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Human placental Na^+,K^+-ATPase α subunit: cDNA cloning, tissue expression, DNA polymorphism, and chromosomal localization

(Southern analysis/RNA transfer blotting/*in situ* hybridization/chromosome 1/butyrate induction)

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ABSTRACT **A 2.2-kilobase clone comprising a major portion of the coding sequence of the Na^+,K^+-ATPase α subunit was cloned from human placenta and its sequence was identical to that encoding the α subunit of human kidney and HeLa cells. Transfer blot analysis of the mRNA products of the Na^+,K^+-ATPase gene from various human tissues and cell lines revealed only one band (≈4.7 kilobases) under low and high stringency washing conditions. The levels of expression in the tissues were intestine > placenta > liver > pancreas, and in the cell lines the levels were human erythroleukemia > butyrate-induced colon > colon > brain > HeLa cells. mRNA was undetectable in reticulocytes, consistent with our failure to detect positive clones in a size-selected (>2 kilobases) λgt11 reticulocyte cDNA library. DNA analysis revealed a polymorphic *Eco*RI band and chromosome localization by flow sorting and *in situ* hybridization showed that the α subunit is on the short arm (band p11-p13) of chromosome 1.**

Na^+,K^+-ATPase is present in the plasma membrane of all animal cells and is responsible for the vectorial transport of Na^+ out and K^+ into the cell. It is an integral transmembrane enzyme complex comprised of two subunits, a catalytic α subunit (110 kDa) and a glycosylated β subunit (50 kDa). The α subunit bears the cytoplasmic ATP binding and phosphorylation sites as well as the extracellularly exposed cardiac glycoside binding site; the function of the β subunit remains unknown. It is generally believed that the active complex is an $\alpha_2\beta_2$ dimer (for review, see ref. 1).

The enzymatic reaction catalyzed by Na^+,K^+-ATPase has been studied extensively and shown to involve a complex sequence of steps whereby specific cation binding and release are tightly coupled to ATP binding, phosphorylation–dephosphorylation, and conformational transitions of the enzyme. The transmembrane disposition and topography have also been characterized (1). More recently, detailed information about the primary structure of the α and β subunits of Na^+, K^+-ATPase from diverse sources has been deduced from the complete cDNA sequences. Thus, the cDNA sequences encoding the α subunit from sheep kidney (2) and the electric ray *Torpedo californica* (3) are 77% homologous, representing >85% amino acid sequence homology, and cDNA sequences encoding the β subunit of mammalian (human HeLa) cells (4) and *T. californica* (5) are 61% homologous. In rat brain, the β-subunit gene encodes four mRNA species that are expressed in a tissue-specific manner (6).

In this paper, we describe the isolation, characterization, expression, and chromosomal mapping of a cDNA** containing a major portion of the coding region for the catalytic α subunit of Na^+,K^+-ATPase derived from human placental tissue. This sequence includes regions coding for the major functional domains of the enzyme—namely, the intracellularly located ATP binding and phosphorylation sites as well as most, if not all, of the extracellular cardiac glycoside binding site.

MATERIALS AND METHODS

Isolation of cDNA. An oligo(dT)-primed human placenta cDNA library in λgt11 (7) was screened according to the method of Benton and Davis (8) with an α-subunit Na^+,K^+-ATPase ^{32}P-labeled cDNA probe from sheep kidney (2), kindly provided by J. B. Lingrel (University of Cincinnati College of Medicine, Cincinnati, OH). Subcloning was performed in the plasmid vector pBS (Stratagene, San Diego, CA).

RNA Isolation and Transfer Blotting. RNA samples from human placenta, intestine, pancreas, liver, and reticulocytes were isolated by the guanidinium thioisocyanate method of Chirgwin *et al.* (9). RNA from cultured human brain glioblastoma cell lines 188 and 126 (10), human colon carcinoma LS174T (11), human epithelial cells (HeLa), and human erythroleukemia cells (HEL) was prepared as described (12). Poly(A)$^+$ mRNA was purified by affinity chromatography on oligo(dT)-cellulose columns (13). Two micrograms of poly-(A)$^+$ RNA from different tissue sources was fractionated by electrophoresis in 1% agarose/2.2 M formaldehyde as described (14, 15). The RNA was transferred onto nitrocellulose filters and hybridized with an α-^{32}P-labeled dCTP nick-translated probe. A nonstringent wash was performed in 0.3 M NaCl/30 mM sodium citrate at 25°C for 45 min; this was followed by autoradiography. A second stringent wash in 15 mM NaCl/1.5 mM sodium citrate at 65°C for 45 min was also performed and followed by autoradiography.

