 12.5-kb *Eco*RI fragment (Fig. 1). Two clones, MG4 and MG15A, each span the entire transcription unit of the mouse β subunit gene, which is estimated to be 10.5 kb. The MG4 clone also carries 28 kb of 5' flanking sequences and 3 kb of 3' flanking sequences, while clone MG15A carries 20 kb of 5' flanking sequences and 7 kb of 3' flanking sequences (Fig. 1*A*).

Structure of the Mouse H,K-ATPase β Subunit Gene. We determined the nucleotide sequence of all exons and ≈1.6 kb of sequences flanking the 5' end of the mouse H,K-ATPase β subunit gene. Sequence analysis indicates that the H,K-ATPase β subunit gene is divided into seven exons (Fig. 1*B*), 57 to 563 bp long, while the length of the six introns varies from 114 bp to ≈3.3 kb (Table 1). All splice donor and acceptor sequences (Table 1) conform to the consensus sequences compiled for other genes (21). The nucleotide sequence of the exons (Fig. 2) shows 94% identity within the coding region, and 81% identity within the 3' untranslated region, to rat H,K-ATPase β subunit cDNA (10). The predicted mouse protein exhibits 95% identity to the rat H,K-ATPase β subunit. Of the 15 amino acid differences between the two proteins, 8 represent conservative substitutions (22).

A projection of the exon/intron boundaries on the nucleotide and predicted amino acid sequence of the mouse H,K-ATPase β subunit is shown in Fig. 2. Exon 1 (137 bp) contains the entire 5' untranslated region of the mRNA and the first 37 amino acid residues, which correspond to the intracellular amino-terminal domain of the β subunit polypeptide. The entire membrane-spanning region (designated M in Fig. 3), and the first 15 residues of the extracellular domain are encoded by sequences contained within exon 2 (129 bp). Exons 3 (114 bp), 4 (200 bp), 5 (57 bp), 6 (111 bp), and 7 (563 bp) contain the sequences encoding the remainder of the β subunit. Exon 7 encodes the 53 amino acid residues at the carboxyl terminus of the protein and 404 bp of 3' untranslated sequence.

Recently, the exon/intron organization of the human Na,K-ATPase β1 (23) and mouse Na,K-ATPase β2 subunit genes (24) has been elucidated. We therefore compared the organization of these genes with that of the H,K-ATPase β subunit gene. The Na,K-ATPase β1 subunit gene is divided by five introns, whereas the Na,K-ATPase β2 and H,K-ATPase β subunit genes are each divided by six introns. A projection of the position of the introns on an alignment of the corresponding proteins is shown in Fig. 3. The H,K-ATPase β and Na,K-ATPase β2 subunit genes show an identical intron/exon arrangement. The positions of introns 1, 2, and 5 are exactly conserved in all three β subunit genes. However, the Na,K-ATPase β1 gene has no counterpart to intron 6 of the H,K-ATPase β and Na,K-ATPase β2 genes. Further, the positions of introns 3 and 4 in the Na,K-ATPase β1 gene differ from the positions of the corresponding introns in the H,K-ATPase β and Na,K-ATPase β2 genes. Nucleotide and predicted amino acid sequence analyses indicate that the H,K-ATPase β and Na,K-ATPase β2 subunits are more closely related to each other than either is to the Na,K-ATPase β1 subunit (10). The similarities in gene organization and amino acid sequence strongly indicate that the H,K-ATPase β and Na,K-ATPase β1 and β2 subunit genes are members of a multigene family and have evolved from a common ancestral β subunit gene. It should be noted in this context that the Na,K-ATPase β subunit of an invertebrate, the brine shrimp *Artemia salinas* (25), shows greater amino acid sequence homology to the rat Na,K-ATPase β1 subunit than it does to the rat β2 subunit. This observation suggests that the gene encoding the Na,K-ATPase β1 subunit most closely resembles the ancestral β subunit gene. According to this view, the H,K-ATPase and Na,K-ATPase β2 genes

