The Na,K-ATPase α4 Gene (*Atp1a4*) Encodes a Ouabain-Resistant α Subunit and Is Tightly Linked to the α2 Gene (*Atp1a2*) on Mouse Chromosome 1†

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ABSTRACT: We have isolated and characterized cDNA clones encoding the murine homologue of a putative fourth Na,K-ATPase α subunit isoform (α4). The predicted polypeptide is 1032 amino acids in length and exhibits 75% amino acid sequence identity to the rat α1, α2, and α3 subunits. Within the first extracellular loop, the α4 subunit is highly divergent from other Na,K-ATPase α subunits. Because this region of Na,K-ATPase is a major determinant of ouabain sensitivity, we tested the ability of the rodent α4 subunit to transfer ouabain resistance in a transfection protocol. We find that a cDNA containing the complete rodent α4 ORF is capable of conferring low levels of ouabain resistance upon HEK 293 cells, an indication that the α4 subunit can substitute for the endogenous ouabain-sensitive α subunit of human cells. Nucleotide sequences specific for the murine α4 subunit were used to identify the chromosomal position of the α4 subunit gene. By hybridizing an α4 probe with a series of BACs, we localized the α4 subunit gene (*Atp1a4*) to the distal portion of mouse chromosome 1, in very close proximity to the murine Na,K-ATPase α2 subunit gene. In adult mouse tissues, we detected expression of the α4 subunit gene almost exclusively in testis, with low levels of expression in epididymis. The close similarities in the organization and expression pattern of the murine and human α4 subunit genes suggest that these two genes are orthologous. Together, our studies indicate that the α4 subunit represents a functional Na,K-ATPase α subunit isoform.

Na,K-ATPase is the membrane-associated enzyme that establishes the low intracellular levels of Na^+ and high intracellular levels of K^+ that are essential for ionic homeostasis in all mammalian cells. The Na,K-ATPase accomplishes this by coupling the hydrolysis of ATP to the active transport of Na^+ and K^+ (*1*). Structurally, the enzyme consists of two subunits, α and *β*, present in equimolar amounts (*2*). The α subunit contains 8−10 hydrophobic segments that anchor the polypeptide in the plasma membrane and provide a pathway for the transport of ions into and out of the cell (*3*). The α subunit is the catalytic subunit of the enzyme and contains both the cation and ATP binding sites (*4*). Although the presence of the *β* subunit is essential for Na,K-ATPase activity, the exact functional role of the *β* subunit has not yet been established.

Initial cDNA cloning experiments revealed the existence of three distinct, highly conserved α subunit isoforms in rodents and primates (*5*). The three α subunit isoforms are encoded by separate genes, each of which is expressed in a distinct tissue- and cell-specific pattern (*6*). The α1 subunit is ubiquitously expressed. The α2 isoform shows predominant expression in brain, heart, and skeletal muscle, whereas expression of the α3 subunit is restricted primarily to tissues of neural origin (*7*). Within the brain, neurons are the primary site of α3 subunit expression, while α2 subunits are found principally in astrocytes (*8*). Chromosomal dispersion of α subunit genes, coupled with their differing expression patterns, suggests specialized functional roles for these isoforms.

Although mice containing a targeted mutation in the α2 subunit gene exhibit deficits in cardiac function (*9*), no prominent biochemical differences between α subunit isoforms have been described. The three well-characterized α subunit isoforms have similar enzymatic properties and affinities for cations (*5*). One functional difference between α subunit isoforms is their differential sensitivity to the cardiac glycoside class of drugs. These drugs, which include ouabain and digitalis, bind to the enzyme and inhibit Na,K-ATPase activity. It has been established that the rodent α1 subunit is resistant to high levels of ouabain (*10*, *11*), whereas the primate α1 subunit and rodent and primate α2 and α3 subunits are inhibited at relatively low drug concentrations (*12*). These differences in ouabain sensitivity form the basis for a gene transfer assay that has been used to analyze structure−function relationships for the Na,K-ATPase α subunit (*13*).

