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Molecular Cloning of Three Distinct Forms of the Na^+,K^+-ATPase α-Subunit from Rat Brain†

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ABSTRACT: Rat brain and kidney cDNA libraries were constructed and screened with a cDNA insert corresponding to the mRNA for the sheep kidney Na^+,K^+-ATPase catalytic subunit. The α-subunit cDNAs isolated from the kidney library were derived from a single class of messenger RNA, and the brain cDNAs were derived from three classes of messenger RNA. The most abundant brain cDNA, which spans 5.1 kilobases, encodes the $\alpha(+)$ form of the enzyme. The second most abundant brain cDNA, which spans 3.65 kilobases, is identical with that of the kidney form and therefore encodes the α isoform. The third class of cDNA, which spans 3.55 kilobases, was present at low abundance and encodes an isoform of the α-subunit, designated αIII, which has not been identified previously. The complete nucleotide sequence and deduced amino acid sequence for each of the brain and kidney cDNAs have been determined. In addition, we have identified a lysine-rich sequence that may function as a movable, ion-selective gate during cation binding and occlusion and have also identified several amino acid sequence variations that appear to explain some of the well-known species and tissue differences in cardiac glycoside sensitivity.

The Na^+,K^+-ATPase is an integral membrane protein that catalyzes the active transport of Na^+ and K^+ across the plasma membrane of animal cells [for reviews see Stahl (1986) and Schwartz et al. (1975)]. The enzyme consists of two subunits, a catalytic subunit (α) of M_r 112000 and a glycoprotein subunit (β) of unknown function with an M_r of 35000 for the protein component. The complete amino acid sequences for the α and β subunits from sheep kidney (Shull et al., 1985, 1986), pig kidney (Ovchinnikov et al., 1986), and *Torpedo californica* electric organ (Kawakami et al., 1985; Noguchi et al., 1986) have been determined from their cDNAs. However, in mammals there are at least two isoforms of the catalytic subunit. Besides the well-characterized renal form, which is designated α, there is an additional form, termed $\alpha(+)$, which has been identified in several tissues. For example, brain tissue contains a form that appears to be identical with the renal form (α) as well as the $\alpha(+)$ form, which has a higher apparent molecular weight on SDS[1]–polyacrylamide gels (Sweadner, 1979). The two forms also differ in cardiac glycoside sensitivity (Sweadner, 1979), N-terminal amino acid sequence (Lytton, 1985a), and Na^+ affinity (Lytton, 1985b). There is evidence that the $\alpha(+)$ form in adipocytes, which may be identical with the brain $\alpha(+)$, is responsive to insulin whereas the α form is not (Lytton, 1985b; Lytton et al., 1985).

The present study was conducted in order to determine the complete primary structure of the rat α and $\alpha(+)$ forms of the catalytic subunit and to identify amino acid sequence differences between these isoforms, which might provide information regarding functional differences such as cardiac glycoside sensitivity. Therefore, we prepared and screened cDNA libraries from rat kidney, which contains the ouabain-resistant α form, and rat brain in which the predominant form is the oubain-sensitive $\alpha(+)$ isoform. As the full sequences of the ouabain-sensitive sheep and pig kidney enzymes

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[1] Abbreviations: SDS, sodium dodecyl sulfate; cDNA, complementary deoxyribonucleic acid; mRNA, messenger ribonucleic acid; SET, 0.15 M NaCl, 20 mM Tris-HCl, pH 7.8, and 1 mM ethylenediaminetetraacetic acid; Denhardt's solution, 0.02% (w/v) bovine serum albumin, 0.02% (w/v) poly(vinylpyrrolidone), and 0.02% (w/v) Ficoll; nt(s), nucleotide(s); Tris, tris(hydroxymethyl)aminomethane; kb, kilobase(s).

