Molecular Cloning and Immunological Characterization of the γ Polypeptide, a Small Protein Associated with the Na,K-ATPase

Robert W. Mercer,* Daniel Biemesderfer,‡ David P. Bliss, Jr.,* John H. Collins,§ and Bliss Forbush III‡

*Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, Missouri 63110; ‡Departments of Cellular and Molecular Physiology and Internal Medicine, Yale University School of Medicine, New Haven, Connecticut 06510; §Department of Biological Chemistry, University of Maryland School of Medicine, Baltimore, Maryland 21201

Abstract. The γ subunit of the Na,K-ATPase is a small membrane protein that copurifies with the α and β subunits of the enzyme. Strong evidence that the γ subunit is a component of the Na,K-ATPase comes from studies indicating that the subunit is involved in forming the site for cardiac glycoside binding. We have isolated and characterized the cDNAs coding the γ subunit from several species. The γ subunit is a highly conserved protein consisting of 58 amino acids with a molecular weight of 6500. Hydropathy analysis reveals the presence of a single hydrophobic domain that is sufficient to cross the membrane. There are no sites for *N*-linked glycosylation. Northern blot analysis revealed that the γ subunit mRNA is expressed in a tissue-specific fashion and is present in all tissues characterized. γ-specific antibodies have been used to verify that the sequenced protein is the same protein labeled by [³H]nitroazidobenzoyl-ouabain (NAB-ouabain), and that this protein, the γ subunit of the Na,K-ATPase, has a distribution pattern along nephron segments that is identical with the α subunit. In addition, coimmuno-precipitation of the α, β and γ subunits demonstrate specific association of the subunits. These results are consistent with the notion that the γ subunit is specifically associated with and may be an important component of the Na,K-ATPase.

THE Na,K-ATPase is a membrane protein that uses the energy from the hydrolysis of ATP to transport Na and K across the plasma membrane. The enzyme consists of at least two polypeptides, a catalytic α subunit of ∼110 kD and a smaller glycosylated β subunit of ∼55 kD. The α subunit contains the binding site for ATP and the cardiac glycoside inhibitor ouabain; it is phosphorylated and undergoes ligand-dependent conformation changes accompanying the binding and translocation of cations across the plasma membrane. All catalytic functions have been assigned to the α subunit, however, the β subunit may play an essential role in either stabilization, maturation, or enzymatic activity of functional Na,K-ATPase molecules (Gerring, 1991). The amino acid sequences of the α and β subunits of the Na,K-ATpase from several species have been determined from their cDNAs (reviewed in Lingrel et al., 1990; Mercer et al., 1989).

Distinct forms of the rat Na,K-ATPase have been described which can be distinguished by SDS-PAGE (Sweadner, 1979), antigenic determinants (Sweadner and Gilkeson, 1985), and differences in affinity for ouabain (Sweadner, 1979). These differences have been ascribed to the presence of two isoforms of the α subunit (α and α[+]). The cDNAs for the α(+) isoform (termed α2) and an α subunit previously unidentified, designated α3, have been isolated and sequenced (Shull et al., 1986; Takeyasu et al., 1990). In addition to the α isoforms, a putative β subunit isoform cDNA, termed β2, has been identified in mammals (Martin-Vasallo et al., 1989). In glia cells this polypeptide functions as an adhesion molecule and is associated with the α2 subunit of the Na,K-ATPase (Gloor et al., 1990). Characterization of α, α isoform, and β mRNAs has demonstrated that the subunits are expressed in a tissue-specific and developmentally regulated fashion (Mercer et al., 1986; Young and Lingrel, 1987; Schneider et al., 1988; Orlowski and Lingrel, 1988; Martin-Vasallo et al., 1989). Thus, the diversity in subunits and their differential expression and regulation suggests that the Na,K-ATPase may have several specialized functional roles.

Besides the other subunits, a small peptide of ∼10 kD, termed the γ subunit (previously "proteolipid component"), has also been identified in purified preparations of the enzyme. Strong evidence that the γ subunit is a component of the Na,K-ATPase and not a contaminant of purification has come from studies of the cardiac glycoside binding site. These studies have shown that the binding site consists of not only the α and β subunits, but the γ subunit as well (Forbush et al., 1978; Rogers and Lazdunski, 1979; Lowndes et al., 1984). For example, the α and γ subunits are labeled by photoaffinity compounds with approximately equal probability when the reactive groups are 23–24 Å from the lactone ring of the cardiotonic steroid (Forbush, 1983). Although small hydrophobic proteins are associated with the H-ATPase of mitochondria (Blondin, 1979), bacteria (Sierra and Tza-

goloff, 1973) and yeast (Navarre et al., 1992), and the Ca-ATPase of sarcoplasmic reticulum (MacLennan et al., 1972), the significance of these proteins has remained unclear. The exact role of the γ subunit in Na,K-ATPase function is unknown, although its necessity for both ATPase and transport activity has been experimentally questioned (Hardwicke and Freytag, 1981; Horowitz et al., 1991; DeTomaso et al., 1991). For several reasons it has been suggested that the γ polypeptide is a breakdown product of the α and β subunits; the amino acid composition of the γ and α subunits is similar (Collins et al., 1982), antibodies raised to γ cross react with both the α and β subunits (Ball et al., 1983), and a peptide derived from the γ polypeptide fraction contains the NH_2-terminus of the β subunit (Harris and Stahl, 1988). Consequently, the labeling of the γ subunit by ouabain derivatives could be explained if it was a portion of the α or β subunits. However, partial peptide sequencing of HPLC-purified γ subunit has clearly established that the polypeptide is different from the α and β subunits, and therefore cannot be a breakdown product of the larger subunits (Collins and Leszyk, 1987).

Here we summarize recent molecular biological and immunological studies of the γ subunit. Using the DNA PCR and an oligonucleotide probe derived from the amino acid sequence, a cDNA corresponding to the sheep γ subunit was isolated. This clone was subsequently used to identify a cDNA clone containing the complete coding region for the rat kidney γ subunit. Using PCR and oligonucleotide primers based on the sheep and rat nucleotide sequences, the cDNAs coding the γ subunit from the mouse and cow were isolated and their deduced amino acid sequences compared. γ-specific antibodies have been used to demonstrate that the sequenced protein is the same protein labeled by [³H]NAB-ouabain, and that this protein, the γ subunit of the Na,K-ATPase, has a distribution pattern along nephron segments that is identical with the α subunit. These results are consistent with the view that the γ subunit is specifically associated with and may be an important component of the Na,K-ATPase.