Recently, cDNA and genomic clones encoding a fourth possible Na,K-ATPase α subunit isoform have been identi-

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fied (*14*, *15*). DNA sequences for the human α4 subunit were initially isolated from a genomic DNA library and found to be physically linked by <30 kb to the Na,K-ATPase α2 subunit gene (*15*). The predicted sequence of the rat α4 subunit deduced from cDNA shows ~75% identity with the three rat Na,K-ATPase α subunits (*14*). In contrast, the rat α1, α2, and α3 subunits exhibit ~85% amino acid sequence identity to each other (*5*). The putative α4 subunit shows significantly greater conservation to Na,K-ATPase α subunit isoforms than other K^+-transporting ATPases, such as H,K-ATPase, suggesting that α4 is more likely to represent a Na,K-ATPase rather than a H,K-ATPase α subunit. However, given the lack of any functional information, the possibility that α4 may represent a P-type ATPase α subunit (*16*) with altered or novel ion transport properties cannot be ruled out.

To further characterize the α4 subunit, we have isolated cDNAs encoding a complete murine α4 polypeptide and used an α4-specific probe to map the position of the α4 gene in the mouse genome. Our data indicate that the α4 subunit maps to mouse chromosome 1, in very close proximity to the Na,K-ATPase α2 gene. We have also used gene transfer to assay the biological activity of the α4 subunit. In these experiments, we find that introduction of rodent α4 cDNA into ouabain-sensitive primate cells can rescue the cells from ouabain cytoxicity. These results indicate that the transfected α4 subunit can functionally substitute for the endogenous α1 subunit of recipient cells. This functional assay strongly implicates α4 as a fourth Na,K-ATPase α subunit isoform.

EXPERIMENTAL PROCEDURES

Isolation and Characterization of Murine α4 cDNA Clones. Two mouse ESTs[1] with sequence homology to the rat Na,K-ATPase α4 subunit were identified in a search of the GenBank EST database. I.M.A.G.E. Consortium clones 567553 (Accession number AA184055) and 937308 (Accession number AA509743) were obtained from Genome Systems (St. Louis, MO) and found to have overlapping sequence homology. The larger of the two ESTs (567553) was found to correspond to nucleotides 957−3443 of the rat α4 sequence, while the shorter clone corresponds to nucleotides 2957−3443 of rat α4 cDNA. Two overlapping cDNA fragments spanning the 5′ end of the α4 subunit were generated from mouse testis RNA by RT-PCR. PCR was carried out using the following primers:

5′-ATCTAGAGATCACATGATGG-3′(α4/metF)

5′-TCAGCAGGGACTTGGTCTCC-3′(α4/715R)

5′-CAGCAAGCTCTGGTAATTCG-3′(α4/609F)

5′-ACACAGTGACAGTGGCCAGC-3′(α4/1137R)

The α4/metF primer overlaps the initiating methionine of the rat α4 sequence. In the remaining primers, the numbers refer to the nucleotide position in the rat α4 sequence that correspond to the first base in each primer. PCR was carried out with Taq DNA polymerase for 30 cycles (30 s at 94 °C, 30 s at 55 °C, and 1 min at 72 °C). The PCR products were inserted into Bluescript and subjected to DNA sequence analysis with an ABI Automated DNA Sequencer. Four independent clones were sequenced from each of the two 5′ α4 PCR products. The nucleotide sequence of the mouse α4 subunit has been submitted to GenBank with accession numbers AF164348, 49, 50.

Chromosomal Localization and Expression of the Murine α4 Subunit. To map the murine α4 subunit gene (*Atp1a4*), sequences specific for the α4 subunit were used in hybridization analysis to screen an overlapping set of bacterial artificial chromosomes (BACs) that had previously been mapped onto distal mouse chromosome 1 (*17*). Details regarding the mapping of the BAC contigs and localization of the Na,K-ATPase α2 gene (*Atp1a2*) to this region have been previously published (*17*). Segments containing 3′-UTR sequences derived from either rat (nt 3176−3446) or mouse (nt 2877−3393) α4 cDNA were used to probe Southern blots containing *Eco*RI- and *Hind*III-digested DNA prepared from a subset of BAC clones. Hybridization conditions for Southern blotting were as previously described (*17*). To localize the mouse α4 gene relative to the α2 gene, Southern blots were also hybridized at high stringency with α2-specific probes consisting of either an ~2 kb segment of the rat α2 cDNA (nt 1104−3380) or a 3′-UTR-containing mouse α2 cDNA (I.M.A.G.E Consortium clone 619484; accession number AA172598) that extends 3′ from position 3049 in the rat α2 cDNA.