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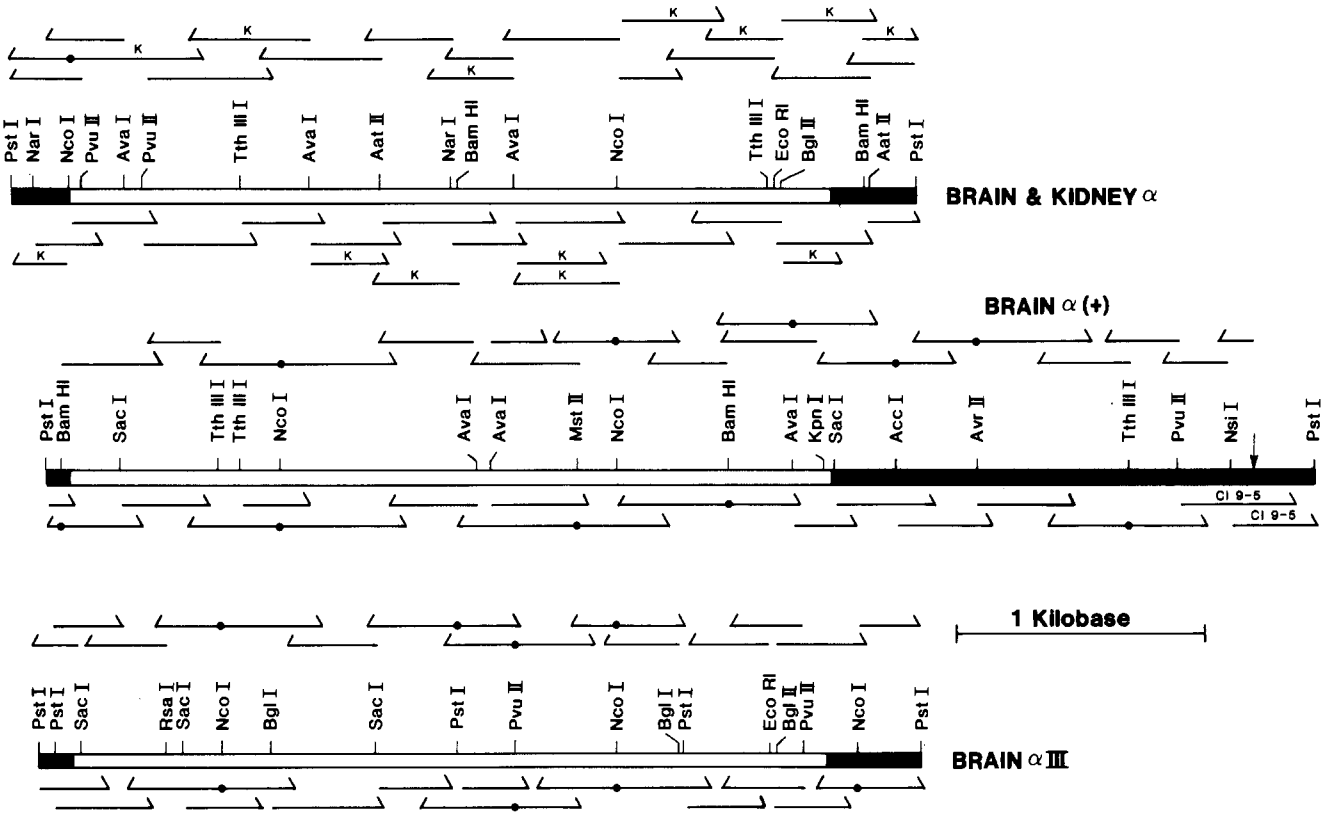

FIGURE 1: Restriction map and sequencing strategy for the Na^+,K^+-ATPase α-subunit isoforms of rat kidney and brain. Open areas represent the open reading frame and darkened areas represent untranslated regions. Restriction sites relevant to the sequencing strategy are shown. The direction and extent of sequencing are shown by arrows (lower arrows, coding strand; upper arrows, noncoding strand). The brain (clone B1-4) and kidney (clone K3-2) α-form cDNAs were virtually identical (see Figure 2 legend), and therefore the kidney cDNA sequence (K) was determined in only one strand. The sequence of $\alpha(+)$ was determined from clone B4-4, which ended at a polyadenylation signal indicated by the vertical arrow; the remaining $\alpha(+)$ sequence was determined from clone B9-5, which ended at a different polyadenylation site. The sequence of brain αIII was determined from clone B3-5.

are known from their cDNAs, the isolation of these rat cDNAs also allows an investigation of species differences in cardiac glycoside sensitivity.

Experimental Procedures

Preparation of Brain and Kidney cDNA Libraries. Poly(A+) RNA was isolated from male rat (CD strain, Charles River Breeding Laboratory) brain and kidney as described previously (Snider et al., 1985). First- and second-strand cDNA syntheses, size fractionation of double-stranded cDNA, tailing of the 3.3–5.5-kb fraction with deoxycytidine 5′-triphosphate, annealing of cDNA to G-tailed *Pst*I-cut pBR322, plating of the libraries, and preparation of replica filters were performed as described previously (Shull et al., 1985). Each library consisted of 50 000 colonies on ten 150-mm plates.

Screening of cDNA Library. The 20 replica filters of each library were prehybridized overnight at 68 °C in 6 × SET, 5 × Denhardt's solution, 0.1% SDS, and 100 μg of denatured salmon sperm DNA/mL and then hybridized with a ^{32}P-labeled (Maniatis et al., 1982) 2.7-kb *Xho*I–*Bgl*II fragment from a sheep kidney Na^+,K^+-ATPase α-subunit cDNA (Shull et al., 1985). Hybridization was performed at 58 °C for 24 h in 60 mL of the same solution containing 5 × 10^6 dpm/mL. The filters were washed 4 times for 30 min each at 58 °C in 6 × SET and 0.1% SDS and then washed for 1 h at 58 °C in 3 × SET and 0.1% SDS. Following autoradiography the filters were washed for 1 h at 63 °C in 1.5 × SET and 0.1% SDS and then subjected to autoradiography a second time. Colonies that gave signals on both replicas were isolated, and plasmid DNA was extracted by the alkaline lysis procedure (Maniatis et al., 1982).

