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Molecular cloning and sequence analysis of human Na,K-ATPase β-subunit

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ABSTRACT

We have isolated a cDNA clone for the β-subunit of HeLa cell Na,K-ATPase, containing a 2208-base-pair cDNA insert covering the whole coding region of the β-subunit. Nucleotide sequence analysis revealed that the amino acid sequence of human Na,K-ATPase exhibited 61% homology with that of Torpedo counterpart (Noguchi et al. (1986) FEBS Lett. in press). A remarkable conservation in the nucleotide sequence of the 3' non-coding region was detected between the human and Torpedo cDNAs. RNA blot hybridization analysis revealed the presence of two mRNA species in HeLa cells. S1 nuclease mapping indicated that they were derived from utilization of two distinct polyadenylation signals in vivo. Total genomic Southern hybridization indicated the existence of only a few, possibly one set of gene encoding the Na,K-ATPase β-subunit in the human genome.

INTRODUCTION

Na,K-ATPase, an enzyme composed of two subunits named α and β, is found in plasma membrane of all animal cells. It regulates the intracellular levels of Na^+ and K^+ against their concentration gradients with the aid of energy released through the hydrolysis of ATP (1). The enzyme is also suggested to play critical roles in cellular phenomena such as homeostasis maintenance or differentiation (2, 3). For the elucidation of these physiologically important roles, it is substantial to understand the regulatory mechanisms of its biosynthesis, assembly and translocation to the plasma membrane. HeLa cell is a favourable model system for these purposes since the turnover and regulation of Na,K-ATPase in cultured HeLa cells have been extensively investigated and well documented (4).

All of the ligand binding sites and enzymatically active sites of this enzyme as an ATPase are known to reside in the α-

subunit. Little is known, however, about the catalytic role of the β-subunit. To understand the molecular architecture of the α-subunit, we and other groups have cloned and sequenced cDNA for α-subunit of Torpedo californica (5) and sheep Na,K-ATPase (6) and elucidated its membrane topology through analysis of the primary structure of the enzyme protein deduced from the nucleotide sequences. Comparison of the primary structure of the two species revealed a remarkable homology between their amino acid sequences. Very recently, we have also succeeded in cloning and sequencing of cDNA for the β-subunit of Torpedo Na,K-ATPase (7). Hydropathic analysis (8) of the primary sequence of the β-subunit revealed one segment possibly running through the membrane. For further understanding of its molecular architecture, it is essential to compare the primary and predicted secondary structures of the β-subunit with those of other species.

With these in mind, we have cloned and sequenced the cDNA for the β-subunit of Na,K-ATPase from HeLa cell cDNA library. Here we report the primary structure, size analysis of mRNA expressed in HeLa cells and the estimation of gene numbers for β-subunit of human Na,K-ATPase.

MATERIALS AND METHODS

Screening and Sequencing of the cDNA clone: HeLa cell cDNA library, a generous gift from Dr. P. Nielsen (Univ. of Basel, Switzerland), was screened with a nick-translated (9) AvaII(83)/HindIII(267) fragment excised from the cDNA encoding β-subunit of Torpedo Na,K-ATPase (7) as a probe. The hybridization was carried out in 3xSSC (1xSSC=0.15M NaCl, 0.015M Na citrate), 10xDenhart's solution at 50°C. The filters were washed sequentially in 3xSSC, 1xSSC, 0.3xSSC and 0.1xSSC at 50°C in the presence of 0.1% SDS before exposing to X-ray films. One of the five positive clones (λHNKB6), apparently harboring the largest cDNA insert, was subcloned into pUC9 and subjected to nucleotide sequence analysis by the dideoxynucleotide chain-termination method (10). DNA sequences were analyzed by GENETYX program (Genetyx Inc., Tokyo).

RNA and DNA Blot Hybridization: RNA was isolated from HeLa cells by the guanidinium isothiocyanate method of Chirgwin et al. (11). Poly(A) RNA was prepared from the total RNA, fractionated by electrophoresis through a 1% agarose gel and transferred to Biodyne membrane (PALL, NY). Hybridization was carried out as described (12). High molecular weight DNA, prepared from human placenta, was digested with restriction enzymes, fractionated by agarose gel electrophoresis, and transferred to nitrocellulose filters (13). Blots were hybridized with a probe which is a nick-translated NcoI(-2)/NcoI(800) fragment excised from pHNKB6.

