

cDNA Cloning of Na ,K -ATPase α -Subunit from Embryos of the Sea Urchin, *Hemicentrotus pulcherrimus*

Authors: Mitsunaga-Nakatsubo, Keiko, Yamazaki, Ken, Hatoh-Okazaki, Midori, Kawashita, Hiroto, Okamura, Chisato, et al.

Source: Zoological Science, 13(6) : 833-841

Published By: Zoological Society of Japan

URL: <https://doi.org/10.2108/zsj.13.833>

The BioOne Digital Library (<https://bioone.org/>) provides worldwide distribution for more than 580 journals and eBooks from BioOne's community of over 150 nonprofit societies, research institutions, and university presses in the biological, ecological, and environmental sciences. The BioOne Digital Library encompasses the flagship aggregation BioOne Complete (<https://bioone.org/subscribe>), the BioOne Complete Archive (<https://bioone.org/archive>), and the BioOne eBooks program offerings ESA eBook Collection (<https://bioone.org/esa-ebooks>) and CSIRO Publishing BioSelect Collection (<https://bioone.org/csiro-ebooks>).

Your use of this PDF, the BioOne Digital Library, and all posted and associated content indicates your acceptance of BioOne's Terms of Use, available at www.bioone.org/terms-of-use.

Usage of BioOne Digital Library content is strictly limited to personal, educational, and non-commercial use. Commercial inquiries or rights and permissions requests should be directed to the individual publisher as copyright holder.

BioOne is an innovative nonprofit that sees sustainable scholarly publishing as an inherently collaborative enterprise connecting authors, nonprofit publishers, academic institutions, research libraries, and research funders in the common goal of maximizing access to critical research.

cDNA Cloning of Na⁺, K⁺-ATPase α -Subunit from Embryos of the Sea Urchin, *Hemicentrotus pulcherrimus*

Keiko Mitsunaga-Nakatsubo^{1,*,**}, Ken Yamazaki^{1,*}, Midori Hatoh-Okazaki¹, Hiroto Kawashita¹, Chisato Okamura¹, Koji Akasaka², Hiraku Shimada² and Ikuo Yasumasu^{1,***}

¹Department of Biology, School of Education, Waseda University, 1-6-1, Nishiwaseda, Shinjuku-ku, Tokyo 169-50, Japan

²Graduate Department of Gene Science, Faculty of Science, Hiroshima University, 1-3-1, Kagamiyama, Higashi-Hiroshima 739, Japan

ABSTRACT—Na⁺, K⁺-ATPase α -subunit cDNA of the sea urchin, *Hemicentrotus pulcherrimus*, was obtained by twice screening prism and gastrula λ gt10 cDNA libraries using an oligonucleotide probe derived from a mostly conserved region, FSBA (5'-*p*-(fluorosulfonyl)-benzoyl-adenosine) binding site of cation transport ATPases. The 5'-end of the non-coding region was determined by primer extension and the region was amplified by 5'-RACE method. The sea urchin α -subunit cDNA consists of 4401 nucleotides and encodes 1038 amino acid residues (MW, 114 kDa). The predicted primary structure, except N-terminal region, has similar degree of high homology to various metazoan Na⁺, K⁺-ATPase α -subunits. Alignment of amino acid sequence and a hydropathy profile also predicts eight putative transmembrane segments at least. The phylogenetic tree suspected from alignment of amino acid sequences of 21 species suggests that sea urchin and vertebrate Na⁺, K⁺-ATPase α -subunits seem to have evolved from a common origin, before vertebrate α -subunit divided into three isoforms.

INTRODUCTION

Na⁺, K⁺-ATPase is a membrane-bound enzyme responsible for active transport of Na⁺ and K⁺ across the plasma membrane in most animal cells. The protein is composed of two subunits; a large catalytic α -subunit and a smaller glycosylated β -subunit (Lingrel and Kuntzweiler, 1994). The α -subunit contains an intracellular ATP-binding site (Farley *et al.*, 1984), a phosphorylation site (Post *et al.*, 1973; Walderhaug *et al.*, 1985), and an extracellular binding site for cardiac glycosides such as digoxigenin and ouabain (Schwartz *et al.*, 1975; Lingrel *et al.*, 1990). Three α -isoforms, α 1, α 2 and α 3, in mammals (Shull *et al.*, 1986; Hara *et al.*, 1987; Herrera *et al.*, 1987) and in chicken (Takeyasu *et al.*, 1988, 1990), two in *Artemia* (Baxter-Lowe *et al.*, 1989; Macías *et al.*, 1991), and only one in *Drosophila melanogaster* (Lebovitz *et al.*, 1989), as well as three β -isoforms, β 1, β 2 and β 3 (β 3 is only found in *Xenopus laevis* (Good *et al.*, 1990)) have been identified so far (Swadner *et al.*, 1989). Each subunit isoform of vertebrates shows different tissue-specific and develop-

mentally regulated expressions (Herrera *et al.*, 1987; Orłowski and Lingrel, 1988).

Enzymatic studies revealed that the plasma membrane of sea urchin eggs and embryos contains an ouabain-sensitive Na⁺, K⁺-ATPase activity (Kinsey *et al.*, 1980; Mitsunaga *et al.*, 1986, 1989), which is stimulated just after fertilization (Ciapa *et al.*, 1984). We have shown the change in the activity of Na⁺, K⁺-ATPase during early development of the sea urchin *Hemicentrotus pulcherrimus* (Mitsunaga-Nakatsubo *et al.*, 1992a). The activity begins to increase at the mesenchyme blastula stage and reaches the maximum at the gastrula stage. The increase at the gastrula stage is actinomycin D-sensitive and is probably due to the increase in ectoderm cells. Northern blot analysis, using a fragment of the *Hemicentrotus* Na⁺, K⁺-ATPase α -subunit cDNA, reveals that the mRNA is about 4.6 Kb long and its maximum expression is at the mesenchyme blastula and the gastrula stages (Mitsunaga-Nakatsubo *et al.*, 1992b).

In this study, we have isolated the sea urchin cDNA clone coding Na⁺, K⁺-ATPase α -subunit, deduced its primary structure from the nucleotide sequence and compared it with those of various metazoans.

* The first and second authors contributed equally to this paper.

** Present address: Graduate Department of Gene Science, Faculty of Science, Hiroshima University, 1-3-1, Kagamiyama, Higashi-Hiroshima 739, Japan.