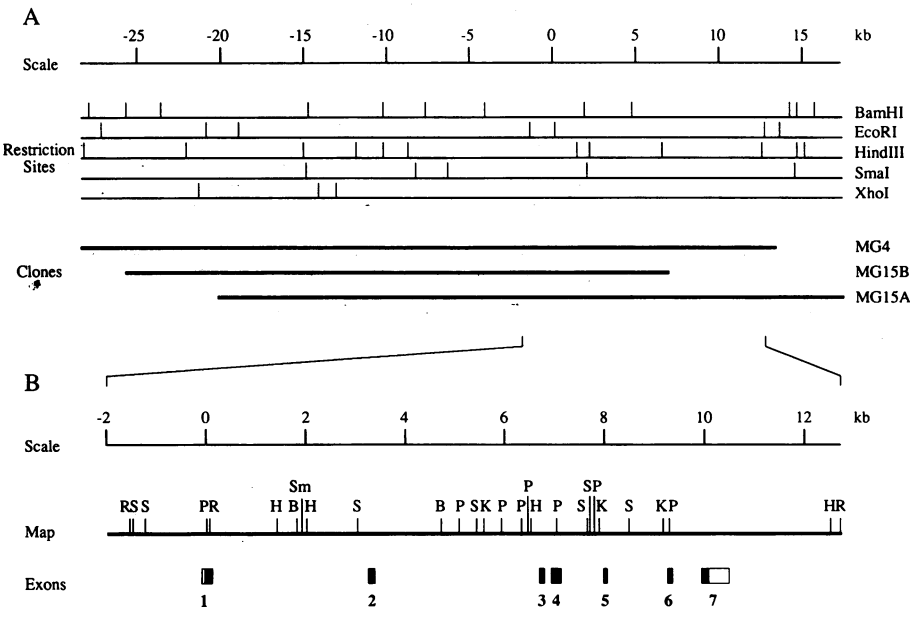

FIG. 1. Physical map of the mouse H,K-ATPase β subunit gene. (*A*) Cosmid clones encompassing the β subunit gene. Recognition sites for restriction enzymes *Bam*HI, *Eco*RI, *Hin*dIII, *Sma* I, and *Xho* I are shown above the cosmid clones. The scale (every 5 kb) is shown at the top. (*B*) Schematic representation of the β subunit gene. The complete transcription unit is contained within the brackets, and the scale (every 2 kb) is shown above the gene. The direction of transcription is from left to right. Exons are represented by boxes numbered 1–7. Closed boxes indicate coding sequences and open boxes indicate 5' and 3' untranslated regions. Recognition sites for restriction enzymes *Eco*RI (R), *Sac* I (S), *Pst* I (P), *Hin*dIII (H), *Sma* I (Sm), *Bam*HI (B), and *Kpn* I (K) are shown on the linear map above the exons.

Table 1. Intron/exon organization of the mouse H,K-ATPase β subunit gene

Exon	Exon position in mRNA	5′ donor	Intron length	3′ acceptor
1	−25 to 112	CGGTGGG/gtaggt... ArgTrp	≈3.1 kb	...ctctcttcacag/TGTGG ValTrp
2	113 to 241	TCACCAG/gtaagc... SerPro	≈3.3 kb	...ctttccttgcag/GGGTA GlyVal
3	242 to 355	TTAGCAG/gtgagt... LeuAla	114 bp	...tgtgttctacag/GCTAT GlyTyr
4	356 to 555	AACAGA/gtgagt... AsnArg	845 bp	...cctttctcctag/ATTGTC IleVal
5	556 to 612	TTCCAG/gtgagc... PheGln	1219 bp	...tctcctccccag/GATGAT AspAsp
6	613 to 723	GCACAG/gtaggt... AlaGln	≈0.6 kb	...cttccacttcag/CCCCAC ProHis
7	724 to 1286			

Positions of exons are numbered from 5′ to 3′ in the direction of transcription, where +1 is the A of the ATG translation initiation codon. Donor and acceptor sequences are shown with the exon sequence in uppercase letters and the intron sequence in lowercase letters. The deduced amino acid sequence at the exon/intron boundary is shown below the exon nucleotide sequence.

diverged from each other more recently than either did from the Na,K-ATPase β1 gene.