S1 Nuclease Mapping: The DNA fragments of pHNKB6, ^{32}P-labelled at the 3'-ends of BglII(1662) and NcoI(800), were digested with EcoRI(2077) and used as probes. HeLa cell total RNA (15 µg) was hybridized with the probe at 50°C overnight and subsequently digested with S1 nuclease as described (14). The hybrids were analyzed by 6% polyacrylamide gel electrophoresis in the presence of 6M Urea.

Enzymes and Chemicals: Restriction endonucleases were obtained from Takara Shuzo, Japan and Nippon Gene, Japan. S1 nuclease and *E. coli* DNA polymerase I were purchased from P-L Biochemicals, USA. DNAse I was a product of Sigma, USA. [α-^{32}P]dCTP was obtained from ICN Radiochemicals, USA.

RESULTS

Isolation and Sequence Analysis of a HeLa cell Na,K-ATPase cDNA Clone

The λgt11 cDNA expression library (15, 16) constructed from HeLa cell mRNA was screened with a cDNA probe encoding the β-subunit of *T. californica* Na,K-ATPase (7). Five hybridization positive clones were obtained from about 200,000 plaques. One of the clones named λHNKB6, apparently carrying the largest insert, was subjected to further analysis.

Nucleotide Sequence of cDNA and Assignment of Protein Sequence

Fig. 1 shows the nucleotide sequence carried by the cDNA insert of clone pHNKB6 encoding the β-subunit of human Na,K-ATPase. The primary structure of human β-subunit was deduced from the nucleotide sequence. The amino terminal sequence of