*** To whom all correspondence should be addressed.

MATERIALS AND METHODS

Culture of embryos

Gametes of the sea urchin, *Hemicentrotus pulcherrimus*, were obtained by an intracoelomic injection of 0.5 M KCl. Eggs were washed with artificial sea water (ASW), inseminated and allowed to develop at 20°C.

Extraction of total RNA from embryos

Total embryonic RNA was extracted from embryos by the guanidium/hot phenol method (Feramisco *et al.*, 1982). Poly(A)⁺ RNA was fractionated using oligo-(dT) cellulose spun columns (Clontech Lab., Inc.) according to the manufacturer's procedure.

Screening

First screening: 10⁵ clones of the cDNA library constructed from the poly(A)⁺ rich RNA of prism stage embryos (Akasaka *et al.*, 1987) were screened with a 47-mer oligonucleotide probe. The probe (5'GCIGGGGAGTCATTGACACCGTCICCGTTACAGCCACAATGGCACC3') corresponds to a FSBA binding site (GAIVAVTGDGVNDSPALKK) at the amino terminal region of rat Na⁺, K⁺-ATPase α -subunit (Shull *et al.*, 1986). This is a highly conserved region among eukaryotes. The oligonucleotide was 5'-end ³²P-labeled with T4-polynucleotide kinase and used as the probe for plaque hybridization as described previously (Mitsunaga-Nakatsubo *et al.*, 1992b). Ten positive clones not containing the amino terminal region were obtained.

Second screening: To obtain longer clones carrying the amino terminal region for Na⁺, K⁺-ATPase α -subunit, another cDNA library of the gastrula stage was constructed using a 20-base synthetic primer (5'TGCAATGTTCTTGGTCTCCA3') corresponding to the antisense strand (at positions 767–786 in Fig. 2) of SUA5, a clone obtained from the 1st screening. The second library was screened with the *EcoRI*-*HindIII* fragment (fragment from position 746 to 1013) of SUA5, which was labeled with [α -³²P]dCTP (3000 Ci/mmol) using a random-primed DNA labelling kit (Nippon Gene, Toyama). Filters were prehybridized at 68°C for 1 hr in hybridization buffer containing 5 x SSPE, 5 x Denhardt's, 0.3% SDS and 100 μ g/ml denatured salmon sperm DNA. Hybridization was performed in hybridization buffer containing the labeled probe for 12–15 hr. The filters were washed with a buffer containing 1 x SSC and 0.1% SDS for 15 min three times at room temperature and then subjected twice to 30-min washes with 0.1 x SSC and 1% SDS at 68°C. A positive overlapping clone (SUA51) was obtained.

Rapid amplification of cDNA ends (RACE) method

The 5'-noncoding region of cDNA was amplified by the RACE method of Frohman *et al.* (1988) to determine its sequence. First strand cDNA was synthesized using the antisense primer (ISOCHK2; 20 mer from the position 1578 to 1597 in Fig. 2). Deoxycytidine was polymerized to the 3'-end of the first strand, using dCTP and terminal deoxynucleotidyl transferase. PCR was performed with an oligo(dG)_{15–18} primer and Primer4 (at positions 334–353 in Fig. 2) located upstream to the ISOCHK2, which was used for first strand cDNA synthesis. PCR-amplified DNA was extracted and ligated with cosmid vector Charomid 9-42 (Nippon Gene, Toyama), to analyze the insert for sequencing.

Nucleotide sequence of Na⁺, K⁺-ATPase α -subunit

The *EcoRI* fragments of the cDNAs (SUA5 and SUA51) were inserted into pUC118 or pUC119 and the overlapping subclones were generated using the stepwise deletion method (Heinkoff *et al.*, 1984). DNA sequencing was performed using the Sequenase version 2.0 7-deaza-dGTP kit (United State Biochemical Corp., USA) and both strands of the cDNA were sequenced.

Sequence alignment

Sequence alignment and numbers of nucleotide substitutions per site were estimated using an algorithm method described by Gotoh (1993). Construction of phylogenetic tree was performed by the neighbor-joining method (Saitou and Nei, 1987).

Chemicals

[γ -³²P]ATP and [α -³²P]dCTP were purchased from Radiochemical Centre, Amersham, Bucks, UK. Restriction endonucleases and the other enzymes were obtained from Takara Shuzo Co., Ltd., Kyoto, Toyobo Co., Ltd., Japan and Nippon Gene Co., Ltd., Japan. Sequenase version 2.0 7-deaza-dGTP kit and random primer labeling kit were the products of the United States Biochem. Co., USA and Nippon Gene Co. Ltd., Japan, respectively. Artificial sea water was obtained from Jamarin laboratory, Osaka, Japan.

RESULTS AND DISCUSSION

Several conserved regions have been reported in active sites of Na⁺, K⁺-ATPase α -subunit: phosphorylation site, FITC-binding site and two FSBA-binding sites. For a probe to screen the cDNA of sea urchin Na⁺, K⁺-ATPase α -subunit, one of the FSBA-binding sites located at the C-terminal region was selected and an antisense oligonucleotide (47 mer) was chemically synthesized based on its amino acid sequence reported in mammals (Shull *et al.*, 1986). 10⁵ recombinant phages from a λ gt10 cDNA library of the prism stage embryos were screened, as described previously (Mitsunaga-Nakatsubo *et al.*, 1992b). Ten positive signals were obtained. They included the nucleotide sequences encoding amino acid sequence of the probe region, whereas none of them had the amino terminal region. Clone SUA5 was selected and its nucleotide sequence was determined (Fig. 1).

To obtain cDNA clones carrying the N-terminal region, a cDNA library of the gastrula stage was constructed using oligonucleotide primer corresponding to the antisense strand sequence near the 5'-end of clone SUA5. 10⁵ recombinant phages were screened with the 5' region of SUA5 (268 bp fragment digested with *EcoRI* and *HindIII* as shown in Fig. 1). A 779 bp cDNA clone with a 42-bp overlap at the 5'-end of clone SUA5 (SUA51) was obtained (as shown in Fig. 1). cDNA fragment including this overlapping region could be amplified by RT-PCR method using two pairs of primers, which locate opposite sides of the region (HNKUP, 689–710 and HNKDOWN, 1498–1518 or ISOCHK2, 1578–1597; nucleotide numbers correspond to Fig. 2). In addition, Northern blot analysis with probes of specific fragment from each cDNA clone revealed identical size and expression patterns of mRNA during development (data not shown). These results suggest that SUA5 and SUA51 are the cDNA clones coding the same mRNA of Na⁺, K⁺-ATPase α -subunit.