Materials and Methods

Isolation of cDNA Clones

The cDNA coding for a portion of the sheep kidney γ subunit was isolated using a modification of the procedure of Frohman et al. (1988). Briefly, poly(A)$^+$ sheep kidney RNA (Davis et al., 1986) was reverse transcribed according to the supplier (GIBCO BRL, Gaithersburg, MD) using a 35-base oligonucleotide containing 17 dT residues and an 18-base adaptor sequence containing three endonuclease recognition sites (dT$_{17}$-adaptor). This reaction mixture was diluted to 0.5 ml with TE buffer (10 mM Tris-Cl, pH 8.0, 1 mM EDTA) and stored at 4°C. 10 μl of the diluted reaction mixture, 50 pmol each of the 18-base adaptor primer and a 38-base γ sequence primer, in 100 μl of PCR buffer (50 mM KCl, 10 mM Tris-Cl, pH 8.3, 1.5 mM MgCl$_2$, 0.2 mM each dNTP, 0.01% [wt/vol] gelatin) were denatured (5 min, 95°C) and cooled to room temperature. The 38-base γ sequence primer, 5'-GAGAA(C,T)GAGGA(C,T)CCITTCTA(C,T)TA(C,T)GA(C,T)TA(C,T)GAGACIGT, was derived from the 13-amino acid NH_2-terminal γ sequence (Collins and Leszyk, 1987). *Thermus aquaticus* (Taq) DNA polymerase (2.5 U; Perkin-Elmer Corp., Norwalk, CT) was added and the mixture overlaid with 100 μl of mineral oil (Sigma Immunochemicals, St. Louis, MO). Using a temperature cycler incubator (Coy Laboratory Products, Grass Lake, MI), the mixture was annealed at 50°C for 2 min, extended at 72°C (3 min), and denatured at 94°C (2 min) for 35 cycles. This was followed by a final extension at 72°C for 15 min. The reaction was electrophoresed (1% agarose) and a band of $\sim$400 bp excised from the gel and

purified using ground glass (Davis et al., 1986). The purified DNA was end repaired using T4 DNA polymerase, digested with EcoRI and ligated into SmaI/EcoRI digested M13mpl8. Several clones were isolated and sequenced using the dideoxy chain termination method of Sanger et al. (1977) as modified by Biggin et al. (1983). One clone, corresponding to the sheep kidney γ sequence was identified, isolated and used to screen a dT-primed λgtll rat kidney library (provided by M. Mueckler, Washington University School of Medicine, St. Louis, MO). Over 10 positive phage clones were plaque purified and their cDNA insert characterized. Based on size all clones appeared to be identical. For the bovine and mouse clones the corresponding mRNA was reverse transcribed as before using oligo dT as a primer. This reaction was diluted to 0.5 ml with TE buffer and used for PCR. The reactions were as described except the 5' primer consisted of 5'-ATGGT(C,G)GC(A,C,T)GT(C,G)CAGG(A,G)(C,G)A(A,C)(C,T)GA(A,G) coding the first eight amino acids and a primer from the 3' untranslated region consisting of 5'-GGCACTGCTGGCTCTC. The PCR reaction was electrophoresed and a band consisting of $\sim$200 bp subcloned into pBluescript II (Stratagene, La Jolla, CA) and sequenced.

RNA Blot Hybridization

RNA was isolated from adult rat tissues, and fractionated by electrophoresis through a 1% agarose formaldehyde gel. The RNA was transferred to nylon (BioTrans; ICN Biomedicals, Costa Mesa, CA) by vacuum (Hoeffer Scientific Instruments, San Francisco, CA) and prehybridized and hybridized as described (Schneider et al., 1985). cDNA probes were labeled employing the Klenow fragment of *E. coli* DNA polymerase I and oligonucleotide primers by the method of Feinberg and Vogelstein (1984).

In Vitro Translation

RNA was prepared using SP6 RNA polymerase as described by the supplier (Promega Corp., Madison, WI). For translations, 3 μl of a 20-μl transcription reaction were added directly to a 30-μl rabbit reticulocyte lysate translation reaction (Promega Corp.), supplemented with 1 μl of dog pancreatic microsomes (Promega Corp.) and containing [³H]leucine (Amersham Corp., Arlington Heights, IL). After 30 min, the reaction was centrifuged in a Beckman Airfuge (Beckman Instruments, Inc., Fullerton, CA), the pellet washed once in 100 μl of 150 mM NaCl, 25 mM Hepes, pH 7.4, and analyzed by SDS-PAGE (Schägger and Jagow, 1987) and fluorography.

Immunological Reagents

The preparation of a mAb (mAb C62.4) to the α subunit of the dog Na,K-ATPase has been previously described (Kashgarian et al., 1985). A polyclonal antibody (Ab-gam) against the γ subunit was raised in rabbits by immunization with dog kidney γ polypeptide obtained by elution from SDS-PAGE gels of purified Na,K-ATPase. Another antibody (Ab-G17) was prepared by immunizing rabbits with hemocyanin-conjugated synthetic peptide prepared to amino acids 6–22 of the sheep γ subunit.

Immunoprecipitation and Immunoblots

Dog renal medullary membranes, enriched in Na,K-ATPase, were prepared as previously described (Forbush and Palfrey, 1983). Membranes were solubilized on ice for 15 min at a concentration of 500 μg/ml in buffer containing 1% detergent (Triton X-100, CHAPS, n-octylglucoside, $C_{12}E_8$ or n-dodecylmaltoside), 50 mM Tris-Cl, pH 7.3, and 100 mM NaCl. Samples were cleared of insoluble material by centrifugation in a microfuge for 10 min at 15,000 g at 4°C. 10 μg of control IgG or 10 μl of mAb C62.4 ascites fluid were added to the cleared sample and allowed to incubate on a Nutator (Clay Adams, Parsippany, NJ) for 1 h at room temperature. Because mouse antibodies have a low affinity for Protein A, 10 μg of rabbit anti-mouse IgG was added, and allowed to incubate for an additional hour. The immune complex was precipitated by the addition of 5 mg swollen Protein A-Sepharose CL-4B to each sample. Samples were washed three times in solubilization buffer, once in the same buffer containing 750 mM NaCl, and once in solubilization buffer diluted 1:10 in water. Samples were boiled for 3 min in 50 μl of sample buffer, separated by SDS-PAGE (Schägger and Jagow, 1987) and electrophoretically transferred to nitrocellulose. Sections of the nitrocellulose were probed using antibodies to the α, β or γ subunits of the Na,K-ATPase as described in the figure legends.

Immunocytochemistry

Kidneys, collected from freshly slaughtered sheep were diced into 3-mm

cubes and fixed in PLP fixative (2% paraformaldehyde, 750 mM lysine, and 10 mM sodium periodate in phosphate buffer, pH 7.4) for 6 h at 4°C. The tissue was cryoprotected by incubating for 1 h in 2.3 M sucrose in phosphate buffer, pH 7.2, with 50% polyvinylpyrrolidone (Tokuyasu, 1991), mounted on aluminum nails, frozen, and stored in liquid nitrogen. Semithin frozen sections (0.5 mm) were prepared with a Reichert Ultracut E ultramicrotome equipped with an FC-4E cryoattachment and mounted on gelatin coated slides (Biemesderfer et al., 1992). Double immunolabeling was carried out as previously described (Biemesderfer et al., 1992). Briefly, semithin cryosections were washed sequentially in PBS and blocking buffer (1% BSA in PBS) for 15 min, after which they were incubated for 1 h with primary antibodies diluted in blocking buffer. Affinity purified anti-γ (Ab-gam) and anti-α (C62.4) were diluted 1:10 and 1:100 respectively. Cryosections were then incubated with FITC-conjugated goat anti-mouse and rhodamine-conjugated goat anti-rabbit F(ab')2, after which they were mounted in 75% glycerol in PBS containing 0.1% p-phenylenediamine to retard fading. To control for cross-reactivity between IgGs, some sections were labeled with each primary antibody alone followed by the combined secondary antibodies. Micrographs were taken using Kodak Tri-X film (Eastman Kodak Co., Rochester, NY) with a Zeiss Axiophot microscope (Carl Zeiss, Inc., Thornwood, NY).