Characterization of cDNAs. Brain and kidney cDNAs were analyzed by restriction mapping according to standard protocols (Maniatis et al., 1982). DNA sequence analysis was performed by the method of Maxam and Gilbert (1980). The nucleotide sequence was determined at least once in both strands for each of the brain isoforms except for nts 1–47 and 4866–5107 of $\alpha(+)$, which were determined from only one strand. The kidney α form cDNA sequence was analyzed in only one strand because it was virtually identical with the brain α form (see Figure 2 legend).

Results

Isolation of Brain and Kidney α-Subunit cDNAs. Screening of rat brain and kidney cDNA libraries with a sheep kidney Na^+,K^+-ATPase α-subunit hybridization probe resulted in 78 positive signals in the brain library and 117 positive signals in the kidney library. Eight kidney and 25 brain cDNAs were colony purified and analyzed by restriction mapping. The kidney cDNAs exhibited a single restriction pattern whereas the brain cDNAs had three distinct patterns (diagramed in Figure 1). The medium-abundance (six isolates) brain class had a 3.65-kb insert, and its restriction pattern was identical with that of the kidney form. The relative abundance of this class and its similarity to the kidney class (Sweadner, 1979; Schmitt & McDonough, 1986) suggested that it was derived from the α-form mRNA. The most abundant class of brain cDNA (16 isolates) was 5.1 kb in length and had a very different restriction pattern from the kidney form. Its relative abundance indicated that it was derived from the $\alpha(+)$ mRNA. The third class of cDNA (three isolates) had a 3.55-kb insert. This class of cDNA is apparently derived from