DNA Isolation and Southern Blotting. Human genomic DNA was isolated from leukocytes as described by Goossens and Kan (16). Two to 10 μg of DNA was cleaved with restriction enzymes and the generated fragments were separated on 0.8% agarose gels and transferred to nitrocellulose filters as described by Southern (17). The hybridization and washing conditions were as described above for RNA transfer blots except with the omission of the nonstringent wash.

Subcloning and DNA Sequencing. The 2.2-kilobase (kb) *Eco*RI insert was first subcloned into the plasmid vector pBS and then into the filamentous bacteriophage vector M13-mp10, and single-stranded DNA was recovered from recombinant particles as described by Messing (18). DNA sequenc-

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Abbreviation: HEL, human erythroleukemia.
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**This sequence is being deposited in the EMBL/GenBank data base (Bolt, Beranek, and Newman Laboratories, Cambridge, MA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J03007).

ing was performed with dideoxynucleotide chain termination using dATP[α-^{35}S] (19) and DNA primers synthesized on an Applied Biosystems DNA synthesizer model 380A.

Chromosomal Localization. Human chromosome suspensions were prepared from a lymphocyte cell line and stained with 4',6-bis[2'-imidazolinyl]-4*H*,5*H*]-2-phenylindole and chromomycin A3 (20). Thirty thousand chromosomes of each type were sorted with a dual-laser fluorescence-activated cell sorter and spotted onto nitrocellulose filters. The chromosomes were alkali denatured *in situ*, neutralized, baked, and hybridized to the Na^+,K^+-ATPase cDNA isolated from the placental cDNA library. Final washing was at 65°C for 45 min in 15 mM NaCl/1.5 mM sodium citrate/0.1% $NaDodSO_4$. *In situ* hybridization was performed by preparing human chromosome spreads from phytohemagglutinin-stimulated peripheral blood lymphocytes synchronized with methotrexate and thymidine. The procedures for *in situ* hybridization using ^{3}H-labeled probes were the same as described (21, 22). For regional mapping of the Na^+,K^+-ATPase gene on chromosome 1, only cells with grains on chromosome 1 were selected for analysis.

RESULTS

Isolation of a Human Placenta α-Subunit cDNA. Using the human placenta cDNA library, ≈200,000 bacteriophages were screened with the sheep kidney cDNA probe pNKA1 (2). Four positive clones were purified and characterized. They all contained a 2.2-kb *Eco*RI fragment (HP.NKα1), which was first subcloned into the plasmid vector pBS and then into the filamentous bacteriophage vector M13mp10; it was next subjected to DNA sequencing.

HP.NKα1 was found to contain an open reading frame of 745 amino acids (Fig. 1). By comparison with the cDNA sequences from the electrical ray, rat, and sheep, the primary structure of HP.NKα1 includes the hydrophobic regions H3–H7 as well as the phosphorylation and ATP binding sites but not the H1, H2, and H8 regions. When the deduced amino acid sequence of HP.NKα1 was compared to its counterparts from rat brain, sheep kidney, and ray electric organ, homologies of 97.3%, 97.5%, and 88.3%, respectively, were found. In this 745-amino acid stretch from the placenta cDNA, the rat and sheep α subunits differ from the human α subunit in 21 and 16 residues, respectively (Table 1).

