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Rat-Brain Na,K-ATPase β-Chain Gene: Primary Structure, Tissue-Specific Expression, and Amplification in Ouabain-Resistant HeLa C^+ Cells

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We deduced the complete amino acid sequence of the rat brain Na,K-ATPase β-subunit from cDNA. The rat brain β-subunit exhibits a high degree of primary sequence and secondary structural homology with the human and *Torpedo* β-subunit polypeptides. Analysis of rat tissue RNA reveals that the β-subunit gene encodes four separate mRNA species which are expressed in a tissue-specific fashion. In ouabain-resistant HeLa C^+ cells, β-subunit DNA sequences are amplified (~20-fold) and β-subunit mRNAs are overproduced relative to levels in parental HeLa cells. These results suggest that the β-subunit plays an important role in Na,K-ATPase structure-function and in the mechanism underlying cellular resistance to the cardiac glycosides.

The Na,K-ATPase is the enzymatic activity responsible for maintaining the high internal K^+ and low internal Na^+ levels characteristic of most animal cells. The enzyme couples the hydrolysis of ATP to the movement of Na and K ions across the plasma membrane, thus producing the electrochemical gradients that are the primary source of energy for the active transport of nutrients, the action potential of excitable tissues, and the regulation of cell volume (9, 12).

In all tissues from which the Na,K–ATPase has been isolated, the enzyme has been shown to consist of two polypeptide subunits, an α-subunit of ~100 kilodaltons (kDa) which contains the binding sites for ATP and the cardiac glycosides such as digoxin and ouabain, and a glycosylated β-subunit with a molecular mass of ~55 kDa (9). The known functions localized to the α-subunit are phosphorylation by ATP and conformational changes associated with the transport of ions (12, 15). Although biochemical studies indicate that the β-subunit associates with the α-subunit in a region close to the ouabain-binding site (11), the relationship of the β-subunit to the activity of the Na,K-ATPase remains unknown.

Here we describe the isolation and characterization of cDNA clones coding for the rat brain Na,K-ATPase β-subunit. Comparison of the deduced amino acid sequence of rat brain β-subunit to the human (13) and *Torpedo* (17) proteins showed a striking degree of structural homology between the β-subunit polypeptides. The rat β-subunit gene encodes four mRNA species which are expressed in a tissue-specific fashion. In the ouabain-resistant HeLa cell line, C^+, β-subunit genomic DNA sequences are amplified and β-subunit mRNAs are overexpressed relative to levels in parental HeLa cells. These results are consistent with the view that the β-subunit polypeptide is an important structural component of the Na,K-ATPase and may play a role in the mechanism of cellular resistance to ouabain.

MATERIALS AND METHODS

An oligo(dT)-primed λgt11 cDNA library constructed from mRNA isolated from 1- and 2-week-old rat brains (provided by N. Davidson and A. Dowsett, California Institute of Technology) was screened with a Na,K-ATPase antibody by the method of Young and Davis (24). The antibody used in the screening was a rabbit antiserum (K3) raised against rat kidney Na,K-ATPase (23). This antibody was provided by K. J. Sweadner (Massachusetts General Hospital). Conditions for antibody screening of the library and protein blotting were as previously described (22).

The cDNA insert from an immunoreactive bacteriophage clone, λrb19, was isolated, subcloned into the plasmid vector pAT153, and used to screen a random-primed λgt11 cDNA library which was constructed from neonatal rat brain mRNA. This library was also provided by N. Davidson and A. Dowsett. Positive phage clones were plaque purified, and their cDNA inserts were characterized by standard restriction endonuclease mapping procedures.

DNA sequence analysis. The cDNA insert of λrb19G was used to generate a sequencing library in the bacteriophage vector M13mp19 by the method of Dale et al. (5). Separate sequencing libraries were constructed from each DNA strand. The DNA sequence of λrb19G was determined by the dideoxynucleotide chain termination method (21) with [^{35}S]dATP and 6% buffer gradient gels as described by Biggin et al. (2). The DNA sequence was compiled and analyzed by using the programs from Staden and the University of Wisconsin Genetics Computer Group.