The 5'-noncoding region was amplified by the 5'-rapid amplification of cDNA ends (RACE) method. One of clones, R4 had a 346 bp overlapping region of SUA51 and extended 7 bp upstream. The extended length of the clone R4 nucleotide sequence corresponded closely to the major transcription start site determined by primer extension (data not shown). Therefore, the nucleotide sequence combining SUA5, SUA51 and R4 together is shown as a single sea urchin Na⁺,

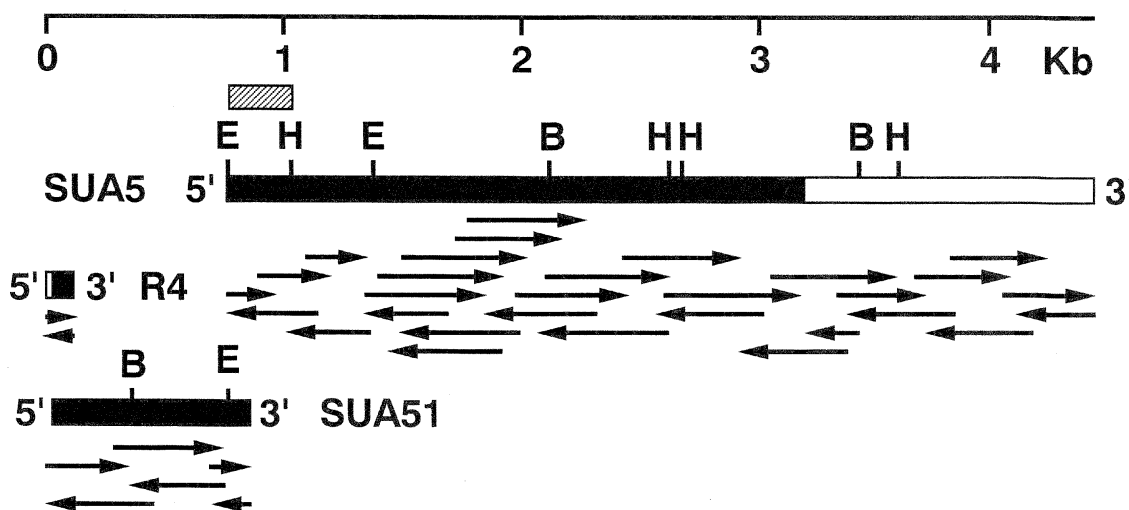


Fig. 1. Restriction maps and sequencing strategy of cDNA clones for Na⁺, K⁺-ATPase α-subunit. The restriction map is a composite from cDNAs' sequences determined. Darkened and open areas represent the coding and noncoding regions, respectively. Arrows indicate directions of sequencing. SUA5, SUA51 and R4 are cDNA clones. B, E and H are *Bgl*II, *Eco*RI and *Hind*III sites, respectively. Shaded box shows 268-bp probe for the second screening.

K⁺-ATPase α-subunit sequence (Fig. 2). The total number of nucleotides is 4401.

The cDNA has a long untranslated sequence in the 3' region with a poly(A) additional signal AATAAA approximately 20 nucleotides upstream from the start of the 3'-poly(A) tail. The first methionine codon within the open reading frame is located upstream of a lysine-rich cluster (at positions 115–168, amino acid residues 37–54), which is reported to be a putative ion-selectable domain of Na⁺, K⁺-ATPase α-subunit. A termination codon is found next to Tyr-1038. The molecular weight of the protein is calculated to be 114 kDa.

As shown in Fig. 3, the overall hydropathy profile of the α-subunit is similar to that of sheep kidney (Shull *et al.*, 1985). Eight major hydrophobic regions are indicated as H1-H8 in the figure. Alignment of amino acid sequences of sea urchin α-subunit with those of diverse species also showed high degrees of sequence similarity in these hydrophobic regions (Fig. 4). Therefore, it is suggested that at least eight putative transmembrane domains also exist in sea urchin Na⁺, K⁺-ATPase α-subunit. The regions between H1-H2, H3-H4, H5-H6 and H7-H8, respectively, are putative short extracellular loops, whereas the domains between H2-H3 and H4-H5, respectively, are to be longer cytoplasmic loops. The amino acid sequence of the most highly conserved region, H4, matches almost perfectly between sea urchin and other species, whereas the average identity of the entire length is about 76%. Signal peptide could not be identified in the amino terminal region. Like other Na⁺, K⁺-ATPase α-subunit reported in diverse species (Kawakami *et al.*, 1985, 1986; Ovchinnikov *et al.*, 1986; Shull *et al.*, 1985), the signal peptide is not included in the sea urchin ATPase.

The catalytic sites, reported to be present in the cytoplasmic regions (Lingrel *et al.*, 1990; Lingrel and Kuntzweiler, 1994), were also identified in the region between

H4 and H5 transmembrane sites, and are all highly conserved in the amino acid sequences (Fig. 4). Phosphorylation site around the aspartyl residue (no. 393) almost perfectly matches with those of other species. ATP binding-site around the Lys (at 523) probed with fluorescein 5'-isothiocyanate (FITC) is also highly homologous. Binding sites of FSBA, another ATP analogue (AX₁VVHGSCLK (I) and QGAIVAVTGDGVNDS-PALX₂K (II)) (Ohta *et al.*, 1986) are also identified in the sea urchin Na⁺, K⁺-ATPase α-subunit. Especially, the latter FSBA-binding site FSBA II, used as a probe for screening, matches almost perfectly except one amino acid residue at the N-terminal end of this region. On the contrary, the deduced N-terminal amino acid sequence for sea urchin differs from the sequences reported in vertebrates (Fig. 4) and is comparatively long like that for *Drosophila melanogaster*.

The activity of sea urchin embryonic Na⁺, K⁺-ATPase is ouabain-sensitive (Kinsey *et al.*, 1980; Ciapa *et al.*, 1984; Mitsunaga *et al.*, 1986, 1989). It has been reported that uncharged amino acids are present at the terminus of H1-H2 extracellular domain of every ouabain-sensitive Na⁺, K⁺-ATPase, and the substitutions of the amino acids into charged amino acids invariably produce a resistant enzyme (Price *et al.*, 1989; Horisberger *et al.*, 1991; Lingrel and Kuntzweiler, 1994). In sea urchin, one of the termini of H1-H2 is negatively charged (Glu at 135), though the other is not (Asn at 146). Relatively high concentration of ouabain necessary for inhibition of sea urchin Na⁺, K⁺-ATPase activity, may be due to the amino acid residues at the termini of the H1-H2 extracellular domain. In Na⁺, K⁺-ATPase of *Artemia*, which have low affinity for ouabain, the same amino acids as in sea urchin are present at the termini of H1-H2.