Expression of α4 subunit mRNA sequences was detected by dot blot analysis of a Mouse RNA Master Blot (Clontech Inc., Palo Alto, CA). The blot was probed with cDNA derived from mouse α4 EST clone 937308. Hybridization was carried out under conditions recommended by the manufacturer.

Cell Culture and DNA Transfection. Human embryonic kidney (HEK) 293 cells were used as recipients for transfection. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). Transfections were performed by the calcium phosphate coprecipitation method described previously (*13*). To generate stable α4 subunit transfectants, cultures were split 1:4 48 h after transfection and medium containing ouabain (0.5 μM) was added 4 h later. Colonies of transfected cells were picked in 2−3 weeks and expanded into cell lines that were maintained in medium containing 0.5 μM ouabain.

Cytotoxicity Assay. The extent of ouabain resistance in α4-expressing cells was measured by examining the survival of transfectants in media containing increasing concentrations of ouabain. Triplicate 1.0 mL cultures containing 10^5 cells were plated in 6-well plates containing 0.01−100 μM ouabain, and without ouabain. After 3 days of incubation, cells were washed free of drug and cellular protein was determined using the cupric sulfate/bicichoninic acid method (BCA reagent, Pierce Chemicals, Rockford, IL). Relative plating efficiency of each clone was determined by dividing the absorbance observed at a given drug concentration by the absorbance detected in the same clone in medium without drug. The IC_{50} is defined as the drug concentration required to reduce the plating efficiency of each clone by 50%. The fold resistance is calculated by dividing the IC_{50} of a transfected clone by the IC_{50} measured for drug-sensitive control HEK 293 cells.

[1] Abbreviations: ORF, open reading frame; BAC, bacterial artificial chromosome; STS, sequence tag site; EST, expressed sequence tag; UTR, untranslated region.

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                    .         .         .         .
mouse α4    MEPGKEKEVEAPGELNQKPRPSTRSSTTNRQPKMKRRKKD  40
            |||||     |       |||||  | | ||||||  ||||||
rat   α4    MEPGK----ETAATSEQKPRPTLRASNTNRQPKVKRRKKD  36
                    .         .         .         .
mouse α4    LEELKKEVVMDDHKLTLDELSAKYSVDLTKGLSVLEAQDI  80
            ||||||||||||||||||||||||||||||||||||  || |
rat   α4    LEELKKEVVMDDHKLTLDELSAKYSVDLTKGLSVTDAQEI  76
                    .         .         .         .
mouse α4    LFQNGPNVLTPPPTTPEWVKFCRQLFGGFSLLLWTGACLC 120
            |  ||||||||||||||| ||| ||||||||||||||  ||
rat   α4    LTLNGPNVLTPPPTTPEWIKFCKQLFGGFSLLLWTGSLLC 116
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FIGURE 1: Comparison of the amino-termini of the mouse and rat Na,K-ATPase α4 subunits. The deduced amino acid sequence of the mouse α4 subunit is shown above the rat α4 subunit. Vertical lines indicate identical amino acid residues, while horizontal dashes in the rat sequence allow optimal alignment for amino acid insertions. Amino acids are numbered to the right of the sequence.

RESULTS

Cloning and Characterization of the Murine Na,K-ATPase α4 Subunit. In a search of the GenBank EST database, we identified two murine ESTs with sequence homology to the rat Na,K-ATPase α4 subunit. One of the cDNA clones (567553) was sequenced on both strands and found to encode the 3′ end (nucleotides 957−3443) of the mouse α4 subunit. The 5′ end of the α4 subunit was generated from mouse testis RNA by RT-PCR. The complete cDNA, commencing at the initiating ATG contains a 3099 nt-long ORF and 281 nt of 3′-UTR sequence. The cDNA codes for a 1032 amino acid polypeptide that exhibits 76−78% identity to the rat Na,K-ATPase α1, α2, and α3 subunits. Sequence comparisons indicate that the rat (*14*) and mouse α4 subunits share 94% amino acid sequence identity and that most of the sequence differences represent conservative substitutions. However, sequences at the N-termini of the α4 subunit are quite divergent between rat and mouse species. As shown in Figure 1, residues 6−16 of the mouse α4 subunit are completely divergent from the corresponding segment of the rat α4 subunit. Within this segment, the mouse α4 subunit contains a four amino acid long insertion compared to the rat polypeptide.