Mapping of the Transcription Start Site and Sequence Analysis of the Promoter Region. To determine the initiation point of H,K-ATPase β subunit mRNA transcription, we performed S1 nuclease mapping and primer extension analysis. First, the transcription startpoint of β subunit mRNA was determined by S1 nuclease mapping using a 541-bp *Eco*RI–*Nsi* I restriction fragment (positions −491 to +50). Mouse stomach RNA strongly protected two tight clusters of fragments, 72–75 and 117–118 nucleotides in length (Fig. 4*A*). Several additional weakly protected fragments, with lengths of up to ≈290 nucleotides, were also detected. In contrast, no protected fragments were observed when mouse kidney RNA was used as a control (Fig. 4*A*). These results suggest that the start sites of β subunit mRNA transcription are located at positions −24 and −67. Primer extension using mouse stomach poly(A)$^+$ RNA as template generated two predominant bands, which comigrated with cytosine residues −24 and −25 in the accompanying DNA sequencing ladder (Fig. 4*B*). The length of these fragments corresponds very well with the downstream cluster of initiation sites identified by S1 nuclease mapping. Primer extension also produced weak bands that correspond to protected fragments at positions −41, −67, and −83 in S1 nuclease mapping. These fragments were also detected when a primer complementary to nucleotides −15 to +10 was used to initiate primer extension (data not shown). Taken together, these results

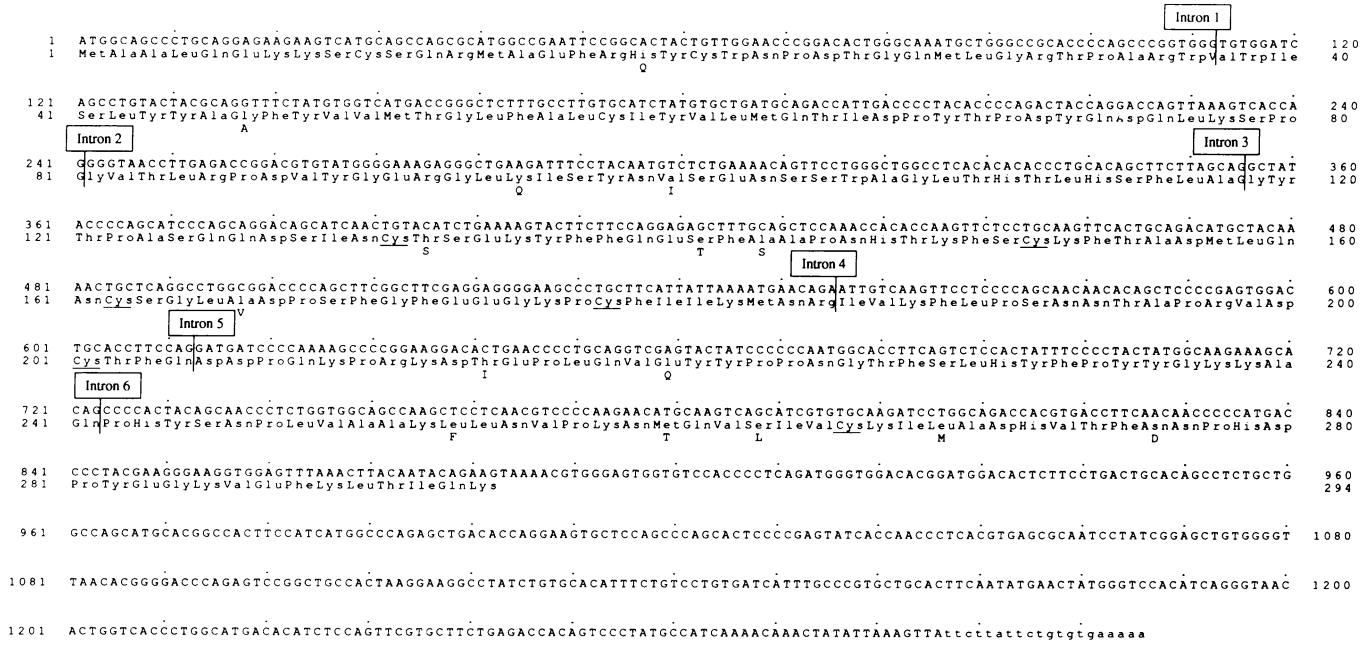

FIG. 2. Nucleotide sequence of the coding and 3′ untranslated regions of the mouse H,K-ATPase β subunit gene and deduced amino acid sequence of the protein. The nucleotide sequence is shown above the deduced amino acid sequence. Nucleotide and amino acid residues are numbered at the left and right. Nucleotide 1 is the A of the ATG codon for the initiator methionine. Residues in the corresponding rat β subunit polypeptide that differ from those in the mouse sequence are indicated by the single-letter amino acid code below the mouse sequence. Positions of the introns are indicated by vertical lines, and conserved cysteines are underlined. The sequence 3′ of the major polyadenylylation site is shown in lowercase letters.