Results

Isolation and Characterization of γ Subunit cDNA Clones

The strategy for the isolation of the cDNA for the γ subunit is based on the procedure of Frohman et al. (1988). mRNA from sheep kidney was isolated and reverse transcribed using a 35-base oligonucleotide with 17 dT residues and an 18-base adaptor sequence containing three endonuclease sites. This reaction creates a single-stranded antisense DNA strand. Next, a 38-base oligonucleotide containing deoxyinosine in two positions (Ohtsuka et al., 1985), and derived from the 13–amino acid NH2-terminal γ peptide sequence (Collins and Leszyk, 1987) using preferred codons, was annealed to the cDNA strand and used as a primer to generate the complementary second strand. Repetitive PCR cycles using the γ subunit primer and the 3' adaptor primer resulted in the specific amplification of the double-stranded γ subunit cDNA. Using this technique a cDNA clone corresponding to a portion of the sheep kidney γ subunit was isolated. This clone was subsequently used to identify and isolate a cDNA containing the complete coding region for the rat kidney γ subunit. Primers based on the sheep and rat DNA sequences were used in PCR reactions to isolate cDNAs from the murine and bovine kidney. The nucleotide and deduced amino acid sequences from rat, sheep, cow, and mouse γ subunits are shown in Fig. 1.

As shown, the rat cDNA consists of 645 nucleotides with an open reading frame beginning from the first ATG triplet downstream of an in-frame termination codon (TGA) beginning at position 6. The initiation codon is found within the consensus sequence thought to be necessary for efficient translation of eucaryotic mRNAs (Kozak, 1989). An open reading frame extends from this ATG triplet to position 174 and codes for a polypeptide of 58 amino acids with a molecular mass of 6500. The γ subunits exhibit ~93% amino acid similarity (72% identity, 21% favored substitutions). There are no sites for N-linked glycosylation. The hydropathy profile (Kyte and Doolittle, 1982; Eisenberg et al., 1984) of the deduced amino acid sequence of the rat kidney γ subunit is shown in Fig. 2. The analysis predicts a single hydrophobic transmembrane domain consisting of 19 amino acids (amino acids 21–39) and highly charged intra- and extracel-

RAT   CGAAATGACAGAGCTGTCAGCTAACC

SHEEP   TTCTATTATGACTATGAGACGGTCCGCAAT
RAT   ATGGTGGCAGTGCAGGGGACGGAGAATCCCTTCGAGTATGACTATGAAACCGTCCGCAAA   60
MOUSE   ATGGTGGCTGTGCAGGGGACTGAGAATCCCTTCGAGTACGACTATGAAACAGTCCGCAAA
BOVINE ATGGTGGCTGTGCAGGGGACCGAGGACCCATTCTACTATGACTACGAGACCGTCCGTAAT

SHEEP   E N E D P F Y Y D Y E T V R N
                                             F Y Y D Y E T V R N
RAT   M V A V Q G T E N P F E Y D Y E T V R K
MOUSE   M V A V Q G T E N P F E Y D Y E T V R K
BOVINE M V A V Q G T E D P F Y Y D Y E T V R N

SHEEP   GGGGGCCTGATCTTCGCCGCCCTGGCCTTCATCGTGGGGCTCGTCATCATCCTCAGCAAA
RAT   GGAGGCCTGATCTTCGAGGGCCTTGCCTTCGTCGTGGGACTCCTCATTCTCCTCAGCAAA   120
MOUSE   GGAGGCCTGATCTTCGCGGGCCTGGCCTTCGTCGTGGGCCTCCTCATCATTCTCAGCAAA
BOVINE GGGGGCCTGATCTTCGCCGCCCTGGCCTTCATCGTGGGGCTCGTCATCATCCTCAGCAAA

SHEEP   G G L I P A A L A F I V G L V I I L
        G G L I P A A L A F I V G L V I I L S K
RAT   G G L I P A G L A F V V G L L I L L S K
MOUSE   G G L I P A G L A F V V G L L I I L S K
BOVINE G G L I P A A L A F I V G L V I I L S K

SHEEP   AGATTCCGCTGCGGGGCCAAGAAGAAGCACCGGCAAATTCCTGAAGATGGGCTCTGAGGA
RAT   AGATTCCGCTGTGGGGGCAGTAAGAAGCATAGGCAGGTCAATGAAGATGAGCTGTGACGG   180
MOUSE   AGGTTCCGCTGTGGGGGCGGTAAGAAACATAGGCAGGTCAATGAAGATGAACTGTGACAG
BOVINE AGATTCCGCTGCGGGGCCAAGAGGCAGCACCGGCAAATTCCTGAAGATGGGCTCTAAGGA

SHEEP   R F R C G A K K K H R Q I P E D G L End
RAT   R F R C G G S K K H R Q V N E D E L End
MOUSE   R F R C G G G K K H R Q V N E D E L End
BOVINE R F R C G A K R Q H R Q I P E D G L End

SHEEP   GAGAGCCAGCCGTGCCCACCCTTGCACCGGGGCTGCTTGGAAGTCTTCCCTGAGCTGCAG
RAT   AAGAGCCGGCAGTGCCACCGGTGCATGGGCACAGTGATGCCACTTCCCCAGCCAGGCACC   240
MOUSE   AAGAGCCAGCAGTGCC
BOVINE GAGAGCCAGCAGTGCC

SHEEP   ACTTCCGTCCCAGCACTGTCAGGCCCCTCGGACTGAAGGAAGCTTCGGGCTCCACGTGAC
RAT   ACGGACCCCACCATGGTCAGCACTGGCTGAAGGAAGTGCCAGGCTTACTGGACCCCTCAT   300

SHEEP   CCCCCGGTGGC
RAT   GGTCATCCCTCATGCCTCTAGATCGTTAACTTCCTCTCTCCCCTCCCCAACCTCTCTTAC   360
        ACCCAACTCCCTATTCCAAGTTGGGGTACATGTGCAATCTCATTGCCCTGGTTTTCAATG   420
        AATCGCTCTACAAGAATCTGCCAACAAAAGCAGGCTCAGTGTGGTCATCTACGGGAGTGT   480
        ATCCTTCGCCCCTGAACAGTTACTCTGGGGACAGTTACCCTGCCTGACTTCTGGGCGCCC   540
        TGCCAAAGTCTGAAGCTGTTAAGATGAAGGTGACTTGGTGGACTGGGTCCTGCTGCTGCT   600
        GCTGCTGCTGCTGCTGCTG

Figure 1. Nucleotide and deduced amino acid sequences of the γ subunit of the Na,K-ATPase. The amino acid sequence for the sheep kidney γ subunit determined from peptide analysis is shown underlined, above the deduced amino acid sequences for the sheep, rat, mouse, and bovine γ subunits. The portion of the cDNA nucleotide sequences corresponding to the PCR primers are underlined. These sequences are available from EMBL under accession numbers X70059 (bovine), X70060 (mouse), 70061 (sheep), and X70062 (rat).

lular domains. Until the site that interacts with the cardiac glycosides is determined it is not known which portions of the subunit are cytoplasmic or extracellular.

Expression of γ Subunit mRNAs in Rat Tissues

Rat kidney γ subunit cDNA was used to analyze the RNA products of γ subunit gene expression in several rat tissues by hybridization to total cellular RNA. As shown in Fig. 3 B the γ subunit gene encoded two RNA species of ~1.5 and 0.8 kb in length. The smaller transcript was more abundant in the kidney, with lower levels in the spleen, lung, and heart. In contrast, the larger transcript was more abundant in the spleen and lung. Thus, the expression of γ subunit mRNA appears to be regulated in a tissue-specific fashion. Moreover, in contrast to α subunit mRNA expression shown in Fig. 3 A, total γ mRNA levels are higher in the spleen and lower in the brain. In the kidney, however, it appears that the level of α1, β1, and γ mRNA expressed is similar, suggesting that the subunits may be coordinately regulated (data not shown).