Expression of the α-Subunit cDNA in Human Tissues. The human placenta α-subunit cDNA (HP.NKαl) was used to analyze the RNA products of Na^+,K^+-ATPase gene expression in various human tissues by RNA transfer blotting. One RNA species of ≈4700 nucleotides was detected in mRNA from human liver, pancreas, placenta, and intestine as well as from tissue culture cells (HEL, HeLa, brain, colon) grown *in vitro* (Fig. 2). Only one band was detected under nonstringent and stringent washing conditions. There was no detectable hybridization in reticulocytes, although equal amounts of poly(A)$^+$ mRNA were loaded in each lane. The same blot was rehybridized with protein 4.1 cDNA, which detected a 5.6-kb mRNA in the reticulocyte lane (data not shown), thus showing that the absence of α-subunit mRNA did not result from RNA degradation but rather from the lack of any detectable mRNA. RNA transfer blotting also showed that the levels of expression of the Na^+,K^+-ATPase α subunit vary in the tissues examined. In RNA derived from human tissues, the levels of the Na^+,K^+-ATPase α-subunit mRNA expression were in the order: intestine > placenta > liver > pancreas. In tissue culture cells, the levels were HEL > colon > brain > HeLa. Whereas cells grown *in vitro* may not reflect the *in vivo* situation, it is clear that in mRNA isolated from fresh tissues, the levels of mRNA expression of the Na^+,K^+-ATPase α-subunit gene are regulated in a tissue-specific manner. Moreover, the induction of the colon cell line with sodium butyrate results in an ≈2-fold increase in the amount of mRNA expressed as compared to the noninduced state.