RNA and DNA blot hybridization. RNA was isolated from adult rat tissues and tissue culture cells by the guanidinium isothiocyanate method of Chirgwin et al. (3). High-molecular-weight DNA was prepared from tissue and cells by the method of Gross-Bellard et al. (10). Conditions for electrophoresis, transfer, and hybridization analysis of DNA and RNA samples were as previously described (22). cDNA probes were labeled (specific activity, 0.5×10^8 to 4×10^8 cpm/μg) by either nick translation (20) (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) or employing the Klenow fragment of *Escherichia coli* DNA polymerase I (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) and oligonucleotide primers (Pharmacia-P-L Biochemical) by the method of Feinberg and Vogelstein (8).

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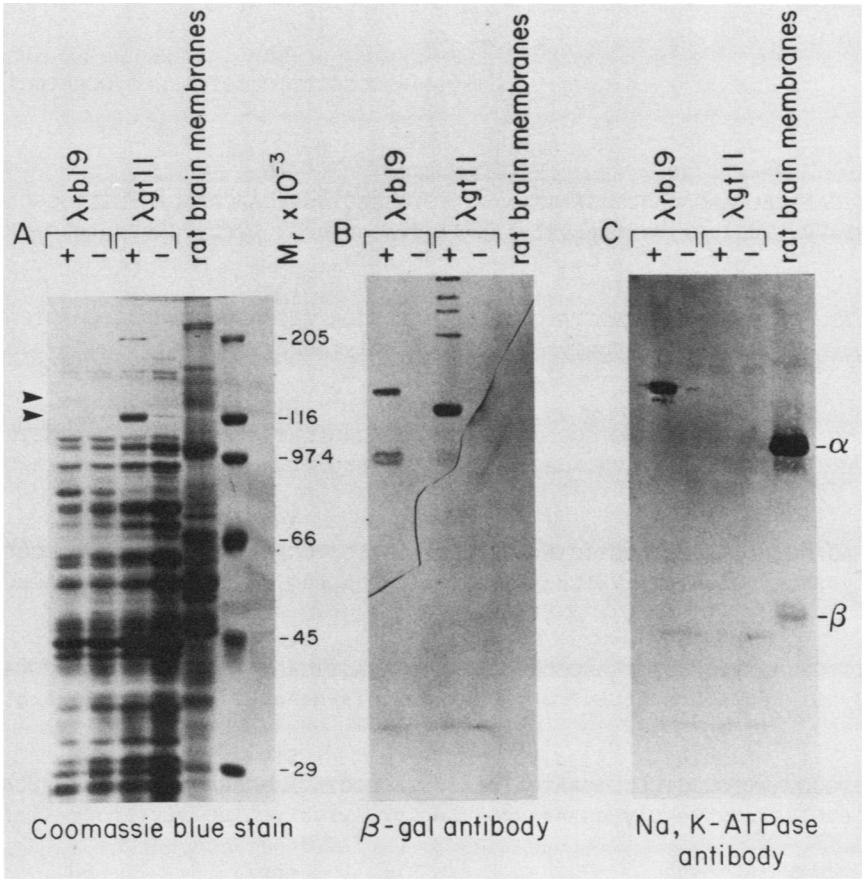

FIG. 1. Immunoblot analysis of λrb19 fusion protein. The phage clones λrb19 and wild-type λgt11 were used to lysogenize *E. coli* Y1089. Production of fusion protein was induced with (+) or not induced (−) with isopropyl-β-D-thiogalatopyranoside. Lysogen proteins were separated on a 7.5% sodium dodecyl sulfate-polyacrylamide gel and transferred to nitrocellulose. (A) Coomassie blue staining of lysogen proteins. (B) Reactivity with β-galactosidase antibody followed by treatment with alkaline phosphatase conjugated to protein A. (C) Reactivity with Na,K-ATPase antibody followed by treatment as in panel B. Molecular weight markers ($\times 10^3$) are shown in panel A. The position of β-galactosidase (lower arrow) was determined from the mobility of purified β-galactosidase. Upper arrow denotes the position of the λrb19 fusion protein. The positions of rat brain Na,K-ATPase α- and β-subunits are shown in panel C.