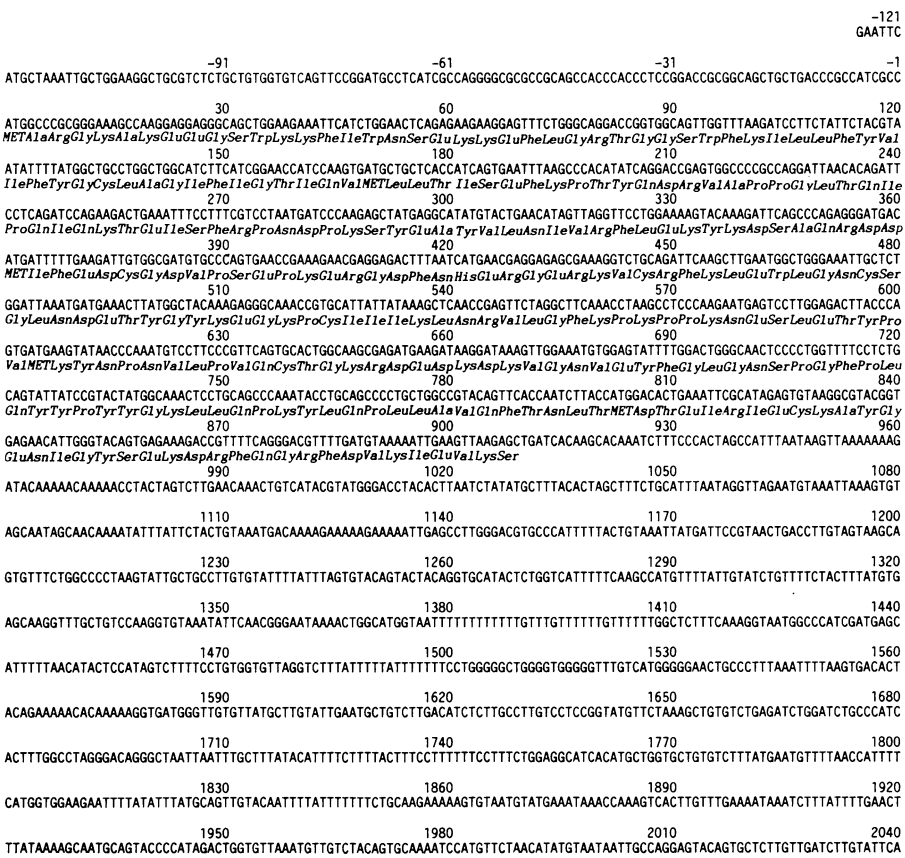

Fig. 1. Nucleotide sequence of the cDNA encoding the β-subunit of human Na,K-ATPase. Nucleotide residues are numbered in the 5' to 3' direction, beginning with the first residue of the ATG triplet encoding the initiating methionine, and the nucleotides on the 5' side of residue 1 are indicated by negative numbers. The deduced amino acid sequence of the Na,K-ATPase β-subunit is shown below the nucleotide sequence, and amino acid residues are numbered beginning with the initiating methionine; the number of the amino acid residue at the right-hand end of each line is given.

human β-subunit agrees fairly well with that of lamb and dog Na,K-ATPase (9 and 33 amino acids, respectively) determined by protein sequencing (17, 18) (Fig. 2A). The molecular weight of the β-subunit composed of 303 amino acid residues (including the initiating methionine) was calculated to be 35,061. The 3' non-

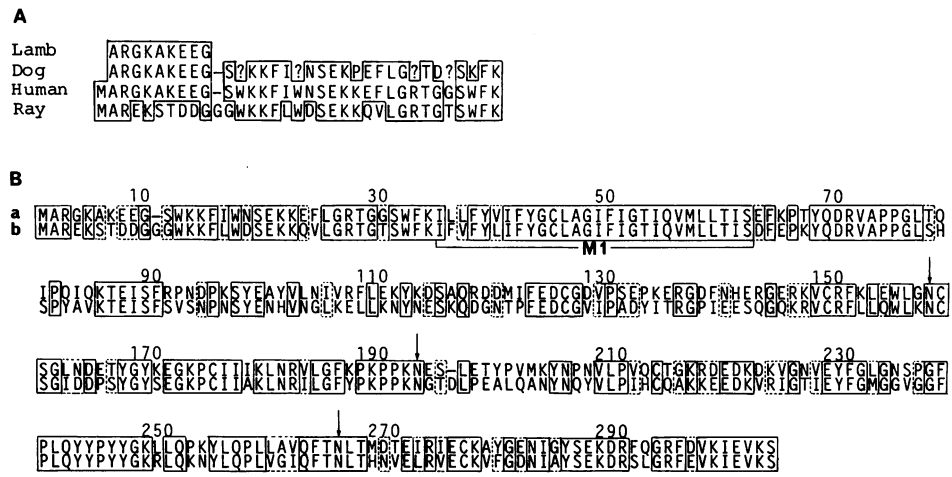

Fig. 2. Alignment of amino acid sequences of Na,K-ATPase β-subunits. (A) N-terminal amino acid sequences of the β-subunits of lamb (17), dog (18), human and Torpedo (Ray)(7) are shown by one-letter amino acid notation. Identical residues at the same position are enclosed with solid lines. (B) Total sequences of human (a) and Torpedo (b) are shown. Pairs of identical residues are enclosed with solid lines, and pairs of residues considered to be favoured amino acid substitutions with dotted lines. Favoured amino acid substitutions are defined as pairs of residues belonging to one of the following groups: S, T, P, A and G; N, D, E, and Q; H, R and K; M, I, L and V; F, Y and W (24). Gaps (-) have been inserted to achieve maximum homology. The positions in the aligned sequences are numbered as defined in human sequence (Fig. 1). The asparagine residues as possible glycosylation sites are marked with arrows. The putative transmembrane segment M1 is indicated.

coding region of the cDNA contains the four polyadenylation signals AATAAA (19), at residues 1359, 1875, 1900, and 2061.