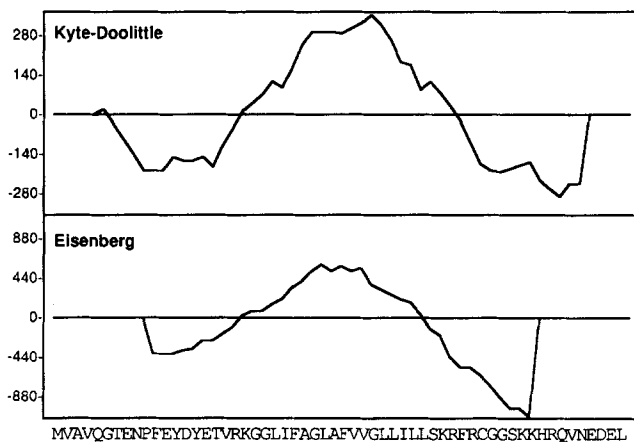MVAVQGTENPFEYDYETVRKGGLIFAGLAFVVGLLILLSKRFRCGGSKKHRQVNEDEL

Figure 2. Hydropathy profiles of the γ subunit of the Na,K-ATPase. Hydropathy profiles were determined using the procedure of Kyte-Doolittle (1982) and Eisenberg et al. (1984). Hydrophobic regions are above the *x*-axis and hydrophilic regions are below. Plots were generated using Geneworks from IntelliGenetics, Inc. (Mountain View, CA).

Immunological Characterization of the γ Polypeptide

The γ subunit was characterized using two polyclonal antibodies to the γ polypeptide; the first of these (Ab-gam) was raised by immunization with dog kidney γ peptide obtained by elution from SDS polyacrylamide gels of purified Na,K-ATPase. The second antibody (Ab-G17) was prepared by immunization with hemocyanin-conjugated synthetic peptide prepared to amino acids 6–22 (G-17) of the sheep γ subunit. For some purposes the antibodies were purified by adsorption to nitrocellulose-immobilized dog kidney γ peptide.

To characterize the specificity of the γ reactive antisera, renal membranes were analyzed by SDS-PAGE (Fig. 4). As shown in Fig. 4 *B* (Ab-gam) and C (Ab-G17), the antisera react to two polypeptides of ∼8.8 and ∼9.9 kD (described in recent reports as 6.7 and 7.6 kD because of an error in commercial molecular weight standards; Kratzin et al., 1989).

Figure 4. Immunological characterization of the γ polypeptide. (*A*) Coomassie-stained SDS gel. (*B*) Corresponding immunoblot with Ab-gam. (*C*) Corresponding immunoblot with Ab-G17. Lanes *1*, Dog renal membranes; lanes *2*, [³H]NAB-ouabain–labeled purified dog kidney Na,K-ATPase; Lanes *3*, purified dog kidney Na,K-ATPase; Lanes *4*, lamb renal membranes; (*D*) In vitro translation using rabbit reticulocyte lysate supplemented with rough microsomes. Lane *1*, rat γ subunit synthetic mRNA; Lane *2*, no RNA control. The labeled protein in both lanes is globin.

The antisera do not recognize either the α and β subunits. It is not known why there is a difference in the molecular weight as deduced from the cDNA from that determined by SDS-PAGE. In addition, the reason γ migrates as a doublet is not clear, but importantly the same protein doublet is seen when synthetic γ subunit RNA is translated in vitro. Using rabbit reticulocyte lysate, synthetic RNA prepared from the γ cDNA was translated in the presence of dog pancreatic microsomes. Microsomal membranes were isolated and analyzed by SDS-PAGE. As shown in Fig. 4 *D* two proteins are translated in the presence of the single message. These bands correspond in size to the immunoreactive proteins identified by the γ antisera. Consequently, it appears that the two immunoreactive γ polypeptides are most likely products of a single RNA message.
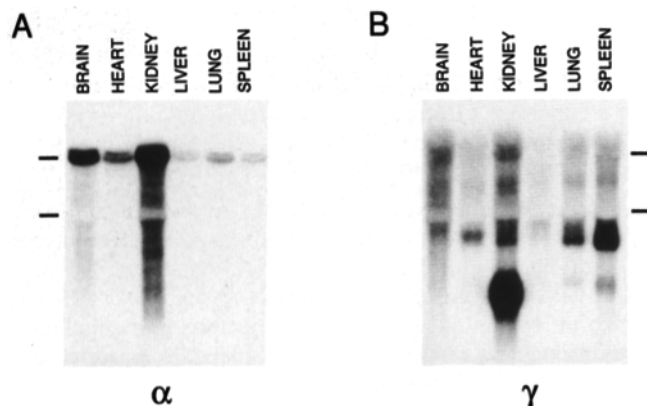

Figure 3. RNA hybridization analysis of the Na,K-ATPase γ subunit gene expression in rat tissues. RNA was prepared from the tissues indicated. Total cellular RNA (10 µg) was fractionated by electrophoresis through a 1% agarose-formaldehyde gel. The RNA was transferred to a nitrocellulose filter and hybridized to (*A*) α or (*B*) γ subunit cDNA. The horizontal bars indicate the positions of the 28S and 18S ribosomal RNAs.
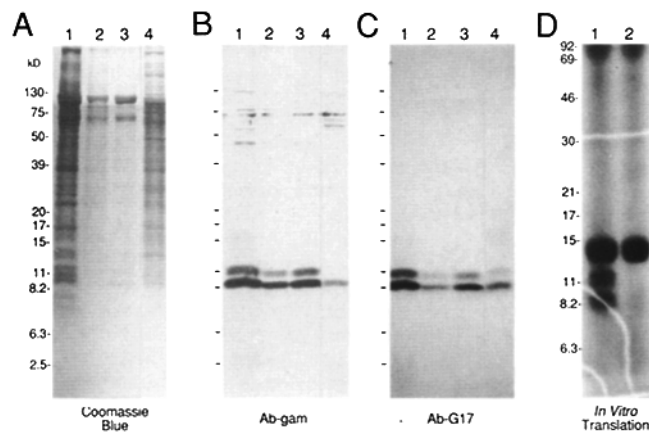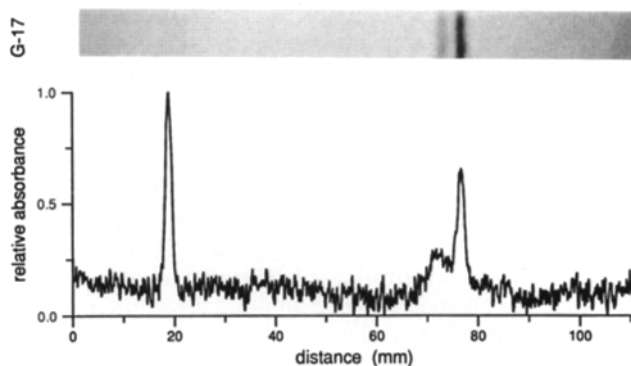