Cell culture. HeLa and C^+ cells were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum (Armour). C^+ cells, provided by J. F. Ash (University of Utah School of Medicine), were maintained in medium containing 1 μM ouabain (Sigma Chemical Co., St. Louis, Mo.). C^- cells were C^+ cells grown in the absence of ouabain for the times indicated in each experiment.

RESULTS

Isolation of cDNA clones. We screened an oligo(dT)-primed λgt11 expression library constructed from neonatal rat brain mRNA with an antibody reactive to rat brain Na,K-ATPase. One phage clone, λrb19, containing a 900-base-pair *Eco*RI cDNA insert, remained immunoreactive through four rounds of screening and plaque purification. This phage clone was used to lysogenize *E. coli* Y1089. A bacterial lysate was prepared from the λrb19 lysogen and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting (Fig. 1).

The reactivity of the bacterial lysates with Na,K-ATPase antibody is shown in Fig. 1C, and the reactivity of the lysates with β-galactosidase antibody is shown in Fig. 1B. The Coomassie blue staining pattern of the lysates is presented in Fig. 1A. The addition of isopropyl-β-D-thiogalactopyranoside (+ lane, Fig. 1A) to the wild-type λgt11 lysogens resulted in the increased expression of β-galactosidase (lower arrow). However, addition of isopropyl-β-D-thiogalactopyranoside to λrb19 lysogens induced the expression of a 145-kDa fusion protein (upper arrow in panel A). This fusion protein was immunoreactive with both the β-galactosidase antibody (panel B) and the Na,K-ATPase antibody (panel C). The λgt11 lysogen was not immunoreactive with the Na,K-ATPase antibody (panel C), and rat brain membranes failed to react with the β-galactosidase antibody (panel B). However, the Na,K-ATPase antibody was reactive with the 55-kDa β-subunit of rat brain Na,K-ATPase (panel C). Thus, the Na,K-ATPase antibody reacts specifically with the λrb19 fusion protein and not with bacterial or phage polypeptides.

The cDNA insert of λrb19 was used as a hybridization probe to screen a random-primed λgt11 cDNA library constructed from neonatal rat brain mRNA. Several positive phage clones were identified and plaque purified, and their cDNA inserts were characterized. One clone, λrb19G, was studied in the greatest detail.

Characterization of a cDNA clone for the Na,K-ATPase β-subunit. The sequence of λb19G is presented in Fig. 2. The