Sequence comparisons indicate that the rodent α4 subunit is also quite divergent from the Na,K-ATPase α1, α2, and α3 subunits in the extracellular domain located between the first two transmembrane (TM) segments of the polypeptide. This extracellular domain has previously been shown to contain key residues that contribute to the ouabain sensitivity of the Na,K-ATPase (*5*). As shown in Figure 2, the first extracellular domain in the rat α subunit is 13 residues in length, whereas this domain is 12 residues long in each of the other three rodent and human α subunit isoforms. Compared to the rat and human α1, α2, and α3 subunits, the rodent α4 subunit exhibits no more than 25% amino acid sequence identity within the first extracellular domain.

Atp1a4 Encodes a Ouabain Resistant Na,K-ATPase. Although sequence comparisons suggest that *Atp1a4* is likely to encode a P-type ATPase α subunit, it is not possible to conclude from DNA sequence analysis alone whether the protein product of *Atp1a4* represents a functional Na,K-ATPase α subunit or an α subunit of a P-type ATPase with different ionic specificity. To help resolve this issue, we tested the ability of rat α4 subunit cDNA to confer ouabain resistance upon ouabain sensitive cells via transfection. In this assay, transfer of ouabain resistance indicates that the transfected α subunit can substitute for the endogenous α subunit of recipient cells. Because endogenous sodium pumps are poisoned in the presence of ouabain, rescue from ouabain cytotoxicity provides clear evidence that the transfected α subunit contributes to a functional Na,K-ATPase.

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mouse alpha4    HVNYYKENANKDN
  rat alpha4    HVSYYQENANKDN

rodent alpha1    RSATEEEPPNDD
 human alpha1    QAATEEEPQNDN

rodent alpha2    LAAMEDEPSNDN
 human alpha2    QAAMEDEPSNDN

rodent alpha3    QAGTEDDPSGDN
 human alpha3    QAGTEDDPSGDN
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FIGURE 2: Sequence comparison of the first extracellular domain of the Na,K-ATPase α subunit. Alignment of sequences in the first (H1−H2) extracellular domain of the human α1 (*29*), rat α1, α2, and α3 (*30*, *31*), and rat α4 (*14*) subunits. Mouse and rat (rodent) α1, α2, and α3 sequences are identical in this region (V. A. Canfield and R. Levenson, unpublished). The sequence of the H1−H2 region in human α4 is unknown. Charged residues at the boundaries of the H1−H2 ectodomain are shown in boldface.

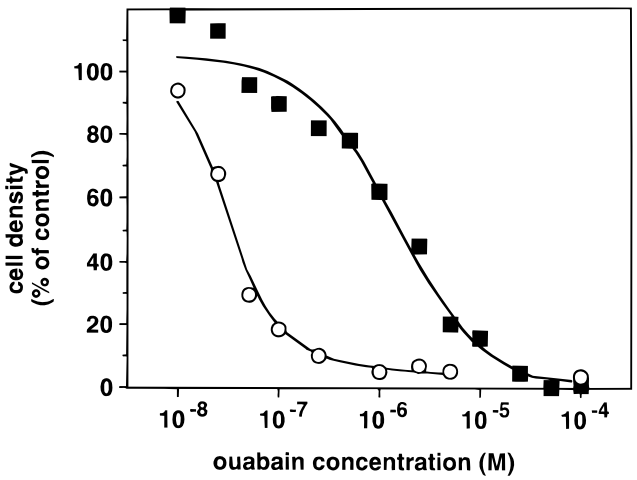

FIGURE 3: Ouabain resistant phenotype of α4 transfectants. HEK 293 cells transfected with the α4 subunit (closed squares) or untransfected 293 cells (open circles) were grown in increasing concentrations of ouabain. Mass cultures exposed to ouabain concentrations ranging from 0.01 to 100 μM were scored for survival after 3 days. Each point is an average of three cultures expressed as a percentage of the control culture which contained no drug.