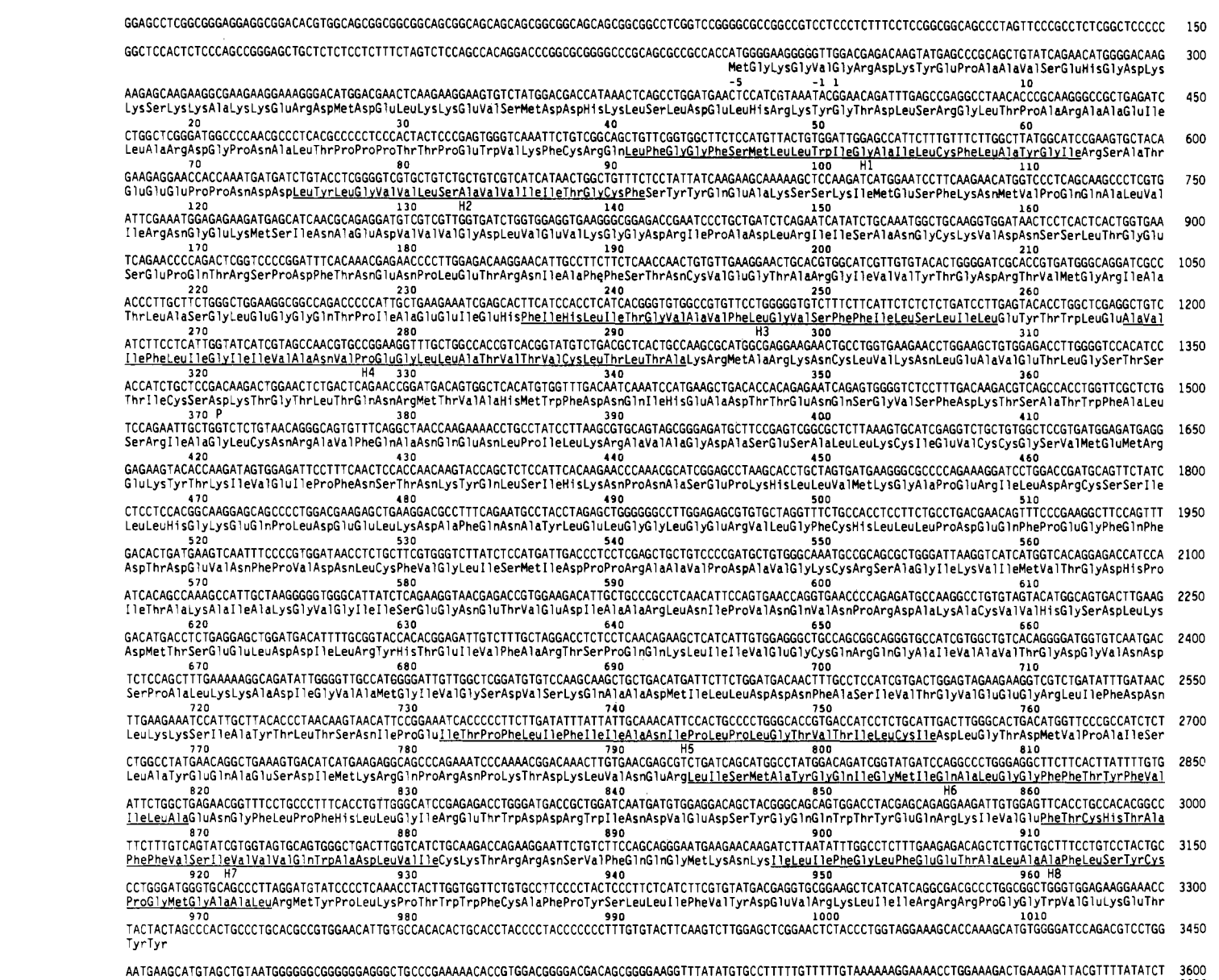

FIGURE 2: Nucleotide sequence of the cDNA for the Na^+,K^+-ATPase α isoform of rat kidney and brain. The sequence shown is for brain cDNA B1-4. Kidney cDNA K3-2 was identical except that it was missing the first three nts of the sequence and had Cs, rather than Ts, at positions 97 and 1680. The deduced amino acid sequence is numbered starting with the N-terminus of the mature protein (Lytton, 1985a) and is preceded by five amino acids occurring in the primary translation product. The phosphorylation site (Asp-371) is indicated by P and the major hydrophobic domains (H1–H8) are underlined.

an mRNA that encodes a low-abundance α-subunit isoform (αIII), which has not been identified previously.

Characterization of cDNAs for Three α-Subunit Isoforms. The restriction map and sequencing strategy for the Na^+,K^+-ATPase α-subunit cDNAs are shown in Figure 1; the nucleotide sequence and deduced amino acid sequence for the α (brain and kidney), α(+), and αIII isoforms are shown in Figures 2, 3, and 4, respectively.

The brain and kidney α-form cDNAs are identical except for several nt differences (see Figure 2 legend), which did not result in amino acid substitutions. They have a 237-nt 5′-untranslated sequence, a 1023 codon open reading frame, and 328 nts of 3′-untranslated sequence. The initiation codon is the first ATG triplet encountered in the sequence and lies in a perfect 9-nt consensus sequence for initiation of translation (Kozak, 1984). Residues 1–14 of the deduced amino acid sequence are identical with those of the N-terminal sequence of the rat brain α form determined previously (Lytton, 1985a); five amino acids, which are apparently removed by post-translational processing, precede the N-terminus of the mature α-subunit. The mature protein consists of 1018 amino acids and has an M_r of 112 573.

The identity of the α(+) form (Figure 3) was confirmed by comparison of the deduced N-terminal amino acid sequence (GREYSPAATTAENG) with that determined by analysis of the rat brain α(+) protein (GREYSPAAEVAEVG) (Lytton, 1985a). Although mismatches occurred at residues 9, 10, and 13, it seems possible that this was due to errors in the original amino acid sequence analysis; in the study cited the problem of a rapidly rising background during each cycle of amino acid analysis was noted. In addition, a hybridization probe consisting of mixed oligonucleotides derived from the sequence AEVAEV, which contained the residues in question, failed to hybridize with any of the 25 brain cDNAs (data not shown). The α(+) cDNA contains a 96-nt 5′-untranslated region, a 1020 codon open reading frame that begins with the first ATG triplet, and 1851 nts of 3′-untranslated sequence. There are three potential polyadenylation signals (beginning

FIGURE 3: Nucleotide sequence of the cDNA for the Na^+,K^+-ATPase $\alpha(+)$ isoform of rat brain. Nts 1–4866 are from clone B4-4 and nts 4530–5107 from clone B9-5. The deduced amino acid sequence is numbered starting with the N-terminus of the mature protein (Lytton, 1985a) and is preceded by five amino acids that occur in the primary translation product. The phosphorylation site (Asp-369) is indicated by P and the major hydrophobic domains (H1–H8) are underlined.

at nts 3293, 4853, and 5085) (Proudfoot & Brownlee, 1976). The primary translation product of $\alpha(+)$, like that of α, has five additional amino acids at its N-terminus. The mature brain $\alpha(+)$ protein consists of 1015 amino acids and has an M_r of 111 736. Although molecular weight determination by SDS gel electrophoresis suggests that $\alpha(+)$ is slightly larger than α (Sweadner, 1979), this would appear to be due to the anomalous migration rates of hydrophobic proteins on SDS gels (de Jong et al., 1978).

The αIII cDNA (Figure 4) has a 141-nt 5′-untranslated sequence, a 1013 codon open reading frame, and a 378-nt 3′-untranslated sequence. The position of the initiation codon is clear because it is the first ATG triplet, it is preceded by an in-frame stop codon at nts 79–81, and it lies within a good consensus sequence for initiation of translation (Kozak, 1984). As the αIII protein has not been identified previously, there are no data available concerning the N-terminus of the mature protein. The primary translation product has 1013 amino acids and an M_r of 111 727. The extensive amino acid homology with α and $\alpha(+)$, discussed below, clearly demonstrates that αIII is an Na^+,K^+-ATPase α-subunit isoform.