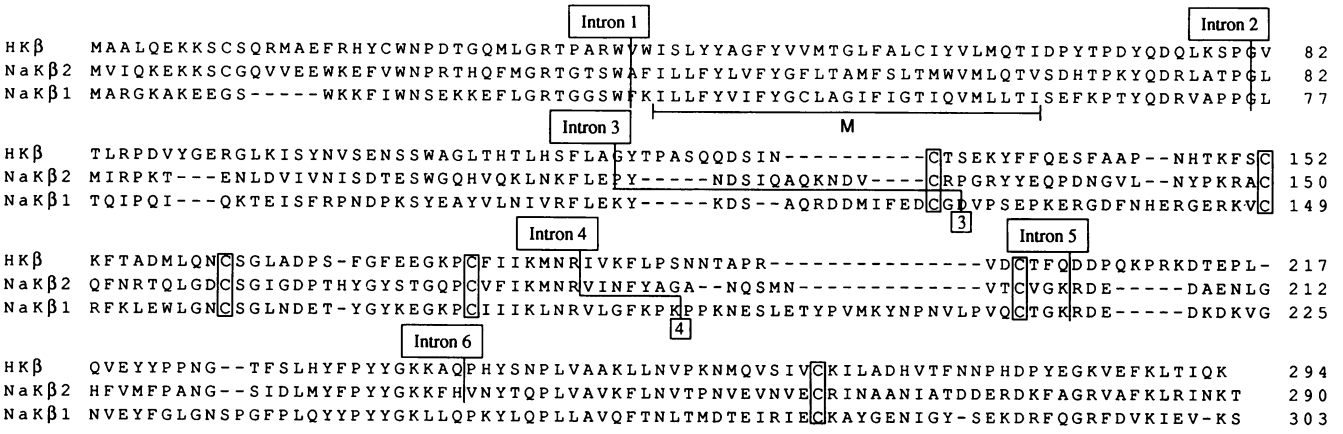

FIG. 3. Projection of intron positions on the H,K-ATPase and Na,K-ATPase β subunit amino acid sequences. The deduced amino acid sequence of the mouse H,K-ATPase β subunit (HKβ, top line) was aligned with the sequences of the rat Na,K-ATPase β2 (NaKβ2, middle line) and human Na,K-ATPase β1 (NaKβ1, bottom line) subunits. Dashes in the sequences allow optimal alignment for amino acid insertions/deletions. Amino acids are numbered on the right. Positions of the introns (numbered boxes) are indicated by vertical lines, and conserved cysteines are boxed. The membrane-spanning region is indicated by the bracket labeled M.

suggest that the downstream cluster of sites (centered at position −24) represents the major initiation point of β subunit mRNA transcription.