Comparison of the Amino-acid and Nucleotide Sequence of Human and T. californica Na,K-ATPase β-subunit

The alignment of the amino acid sequences of the β-subunit of human and Torpedo Na,K-ATPase is given in Fig. 2B. The degree of sequence homology between the two β-subunits evaluated on the basis of the alignment of the amino acid sequences was 61%, gaps being counted as one substitution regardless of their length. Hydropathy profile (8) of the β-subunit of human Na,K-ATPase was similar to that of Torpedo enzyme except that the amino acid positions 118-147 were more hydrophilic in human enzyme (data

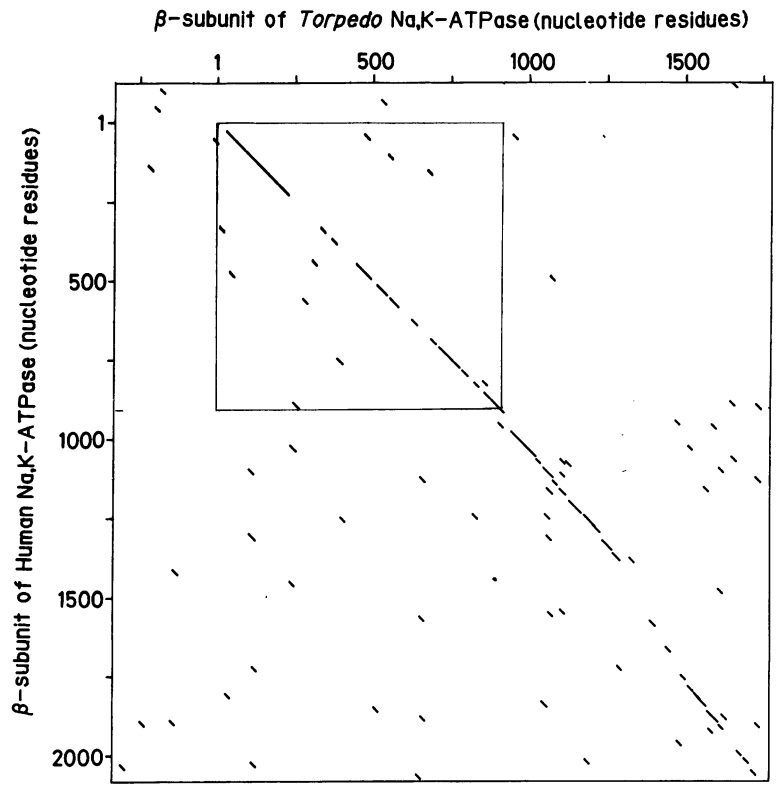

Fig. 3. Dot matrix analysis of cDNA sequences between human and Torpedo β-subunit of Na,K-ATPase. The nucleotide numbering is in the direction of from 5' to 3' in the message strand beginning with the initiation codon of β-subunit cDNA. A computer program (Genetyx, SDC Inc., Tokyo) was used to generate a matrix of dots displaying segments of homology. The program prints a bar if 12 nucleotides out of 15 nucleotides are identical between two β-subunit cDNAs. The coding region is enclosed with solid lines.

not shown). The putative transmembrane segment M1 contained the highly conserved region composed of a 22-amino acid strech. Three of the four potential N-glycosylation sites (20) found in Torpedo are conserved in the human sequence (asparagine residues 158, 193 and 265). The putative nucleotide binding sequence Gly-X-Gly-X-X-Gly (X can be any amino acid) (21) was found in the Torpedo sequence (7) but not conserved in the human sequence (residues corresponding to 231-236). Dot matrix analysis (Fig. 3) detected highly homologous nucleotide sequences not only in

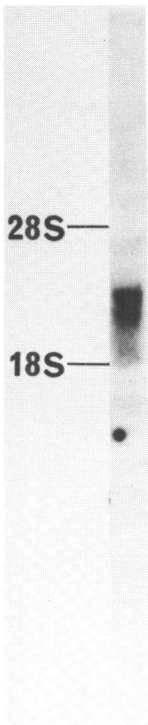

Fig. 4. Blot hybridization analysis of poly(A) RNA from HeLa cells. 5 µg of poly(A) RNA was analyzed as described in "MATERIALS AND METHODS". The positions of size markers (28S and 18S ribosomal RNA) are indicated.

the protein coding region but also in the 3' non-coding region (residues around 970-1390 and 1780-1930). A remarkably homologous stretch comprising of 74 nucleotides was found at residues 981-1054 (96% homology with only three substitutions). The implication of the remarkable coincidence is not clear at present. No similar homology was found among the cDNA of human, sheep and Torpedo Na,K-ATPase α-subunits (Kawakami, unpublished results).