A full length rat α4 subunit cDNA cloned into the mammalian expression vector pCB6 was transfected into ouabain-sensitive HEK 293 cells, and transfectants were selected in 0.5 μM ouabain. Using this approach, we were able to isolate stable, ouabain-resistant clones after three weeks of selection in drug. To determine the extent of ouabain resistance, populations of cells expressing the α4 subunit were plated in increasing concentrations of ouabain (Figure 3) and the drug concentration required to reduce the plating efficiency of each population by 50% (IC_{50}) was estimated. Wild-type HEK 293 cells exhibited an IC_{50} for ouabain of ∼30 nM. In contrast, cells expressing the rat α4 subunit demonstrated an IC_{50} for ouabain of ∼1.5 μM, a ∼50-fold increase in ouabain resistance compared to untransfected HEK 293 cells. Cells transfected with the

naturally ouabain-resistant rat α1 subunit exhibited an IC_{50} for ouabain of >100 μM (data not shown). These results indicate that the rat Na,K-ATPase α4 subunit is capable of conferring ouabain resistance upon ouabain-sensitive HEK 293 cells. The ability of the transfected α4 subunit to transfer the ouabain resistant phenotype provides compelling evidence that the α4 polypeptide represents a functional Na,K-ATPase α subunit isoform.

Chromosomal Localization and Expression of the Murine α4 Subunit Gene (Atp1a4). Genomic cloning studies have revealed that the gene encoding the α4 subunit is physically linked to the α2 subunit gene in the human genome. These two genes are separated by no more than 30 kb and have been mapped on human chromosome 1 (*15*). We have previously localized the Na,K-ATPase α2 subunit gene to the distal region of mouse chromosome 1, a region that is syntenic with a segment of human chromosome 1 containing the α2 subunit gene (*17, 18*). We asked, therefore, whether the α2 and α4 genes were closely linked in the mouse genome. To address this question, we utilized a panel of BAC clones representing a 700 kb region of mouse chromosome 1 that spans the *Looptail* (*Lp*) locus and the Na,K-ATPase α2 subunit gene (*17*).

In an initial series of experiments, cDNA probes encoding portions of either the rat α2 (nt 1104−3380) or rat α4 (3′-UTR) subunits were hybridized to a Southern blot containing DNAs prepared from several BAC clones. As shown in Figure 4A, the α2 subunit probe hybridized most intensely to BAC clone b4. BAC clone b4 has previously been shown to contain exons 1−13 of *Atp1a2*, the gene encoding the Na,K-ATPase α2 subunit (*17*). In addition, the α2 probe hybridized less intensely to a distinct set of DNA fragments in BAC clones b5, b6, and b7 (Figure 4A). STS content mapping indicates that BACs b5, b6, and b7 do not overlap b4, suggesting the possibility that these signals represent cross hybridization of the α2 probe with another Na,K-ATPase α subunit. Consistent with this notion, a 3′-UTR α4 cDNA probe hybridized strongly with an informative set of DNA bands in BAC clones b5, b6, and b7, but not b4 (Figure 4A, lower panel). These hybridizing bands were clearly distinct from the DNA fragments detected with the α2 probe in BAC b4. Neither the α2 or α4 probes hybridized to BACs b8 or b9, which overlap the proximal boundaries of BACs b3 and b6. These results suggest that segments of the murine α4 subunit gene are contained within BAC clones b5, b6, and b7.

To more precisely map the relative location of α2 and α4 sequences, additional BACs were analyzed using murine cDNA probes that are specific for the α2 or α4 3′-UTR regions. As shown in Figure 4B, the α2-specific probe hybridized to a single band in BAC clones b3, b10, and b11. This is consistent with previous mapping studies (*17*) in which BAC b3 was found to contain exons 4−23 of *Atp1a2*, which includes the 3′-UTR. In contrast, the α4-specific probe hybridized to a single band in BACs b3 and b6, but did not hybridize to b10 or b11. These results suggest that clones b10 and b11 contain segments of the murine α2 subunit gene, that clone b6 contains a portion of the α4 subunit gene, and that sequences for both α2 and α4 genes are present on BAC b3.