Figure 5. Identification of [³H]NAB-ouabain–labeled γ polypeptide. [³H]NAB-ouabain–labeled dog kidney Na,K-ATPase was separated by SDS-PAGE and electrophoretically transferred to nitrocellulose. The autoradiograph of the blot was scanned and plotted relative to the labeling of the α subunit. The immunoblot with antibody G-17 is shown above.
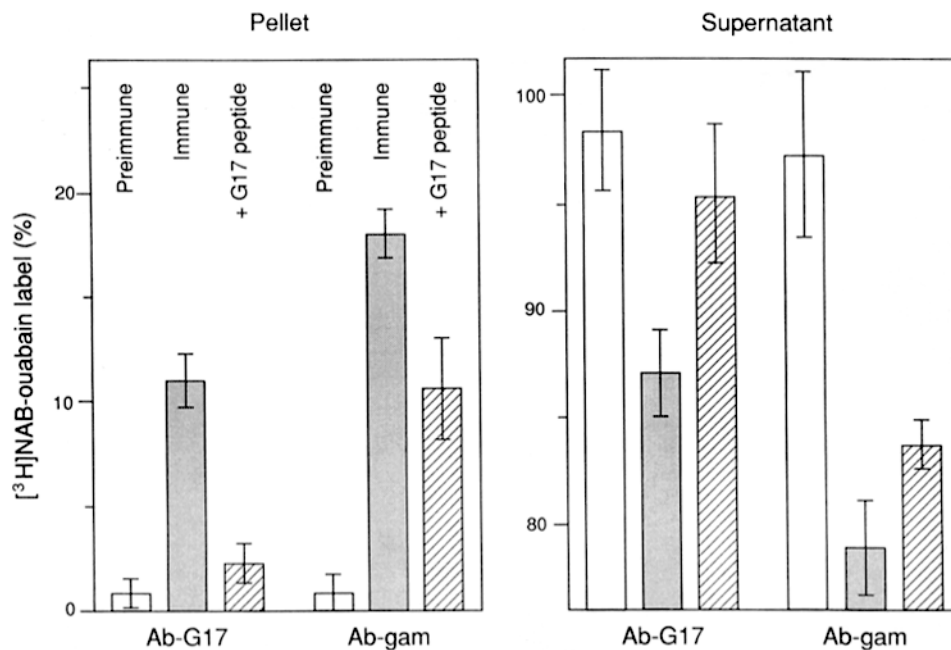
Figure 6. Immunoprecipitation of [³H]NAB-ouabain–labeled γ polypeptide. [³H]NAB-ouabain-labeled γ polypeptides were immunoprecipitated with either Ab-gam, Ab-G17 or preimmune antisera. The radioactivities of the precipitates and supernatants were determined and expressed as the percent of total labeled protein. Where indicated 15 µg of G17 peptide was added.

Identification and Immunoprecipitation of [³H]Nitroazidobenzoyl-ouabain–Labeled γ Polypeptide

The photoaffinity derivative of ouabain, nitroazidobenzoyl-(NAB)[1]-ouabain, specifically inhibits and covalently labels the Na,K-ATPase (Forbush et al., 1978). The results of an experiment designed to test if the γ antisera recognize polypeptides that have been specifically labeled with [³H]NAB-ouabain are shown in Fig. 5. [³H]NAB-ouabain–labeled polypeptides were separated by SDS-PAGE, transferred to nitrocellulose and analyzed for reactivity to the G-17 antiserum. As shown, the major polypeptides labeled by NAB-ouabain correspond to the α and γ subunits of the Na,K-ATPase, however, only the polypeptides that correspond to the labeled γ subunit of the Na,K-ATPase are immunoreactive. Additional evidence that the antisera identifies polypeptides that are specifically labeled by [³H]NAB-ouabain is shown in Fig. 6. Labeled γ polypeptides were immunoprecipitated with either Ab-gam or Ab-G17 antisera. When compared with preimmune sera, both antisera immunoprecipitate solubilized NAB-ouabain–labeled polypeptides. Moreover, the immunoprecipitation of labeled γ polypeptide is specific since it can be partially (Ab-gam) or completely blocked (Ab-G17) by the presence of the G-17 peptide.

The γ and α subunits of the Na,K-ATPase Colocalize in the Sheep Kidney

To immunolocalize both γ and α subunits of the Na,K-ATPase in the kidney, double-label immunofluorescence experiments were carried out using antibodies specific for each subunit. As shown in Fig. 7, antibodies to both proteins colocalized to the basolateral membranes of the tubular epithelial cells in sheep kidney. In addition, staining of both antibodies varies axially along the nephron with cells of the distal convoluted tubule, connecting tubule cells and principal cells having the strongest staining while the proximal segments were stained less intensely. Both antibodies failed to stain a subpopulation of cells in connecting tubules and collecting ducts presumed to be intercalated cells. Similar results were obtained in rat kidney using the Ab-gam antibody. These results are in agreement with previous studies showing that the expression of the Na,K-ATPase is restricted to the basolateral membrane in renal epithelial cells and varies in its level of expression along the nephron (Kashgarian et al., 1985). As shown in Fig. 7, the γ subunit specifically codistributes with the α subunit and is present or absent only in conjunction with the α subunit, supporting the notion that the polypeptide is specifically associated with the Na,K-ATPase.

The γ subunit Coimmunoprecipates with the α and β subunits of the Na,K-ATPase

Having demonstrated that γ colocalized with the Na,K-ATPase in renal epithelial cells, biochemical experiments were carried out in order to establish direct molecular interaction of the γ with the Na,K-ATPase. For this purpose the subunit composition of immunopurified enzyme was analyzed following solubilization in various detergents (Fig. 8). The Na,K-ATPase was immunoprecipitated using a mAb to the α subunit (C62.4) and the resulting immunoprecipitate probed on nitrocellulose with anti-α, -β and -γ antibodies. Fig. 8 shows that α, β, and γ subunits were coprecipitated after solubilization in CHAPS. However, when the enzyme was solubilized using n-octylglucoside only the α and β subunits were detected. Only the α and β subunits were detected when membranes were solubilized with Triton X-100, $C_{12}E_8$ or n-dodecylmaltoside (data not shown). These results demonstrate that the γ subunit forms a molecular assembly with the α and β subunits of the Na,K-ATPase via interactions that can be disrupted upon detergent solubilization.

Discussion

We have isolated the cDNAs from several species coding the

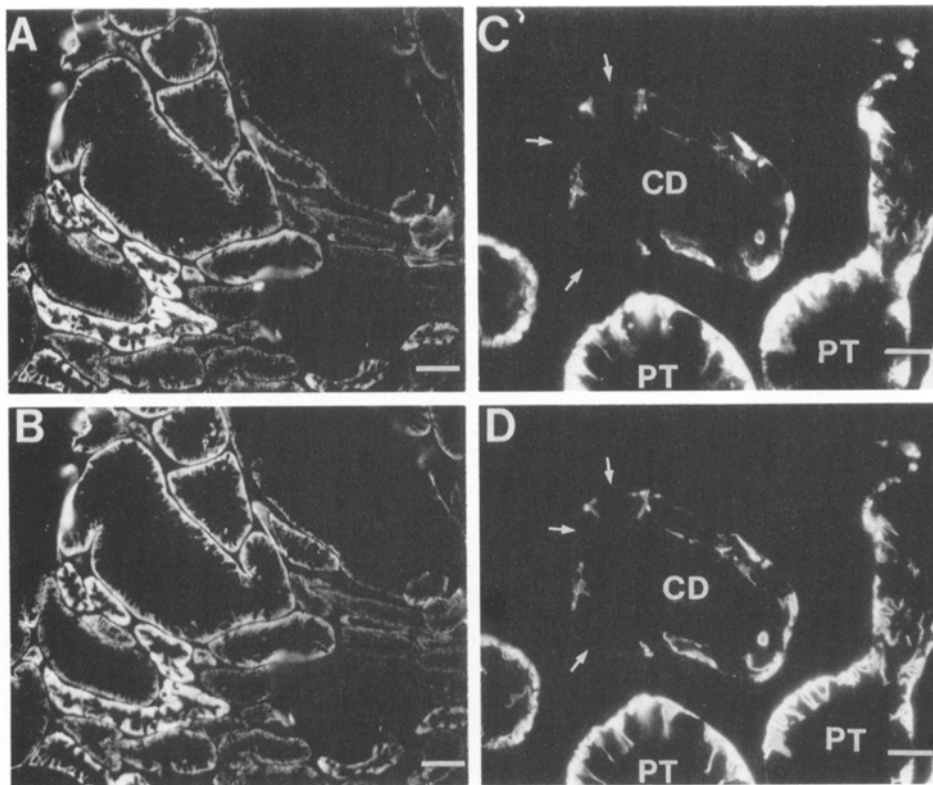1. *Abbreviation used in this paper:* NAB, nitroazidobenzoyl.