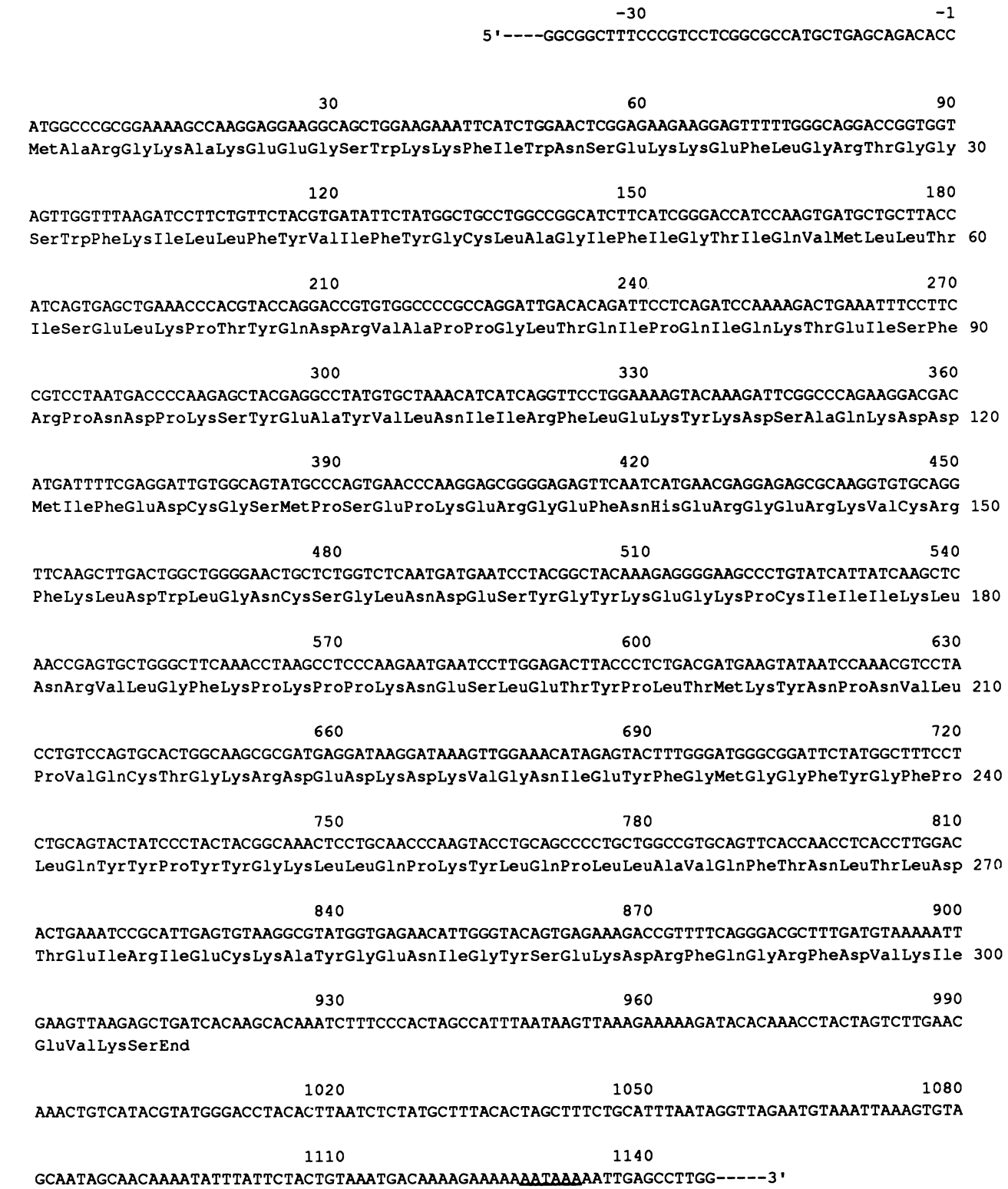

FIG. 2. Nucleotide sequence of rat brain Na,K-ATPase β-subunit cDNA and the deduced amino acid sequence of the protein. Nucleotide residues are numbered above the sequence in the 5′-to-3′ direction. Nucleotide 1 is the A of the ATG codon for the initiator methionine. Negative numbers refer to the 5′-untranslated region and are numbered in the 3′-to-5′ direction. The deduced amino acid sequence is shown beneath the nucleotide sequence. The amino acids are numbered on the right side of the sequence and begin with the initiator methionine. The polyadenylation signal AATAAA that begins at residue 1128 is underlined.