Organization of the α-Subunit Isoforms. The general organization of all three isoforms appears to be identical with that of the sheep (Shull et al., 1985), pig (Ovchinnikov et al., 1986), and *T. californica* enzymes (Kawakami et al., 1985) described previously. Four major hydrophobic domains (H1–H4), determined by hydropathy analysis (Kyte & Doolittle, 1982), occur in the N-terminal third of the protein, and each domain appears to represent a single transmembrane pass. The phosphorylation site (Bastide et al., 1973) occurs

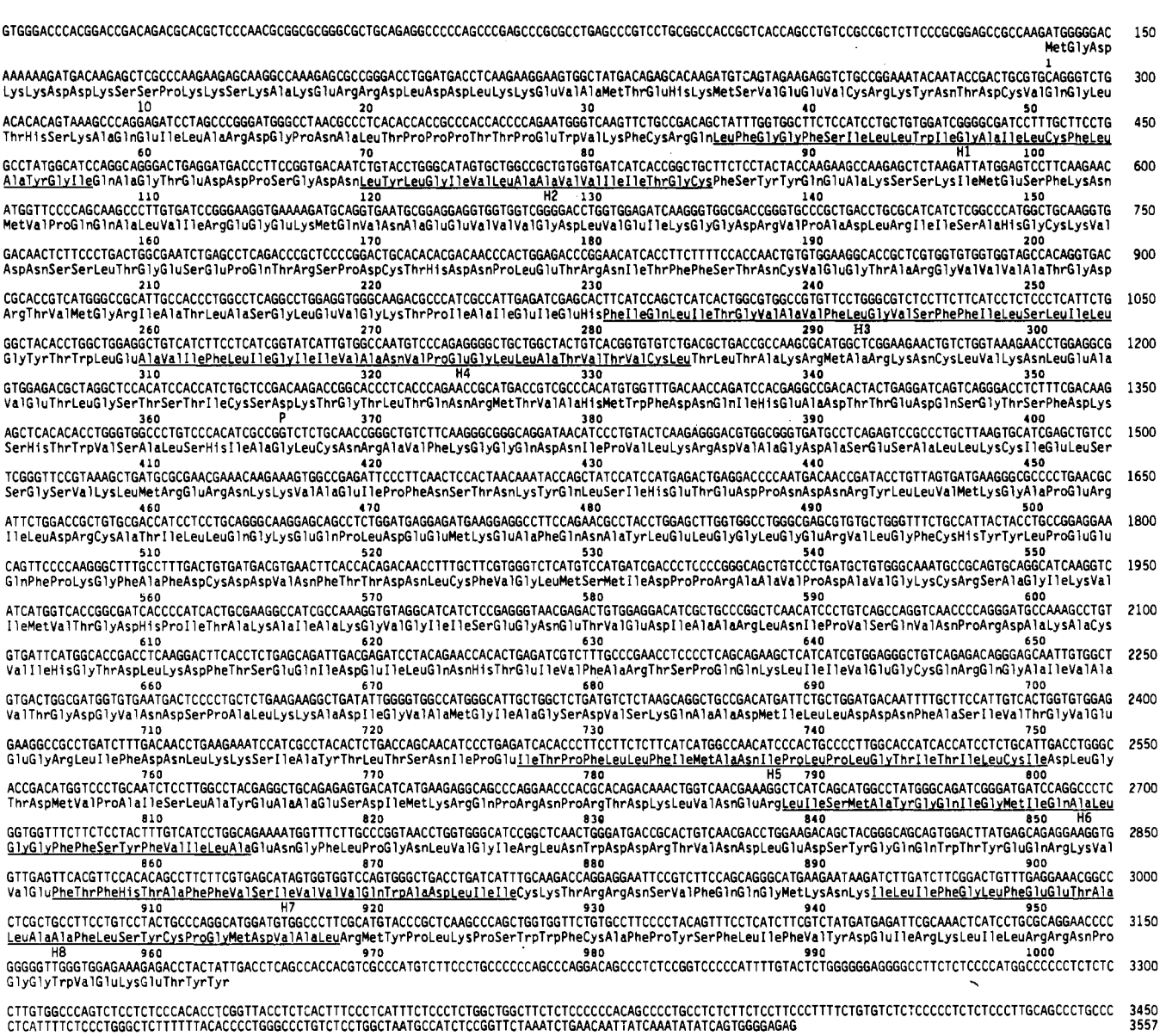

FIGURE 4: Nucleotide sequence of the cDNA (B3-5) for the Na^+,K^+-ATPase αIII isoform of rat brain. The deduced amino acid sequence is numbered by starting with the initiating methionine. The phosphorylation site (Asp-366) is indicated by P and the major hydrophobic domains (H1–H8) are underlined.

around amino acid 370 in each protein and is followed by a cytoplasic domain of over 400 amino acids. There are four major hydrophobic domains (H5–H8) in the C-terminal regions of the protein, and each may represent a single transmembrane pass. However, H5 could be a double pass, as suggested for the Ca^{2+}-ATPase (MacLennan et al., 1985), and a short hydrophobic sequence following H8 might also cross the membrane. A possible clue regarding the transmembrane organization of the C-terminal region of the protein is the presence of a potential phosphorylation site for a cAMP-dependent protein kinase (Krebs & Beavo, 1979) in the H7–H8 junction (Arg-Arg-Asn-Ser-Val; see Figure 6). There is evidence that the Na^+,K^+-ATPase is regulated by cAMP (Lingham & Sen, 1982), and if this site is involved, then it should be on the cytoplasmic side.