Three kinds of Na⁺, K⁺-ATPase α-subunit isoforms, which are products of different genes, were demonstrated in chicken (Takeyasu *et al.*, 1988, 1990), rat (Shull *et al.*, 1986) and

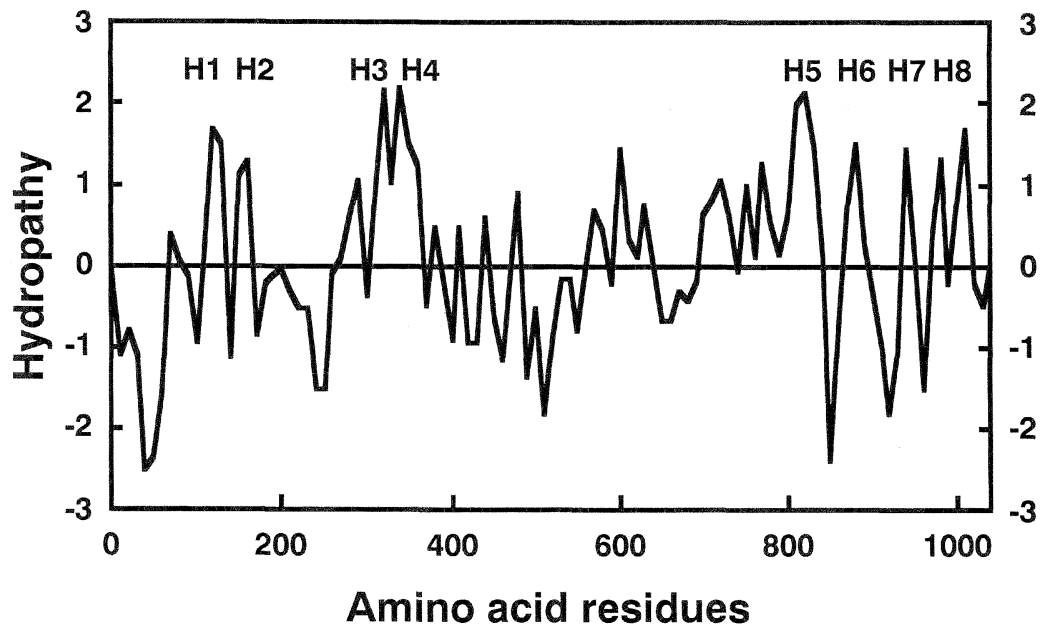


Fig. 3. Kyte-Doolittle hydropathy plot of Na⁺, K⁺-ATPase α -subunit (Kyte and Doolittle, 1982). The window was set at 20 amino acids. On the abscissa, the position in the primary amino acid sequence is indicated. The numbers on the ordinate are the relative hydrophobicity values, hydrophobic being positive and hydrophilic being negative. The hydrophobic peaks corresponding to the proposed transmembrane regions are indicated (H1-H8).

1991). However, biochemical analysis of *Hydra* Na⁺, K⁺-ATPase reveals the existence of two types of ouabain-sensitive ATPase (Canfield *et al.*, 1992). Analysis of the isoforms in sea urchin embryos is now under investigation.

ACKNOWLEDGMENTS

The authors wish to express their hearty thanks to Dr. O. Gotoh of Saitama Cancer Center for his kind help of constructing the phylogenetic tree and alignment of Na⁺, K⁺-ATPase. We are also grateful to Dr. H. Yonekawa of the Tokyo Metropolitan Institute of Medical Science for his helpful advice on the construction of phylogenetic tree and evolution.

REFERENCES

- Akasaka K, Taira M, Shiroya T, Shimada H (1987) Cloning of stage specific gene sequences from a cDNA library representing poly(A)⁺ RNA of sea urchin prism-embryos. *Zool Sci* 4: 739–742
- Baxter-Lowe LA, Guo JZ, Bergstrom EE, Hokin LE (1989) Molecular cloning of the Na, K-ATPase α -subunit in developing brine shrimp and sequence comparison with higher organisms. *FEBS Lett* 257: 181–187
- Canfield VA, Xu KY, D'Aquila T, Shyjan AW, Levenson R (1992) Molecular cloning and characterization of Na,K-ATPase from *Hydra vulgaris*: Implications for enzyme evolution and ouabain sensitivity. *New Biol* 4: 339–348
- Ciapa B, De Renzis G, Girard JP, Payan P (1984) Sodium-potassium exchange in sea urchin egg. I. Kinetic and biochemical characterization at fertilization. *J Cellular Physiol* 121: 235–242
- Farley RA, Tran C M, Carilli CT, Hawke D, Shively JE (1984) The amino acid sequence of a fluorescein-labeled peptide from the active site of (Na, K)-ATPase. *J Biol Chem* 259: 9532–9535
- Feramisco JR, Smart JE, Burridge K, Helfman DM, Thomas GP (1982) Co-existence of vinculin and a vinculin like protein of higher molecular weight in smooth muscle. *J Biol Chem* 257: 11024–11031
- Frohman MA, Dush MK, Martin GR (1988) Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. *Proc Natl Acad Sci USA* 85: 8998–9002
- Good PJ, Richter K, Dawid IB (1990) A nervous system-specific isotype of the β subunit of Na⁺, K⁺-ATPase expressed during early development of *Xenopus laevis*. *Proc Natl Acad Sci USA* 87: 9088–9092
- Gotoh O (1993) Optimal alignment between groups of sequences and its application to multiple sequence alignment. *Compt Appl Biosci* 9: 361–370
- Hara Y, Urayama O, Kawakami K, Nojima H, Nagamune H, Kojima T, Ohta T, Nagano K, Nakao M (1987) Primary structures of two types of alpha-subunit of rat brain Na⁺, K⁺-ATPase deduced from cDNA sequences. *J Biochem* 102: 43–58
- Heinkoff S (1984) Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. *Gene* 28: 351–359
- Herrera VLM, Emanuel JR, Ruiz-Opazo N, Levenson R, Nadal-Ginard B (1987) Three differentially expressed Na, K-ATPase α subunit isoforms: structural and functional implications. *J Cell Biol* 105: 1855–1865
- Horisberger J-D, Lemas V, Kraehenbühl J-P, Rossier BC (1991) Structure-function relationship of Na, K-ATPase. *Annu Rev Physiol* 53: 565–584
- Iwabe N, Kuma K, Miyata T (1996) Evolution of gene families and relationship with organismal evolution: rapid divergence of tissue-specific genes in the early evolution of chordates. *Mol Biol Evol* 13: 483–493
- Jaisser F, Canessa CM, Horisberger JD (1992) Primary sequence and functional expression of a novel ouabain-resistant Na, K-ATPase. The β subunit modulates potassium activation of the Na, K-pump. *J Biol Chem* 267: 16895–16903
- Kano I, Nagai F, Satoh K, Ushiyama K, Nakao T, Kano K (1989) Structure of the α 1 subunit of horse Na, K-ATPase gene. *FEBS Lett* 250: 91–98