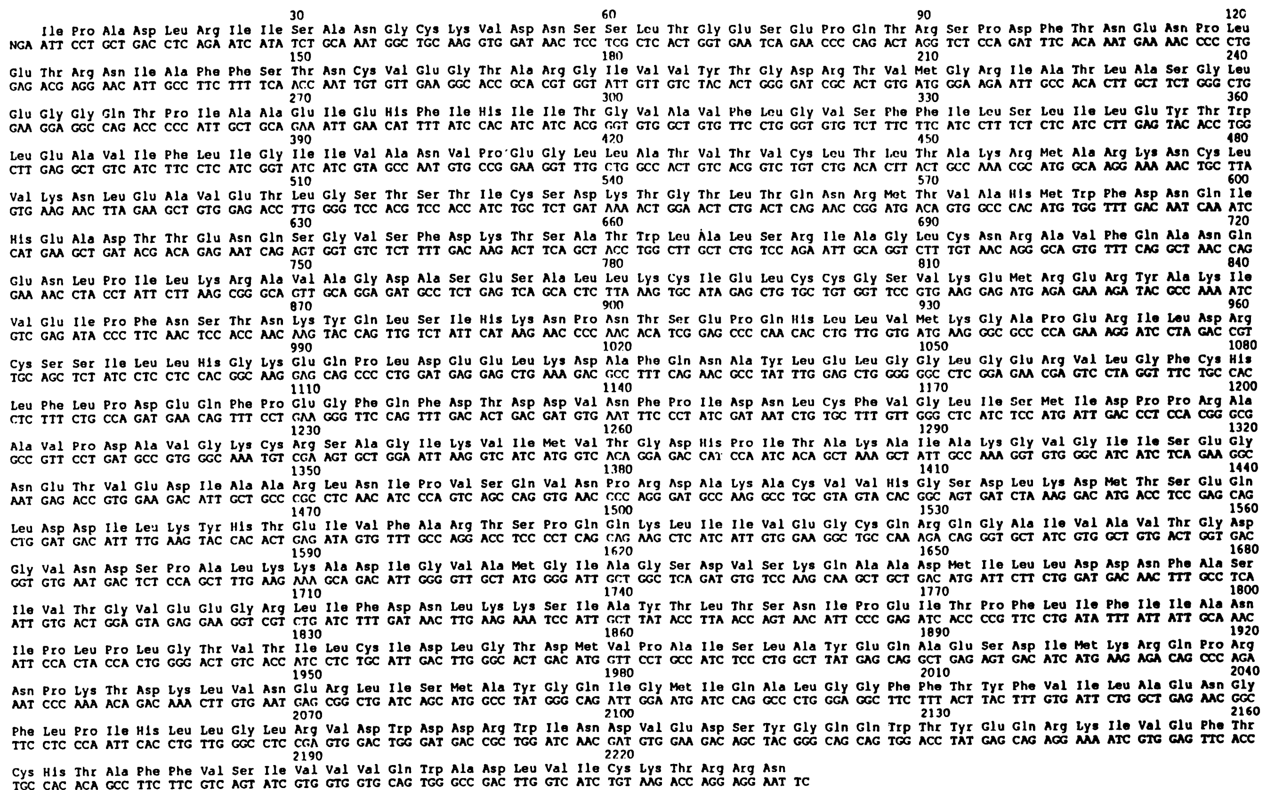

FIG. 1. Nucleotide and deduced amino acid sequence of HP.NKα1. The sequence starts and ends with an *Eco*RI site (GAATTC) and the numbers refer to nucleotides in the clone.

Table 1. Positions of the amino acid residue where the sheep and rat Na^+,K^+-ATPase α subunit differ from the human α subunit

Residue	Rat	Human	Sheep
279	**E**	A	A
287	**L**	I	I
412	**F**	L	L
431	E	E	**D**
455	**V**	L	**V**
461	**M**	K	K
466	**K**	R	R
468	**T**	A	A
489	P	P	**A**
491	**A**	T	**A**
492	S	S	**G**
495	**K**	Q	**R**
516	L	L	**I**
552	**L**	F	**M**
568	**E**	D	D
573	**V**	I	**V**
646	**N**	S	S
654	K	K	**R**
668	S	S	**P**
670	**E**	Q	Q
676	**R**	L	L
730	**V**	A	A
833	**L**	K	**Q**
866	L	L	**M**
874	**F**	I	**N**
879	**I**	L	**I**
881	**E**	V	V
882	**T**	D	**T**

Residue number is according to the sheep Na^+,K^+-ATPase α subunit (2). Residues in bold type indicate differences from the human residue.

DNA Analysis. Southern blot analysis of human DNA digested with *Eco*RI uncovered a variable 6.5-kb fragment (Fig. 3). When the same blot was hybridized with a β-globin probe, the expected globin bands were detected with equal intensities (data not shown). Hence, the variable 6.5-kb *Eco*RI fragment did not result from incomplete digestion but rather from a restriction fragment length polymorphism.

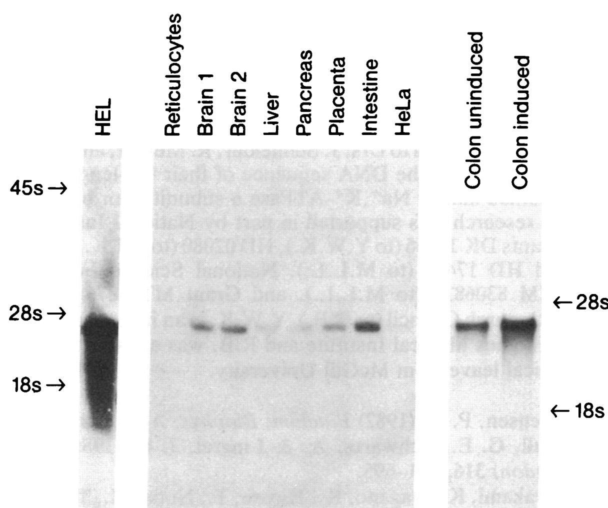

FIG. 2. Transfer blot analysis of RNA. Two micrograms of poly(A)$^+$ RNA was loaded into each lane except 1.5 μg was loaded for HEL cells. Brain 1 and 2 represent two glioblastoma cell lines. The filter was hybridized with the nick-translated ^{32}P-labeled HP.NKα1 and washed at 65°C in 15 mM NaCl/1.5 mM sodium citrate/0.1% $NaDodSO_4$. A nonstringent wash in 0.3 M NaCl/30 mM sodium citrate at 25°C yielded identical RNA bands.

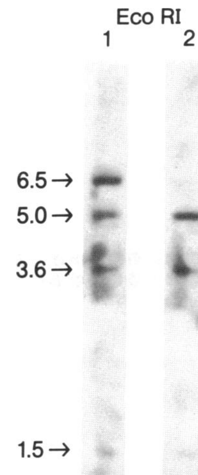

FIG. 3. Southern blot analysis of genomic DNA. Ten micrograms of human genomic DNA from two individuals was digested with *Eco*RI and hybridized to a nick-translated ^{32}P-labeled HP.NKα1 probe. Lane 1 contains an additional 6.5-kb band representing a restriction fragment length polymorphism.