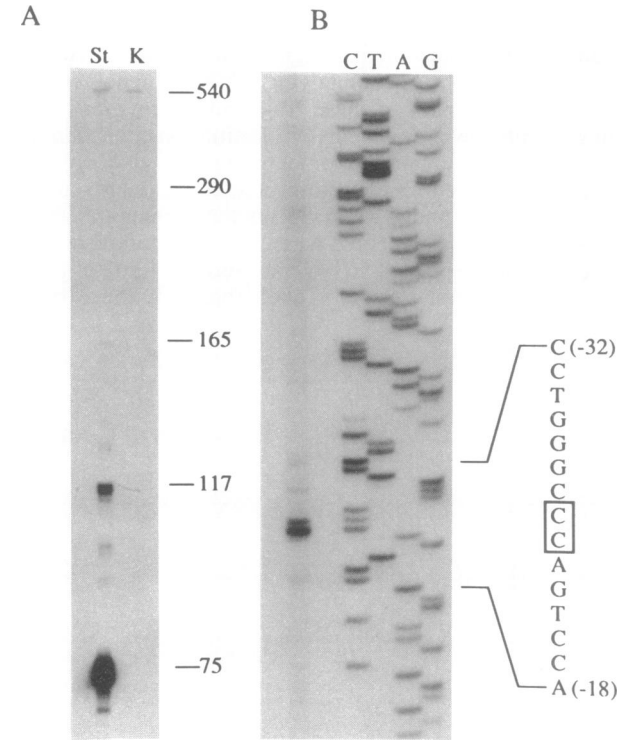

FIG. 4. Mapping of the transcription initiation site. (*A*) S1 nuclease mapping. An end-labeled 541-bp *Eco*RI–*Nsi* I genomic fragment (−491 to +50) was hybridized to total RNA prepared from mouse stomach (St) and kidney (K). Hybrids were digested with S1 nuclease and the products were analyzed on a denaturing polyacrylamide gel. The 541-bp band is the incompletely digested probe. The sizes of the protected fragments are shown in bp. (*B*) Primer extension analysis. A 25-base oligonucleotide (+13 to +37) served as a primer for reverse transcriptase using 2 μg of mouse stomach poly(A)$^+$ RNA as a template. The sizes of the primer extension products were deduced from a sequencing ladder (lanes C, T, A, and G on the right) derived from a fragment of mouse β subunit genomic DNA using the same oligonucleotide primer. Brackets at right enclose a portion of the sequencing ladder from positions −32 to −18. The boxed cytosine residues represent the major site of transcription initiation.

The sequence flanking the 5′ end of the mouse H,K-ATPase β subunit gene is shown in Fig. 5. The 5′ flanking sequence has a TATA-like sequence, TATAA, from −53 to −49, and an AP2 element, CCCCAGGC, from −765 to −758. Sequences corresponding to other regulatory elements, such as CAAT or GC boxes, were not present within the 1600-bp segment analyzed. In many eukaryotic class II gene promoters, the TATA element is located ≈25–30 bp upstream of the transcription start site (26, 27), and interaction of a factor or factors with this sequence specifies the site of transcription initiation (28). For the H,K-ATPase β subunit gene, S1 nuclease protection and primer extension analysis identified a cluster of transcription start sites (position −24 to −25) ≈30 bp downstream from the TATA box. These results are consistent with the view that this region represents the major initiation site of β subunit mRNA transcription.

The regulation of acid secretion in the parietal cell by secretagogues such as gastrin and acetylcholine is thought to be mediated by an increase in intracellular cAMP (reviewed in ref. 16). DNA sequence analysis of the promoter region of the human H,K-ATPase α subunit gene (8) reveals the presence of a putative cAMP response element, and treatment of isolated canine parietal cells with secretagogues results in an increase in the abundance of H,K-ATPase α subunit mRNA (29). However, a computer analysis of the region flanking the 5′ end of the H,K-ATPase β subunit gene failed to reveal the presence of consensus cAMP or hormone-response elements. It will clearly be of interest to determine whether cAMP and/or secretagogues play a direct role in the regulation of H,K-ATPase β subunit gene expression.

The genes encoding the H,K-ATPase α and β subunits appear to be expressed exclusively in parietal cells of the gastric mucosa (10, 13, 17). Although the factors that underlie the cell-specific activation of the α and β genes are unknown, it seems likely that these two genes may share regulatory elements. A computer search of the region flanking the 5′ end of the H,K-ATPase α and β subunit genes identified numerous short segments of nucleotide sequence identity (149 segments showing 10 base matches in a window of 12 bases). However, it is not possible with this approach to distinguish between sequences that may represent shared regulatory elements and sequence matches that occur by chance. Sequence analysis of the human H,K-ATPase α subunit promoter (8) revealed the presence of sequence motifs that were also present in the promoter region of the human and rat pepsinogen genes (30). Because the pepsinogens are expressed in chief and mucous neck cells (and not in mature

FIG. 5. Nucleotide sequence of the promoter region of the mouse H,K-ATPase β subunit gene. The sequence shown has the same polarity as the mRNA. Nucleotides are numbered with +1 being the A of the ATG initiation codon. The deduced amino acid sequence of a portion of the first exon is shown below the nucleotide sequence. The TATA and AP2 elements are boxed, and the sequence complementary to the synthetic oligonucleotide used for primer extension is underlined. The ends of the fragment used for S1 nuclease mapping are indicated by asterisks. The strong site of transcription initiation is indicated by the heavy arrow, while minor transcription start sites are indicated by light arrows.

parietal cells) of the gastric mucosa (31), this observation suggests the potential for stomach-specific transcriptional control elements. However, we were unable to identify similar sequence motifs within the region flanking the 5′ end of the H,K-ATPase β subunit gene. The availability of cosmid clones containing 28 kb of sequences flanking the 5′ end of the mouse H,K-ATPase β subunit gene should allow us to study the factors implicated in the stomach and parietal cell-specific activation of this gene.

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