Amino Acid and Nucleotide Sequence Similarities. Pairwise amino acid homology comparisons between the different isoforms gave the following values for percent identity: α vs. α(+), 86.3%; α vs. αIII, 85.3%; α(+) vs. αIII, 86.3%. A comparison of the three rat isoforms with the α-subunit of *T. californica* electric organ (Kawakami et al., 1985) gave the following values: α, 86.6%; α(+), 83.3%; αIII, 83.0%. The most substantial differences in amino acid sequence between the three rat α-subunit isoforms occur in the N-terminal region and in the region between the phosphorylation site and the peptide that binds fluorescein isothiocyanate (amino acids 498–508 in α; Kirley et al., 1984; Farley et al., 1984). The greatest similarities occur around the phosphorylation site (perfect identity for 85 amino acids or more), in the major hydrophobic domains (94–95% are identical), and in the 200 residues that precede H5 (94–96% are identical). The latter region includes two peptides, which bind 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine that may form part of the ATP binding site (amino acids 650–666 and 704–722 in α; Ohta et al., 1986). Pairwise nucleotide homology comparisons of the protein coding regions gave the following values for percent identity: α vs. α(+), 76.5%; α vs. αIII, 76.2%; α(+) vs. αIII, 79.0%. Comparisons of the 5′ and 3′-untranslated sequences revealed no extended regions of similarity.

Presence of a Lysine-Rich Sequence in the N-Terminal Region. A comparison of the N-terminal sequences of the three rat α-subunit isoforms and the α-subunits of sheep (Shull et al., 1985) and pig (Ovchinnikov et al., 1986) kidney, *T. californica* electric organ (Kawakami et al., 1985), and the

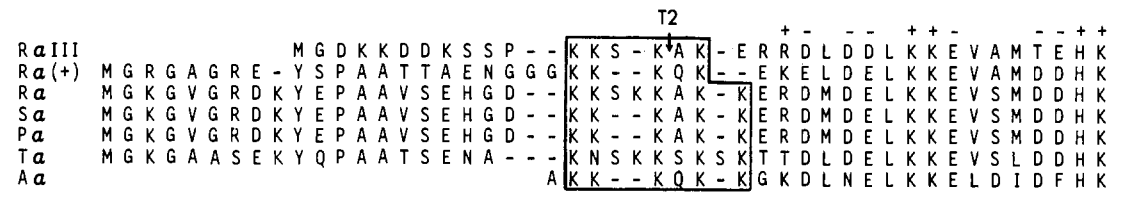

FIGURE 5: Comparison of the N-terminal amino acid sequences of the Na^+,K^+-ATPase α-subunit isoforms from several species. The sequences shown are for the rat isoforms [RαIII, Rα(+), and Rα], sheep kidney α [Sα; data from Shull et al. (1985)], pig kidney α [Pα; data from Ovchinnikov et al. (1986)], *Torpedo californica* electric organ α [Tα; data from Kawakami et al. (1985)], and the α-subunit of the brine shrimp *Artemia salina* [Aα; data from Morohashi and Kawamura (1984)]. T2 indicates the possible location of trypsin site 2, which is rapidly cleaved when the enzyme is in the E_1Na conformation (Jørgensen et al., 1982b). The box encloses a lysine-rich sequence that may serve as a movable, positively charged gate during cation binding and occlusion (see Discussion). The consensus charge on the residues following the lysine-rich sequence is indicated.