SU : MITMAARVEKSGEGRGESYRVAVQTEVPDDGRNAAGPKSKKKEKRDLEELKKEMEFDDHKISLEDLVARLDSNITTGL 80
 HD : MADP·DLESRGKADSYSVAEKKS·P·K I·NAN·AK·D·L·MTE·SMK·S·LSMYETSLEK· 69
 AR : MAKG·QK·GK·N·LDI·F·PI·ECYQ·G·PE· 43
 DM : MALRSDYEHGRADSY·VATVIATDDDNRTA·QYKSRKMPA·VN·EN·DD·Q·LDI·F·P·EMYQ·FQTHPEN· 81
 TC : MGKG·ASEKYQPAATSENA·NSK·S·S·TT·D·VSL·LN·DE·HQKYGTDL·Q· 63
 C1 : MGKGAGRDKYEPATSEHGTK·A·E·MD·ISM·L·DE·HRKYGTDL·SR· 61
 C2 : MDGREYSPAATTSENGGRR·Q·EKE·D·VNL·L·DE·GRKYQV·DL·SR· 58
 C3 : MGDKGE·ESP·GKG·DD·VAMTE·M·I·EVC RKYNTDCVQ· 50
 SP : MGKGVGRDKYEPAAVSEHGDK·AK·E·MD·VSM·L·DE·HRKYGTDL·NR· 61

SU : TVAQAAHVLRADGPNLSTPPPK-TPEWIKFCQQLFGGFATLLWMGSILCFLAYTIEAATKDE-PNSDNLYLGVLASVVTI 159
 HD : SENIV·RN·E·L·A·Q·V·K·M·SM·I·A·F·FG·R·VRDTN·M·E·SV· 148
 AR : N·RSNIE·C·T·KN·L·T·A·G·SSGN·DMLK·T·V 123
 DM : SH·R·KEN·E·LTP·Q·V·ED·VAM·I·A·V·S·Q·S·SE·AD·SA·V 159
 TC : P·R·KEI·A·T·R·SI·T·A·G·QV·V·N·AN·V·ST· 142
 C1 : T·R·EI·T·T·V·R·SL·I·L·G·TSMEG·V·A· 140
 C2 : SN·R·E·Q·A·T·V·R·SI·I·A·G·Q·ME·SN·V·A·V 137
 C3 : HSK·QEI·A·T·V·R·SI·I·A·G·Q·G·E·SN·V·A· 129
 SP : T·R·EI·A·T·V·R·SM·I·AV·G·Q·EE·QN·V·SA· 140

H1

H2

SU : TGCPSYYQEAQSSKIMESFKNMVPQALVLRAGEWHSINAVNVVRGDIVEVKGDRIPADIRVVESKSFVKDNSSLTGESE 240
 HD : S·K·I·D·KKIT·EQ·V·V·F·F·I·C·GL· 229
 AR : I·N·R·D·L·Y·A·E·QRVTLK·EELTM·F·V·L·L·AR· 204
 DM : V·S·N·F·T·I·E·KP·LR·EDL·L·VL·LEF·L·LVY·I·ARD· 240
 TC : D·Q·I·D·KS·EQ·V·L·L·I·SAC·C· 223
 C1 : Q·V·N·KM·EG·V·L·L·I·SAHGC· 221
 C2 : D·Q·I·E·KIQ·E·V·L·V·M·I·S·HGC· 218
 C3 : Q·I·E·KMQL·EE·V·L·V·L·I·SAHGC· 210
 SP : Q·I·N·KM·EE·V·L·L·I·SANGC· 221

SU : PQRSPEFTSDNPLETKNIAFFSTNASEGTCRGIVISTGDNTVMGRIAGLASGLDVGDTPIAKEIEHFIHITTA VAVFLGV 321
 HD : AVD·HE·I·L·V·AT·VRI·N·GS·K·L·V·G· 310
 AR : A·N·L·V·M·G·I·T·E·A·G· 285
 DM : GA·HE·L·V·ALPK·V·C·H·A·T·H·L·G· 320
 TC : YS·E·CV·A·NI·H·T·E·Q·A·G· 304
 C1 : T·D·SNE·R·CV·AV·R·S·EG·K·M·L·G· 302
 C2 : T·HE·R·C·CV·A·R·S·E·R·M·RL·G·L· 299
 C3 : T·DC·H·R·T·CV·A·V·A·R·T·E·K·V·QL·G·I· 291
 SP : T·D·NE·R·CV·A·VY·R·T·E·G·Q·A·G· 302

H3

SU : SFFFLSFPQGYTWLACVFLIGITIVANVPEGLLATVTVCLTLTAKRMAHKNCLVKNLEAVETLGTSTICSDKTGTLTQNR 402
 HD : LII·LAM·H·II·K·K·H·V· 391
 AR : T·IIA·V·H·D·V·S· 366
 DM : T·VIA·I·H·D·VI·S· 401
 TC : I·LI·VI·R· 385
 C1 : I·LI·E·VI·R·G· 383
 C2 : I·LI·VI·R· 380
 C3 : V·LI·VI·R· 372
 SP : I·LI·E·VI·R· 383