A composite map of the relative positions of the α2 and α4 genes is shown in Figure 4C. The BAC array is based

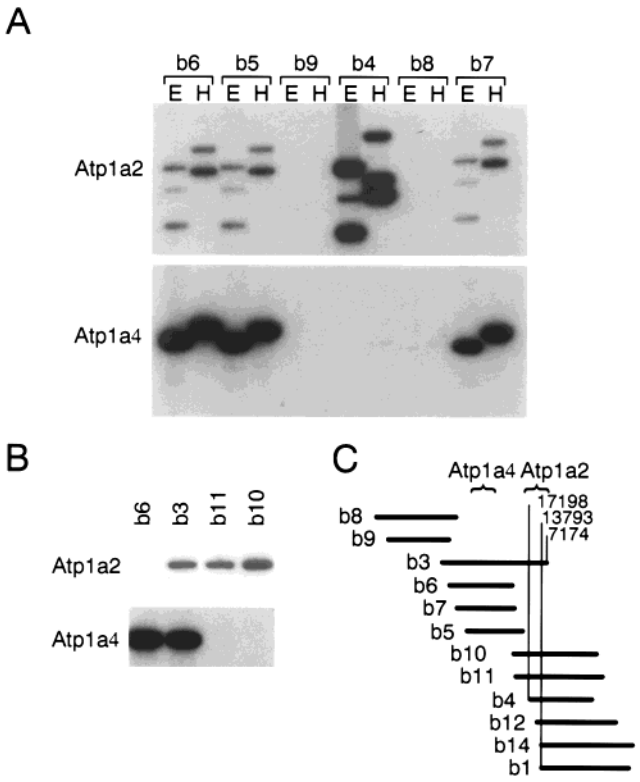

FIGURE 4: Mapping of *Atp1a4* to mouse chromosome 1. α4 gene sequences were mapped onto a BAC contig of the mouse *Looptail* region. A. Upper panel. Southern blot of DNA prepared from BAC clones b4−b9, digested with either *Eco*RI (E) or *Hind*III (H) and hybridized with a rat α2 (nt 1104−3380) cDNA fragment. Lower panel. The same blot, stripped and rehybridized with a rat α4 3′-UTR probe. B. Upper panel. Southern blot of DNA prepared from BAC clones b3, b6, b10, and b11, digested with *Eco*RI and hybridized with a murine, α2-specific, 3′-UTR probe. Lower panel. The same blot, stripped and rehybridized with a murine, α4-specific, 3′-UTR probe. C. Location of *Atp1a4* and *Atp1a2* sequences within the BAC contig of the mouse *Looptail* region. The BAC contig and linear relationship of BAC ends has been previously described (*17*). Vertical lines indicate the position of BAC end sequences (and corresponding nucleotide number) in the homologous human α2 gene ATP1A2.

on previous BAC-end sequencing and STS-mapping experiments (*17*). Here we show by hybridization analysis that *Atp1a4* maps to BACs b3, b5, b6, and b7, whereas *Atp1a2* maps to BACs b3, b4, b10, and b11. These results place *Atp1a4* centromeric to *Atp1a2* on mouse chromosome 1. Our earlier mapping studies have shown that BACs b8 and b9 overlap the proximal boundaries of clones b3 and b6 (*17*). The absence of hybridizing α4 subunit sequences on BACs b8 and b9, therefore, suggests that these BACs define the proximal boundary for the position of *Atp1a4* within this BAC contig. The presence of *Atp1a4* and *Atp1a2* sequences on BAC b3 strongly indicates that these two genes are in close proximity to one another on mouse chromosome 1. Since the DNA insert within BAC b3 is ~170 kb, the α2 and α4 subunit genes could be separated by a maximal distance of 170 kb, although the absence of α4 sequences on overlapping BACs (b8, b9, b10, and b11) suggests they are likely to be much closer. Taken together, our mapping data show that *Atp1a2* and *Atp1a4* are closely linked on the distal portion of mouse chromosome 1.

We examined expression of *Atp1a4* in a panel of adult and embryonic mouse tissues. A dot blot containing poly

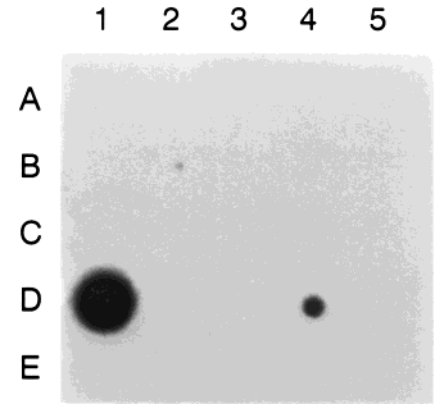

FIGURE 5: Expression of α4 subunit mRNA in mouse tissues and during embryogenesis. A murine RNA Master Blot (Clontech) containing poly A^+ RNA from a panel of mouse tissues and embryos was probed with cDNA derived from mouse α4 EST clone 937308 (nt 2957−3443). Key: A1, brain; A2, eye; A3, liver; A4, lung; A5, kidney; B1, heart; B2, skeletal muscle; B3, smooth muscle; C1, pancreas; C2, thyroid; C3, thymus; C4, submaxillary gland; C5, spleen; D1, testis; D2, ovary; D3, prostate; D4, epididymis; D5, uterus; E1, embryonic day 7; E2, embryonic day 11; E3, embryonic day 15; E4, embryonic day 17.