Chromosomal Localization and Regional Mapping of HP.NKα1. Hybridization of HP.NKα1 to chromosome spot blots revealed an autoradiographic signal in the chromosome 1 fraction (Fig. 4). For the sublocalization of this signal, 62 chromosome spreads with grains on human chromosome 1 were analyzed, with an average of 4 grains per spread (14-day exposure). Among 93 grains found on chromosome 1, 60% (56/93) of the grains were clustered in the proximal short arm close to the centromere, in the region 1p11-1p13 (Fig. 5). Thus, the Na^+,K^+-ATPase α-subunit gene is regionally assigned to 1p11-1p13.

DISCUSSION

The high degree of nucleotide sequence homology obtained for the cDNA clone of human placental Na^+,K^+-ATPase with the corresponding region of the Na^+,K^+-ATPase α subunit of sheep kidney (2) and *T. californica* (3) provides unequivocal evidence that this cDNA clone contains the major portion of DNA coding for the human catalytic subunit. The striking homology in the primary structure of the α-subunit Na^+,K^+-ATPase in the electric ray, sheep, and human lends support to the idea that slow evolutionary

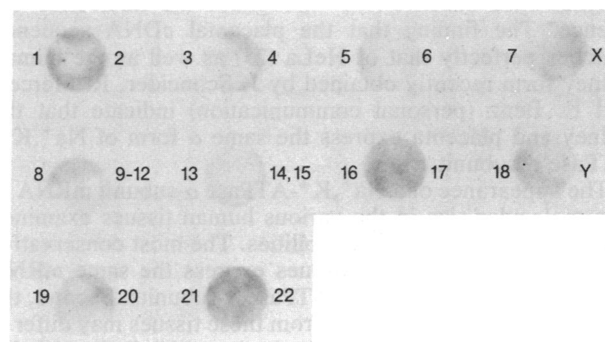

FIG. 4. Chromosomal localization of the Na^+,K^+-ATPase α-subunit gene. A panel of 25-mm (diameter) nitrocellulose filters containing human sorted chromosomes was hybridized to ^{32}P-labeled HP.NKα1. The only positive signal was on the filter containing chromosome 1.

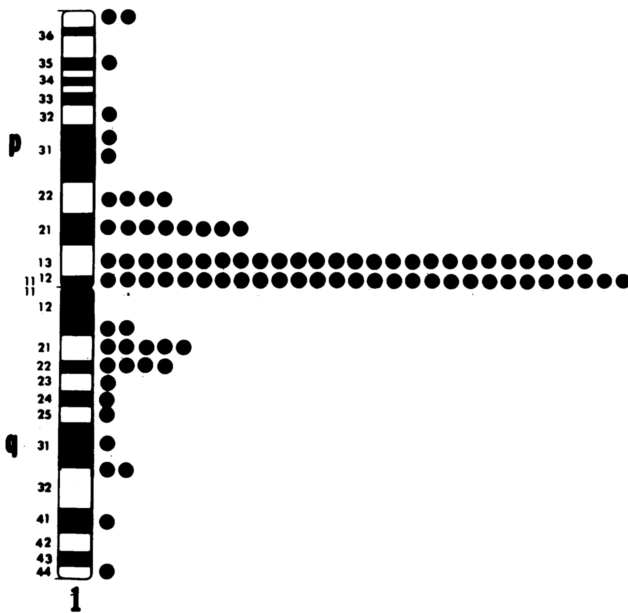

FIG. 5. Sublocalization of the Na^+,K^+-ATPase α-subunit gene. The histogram shows a grain distribution on human chromosome 1 after *in situ* hybridization of the ^3H-labeled HP.NKα1 to human lymphocyte metaphase spreads.

diversity occurs in proteins that interact with other macromolecules. Thus, the slower evolutionary rate for cytochrome *c* compared to the globins has been attributed primarily to the fact that cytochrome *c* interacts with other macromolecular complexes, whereas the primary function of hemoglobin is to bind only O_2 (23).