H4

P

SU : MTVAHMWFDNTIVEADTTEDQSGGQFDKSSPTWMLARIAACLCNRSEFKAGQENVPILKRDTTGDASESALVKCVELCMFN 483
 HD : KM·IAH·G·L·KS·KV·A·S·A·PN·ND·AV·RKEC·T·IL·F·SVG· 472
 AR : G·T·A·AG·K·VK·A·S·A·PN·STT·EV·A·IL·TTGE· 447
 DM : Q·I·V·Y·RT·GFK·S·T·A·G·DG·KEVS·A·L·M·ALGD· 482
 TC : Q·H·N·IS·T·LS·N·S·A·AV·Q·DS·SVA·L·I·CGS· 466
 C1 : Q·H·N·AS·A·L·S·G·AV·Q·N·AVA·L·I·CGS· 464
 C2 : Q·H·N·AT·R·A·S·G·AV·P·IS·S·A·L·I·Q·SCGS· 461
 C3 : Q·H·N·TS·A·V·SH·G·AV·G·VA·L·I·SSGS· 453
 SP : Q·H·N·VS·T·A·L·S·G·AV·Q·N·D·L·AVA·L·I·V·CGS· 464

SU : VTEYRKNKKVCEIPFNSTNKYQVSIHETEGD-D-RNLLVMKGAPERILDRCTTILIKGKEVDMNDEMOTA FNAYLELGG 562
 HD : MDI·A·T·V·Q·NSS·G·Y·KV·E·S·N·E·QPLK·DVIEIY·K·D· 551
 AR : TEAI·R·I·A·F·N·DKS·G·Y·E·S·FMN·I·TE·LKE·M· 528
 DM : MNI·R·IA·V·DTN·P·Y·E·S·F·N·KVLDE·KE·M· 563
 TC : SQM·DR·P·IV·L·NDKA·S·Y·S·LN·EDKPL·E·KE·Q· 546
 C1 : K·M·ERYP·V·L·KNANAGES·H·DS·H·VQPLDE·IKD·Q· 545
 C2 : KKM·D·P·T·L·R·E·PQGH·I·E·SR·LQ·Q·PLDE·KE·Q· 541
 C3 : KVM·ER·A·L·DPN·N·Y·S·LQ·Q·QPLDE·KE·Q· 534
 SP : K·M·ERYA·IV·L·KNANAGEP·H·SS·H·QPLDE·LKD·Q· 545

FITC

SU :	LGERVLGFCHCYLPADQFPLGFAFDADEV-NFPLEKLCFVGLMSMIDPPRAAVPDAVGGKCRSAGIKVIMVTGDHPITAKAI	642
HD :Y..V..Y..K..L..KTE..EQ.....G...L..L.....S.....	632
AR :DYL..L..KY..H...N..DA...TG..R..A.....A.....	608
DM :DFM..S..KY..N..K..NT..DI...IDN..R.....A.....	643
TC :LK..STSK..E..YP..VE..P...ITD.....	626
C1 :LA..D...E...Q..T...-...V.....	625
C2 :L..P..K..R..R...-...TSD.....	621
C3 :F...EE..Y..K...C..D...-ATDN.....	614
SP :LM..DE...E...Q..T..D...-VDN.....I.....	625
SU :	AKGVGI ISEGNETRDDI AQRGLG IPIE---QVNPKDAKAI VVHGSELKD IARELDLAILADHPEIVFARTSPQQKLI IVEGC	720
HD :CEE...L..N..L..DLSAQK..S...C..I..AK...KN..E..K..C..T.....	713
AR :	..S.....VE...A..N..V---SE...R...A...G..R..TPDA..E..RH.....	686
DM :	..S.....VE...A..N..V---SE...RE...A...A..R..VSSDQ..E..RY..T.....	721
TC :VE...A..N..V---N...R...C...TD...LSH..N..D..HY..T.....	704
C1 :D...VE...A..N..V---S...R...C...D...MTS..Q..D..LH..T.....	703
C2 :VE...A..N..V---S...RE...C...D...MTA..Q..E..RN..T.....	699
C3 :VE...A..N..V---S...R...C..I..TD...MSS..QI..E..QN..T.....	692
SP :VE...A..N..V---S...R...R..C...D...MTP..Q..D..KY..T.....	703
FSBA I		
SU :	QRAGAIVAVTGDGVNDSPALKKADIGVAMG IAGSDVSKQAADM ILLDDNFASIVTGVEEGR LIFDNLKKS IAYTLTSN IPE	801
HD :	..Q.....	794
AR :	..Q.....	767
DM :	..M.....	802
TC :	..Q.....	785
C1 :	..Q.....	784
C2 :	..Q.....I.....	780
C3 :	..Q.....R.....	773
SP :	..Q.....	784
FSBA II		
SU :	<u>I</u> SPFLIFILASIPPLGVVITLCTD <u>L</u> GTDLVPAISLAYEEAESD IMKRRPRDPQNDKLVNER <u>L</u> ISVSYGQIGMIQASAGFF	882
HD :M...FG.....TI.....M.....K.....H..N..IR.....LA...M..T.....	875
AR :L..FD.....T.....M.....N..VT.....LA.....	848
DM :AS..CD.....T.....M.....DH..A...P...F...S...MA.....A.....	883
TC :	..T...V...I..NV...T...T...M.....R.....Q..N..KT.....MA.....LG...	866
C1 :	..T...V...I..N...TC...T...M.....Q.....Q..N..KT.....MA.....LG...	865
C2 :	..T...L...I..N...T...T...M.....A.....Q..N..RT.....MA.....LG...	861
C3 :	..T...L...M..N...TI...T...M.....A.....Q..N..RS.....MA.....LG...	854
SP :	..T...T...I..N...T...T...M.....Q.....Q..N..T.....MA.....LG...	865
H5		
SU :	AYFVIMGENGFLPNDLIMLRSRWDDKAVLNVEDSYGQQWGYTQRKQL EYTCHTAFFASIVVVQWADVITICKTRRNSLIHQG	963
HD :	T...I..LA...S...Y..FG...Q...MSNN..LL..F..SE..T..F...E..I..L..Q...TT...L...S...L..FQ...	956
AR :	V...A...C...W...FG...KH...SR...NDLT...E..T..DA...SS...Y..V...I...L...S...VFQ...	929
DM :	V...A...A...KK..FGI..KM..S...NDLT...E..T..RD...T...I...L...IFQ...	964
TC :	S...LA...I...GI..EK..ELWTQDL...T..E...IV...S...V...I...L...IFQ...	947
C1 :	T...A...A...SG..VGI..LQ...RWIND...TFE...IV..F...V...L...VFQ...	946
C2 :	T...LA...A...AR..LGV..LA...RSTNDL...E..T..E...VV..F...L...VFQ...	942
C3 :	S...LA...A...SC..VGI..LS...RTINDL...T..E...VV..F...V...L...VFQ...	935
SP :	T...A...A...H..LGI..VT...RWIND...T..E...IV..F...V...L...VFQ...	946
H7		
SU :	MNNWV LNFGLFFETALAAFLSYCPGLENGI RMYPLRIGWVFAFPFSL I FVYDECRRF I LRHNPGGWVELETYY	1038
HD :	T...F...Q..T..VNT...LR..MNFT...LPGL...I..YL..K...K...	1031
AR :	R..N...A..V...C...T...MDK...K..N...P..L...F...A..K...R...Q...	1004
DM :	R..A...V...V...M..K...KLV...P..I..A..A...I...T...Y..R...L..Q...	1039
TC :	K..K...I...E...T...TDIA...KPS...C...Y...I..L...A...R...Q...	1022
C1 :	K..K...I...E...MDVA...KPT...C...Y...L...I..KL..I..R...R...	1021
C2 :	K..K...I...LE...MGVA...KVT...C...Y...A...V..KL...RY...K...	1017
C3 :	K..K...I...E...MDVA...KPS...C...Y..F...I..KL...R...K...	1010
SP :	K..K...I...E...MGVA...KPT...C...Y...V..KL..I..RR...K...	1021
H8		