A^+ RNA prepared from 18 different mouse tissues, as well as 4 stages of embryonic mouse development, was hybrized with a probe derived from the 3′ end of the mouse α4 subunit cDNA. As shown in Figure 5, α4 subunit mRNA was detected at high abundance in testis and at lower abundance within epididymis. Expression of α4 mRNA was undetectable in any other mouse tissue or during fetal mouse development. Testis-specific expression of the α4 subunit has been reported in the rat and human (*14*). Our results provide the first evidence of α4 expression in a tissue other than testis. It will clearly be of interest to determine whether α4 subunits are present predominantly in spermatocytes, or whether this ATPase subunit is expressed in additional compartments within testis and epididymis.

DISCUSSION

Sequences encoding a potential fourth Na,K-ATPase α subunit were originally identified in a genomic screen designed to identify genes exhibiting high sequence similarity to Na,K-ATPase catalytic subunits (*15*). However, the functional status of the α4 gene has not yet been investigated. By mapping α4 gene sequences onto a panel of BAC clones, we show very tight linkage of the Na,K-ATPase α4 and α2 genes in mouse. The α4 and α2 genes have also been shown to be closely linked in humans (*15*), suggesting a common evolutionary history for these two genes in mice and man. Expression of the murine α4 subunit gene is restricted primarily to testis, an expression pattern also manifested in rat and humans (*14, 15*). Similarities in genomic organization and expression patterns suggest that the mouse and human α4 genes are likely to be orthologous. Using an assay that measures the biological activity of transfected ATPase genes, we found that rat α4 subunit cDNA conferred ouabain resistance upon ouabain sensitive cells. The ability of the transfected α4 subunit to substitute for the endogenous ouabain sensitive α subunit of HEK 293 cells provides strong support for the view that the α4 gene encodes a functional and ouabain-resistant Na,K-ATPase α subunit isoform.

We localized *Atp1a4* to distal mouse chromosome 1 by hybridization of an α4-specific probe to a panel of overlapping BAC clones previously mapped onto this region of the mouse genome. The BAC clones form a contig that spans a 700 kb region overlapping the mouse *Looptail* (*Lp*) mutation (*17*). Our data indicate that sequences specific to both the *Atp1a2* and *Atp1a4* genes are present within this contig. In humans, the α2 and α4 genes were originally localized on overlapping λ phage clones, ∼25−30 kb apart (*15*). Localization of α2 and α4 sequences on the same BAC clone establishes a maximal physical distance between the murine genes of ∼170 kb. However, recent transcription mapping studies indicate that *Atp1a2* and *Atp1a4* are likely to be located in much closer proximity to each other (Underhill et al., unpublished results). Additional physical mapping of BAC clone b3 should establish more precisely the physical distance separating *Atp1a2* and *Atp1a4* on chromosome 1. The close physical proximity of *Atp1a2* and *Atp1a4* in the mouse and human genomes, together with the high degree of amino acid sequence similarity in the α2 and α4 polypeptides, is consistent with the view that *Atp1a2* and *Atp1a4* evolved from a common ancestral gene via gene duplication. However, the fact that these two genes exhibit markedly different expression patterns suggests that the enzyme encoded by each gene may have functional properties selected in response to different physiological demands.

The mapping of *Atp1a4* to distal mouse chromosome 1 raises an additional point of interest. Our physical mapping data places *Atp1a4* within the nonrecombinant interval encompassing the mouse *Lp* mutation. *Atp1a4* can, therefore, be considered a new positional candidate for *Lp*. The *Lp* mutation is manifested by an open neural tube extending from midbrain to the tip of the tail (*19, 20*). In wild-type mice, we show that α4 mRNA does not appear to be expressed at embryonic day 7 or at later embryonic stages (Figure 5). It is not yet known whether *Atp1a4* is expressed during the time of neural tube closure (day 8.5 post conception in mouse), or during gastrulation when defects in neural patterning can first be detected in *Lp* embryos (*21*). However, the fact that expression of *Atp1a4* has so far only been detected in adult mouse testis makes it difficult to reconcile a possible role for *Atp1a4* in manifestation of the *Lp* phenotype.