Figure 7. Immunofluorescence microscopy demonstrating the localization of the γ and α subunits of the Na,K-ATPase in sheep renal cortex. Semithin (0.5 μm) cryosections of PLP-fixed sheep kidney were double labeled with affinity purified Ab-G17 (*A* and *C*) and detected with a rhodamine-conjugated secondary antibody and with mAb C62.4 (*B* and *D*) which was detected with a fluorescein-conjugated secondary antibody. *A* and *B* show labeling with the two antibodies at low magnification. *C* and *D* demonstrate staining with both antibodies in the cortical collecting duct (*CD*) and in the proximal tubules (*PT*). Arrows indicate collecting duct cells that are negative or weakly stained relative to the other cells in this segment. Bars: (*A* and *B*) 100 μm; (*C* and *D*) 20 μm.

γ subunits of the Na,K-ATPase. As deduced from the nucleotide sequence the primary structure of the subunit consists of 58 amino acids with a molecular mass of 6,500. The deduced sheep amino acid sequence is identical with the partial sequence obtained by peptide analysis of the sheep γ sub-
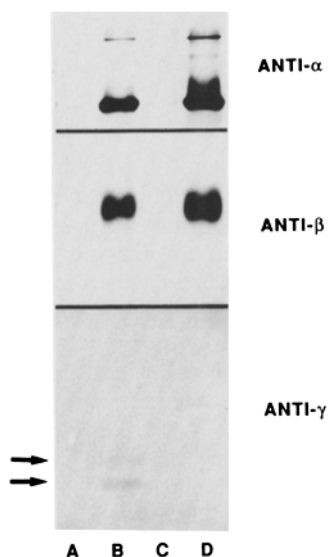

Figure 8. γ coimmunoprecipitates with both the α and β subunits of the Na,K-ATPase. Dog renal membranes enriched in Na,K-ATPase were solubilized in either CHAPS (lanes *A* and *B*) or octylglucoside (lanes *C* and *D*). Immunoprecipitation was carried out with either a control mAb (lanes *A* and *C*) or with a mAb to the α subunit to the Na,K-ATPase (C62.4) (lanes *B* and *D*). The immune complexes were separated by SDS-PAGE on a 16% gel and electrophoretically transferred to nitrocellulose. The nitrocellulose was cut into three sections corresponding to regions known from previous studies to contain the α, β, or γ subunits. Each section was immunoblotted with anti-α, anti-β, or anti-γ antibodies, respectively. The α and β subunits are seen as 100 and 50 kD proteins, respectively (lanes *B* and *D*). γ was detected as a doublet (*arrows*) only in samples solubilized with CHAPS (lane *B*). Note that no proteins were stained in control *A* and *C*.

unit. Hydropathy analysis of the amino acid sequence suggests the presence of one transmembrane domain. There are no sites for N-linked glycosylation.

In the rat, the γ subunit gene encodes two mRNA species that are expressed in a tissue-specific manner. The tissue-specific pattern of expression of the two mRNAs suggests that their noncoding sequences may contain functional information important in the regulation of enzyme biosynthesis. As has been shown for the β subunit (Noguchi et al., 1986), the heterogeneity of γ subunit mRNAs may result from multiple transcription initiation or polyadenylation sites. Though the levels vary considerably, RNAs encoding the γ subunit are present in all tissues examined. Highest levels of γ RNA was expressed in the kidney and spleen. However, in marked contrast to the α and β subunits, there is little γ mRNA expression in the brain. This implies that translational or posttranslational regulation of the subunits maintains the necessary quantities of the Na,K-ATPase polypeptides. Alternatively, the possibility that an isoform of the γ subunit may be present cannot be excluded.

Comparison of the γ amino acid sequences demonstrates a high degree of homology (93% amino acid similarity, 72% identity) between the subunits. The high degree of homology is consistent with the view that the γ subunit may be an essential component of Na,K-ATPase structure or function. Strong evidence that the identified and sequenced γ polypeptide is specifically associated with the Na,K-ATPase is provided by the immunological characterization of the subunit. Antibodies raised against either the purified γ subunit from dog kidney or to a synthetic peptide from the sheep γ sequence react with only two kidney membrane proteins of ∼8.8 and ∼9.9 kD. Moreover, similar size polypeptides are produced by the in vitro expression of synthetic γ subunit

mRNA. Also, the γ subunit antisera is immunoreactive with the in vitro synthesized γ protein and both antisera immunoprecipitate solubilized [³H]NAB-ouabain–labeled γ subunit.

In the kidney the Na,K-ATPase is confined to the basolateral membrane of the renal tubular epithelial cells, and varies in density and distribution along the nephron. The strongest staining of the Na,K-ATPase α subunit is found in the thick ascending limb of Henle's loop and in the distal convoluted tubule (Kashgarian et al., 1985). Immunofluorescence microscopy using purified antibody to the dog kidney γ subunit indicates that the subunit is found only on the basolateral membrane of the tubular epithelium in rat kidney. In addition, double immunofluorescence using the γ subunit antisera and an antiserum specific to the α subunit demonstrates that the γ subunit has a distribution pattern identical with the α subunit. Coimmunoprecipitation experiments demonstrated a direct association of γ with the α and β subunits. These results strongly suggest that the γ subunit is an integral component of the Na,K-ATPase.

There are a growing number of proteins associated with, or a component of, the Na,K-ATPase. For example, ankyrin, a linker protein that in erythrocytes mediates the attachment of the spectrin-actin cytoskeleton to the anion transporter in the plasma membrane, specifically binds to the α subunit of the Na, K-ATPase (Nelson and Veshnock, 1987; Morrow et al., 1989; Koob et al., 1990). The colocalization of ankyrin and spectrin homologues with the Na,K-ATPase at the basolateral surface of kidney epithelial cells has suggested a mechanism for the maintenance of the polarized distribution of this enzyme (Nelson and Veshnock, 1987; Hammerton et al., 1991). Moreover, a putative isoform of the β subunit, termed β2, has been identified as an adhesion molecule of glial (AMOG) cells (Martin-Vasallo et al., 1989; Gloor et al., 1990). AMOG is a Ca²⁺-independent adhesion molecule, which in glia cells, mediates neuron–astrocyte adhesion. Consequently, it appears that the Na,K-ATPase may function as a linkage between extracellular adhesion and the intracellular cytoskeleton. In addition, two other proteins have been shown to be associated with the Na,K-ATPase (Kraemer et al., 1990). Pasin 1 and pasin 2 are peripheral membrane proteins with apparent molecular masses of 77 and 73 kD, respectively, which purify and immunoprecipitate with the Na,K-ATPase. In tissue sections, pasin 1 is colocalized with the Na,K-ATPase to the basolateral surface of columnar epithelial cells (Kraemer et al., 1990). The role of these peripheral proteins in Na,K-ATPase function is unknown.