Amino acid substitutions often occur at the same positions in sheep, rat, and humans. It is interesting to consider whether certain of the amino acid substitutions in mammalian α subunits are relevant to differences in ouabain sensitivity (see Table 1). Thus, of the 12 residues common to the ouabain-sensitive human and sheep α subunits, 6 are common also to the more phylogenetically diverse ouabain-sensitive *T. californica* enzyme but not to the relatively ouabain-insensitive rat enzyme. Surprisingly, these residues (positions 279, 287, 461, 466, 676, and 730) are located in the putative cytoplasmic domain between H2 and H3 and between H4 and H5 rather than at the extracellular ouabain binding domain.

Recently, three distinct forms (α, α^+, and αIII) of the Na^+,K^+-ATPase α subunit from rat brain were cloned and sequenced (24). All three cDNAs differ from each other by internal base substitutions throughout the nucleotide sequence. The finding that the placental cDNA sequence matches perfectly that of HeLa (25) as well as the human kidney form recently obtained by J. Schneider, R. Mercer, and E. Benz (personal communication) indicate that the kidney and placenta express the same α form of Na^+,K^+-ATPase α subunit.

The appearance of a Na^+,K^+-ATPase α-subunit mRNA of one molecular size in the various human tissues examined could be due to several possibilities. The most conservative explanation is that these tissues express the same mRNA encoding identical Na^+,K^+-ATPase α subunits. Second, the Na^+,K^+-ATPase α isoforms from these tissues may differ in primary structure but are similar in size and indistinguishable on RNA transfer blotting. This interpretation is based on the finding that transfer blotting of RNA from rat brain revealed one molecular RNA species (26), whereas three different Na^+,K^+-ATPase α-subunit isoforms have been cloned and sequenced from the same tissue (24). For the rat, the two isoforms encoding α and αIII are the same size, whereas α^+ is larger. Moreover, Schull and Lingrel (27) recently identified two human genes, αA and αB, of $\approx$20–25 kb that code for the α and α^+ catalytic subunits, respectively, thus implying that at least two isoforms are expressed in humans. A third possibility is that the human placenta Na^+,K^+-ATPase α-subunit cDNA is sufficiently unique in nucleotide sequence that it does not cross-hybridize with another α-subunit isoform mRNA. This is unlikely since rat brain α- and α^+-subunit cDNAs are 76.5% homologous (24) and do hybridize to the human genomic DNA (26). Thus, some degree of substantial homology and consequently cross-hybridization would be expected between the human α- and α^+-subunit mRNAs. Based on the recent molecular cloning of two human genes encoding the α and α^+ isoforms, we tend to favor the second hypothesis.

The finding that reticulocytes did not show any detectable mRNA is consistent with our earlier failure to detect positive clones in a λgt11 cDNA library constructed from size-selected (>2 kb) reticulocyte poly(A)$^+$ mRNA (unpublished). In contrast, mRNA for the α subunit is particularly abundant in immature HEL cells. Though Na^+,K^+-ATPase activity can readily be demonstrated even in the mature erythrocyte, it is likely that erythrocytes at the penultimate reticulocyte stage are devoid of mRNA encoding the α subunit and are no longer capable of synthesizing Na^+,K^+-ATPase. Also, the increased mRNA in the induced colon cells implies that the known increased transepithelial cation transport following induction by butyrate is due to the induction of mRNA transcription.

We detected a 6.5-kb polymorphic *Eco*RI band in the genomic DNA from some individuals. Schneider *et al.* (26) also detected this band in a HeLa cell line resistant to ouabain but not in a ouabain-insensitive line. Although they attributed the 6.5-kb band to gene amplification and DNA rearrangement in the resistant line, our data indicate that the 6.5-kb band could be due to DNA polymorphism. This *Eco*RI polymorphism may prove to be useful in the investigation of the suspected human Na^+,K^+-ATPase-related disorders such as familial high blood pressure. It also remains to be determined whether any human disease characterized by altered sodium permeability relates to the Na^+,K^+-ATPase gene(s).

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