Our transfection experiments have revealed a previously unrecognized aspect of α4 subunit function. Introduction of α4 subunit cDNA was found to confer ouabain resistance upon ouabain-sensitive primate cells. HEK 293 cells stably expressing the rat α4 subunit exhibit an ∼50-fold increase in ouabain resistance compared to untransfected wild-type 293 cells. These results indicate that the transfected α4 subunit can substitute for the endogenous ouabain-sensitive α subunit of 293 cells and contribute to a functional Na,K-ATPase. The low level of ouabain resistance of the α4 subunit observed here is consistent with recent measurements of ouabain binding to α4 subunits expressed in murine 3T3 cells (*22*).

The ability of the α4 subunit to transfer ouabain-resistance in our assay is interesting for the following reasons. Naturally ouabain-resistant α subunits, such as the rodent α1 isoform and the hydra α subunit, contain a sequence motif associated with the ouabain resistant phenotype (*23, 24*). This motif is characterized by the presence of charged residues at the

boundaries of the first extracellular domain of the α subunit polypeptide (Figure 2). Sequence analysis indicates that the α4 subunit contains two neutral residues, histidine and asparagine, at the boundary positions. Although histidine can be protonated at slightly acidic pHs, it is not clear whether it is charged or uncharged within the overall context of the α subunit. Thus, it is possible that the presence of histidine at one of the boundary positions could account for the ouabain resistant properties of the α4 subunit. If this is the case, we would expect that the ouabain sensitivity of the α4 subunit may vary as a function of extracellular pH. Alternatively, it is possible that other residues within the α4 subunit may be responsible for ouabain resistance. We have previously demonstrated that sequences other than the border residues within the first extracellular loop of the α subunit can contribute to ouabain resistance (*13*). In addition, mutation of individual residues located within the second extracellular domain and the carboxyl-terminal third of the polypeptide (*25*) have been found to produce a ouabain resistant α subunit. Expression of chimeric α subunits could help to pinpoint segments within the α4 polypeptide that contribute to the ouabain resistance of this isoform.

The studies presented here are consistent with the view that *Atp1a4* encodes a functional Na,K-ATPase α subunit isoform. This conclusion is based on the ability of the α4 subunit to rescue transfected cells from ouabain cytotoxicity, an indication that the transfected α4 subunit can substitute for the endogenous α subunit of HEK 293 cells. Rescue from ouabain cytotoxicity provides strong evidence that the transfected α4 subunit contributes to a functional Na,K-ATPase. The identification of α4 as a catalytic subunit of the Na,K-ATPase suggests the potential for greater isoenzyme heterogeneity than was previously recognized. Several lines of evidence suggest that Na,K-ATPase α/*β* subunit interaction is promiscuous (*26*−*28*) and that each α subunit is capable of association with any of the three identified *β* subunits to produce functional Na,K-ATPase. The existence of four different mammalian α subunits and three *β* subunits raises the possibility of twelve potential Na,K-ATPase isoenzymes. It will now be of interest to determine whether α4 also is promiscuous, or whether this catalytic subunit exhibits specificity with regard to *β* subunit interaction.

The existence of four Na,K-ATPase α subunits, each with a distinct expression pattern, suggests specialized functional roles for these isoforms. Recent knockout experiments have pointed to a specific functional role for the Na,K-ATPase α2 subunit in Ca^{2+} signaling during cardiac contraction (*9*). Mice carrying a targeted deletion in one of the two *Atp1a2* alleles exhibit hypercontractility as a result of increased calcium currents during the myocardial contractile cycle (*9*). The restricted expression pattern of α4 subunits suggests that this α subunit isoform is likely to play a specialized role in testis and epididymis. The development of α4-specific antibodies and localization of the α4 polypeptide to specific cell types or compartments within the testis and epididymis may provide clues as to the role of α4 in testicular function or spermatogenesis. Generation of mice carrying a targeted mutation in *Atp1a4* should also help elucidate the role of this P-type ATPase in testis, and its possible association with the *Looptail* phenotype.

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