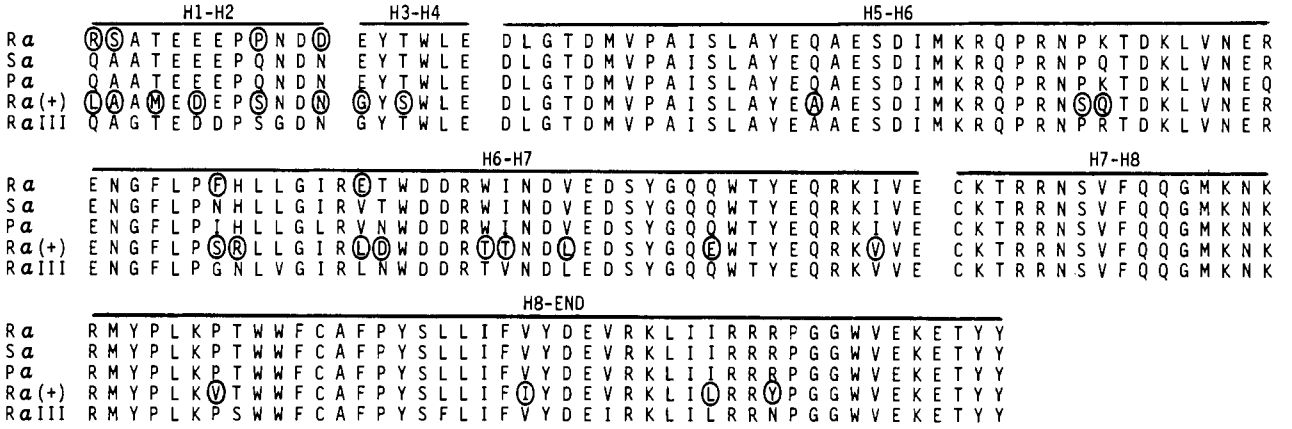

FIGURE 6: Comparison of potential extracellular sequences of the Na^+,K^+-ATPase α-subunit isoforms from several mammalian species. H1–H2 etc. refer to the junction between the major hydrophobic domains H1 and H2 etc. (see Figures 2–4). The sequences shown are for the rat isoforms [Rα, Rα(+), and RαIII], sheep kidney α [Sα; data from Shull et al. (1985)], and pig kidney α [Pα; data from Ovchinnikov et al. (1986)]. The H1–H2 and H3–H4 junctions are known to be on the extracellular side; the location of the remaining sequences are not known. Two comparisons are illustrated: (1) The circled Rα residues differ from both the Sα and Pα residues; some of these amino acid variations may account for the species differences in cardiac glycoside sensitivity of the renal enzyme. (2) The circled Rα(+) residues differ from the Rα residues; some of these variations may account for the tissue differences in cardiac glycoside sensitivity.

brine shrimp *Artemia salina* (Morohashi & Kawamura, 1984) is shown in Figure 5. The first five amino acids of rat, sheep, and pig α and rat α(+) are known to be removed by post-translational processing (Lytton, 1985a; Shull et al., 1985; Ovchinnikov et al., 1986), and judging by the amino acid homology, it is likely that this is also true for the *Torpedo* enzyme. The first 11 amino acids of αIII are not homologous to the other forms, and the brine shrimp enzyme is missing approximately 15 amino acids of the initial sequence. Lysine-16 of the mature rat, sheep, and pig α form is the first residue of a sequence (boxed in Figure 5) that is highly variable in the different forms and species yet retains the distinctive feature of richness in lysine residues. Interestingly, the H^+,K^+-ATPase from rat stomach (Shull and Lingrel, in press) has a similar lysine-rich sequence (KKKAGGGGGKKEK) near its N-terminus. The boundary of this domain on the N-terminal side is apparent from the amino acid homology but may extend beyond the boxed residues on the C-terminal side. The sequence following the lysine-rich sequence is highly conserved. A consensus charge is apparent in a number of positions, and there are three additional lysine residues that are conserved in all of the forms.

Identification of Amino Acid Residues That May Affect Cardiac Glycoside Sensitivity. The availability of full sequence for ouabain-sensitive and -insensitive forms of the Na^+,K^+-ATPase allows an investigation of the residues that may be involved in the well-known species and tissue differences in ouabain sensitivity. The rat kidney enzyme is resistant to cardiac glycosides; the sheep and pig kidney enzymes and the rat brain α(+) isoform are sensitive to cardiac glycosides (Periyasamy et al., 1983; Wallick et al., 1980; Ahmed et al., 1983; Sweadner, 1979). As the ouabain binding site is known to be located on the extracellular side of the membrane, we have compared the potential extracellular sequences for these enzymes and the rat αIII isoform (Figure 6). Because there is no conclusive evidence regarding the transmembrane organization in the C-terminal regions of the protein, the C-terminus and all of the junctions between the major hydrophobic domains in this part of the catalytic subunit have been included in the comparisons.

There are 24 amino acids in rat α that differ from both sheep and pig α, and only 6 of these residues (circled in the rat α sequence in Figure 6) are in potential extracellular domains. Four of them are in the H1–H2 junction and the remaining two are in the H6–H7 junction. If the species differences in cardiac glycoside sensitivity are due to sequence changes at the ouabain binding site [as suggested by the work of Periyasamy et al. (1983)] rather than at some site that affects binding from a distance, then one or more of these six amino acids would appear to be involved. There are 140 amino acids in rat α(+) that differ from rat α, and 24 [circled in the α(+) sequence in Figure 6] are in potential extracellular domains. The variations in the C-terminal regions are unlikely to account for the sensitivity differences between the two isoforms. In almost all of the positions in this region that differ between rat α and α(+), the resistant α form has the same amino acid as the sheep and pig enzymes, which are sensitive. The same is true for the two amino acid differences in the H3–H4 junction. However, in the H1–H2 junction rat α differs from α(+) at all of the positions at which it differed from sheep and pig α. Although α(+) differs from sheep and pig α at four positions, it is similar in that it has uncharged

amino acids adjacent to the transmembrane domains.