Fig. 4. Alignment of amino acid sequences of α -subunits of the Na⁺, K⁺-ATPase from the sea urchin *Hemicentrotus pulcherrimus* (SU), *Hydra vulgaris* (HD; Canfield *et al.*, 1992), the brine shrimp *Artemia franciscana* (AR; Macías *et al.*, 1991), *Drosophila melanogaster* (DM; Levobitz *et al.*, 1990), the electric ray *Torpedo californica* (TC; Kawakami *et al.*, 1985), chicken (C1, C2 and C3; α 1, α 2 and α 3 isoforms respectively, Takeyasu *et al.*, 1988, 1990) and sheep (SP; Shull *et al.*, 1985). Sequences are shown in one-letter amino acid code. Gaps (-) are inserted in the sequences to achieve maximum alignment. Identical amino acids, relative to the *H. pulcherrimus* sequence, are indicated by dots. The eight putative transmembrane segments proposed by Shull *et al.* (1985) for the sheep kidney α -subunit are underlined and labeled with H1-H8. The active sites identified by homology with other ATPases are indicated by underlines (P: phosphorylation site, FITC: FITC-binding region, FSBA I, II: FSBA-binding regions).

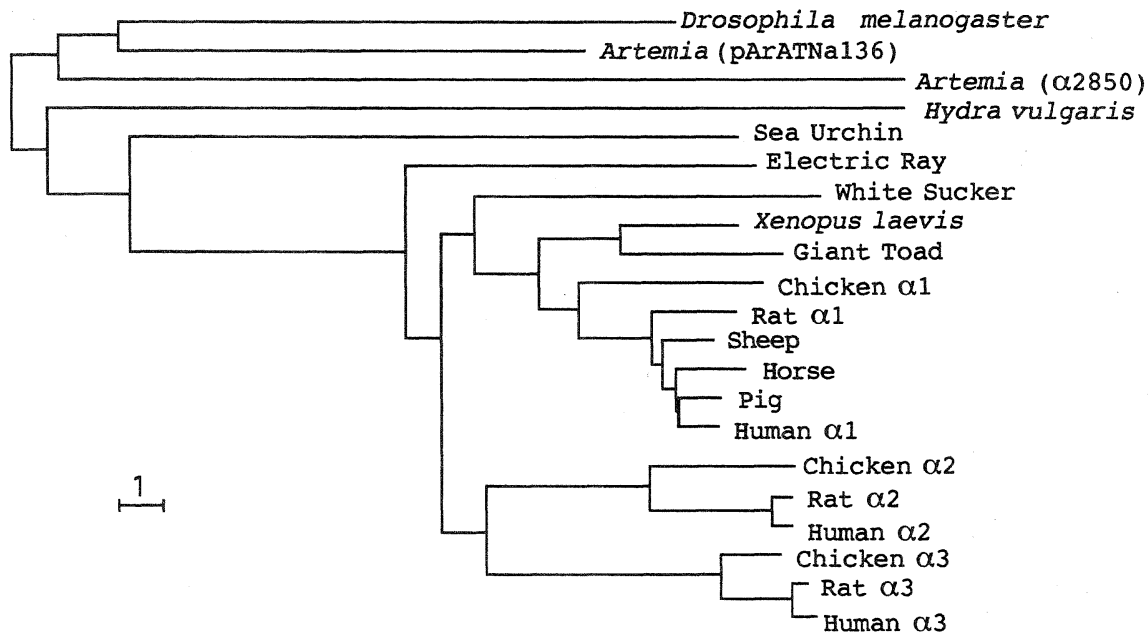


Fig. 5. Phylogenetic analysis of Na^+ , K^+ -ATPase α -subunit amino acid sequences. The tree was built using the neighbor-joining method applied to a categories distance matrix on the basis of the deduced amino acid sequences coded by sea urchin, *Hydra vulgaris* (Canfield *et al.*, 1992), *Artemia* cDNA clones α 2850 and pArATNa136 (Baxter-Lowe *et al.*, 1989; Macias *et al.*, 1991), *Drosophila melanogaster* (Levobitz *et al.*, 1990), electric ray (Kawakami *et al.*, 1985), white sucker (Schönrock *et al.*, 1991), *Xenopus laevis*, *Xenopus laevis* (Verrey *et al.*, 1989), giant toad (Jaisser *et al.*, 1992), chicken α 1-3 (Takeyasu *et al.*, 1988; 1990), rat α 1-3 (Shull *et al.*, 1986), sheep (Shull *et al.*, 1985) and human α 1-3 (Kawakami *et al.*, 1986; Shull *et al.*, 1989; Ovchinnikov *et al.*, 1988). The horizontal bar represents 1 amino acid replacements per 100 sites.