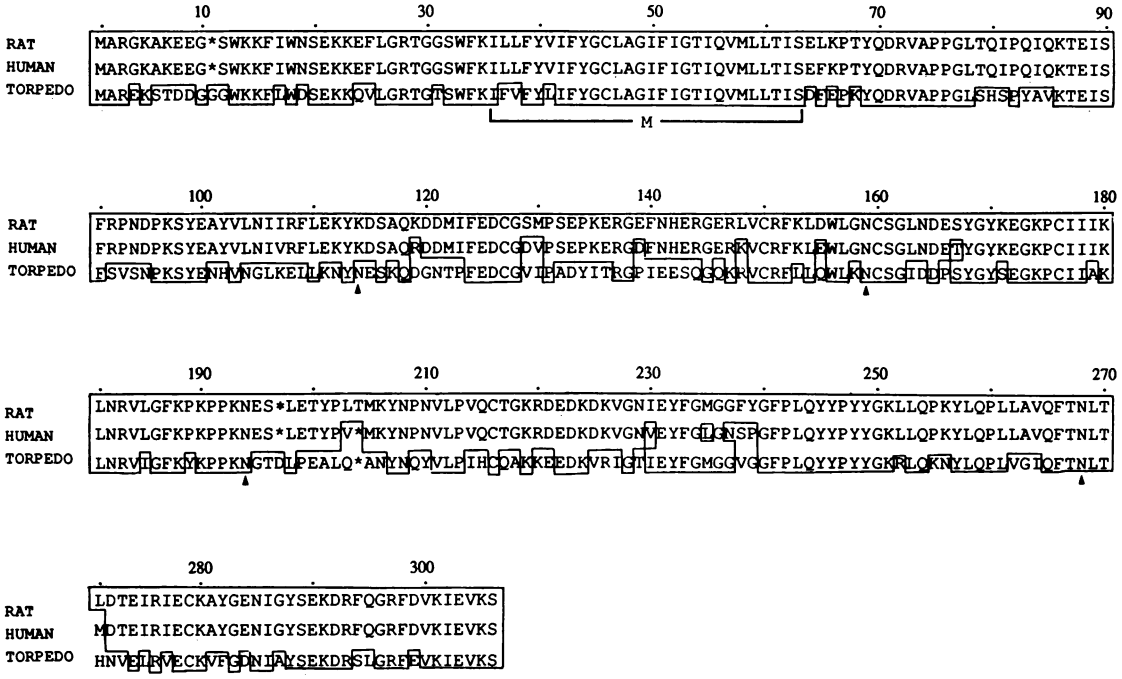

FIG. 3. Amino acid sequences of rat, human, and *Torpedo* β-subunits are shown by the one-letter amino acid notation. Identical residues are enclosed. The asparagine residues which are possible sites for N-linked glycosylation are identified with arrows. The putative transmembrane-spanning region from amino acid residues 36 through 63 is indicated by the bracket labeled M.

initiation codon (residues 1 to 3) was assigned to the first ATG triplet downstream of the in-frame termination codon (TGA) beginning at position −12. The initiation codon is found within the consensus sequence (5′-CCATGG-3′) thought to be necessary for efficient translation of eucaryotic mRNAs (14). An open reading frame commencing with this ATG continues to position 912 and codes for a polypeptide of 304 amino acids with a molecular weight of 34,639. This value is in reasonable agreement with the estimated size of the deglycosylated rat brain Na,K-ATPase β-subunit (23). The translation termination codon which begins at position 913 is followed by 231 base pairs of 3′-untranslated sequence including a polyadenylation signal (AATAAA) (19) beginning at position 1128. The amino acid sequence of the protein deduced from λrb19G cDNA was virtually identical with that reported for the human Na,K-ATPase β-subunit (13). These results demonstrate that λrb19G cDNA represents the entire coding region for the rat Na,K-ATPase β-subunit.

Structure of the rat Na,K-ATPase β-subunit. A comparison of the amino acid sequence of the rat brain β-subunit with its human and *Torpedo* counterparts is shown in Fig. 3. The rat brain β-subunit consists of 304 amino acid residues, whereas the *Torpedo* and human β-subunits are composed of 305 and 303 amino acid residues, respectively. When compared with the human β-subunit, the *Torpedo* polypeptide has an additional glycine residue at position 11 and an additional aspartic acid residue at position 197, whereas the rat β-subunit has an additional threonine residue at position 204. The asparagine residues marked with arrows represent potential sites of N-linked glycosylation. Three of the four potential N-linked glycosylation sites found in the *Torpedo* sequence are conserved in the rat and human sequences. A substitution in the rat sequence at amino acid 114 converts an asparagine residue to leucine, thus eliminating the most N-terminal glycosylation site. The rat brain β-subunit exhibits 95 and 62% amino acid sequence homology, respectively, with the human and *Torpedo* β-subunits. A total of 42% of the 114 amino acid substitutions between rat and *Torpedo*