RNA blot Hybridization Analysis and S1 Nuclease Mapping

Blot hybridization analysis for HeLa cell poly(A) RNA probed by Na,K-ATPase β-subunit cDNA identified two hybridizable RNA species (Fig. 4). To examine a possibility that they are derived from the usage of multiple polyadenylation signals found in the 3' non-coding region of pHNKB6 at the positions of 1359, 1875, 1900 and 2061, the 3'-end labelled anti-sense strand DNA covering the four or three of the signal sites were prepared and

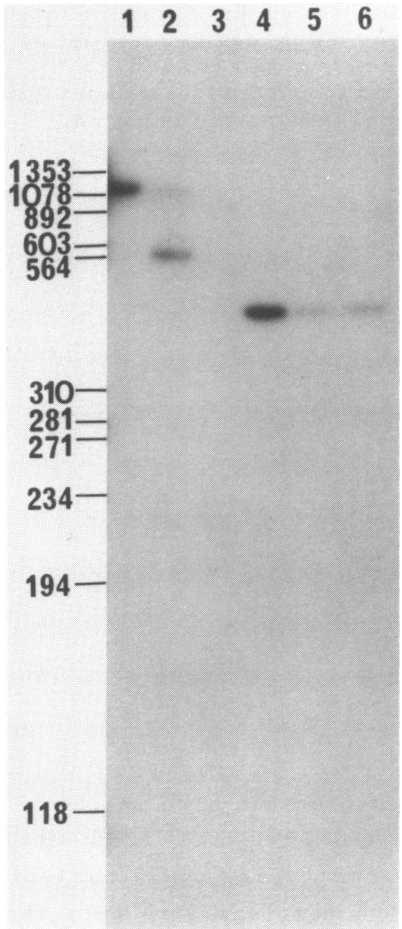

Fig. 5. S1 nuclease mapping of RNA from HeLa cells. The 3'-end labelled DNA probes described in "MATERIALS AND METHODS" were hybridized with HeLa cell total RNA (lanes 2 and 5), or tRNA (lanes 3 and 6) and subsequently digested with S1 nuclease. Lanes 1 and 4 are non-digested DNA probe. NcoI(800)/EcoRI(2077) fragment was used in lanes (1-3) and BglII(1662)/EcoRI(2077) fragment was used in lanes (4-6).

used as the probes for S1 nuclease mapping (Fig. 5). The NcoI(800)/EcoRI(2077) fragment which covers all of the four potential polyadenylation signals was digested with S1 nuclease to 570-nucleotide(nt) fragment, whose tail corresponded to about ten nucleotides downstream from the first polyadenylation signal

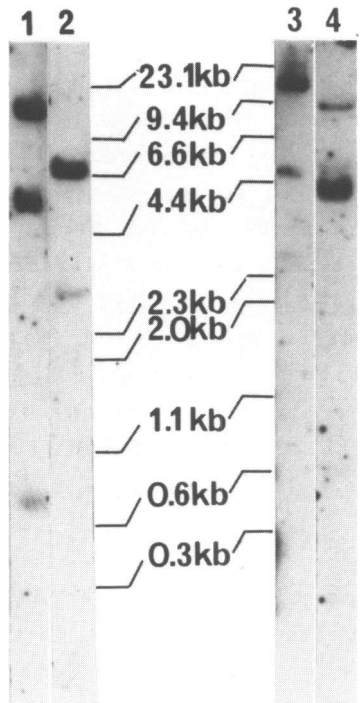

Fig. 6. Southern hybridization for human placenta DNA. High molecular weight DNA was prepared from human placenta and digested with restriction enzymes; (1)BamHI, (2)BglI, (3)ApaI, (4)XbaI. DNA fragments (each 25 µg) were electrophoresed through 1.0% agarose gel and transferred to a nitrocellulose filter. NcoI(-2)/NcoI(800) fragment excised from pHNKB6 was labelled with nick-translation and used as the hybridization probe. Hybridization and washing were done at 65°C. The positions of DNA size markers are indicated. Lanes 1-2 and lanes 3-4 are from different gels.