The exact role of small membrane proteins in the regulation of cation transport is unclear. However, the Ca-ATPase of the sarcoplasmic reticulum is under regulation by phospholamban, a small membrane protein with a molecular weight of 6,000. In reconstituted vesicles, inclusion of phospholamban with the Ca-ATPase inhibits Ca uptake. Phosphorylation of phospholamban by the catalytic subunit of cAMP-dependent protein kinase reverses the inhibitory effect of phospholamban (James et al., 1989). Studies using a cross-linking agent (James et al., 1989) and a synthetic peptide derived from phospholamban (Kim et al., 1990) suggest that phospholamban interacts directly with the Ca-ATPase. The mechanism of regulation of the Ca-ATPase by phospholamban remains unknown. Although phospholamban and the γ subunit are of similar size they have no se-

Figure 9. Amino acid sequence homology between the γ subunit of the Na,K-ATPase and phospholemman. The amino acid sequences of the rodent Na,K-ATPase γ subunit (residues 8–57) and canine phospholemman (6–55; from Palmer et al., 1991) are shown. Identical residues are boxed and conserved residues marked by a line.

quence homology (Fujii et al., 1987), therefore, it seems likely that the γ subunit has a function separate from the role of phospholamban. Interestingly, another small membrane protein with a structure similar to phospholamban and containing a region homologous to the γ subunit has been recently identified (Palmer et al., 1991). This 8.4-kD protein, called phospholemman, is a membrane protein that in myocardium is the major plasma membrane substrate for cAMP-dependent protein kinase and protein kinase C. Recently, phospholemman has been shown to function as a chloride channel (Moorman et al., 1992). A stretch of 50 amino acids that contains the transmembrane domain of the polypeptides exhibits 48% sequence similarity with the γ subunit (Fig. 9). The region of homology excludes the phosphorylation sites of phospholemman suggesting that γ subunit has a function not requiring regulation by multisite phosphorylation.

Although the function of the γ subunit must await further investigation, our results are consistent with the notion that the polypeptide is a component of the Na,K-ATPase. The γ subunit is specifically labeled by photoreactive inhibitors of the Na,K-ATPase, it is evolutionarily conserved across species, and it colocalizes with the α subunit in the kidney. The availability of γ cDNA and antibodies should be useful for studies designed to understand the possible roles of the γ subunit in Na,K-ATPase structure, function and assembly.

References

Ball, W. J., J. H. Collins, L. Land, and A. Schwartz. 1983. Antigenic properties of the α-, β-, and γ-subunits of Na,K-ATPase. *In* Current Topics in Membranes and Transport. Vol. 19. J. F. Hoffman and B. Forbush, editors. Academic Press, New York. 781–785.

Biemesderfer, D., G. Dekan, P. S. Aronson, and M. G. Farquhar. 1992. Assembly of distinct coated pit and microvillar microdomains in the renal brush border. *Am. J. Physiol.* 262:F55–F67.

Biggin, M. D., T. J. Gibson, and G. F. Hong. 1983. Buffer gradient gels and ³⁵S label as an aid to rapid DNA sequence determination. *Proc. Natl. Acad. Sci. USA.* 80:3963–3965.

Blondin, G. A. 1979. Resolution of the mitochondrial N,N'-dicyclohexylcarbodiimide binding proteolipid fraction into three similar sized proteins. *Biochem. Biophys. Res. Commun.* 87:1087–1094.

Collins, J. H., and J. Leszyk. 1987. The "γ subunit" of Na,K-ATPase: a small, amphiphilic protein with a unique amino acid sequence. *Biochemistry.* 26:8665–8669.

Collins, J. H., B. Forbush, L. K. Lane, E. Schwartz, and A. S. Zot. 1982. Purification and characterization of an (Na⁺+K⁺)-ATPase proteolipid labeled with a photoaffinity derivative of ouabain. *Biochim. Biophys. Acta.* 686:7–12.

Davis, L. G., M. D. Dibner, and J. F. Battey. 1986. Basic Methods in Molecular Biology. Elsevier Science Publishing Co., New York. 123–125.

De Tomaso, A. W., P. Zdankiewicz, and R. W. Mercer. 1991. Functional expression of the Na,K-ATPase in *Spodoptera frugiperda* cells. *In* The Sodium

Pump. P. De Weer and J. H. Kaplan, editors. Rockefeller University Press, New York. 69–73.

Eisenberg, D., E. Schwarz, M. Komaromy, and R. Wall. 1984. Analysis of membrane and surface protein sequences with the hydrophobic moment plot. *J. Mol. Biol.* 179:125–142.

Feinberg, A., and B. Vogelstein. 1984. Addendum. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* 167:266–267.

Forbush III, B. 1983. Cardiotonic steroid binding to Na,K-ATPase. *In* Current Topics in Membranes and Transport. Vol. 19. J. F. Hoffman and B. Forbush, editors. Academic Press, New York. 167–201.

Forbush III, B., and H. C. Palfrey. 1983. [³H]Bumetanide binding to membranes isolated from dog kidney outer medulla. *J. Biol. Chem.* 258: 11787–11792.

Forbush III, B., J. H. Kaplan, and J. F. Hoffman. 1978. Characterization of a new photoaffinity derivative of ouabain: labeling of the large polypeptide and of a proteolipid component of the Na,K-ATPase. *Biochemistry.* 17:3667–3676.

Frohman, M. A., M. K. Dush, and G. R. Martin. 1988. Rapid production of full-length cDNAs from rare transcripts: Amplification using single gene-specific oligonucleotide primer. *Proc. Natl. Acad. Sci. USA.* 85:8998–9002.

Fujii, J., A. Ueno, K. Kitano, S. Tanaka, M. Kadoma, and M. Tada. 1987. Complete complementary DNA-derived amino acid sequence of canine cardiac phospholamban. *J. Clin. Invest.* 79:301–304.

Geering, K. 1991. The functional role of the β-subunit in the maturation and intracellular transport of Na,K-ATPase. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 285:189–193.

Gloor, S., H. Antonicek, K. J. Sweadner, S. Pagliusi, R. Frank, M. Moos, and M. Schachner. 1990. The adhesion molecule on glia (AMOG) is a homologue of the β subunit of the Na,K-ATPase. *J. Cell Biol.* 110:165–174.

Hammerton, R. W., K. A. Krzeminski, R. W. Mays, T. A. Ryan, D. A. Wollner, and W. J. Nelson. 1991. Mechanism for regulating cell surface distribution of Na⁺,K⁺-ATPase in polarized epithelial cells. *Science (Wash. DC).* 254:847–850.

Hardwicke, P. M. D., and J. W. Freytag. 1981. A proteolipid associated with Na,K-ATPase is not essential for ATPase activity. *Biochem. Biophys. Res. Commun.* 102:250–257.

Harris, W. E., and W. L. Stahl. 1988. Origin of the γ polypeptide of the Na⁺/K⁺-ATPase. *Biochim. Biophys. Acta.* 942:236–244.

Horowitz, B., K. A. Eakle, G. Scheiner-Bobis, G. R. Randolph, C. Y. Chen, R. A. Hitzeman, and R. A. Farley. 1991. Synthesis and assembly of functional mammalian Na,K-ATPase in yeast. *J. Biol. Chem.* 265:4189–4192.

James, P., M. Inui, M. Tada, M. Chiesi, and E. Carafoli. 1989. Nature and site of phospholamban regulation of the Ca²⁺ pump of sarcoplasmic reticulum. *Nature (Lond.).* 342:90–92.

Kashgarian, M., D. Biemesderfer, M. Caplan, and B. Forbush III. 1985. Monoclonal antibody to Na,K-ATPase: immunocytochemical localization along nephron segments. *Kidney Int.* 28:293–301.