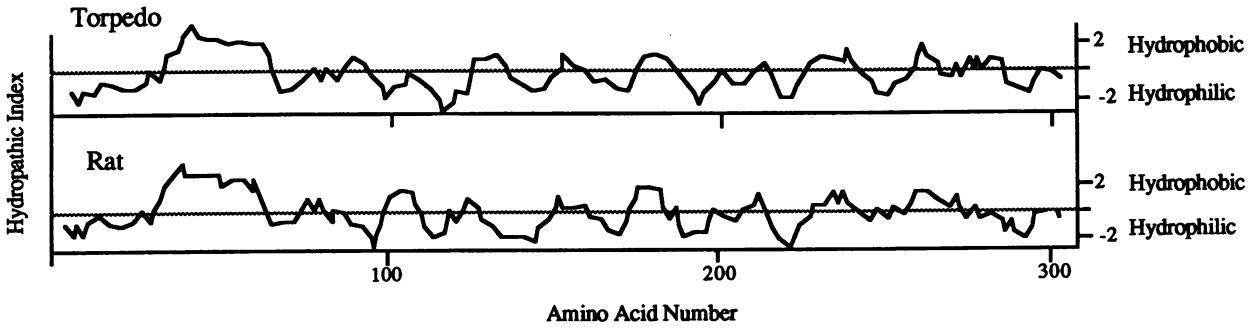

FIG. 4. Comparison of the hydropathy profiles of rat brain and *Torpedo* electroplax Na,K-ATPase β-subunits. Hydropathy plots of the *Torpedo* electroplax (top) and rat brain (bottom) β-subunits were obtained by using the algorithm and hydropathy values of Kyte and Doolittle (16). Hydrophobic regions are above the hatched lines and hydrophilic regions are below.

sequences are conservative. A similar comparison between rat and human β-subunits revealed that 9 of the 15 amino acid substitutions were conservative. A putative transmembrane segment, designated M, located between residues 35 and 62 is highly conserved in all three species.

Hydropathy profiles of the rat brain and *Torpedo* β-subunit sequences obtained by using the algorithm of Kyte and Doolittle (16) are shown in Fig. 4. This analysis predicts that the Na,K-ATPase β-subunits of rat brain and *Torpedo* species contain a highly charged cytoplasmic amino terminus followed by a single hydrophobic transmembrane region of 28 amino acids and a large extracellular carboxy-terminal domain. The secondary structures of rat, human, and *Torpedo* β-subunits also appeared to be virtually identical when analyzed by the method of Chou and Fasman (4; data not shown).

Expression of β-Subunit mRNAs in rat tissues. Rat brain β-subunit cDNA was used to analyze the RNA products of β-subunit gene expression in various rat tissues by hybridization to total cellular RNA on Northern blots. The probe hybridized to mRNAs of ~3, 2.8, 2.6, and 2.4 kilobases (kb) in length in rat brain, bladder, kidney, lung, testis, and heart (Fig. 5). Interestingly, these was no detectable hybridization of the probe to an RNA species in the liver, despite the fact that equivalent amounts of RNA were loaded in each lane. Moreover, when the blot was reprobed with Na,K-ATPase α-subunit cDNA (22), a 5-kb mRNA species was detected in all tissues (data not shown). This result suggests that the absence of β-subunit transcripts in rat liver did not result from degradation of liver RNA.

The 3-kb RNA is the predominant species in rat kidney, whereas the 2.8-kb RNA is the most abundant mRNA in bladder (Fig. 5). In rat brain, the 2.4-kb RNA appears to be the predominant species, while in lung, testis, and heart the 3- and 2.8-kb mRNAs predominate and are of equal abundance. Thus, the expression of β-subunit mRNAs appears to be regulated in a tissue-specific manner.