at the position of 1359. In contrast, the BglII (1662)/EcoRI(2077) fragment which covers three possible signals (positions 1875, 1900 and 2061) was not digested with S1 nuclease. These results indicated that two of the polyadenylation signals at the positions of 1359 and 2061 were utilized in HeLa cell. The products derived from the positions of 1875 and 1900 were not detected at least in our experimental conditions, and probably not used in vivo. It is likely that the two species detected in RNA blot hybridization analysis were

derived from the usage of these two polyadenylation signals at positions 1359 and 2061.

Total Southern Hybridization Analysis

To estimate how many genes exist for Na,K-ATPase β-subunit in the human genome, high molecular weight DNA from human placenta was digested with several kinds of restriction enzymes and analyzed by Southern blotting. Nick-translated NcoI(-2)/NcoI(800) fragment was used as a hybridization probe. Two bands were identified in BglI, ApaI and XbaI digests (Fig. 6, lanes 2-4), while three bands in BamHI digest (Fig. 6, lane 1). Since there is no restriction sites for BamHI, ApaI and XbaI in the DNA fragment used as the probe and one restriction site (at position 134) for BglI, it is concluded that there are at most two, probably one, gene for Na,K-ATPase β-subunit.

DISCUSSION

We have isolated a cDNA clone encoding the β-subunit of human Na,K-ATPase, analyzed the nucleotide sequence and deduced the primary structure of the subunit. The N-terminal sequence of the human enzyme is identical to that of the lamb enzyme (amino acid residues 2-10), and quite similar to that of dog enzyme (26 out of 33 residues at positions 2-34 are identical), both of which sequences were determined by protein analysis (17, 18). Hydrophobic amino terminal sequence characteristic of the signal peptide (22) was absent as is the case for Torpedo enzyme. Three of the four potential N-glycosylation sites found in the β-subunit of Torpedo enzyme were conserved in the human enzyme, suggesting that these sites may be glycosylated. The most conserved region between the two sequence is found in the putative transmembrane segment M1 at positions 35-62. This suggests that the transmembrane segment not only anchor the β-subunit into the membrane but also has some functional role such as formation of ion conducting pathway or interactions with the transmembrane segments of the α-subunit. The sequence Gly-X-Gly-X-X-Gly, characteristic to the nucleotide binding region of various nucleotide binding proteins, was found in the Torpedo sequence but not conserved in the human sequence. The possibility is not ruled out that the change of amino acid

sequence in this position is not a feature of the human Na,K-ATPase in general but specific to the enzyme of transformed cells such as HeLa cells. This point should be examined in our future experiments. Freytag suggested that the β-subunit is not required for the expression of ATPase activity based on the experiment of papain digestion (23). However, the conservation of the hydropathy profile, three potential glycosylation sites and the positions of 7 cystein residues between human and Torpedo enzymes suggests that the transmembrane topology and the overall integrity of the β-subunit are similar in the two species. Taken together with the overall homology in primary structure (61%) between the two species, the Na,K-ATPase β-subunit is expected to have some indispensable catalytic roles in the Na,K-ATPase activity. The degree of sequence homology between the human and Torpedo β-subunits is lower than that between the α-subunits of the two species (87%). This may suggest that the rate of evolution is different for the α and β subunits of the Na,K-ATPase.

S1 nuclease mapping revealed that the two of the four potential polyadenylation signals are used in vivo. It is likely that the two species of mRNA identified in the RNA blot hybridization analysis corresponded to those detected in S1 nuclease mapping. The functional implications of the presence of two mRNA species will be one of the subjects of our future experiments.

Total Southern hybridization analysis revealed the presence of one set of Na,K-ATPase gene coding for β-subunit. The gene coding for α-subunit was also found to be unique (Kawakami, unpublished results). This implies that there is no isozyme having extensive homology with the reported Na,K-ATPase α- and β-subunits. This does not exclude a possibility of the existence of isozyme genes which do not cross hybridize with the cloned cDNA at the stringency used in our experiment.

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