Kim, H. W., N. A. Steenaart, D. G. Ferguson, and E. G. Kranias. 1990. Functional reconstitution of the cardiac sarcoplasmic reticulum Ca²⁺-ATPase with phospholamban in phospholipid vesicles. *J. Biol. Chem.* 265: 1702–1709.

Koob, R., D. Kraemer, G. Trippe, U. Aebi, and D. Drenckhahn. 1990. Association of kidney and parotid Na⁺,K⁺-ATPase microsomes with actin and analogs of spectrin and ankyrin. *Eur. J. Cell Biol.* 53:93–100.

Kozak, M. 1989. The scanning model for translation: An update. *J. Cell Biol.* 108:229–241.

Kraemer, D., R. Koob, B. Friedrichs, and D. Drenckhahn. 1990. Two novel peripheral membrane proteins, Pasin 1 and Pasin 2, associated with Na⁺,K⁺-ATPase in various cells and tissues. *J. Cell Biol.* 111:2375–2383.

Kratzin, H. D., J. Wiltfang, M. Karas, V. Neuhoff, and N. Hilschmann. 1989. Gasphase sequencing after electroblotting on polyvinylidene difluoride membranes assigns correct molecular weights to myoglobin molecular weight markers. *Anal. Biochem.* 183:1–8.

Kyte, J., and R. F. Doolittle. 1982. A simple method for displaying the hydrophobic character of a protein. *J. Mol. Biol.* 157:105–132.

Lingrel, J. B., J. Orlowski, M. M. Shull, and E. M. Price. 1990. Molecular genetics of Na,K-ATPase. *Prog. Nucleic Acid Res.* 38:37–89.

Lowndes, J. M., M. Hokin-Neaverson, and A. E. Ruoho. 1984. Photoaffinity labeling of (Na⁺ + K⁺)-ATPase with [¹²⁵I] iodoazidocymarin. *J. Biol. Chem.* 259:10533–10538.

MacLennan, D. H., C. C. Yip, G. H. Iles, and P. Seeman. 1972. Isolation of sarcoplasmic reticulum proteins. *Cold Spring Harbor Symp. Quant. Biol.*

37:469–477.

Martin-Vasallo, P., W. Dackowski, J. R. Emanuel, and R. Levenson. 1989. Identification of a putative isoform of the Na,K-ATPase β subunit. *J. Biol. Chem.* 264:4613–4618.

Mercer, R. W., J. W. Schneider, and E. J. Benz, Jr. 1989. Na,K-ATPase Structure. *In* Red Blood Cell Membrane. P. Agre and J. C. Parker, editors. 135–185.

Mercer, R. W., J. W. Schneider, A. Savitz, J. Emanuel, E. J. Benz, Jr., and R. Levenson. 1986. Rat brain Na,K-ATPase β-subunit gene: Primary structure, tissue-specific expression and amplification in ouabain-resistant HeLa cells. *Mol. Cell. Biol.* 6:3884–3890.

Moorman, J. R., C. J. Palmer, J. E. John III, M. E. Durieux, and L. R. Jones. 1992. Phospholemman expression induces a hyperpolarization-activated chloride current in *Xenopus* oocytes. *J. Biol. Chem.* 267:14551–14554.

Morrow, J. S., C. D. Cianci, T. Ardito, A. S. Mann, and M. Kashgarian. 1989. Ankyrin links fodrin to the alpha subunit of Na,K-ATPase in Madin-Darby canine kidney cells and in intact renal tubule cells. *J. Cell Biol.* 108:455–465.

Navarre, C., M. Ghislain, S. Leterme, C. Ferroud, J. P. Dufour, and A. Goffeau. 1992. Purification and complete sequence of a small proteolipid associated with the plasma membrane H⁺-ATPase of *Saccharomyces cerevisiae. J. Biol. Chem.* 267:6425–6428.

Nelson, W. J., and P. J. Veshnock. 1987. Ankyrin binding to (Na⁺ + K⁺) ATPase and implications for the organization of membrane domains in polarized cells. *Nature (Lond.).* 328:533–535.

Noguchi, S., M. Noda, H. Takhashi, T. Ohta, K. Nagano, T. Hirose, S. Inayama, M. Kawamura, and S. Numa. 1986. Primary structure of the β-subunit of *Torpedo californica* (Na⁺ + K⁺)-ATPase deduced from the cDNA sequence. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 196:315–320.

Ohtsuka, E., S. Matsuki, M. Ikehara, Y. Takahashiand, and K. Matsubara. 1985. An alternative approach to deoxyoligonucleotides as hybridization probes by insertion of deoxyinosine at ambiguous codon positions. *J. Biol. Chem.* 260:2605–2608.

Orlowski, J., and J. B. Lingrel. 1988. Tissue-specific and developmental regulation of rat Na,K-ATPase catalytic α isoform and β subunit mRNAs. *J. Biol. Chem.* 263:10436–10442.

Palmer, C. J., B. T. Scott, and L. R. Jones. 1991. Purification and complete sequence determination of the major plasma membrane substrate for cAMP-dependent protein kinase and protein kinase C in myocardium. *J. Biol. Chem.* 266:11126–11130.

Rogers, T. B., and M. Lazdunski. 1979. Photoaffinity labeling of the digitalis receptor in the (sodium + potassium)-activated adenosinetriphosphatase. *Biochemistry.* 18:135–140.

Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain terminating inhibitors. *Proc. Natl. Acad. Sci. USA.* 74:5463–5467.

Schägger, H., and G. Von Jagow. 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* 166:368–379.

Schneider, J. W., R. W. Mercer, M. Caplan, J. R. Emanuel, K. J. Sweadner, E. J. Benz, Jr., and R. Levenson. 1985. Molecular cloning of rat brain Na,K-ATPase α-subunit cDNA. *Proc. Natl. Acad. Sci. USA.* 82:6357–6361.

Schneider, J. W., R. W. Mercer, M. Gilmore-Hebert, M. F. Utset, C. Lai, A. Greene, and E. J. Benz, Jr. 1988. Tissue specificity, localization in brain, and cell-free translation of mRNA encoding the A3 isoform of Na,K-ATPase. *Proc. Natl. Acad. Sci. USA.* 85:284–288.

Shull, G. E., J. Greeb, and J. B. Lingrel. 1986. Molecular cloning of three distinct forms of the Na⁺,K⁺-ATPase α-subunit from rat brain. *Biochemistry.* 25:8125–8132.

Sierra, H. F., and A. Tzagoloff. 1973. Assembly of the mitochondrial membrane system. Purification of a mitochondrial product of the ATPase. *Proc. Natl. Acad. Sci. USA.* 70:3155–3158.

Sweadner, K. J. 1979. Two molecular forms of (Na⁺ + K⁺)-stimulated ATPase in brain. Separation and difference in affinity for strophanthidin. *J. Biol. Chem.* 254:6060–6067.

Sweadner, K. J., and R. C. Gilkeson. 1985. Two isozymes of the Na,K-ATPase have distinct antigenic determinants. *J. Biol. Chem.* 260:9016–9022.

Takeyasu, K., V. Lemas, and D. M. Fambrough. 1990. Stability of Na⁺-K⁺-ATPase α-subunit isoforms in evolution. *Am. J. Physiol.* 259:C619–C630.

Tokuyasu, K. T. 1991. Use of polyvinylpyrrolidone and polyvinyl alcohol for cryoultramicrotomy. *Histochem. J.* 21:163–171.

Young, R. M., and J. B. Lingrel. 1987. Tissue distribution of mRNAs encoding the α isoforms and β subunit of rat Na⁺ + K⁺-ATPase. *Biochem. Biophys. Res. Commun.* 145:52–58.