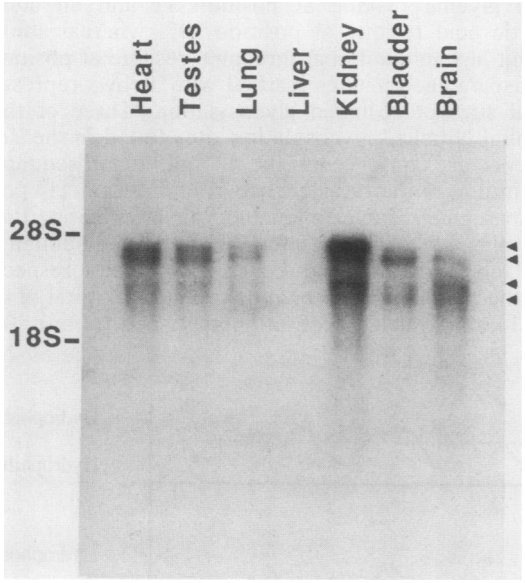

FIG. 5. RNA hybridization analysis of Na,K-ATPase β-subunit gene expression in rat tissues. RNA was prepared from the tissues indicated. Total cellular RNA (10 μg) was fractionated by electrophoresis through a 1% agarose–formaldehyde gel. The RNA was transferred to a nitrocellulose filter and hybridized with 2×10^7 cpm of radiolabeled λrb19 cDNA. The positions of the 28S and 18S RNA markers are indicated on the left. The arrows at the right denote the positions of the β-subunit mRNA sequences.

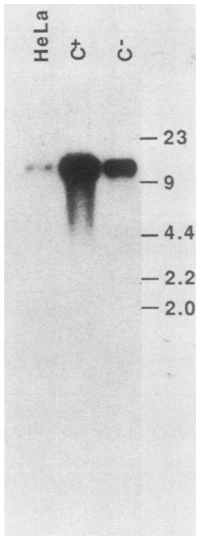

FIG. 6. Amplification of Na,K-ATPase β-subunit genomic DNA sequences in C^+ cells. Total cellular DNA was prepared from HeLa, C^+, and C^- (C^+ cells grown in the absence of ouabain for 4 weeks) cells. DNA was digested with *Eco*RI and electrophoresed through a 0.8% agarose gel. A 10-μg sample of DNA was loaded in each lane. The DNA fragments were transferred to a Zetabind filter (AMF, Meriden, Conn.) and hybridized with 5×10^6 cpm of radiolabeled λrb19 cDNA. Molecular weight markers are shown at the right.

Amplification of β-subunit DNA and mRNA sequences in ouabain-resistant C^+ cells. We used β-subunit cDNA in hybridization experiments to analyze β-subunit DNA content and mRNA expression in C^+ cells. C^+ cells are a ouabain-resistant derivative of HeLa cells (1) and contain amplified levels of Na,K-ATPase α-subunit DNA, mRNA (6, 22), and polypeptide sequences (18).

Southern hybridization analysis of genomic DNA prepared from HeLa, C^+, and C^- (C^+ cells grown in the absence of ouabain for 4 weeks) cells is shown in Fig. 6. In each sample, *Eco*RI digestion generated a single ~12-kb restriction fragment that was reactive with the β-subunit probe and was amplified in C^+ and C^- cells relative to levels in HeLa cells. Quantitation of β-subunit DNA sequences by slot-blot analysis revealed that β-subunit DNA sequences were amplified ~20-fold in C^+ cells and ~5-fold in C^- cells compared with levels in HeLa cells (data not shown).

The mRNA products of β-subunit gene expression in HeLa, C^+, and C^- cells were analyzed by hybridizing β-subunit cDNA to total cellular RNA on Northern blots. β-Subunit cDNA hybridized to mRNA species of ~3, 2.8, 2.6, and 2.4 kb, and each mRNA species appeared to be more abundant in C^+ and C^- cells relative to levels in HeLa cells (Fig. 7). These results clearly demonstrate that Na,K-ATPase β-subunit DNA sequences are amplified in C^+ cells and suggest that the β-subunit plays an important role in the acquisition or expression or both of the ouabain-resistant phenotype.