- Kawakami K, Noguchi S, Noda M, Takahashi H, Ohata T, Kawamura M, Nojima H, Nagano K, Hirose T, Inayama S, Hayashida H, Miyata T, Numa S (1985) Primary structure of the α -subunit of *Torpedo californica* (Na^+ + K^+) ATPase deduced from cDNA sequence. *Nature* 316: 733–736
- Kawakami K, Ohta T, Nojima H, Nagano K (1986) Primary structure of the α -subunit of human Na, K-ATPase deduced from cDNA sequence. *J Biochem* 100: 389–397
- Kinsey WH, Decker GL, Lennarz WJ (1980) Isolation and partial characterization of the plasma membrane of the sea urchin egg. *J Cell Biol* 87: 248–254
- Kyte J, Doolittle RF (1982) A simple method for displaying the hydropathic character of a protein. *J Mol Biol* 157: 105–132
- Levobitz RM, Takeyasu K, Fambrough DM (1989) Molecular characterization and expression of the (Na^+ + K^+)-ATPase alpha-subunit in *Drosophila melanogaster*. *EMBO J* 8: 193–202
- Lingrel JB, Orłowski J, Shull MM, Price EM (1990) Molecular genetics of Na, K-ATPase. In "Prog. Nucleic Acid Res Mol Biol Vol 38" Ed by WE Cohn, K Moldave, Academic Press, New York, pp 37–89
- Lingrel JB, Kuntzweiler T (1994) Na^+ , K^+ -ATPase. *J Biol Chem* 269: 19659–19662
- Macias M-T, Palmero I, Sastre L (1991) Cloning of a cDNA encoding an *Artemia franciscana* Na/K ATPase α -subunit. *Gene* 105: 197–204
- Mitsunaga K, Fujino Y, Yasumasu I (1986) Change in the activity of Cl^- , HCO_3^- -ATPase in microsome fraction during early development of the sea urchin, *Hemicentrotus pulcherrimus*. *J Biochem* 100: 1607–1615
- Mitsunaga K, Fujiwara A, Fujino Y, Yasumasu I (1989) Changes in the activities of H^+ , K^+ -ATPase and Na^+ , K^+ -ATPase in cultured cells derived from micromeres of sea urchin embryos with special reference to their roles in spicule rod formation. *Develop Growth Differ* 31: 171–178
- Mitsunaga-Nakatsubo K, Fujiwara A, Yasumasu I (1992a) Change in the activity of Na^+ , K^+ -ATPase in embryos of the sea urchin, *Hemicentrotus pulcherrimus*, during early development. *Develop Growth Differ* 34: 379–385
- Mitsunaga-Nakatsubo K, Kanda M, Yamazaki K, Kawashita H, Fujiwara A, Yamada K, Akasaka K, Shimada H, Yasumasu I (1992b) Expression of Na^+ , K^+ -ATPase α -subunit in animalized and vegetalized embryos of the sea urchin, *Hemicentrotus pulcherrimus*. *Develop Growth Differ* 34: 677–684
- Ohta T, Nagano K, Yoshida M (1986) The active site structure of Na^+ / K^+ transporting ATPase: Location of the 5'-(*p*-fluorosulfonyl) benzoyl adenosine binding site and soluble peptides released by trypsin. *Proc Natl Acad Sci USA* 83: 2071–2075
- Orłowski J, Lingrel JB (1988) Tissue-specific and developmental regulation of rat Na, K-ATPase catalytic α isoform and β subunit mRNAs. *J Biol Chem* 263: 10436–10442
- Ovchinnikov YA, Modyanov NN, Broude NE, Petrukhin KE, Grishin AV, Arzamazova NM, Aldanova NA, Monastyrskaya GS, Sverdlov ED (1986) Pig kidney Na^+ , K^+ -ATPase. Primary structure and spatial organization. *FEBS Lett* 201: 237–245
- Ovchinnikov YA, Monastyrskaya GS, Broude NE, Ushkaryov YA, Melkov AM, Smirnov YV, Malyshev IV, Allikmets RL, Kostina MB, Dulubova IE, Kiyatkin NI, Grishin AV, Modyanov NN, Sverdlov ED (1988) Family of human Na^+ , K^+ -ATPase genes. Structure of the gene for the catalytic subunit (α III-form) and its relationship with structural features of the protein. *FEBS Lett* 233: 87–94
- Post RL, Kume S (1973) Evidence for an aspartyl phosphate residue at the active site of sodium and potassium ion transport adenosine triphosphatase. *J Biol Chem* 248: 6993–7000
- Price EM, Rice DA, Lingrel JB (1989) Site-directed mutagenesis of a conserved extracellular aspartic acid residue affects the ouabain sensitivity of sheep Na, K-ATPase. *J Biol Chem* 264: 21902–21906

- Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4: 406–425
- Schönrock C, Morley SD, Okawara Y, Lederis K, Richter D (1991) Sodium and potassium ATPase of the teleost fish *Catostomus commersoni*. Sequence, protein structure and evolutionary conservation of the alpha-subunit. *Biol Chem Hoppe-Seyler* 372: 279–286
- Schwartz A, Lindenmayer GE, Allen JC (1975) The sodium-potassium adenosine triphosphatase: pharmacological, physiological and biochemical aspects. *Pharmacol Rev* 27: 3–134
- Shull GE, Schwartz A, Lingrel JB (1985) Amino-acid sequence of the catalytic subunit of the (Na⁺ + K⁺)ATPase deduced from a complementary DNA. *Nature* 316: 691–695
- Shull GE, Greeb J, Lingrel JB (1986) Molecular cloning of three distinct forms of the Na⁺, K⁺-ATPase α -subunit from rat brain. *Biochemistry* 25: 8125–8132
- Shull MM, Pugh DG, Lingrel JB (1989) Characterization of the human Na, K-ATPase α 2 gene and identification of intragenic restriction fragment length polymorphisms. *J Biol Chem* 264: 17532–17543
- Sweadner KJ (1989) Isozymes of the Na⁺/K⁺-ATPase. *Biochim Biophys Acta* 988: 185–220
- Takeyasu K, Tamkun MM, Renaud KJ, Fambrough DM (1988) Ouabain-sensitive (Na⁺ + K⁺)-ATPase activity expressed in mouse L cells by transfection with DNA encoding the α -subunit of an avian sodium pump. *J Biol Chem* 263: 4347–4354
- Takeyasu K, Lemas V, Fambrough DM (1990) Stability of Na⁺, K⁺-ATPase α -subunit isoforms in evolution. *Am J Physiol* 259: C619–C630
- Verrey F, Kairouz P, Schaerer E, Fuentes P, Geering K, Rossier BC, Kraehenbuhl J-P (1989) Primary sequence of *Xenopus laevis* Na⁺-K⁺-ATPase and its localization in A6 kidney cells. *Am J Physiol* 256: F1034–F1043
- Walderhaug MO, Post RL, Saccocmani G, Leonard RT, Briskin DP (1985) Structural relatedness of three ion-transport adenosine triphosphatases around their active sites of phosphorylation. *J Biol Chem* 260: 3852–3859

(Received June 24, 1996 / Accepted August 12, 1996)