Discussion

The existence of multiple forms of the Na^+,K^+-ATPase has been known for many years, but questions have remained concerning the number of isoforms, their genetic basis, and their functional and structural differences. In order to address these questions, we isolated and characterized Na^+,K^+-ATPase α-subunit cDNAs from rat kidney and brain. In addition to cDNAs for the well-known α and $\alpha(+)$ forms we isolated cDNAs for a third brain form, designated αIII, that had not been identified previously. The αIII mRNA is clearly not the product of a pseudogene as the sequence shows that it has the translation start and stop signals and open reading frame of over 1000 codons expected for a functional α-subunit mRNA. The existence of αIII, which was not predicted by the available biochemical data, raises the possibility that there may be additional isoforms besides the three presented here. A definitive answer regarding the genetic basis for the multiple α-subunit isoforms will require the isolation and characterization of their genomic sequences. However, on the basis of the data presented here it seems clear that they are the products of separate genes. If they were the result of differential processing of a single gene, then there should be some coding regions in common. This is not the case. Although amino acid sequence comparisons reveal extensive regions of perfect identity, the codon usage within these regions differs. The nucleotide homology in the protein coding region is less than 80%, and there is little detectable homology in the untranslated regions. As the amino acid homology between the three rat isoforms is about the same as that with the *Torpedo* enzyme, it seems likely that divergence of the genes encoding the three isoforms was an ancient evolutionary event.

It is known that Na^+ and K^+ are occluded within the Na^+,K^+-ATPase during each turnover of the pump and that occlusion requires conformational changes in the enzyme (Beaugé & Glynn, 1979; Glynn et al., 1984). One of the lysine residues that lies within the lysine-rich sequence appears to correspond to trypsin site 2 (see Figure 5), which is highly accessible in the E_1Na conformation but much less accessible in the E_2K form [for a review see Jørgensen et al. (1982a,b)]. Proteolytic cleavage at this site alters the equilibrium between the E_1 and E_2 conformations (Jørgensen & Karlish, 1980). Thus, it appears that the conformational shift that results in cation occlusion involves movement of the lysine-rich sequence. A possible function of this domain is to serve as a movable, ion-selective gate, which controls the passage of Na^+ and K^+ to and from the binding sites occupied during certain stages of the transport process. Positively charged amino acid side chains would be a logical feature of a gate controlling passage of cations.

Species and isoform differences in cardiac glycoside sensitivity are known to be due to large differences in the dissociation rate rather than in the association rate (Tobin & Brody, 1972). For the sensitive enzymes, but not for the resistant forms, binding of the drug is apparently followed by a conformational change that results in a stable, enzymatically inactive complex. Akera et al. (1979a) proposed that the resistant species lack a "lipid barrier" that regulates the release of cardiac glycosides. This hypothesis was based on the observation that, for the sensitive enzymes, K^+ lowers the rate of release of the more hydrophilic drugs but has little effect on the rate of release of the more hydrophobic compounds (Akera & Brody, 1971; Akera et al., 1974, 1978, 1979b). This proposal is consistent with the observed amino acid differences between rat α and the sensitive forms (Figure 6). It is possible that conformational changes occurring after ouabain binding to the sensitive forms involve alterations in the relationship of the H1–H2 junction with the lipid bilayer and that these alterations allow the formation of a stable complex. In the case of rat α the presence of the charged amino acids Arg and Asp at the boundaries of the H1–H2 junction might interfere with such an alteration. Hence, the lipid barrier that prevents dissociation from a sensitive enzyme does not form in the resistant enzyme. It is possible that one or more of the other changes in rat α, such as the Pro in the H1–H2 junction, are compensatory changes that allow the necessary range of conformations that must occur during the catalytic cycle.

Previous studies using photoaffinity and other types of derivatives of ouabain (Fortes, 1977; Goeldner et al., 1983; Jørgensen et al., 1982a) and examination of the amino acid sequence derived from a cDNA (Shull et al., 1985) indicate that the ouabain binding site may be composed of several regions distantly separated in the primary sequence. From these previous studies it appears that ouabain binds to sequences in the C-terminal regions and to the H3–H4 junction. The results of the present study suggest that the H1–H2 junction is also involved. It could conceivably interact with the lactone ring. Thomas et al. (1974) have suggested that the lactone ring may fit into a cleft on the surface of the enzyme that contains one or more anionic sites. With the availability of cDNAs containing the entire coding regions of the sensitive and insensitive forms of the enzyme, it is now possible to test these and other hypotheses concerning the molecular basis of cardiac glycoside binding and sensitivity by using site-directed mutagenesis techniques.

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