DISCUSSION

Our results reveal several surprising aspects of the Na,K-ATPase β-subunit. First is the complex pattern of β-subunit gene expression found in most rat tissues and the apparent absence of β-chain mRNA sequences in rat liver. Second is

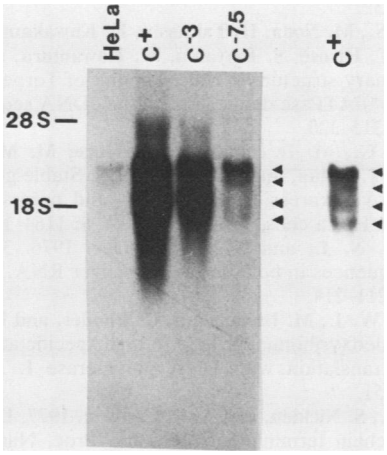

FIG. 7. RNA blot analysis of Na,K-ATPase β-subunit gene expression in HeLa, C^+, and C^- cells. RNA was prepared from HeLa and C^+ cells and C^- cells grown in the absence of ouabain for 3 weeks (C^{-3}) and 7.5 weeks ($C^{-7.5}$). Total cellular RNA (20 μg) was electrophoresed through a 1% agarose–formaldehyde gel. The RNA was transferred to a Zetabind filter and probed with 10^7cpm of radiolabeled λrb19 cDNA. The positions of the 28S and 18S RNA markers are indicated on the left. A lighter exposure of the C^+ lane is shown to the right. When the same blot was reprobed with human actin cDNA, an RNA species which hybridized with approximately equal intensity was observed in all three lanes.

the amplification of β-chain DNA sequences and the overexpression of β-subunit mRNAs in ouabain-resistant C^+ cells. Third is the striking degree of structural homology between the β-subunit polypeptides of three distantly related species: rat, human, and *Torpedo*.

In *Torpedo* species, the β-subunit gene appears to encode only a single mRNA species regardless of which tissue is examined (13; R. W. Mercer, J. W. Schneider, and E. J. Benz, Jr., unpublished data). However, in most rat tissues, rat β-subunit cDNA hybridizes to four distinct mRNA species. The abundance of a particular mRNA species varies from tissue to tissue, suggesting transcriptional or posttranscriptional regulation of β-subunit gene expression. We do not yet know whether these mRNAs arise via differential splicing of a single β-subunit mRNA species or whether they encode different β-subunit isoforms. Further characterization of β-subunit mRNA sequences will be required to resolve this issue.

Hybridization analysis failed to detect β-subunit mRNA transcripts in rat liver. Several potential mechanisms can be suggested to account for this result. One possibility is that the steady-state level of β-subunit mRNA in the liver is below the level of sensitivity of Northern blot hybridization. Alternatively, the liver may contain β-subunit isoforms that do not hybridize with the brain cDNA probe. Our results are consistent with evidence presented by Hubert et al. (11a) suggesting that the liver contains structural variants of the Na,K-ATPase β-subunit. It it interesting to speculate that alternative forms of the β-subunit in the liver may be related to the role of the Na,K-ATPase in bile secretion (7).

Hybridization analysis of HeLa and C^+ revealed the presence of four β-subunit mRNAs. Although the rat β-subunit probe used here does not contain the entire 3′ noncoding region of β-subunit mRNA, previous work has shown that human β-subunit mRNA contains four potential polyadenylation sites (13). Thus, it seems likely that the multiple β-subunit mRNAs in human cells and rat tissues arise by utilization of alternative polyadenylation signals. In this regard, it is interesting that the 3′-untranslated regions of the rat, *Torpedo*, and human β-subunit mRNAs are highly conserved. For example, the rat β-subunit sequence from nucleotides 972 to 1127 exhibits only one nucleotide substitution when compared with the corresponding human sequence (13). This high degree of nucleotide sequence conservation suggests that the 3′-untranslated region plays an important role in β-subunit biogenesis.

Amplification of β-subunit DNA and RNA sequences in ouabain-resistant C^+ cells supports the view that the β-subunit is an essential component of the Na,K-ATPase . The fact that both α- and β-subunits of the enzyme are amplified in C^+ cells implies that both subunits of the ATPase are required for expression of the ouabain-resistant phenotype. The high degree of structural homology between rat, human, and *Torpedo* β-subunit polypeptides further supports the hypothesis that the β-subunit is important for Na,K-ATPase structure and function. The availability of the β-subunit cDNA should allow us to perform experiments designed to elucidate the biochemical role of the β-subunit polypeptide.

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