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

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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)-benzoyladenosine) 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 Kuntzveiler, 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, $\alpha 1$, $\alpha 2$ 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 (Sweadner et al., 1989). Each subunit isoform of vertebrates shows different tissue-specific and developmentally regulated expressions (Herrera *et al.*, 1987; Orlowski 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.

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MATERIALS AND METHODS

Culture of embryos

Gametes of the sea urchin, *Hemicentrotus pulcherrimus*, were obtained by an intracoelomic injection of 0.5 M KCI. 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 manufacture'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 (5GCIGGGGAGTCATTGACACCGTCICCIGTTACAGCCACAATGGCACC3') 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 $[\alpha$ -³²P]dCTP (3000 Ci/mmol) using a randomprimed 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 µg/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). Deoxycitidine 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 *Eco*RI 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 in algorithm method described by Gotoh (1993). Construction of phylogenetic tree was performed by the neighbor-joining method (Saitou and Nei, 1987).

Chemicals

 $[\gamma^{-32}P]$ ATP and $[\alpha^{-32}P]$ 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, FITCbinding 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 *Bg*/II, *Eco*RI and *Hin*dIII 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 (AX₁VVHGSDLK (I) and QGAIVAVTGDGVNDS-PALX₂K (II)) (Ohta *et al.*, 1986) are also identified in the sea urchin Na⁺, K⁺-ATPase α -subunit. Especially, the latter FSBAbinding site FSBA II, used as a probe for screening, matches almost perfectly except one amino acid residue at the Nterminal end of this region. On the contrary, the deduced Nterminal 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

TCATTGATGATGATGACGATGGCTGCCCGTGTAGAGAAAAGCGGAGGGCGCGGGGGGGG	140 45
GANGAGGGACTTGGAAGAGCTGAAGAAATGGAAATTTGATGATCACAAGATCTCACTTGAAGATTTGGTCGCTCGGATTCCAACACCACCGGTTTGACTGTAGCTCAAGCAGCACAATGTGCTCGCCCGAG K R D L E E L K K E M E F D D H K I S L E D L V A R L D S N I T T G L T V A Q A A H V L A R D	280 92
ATGGACCTAATTCCTTGGACCCCACCTCCTAAGACGCGGATGGAT	420 139
AAGGATGAACCCAACAGTGACAAC <u>TTGTACCTGGGTATTGTGCTTGGCAAGGTGTTGTCATCACGGAGGTTT</u> TCTCATACTATCAAGAAGGTTCTAAGATCATGGAATCTTTCAAGAACATGGTTCCTCAGGA K D E P N S D N L Y L G I V L A S V V I I T G C F S Y Y Q E A K S S K I M E S F K N M V P Q E	560 185
AGCCCTTGTGCTACGTGCCGGTGAATGGCACTCTATCAATGCCGTGGTGGTGGTGGTGGTGGTGGATGGGGGGGG	700
ACTCATCTCTGACTGGAGGGCTCGGAGCCTCGGCGCGGGAGTTCACAGGGGATACCCTCGGGGGGGG	840 279
GGTGATAACACCGTCATGGGACGTATGCTGGGCCTCTGGACTGGACGTTGGTGACGCCCATCGCCAAGGAGATCGACCA <u>TTCATCACACAGCTGTAGCCGTCTTCTTGGGTGTATCTTTCTT</u>	980 325
$\frac{\text{CCTGTCTTTCCCTCTTGGATACACCTGGCTTGTAGCTTGTGTTTTCCTCATTGGATCATTGTGGCTAATGTCCTGGGGGGTCCTCGGCCACTGTCACGTATGTCTGACCTTGACGTATGGCTAAGGTCCACAAGA \frac{\text{L S F P L}}{\text{L S F P L G Y T W L E A C V F L I G I I V A N V P E G L L A T V T V C L T L T A K R M A H K N }$	1120 372
ACTGTCTTGTGAAGAACCTTGAGGCCGTAGAGACCCTTGGTAGTACCTCCCACTATCTGCTCTGATAAGACTGGTCCCAAACAGGATGACCGTAGCTCCACATGTGGTTTGATAACACCATCGTAGAGGCGAGAC C L V K N <u>L E A V E T L G S T S T I C S D K T G</u> T L T Q N R M T V A H M W F D N T I V E A D D D	$\begin{smallmatrix}1&2&6&0\\&&4&1&9\end{smallmatrix}$
ACAACAGAGGATCAGAGTGGTGGTCAATTTGACAAGTCTTCACCAACCTGGATGGCCTTAGCTCGTTTGCTTGTCCACCGATCTGAATTCAAGGCTGGACAAGAAAATGTTCCCATCCTAAAGAGAGATACTAC T T E D Q S G G Q F D K S S P T W M A L A R I A C L C N R S E P K A G Q E N V P I L K R D T T	$\begin{smallmatrix}&1&4&0&0\\&&4&6&5\end{smallmatrix}$
TGGAGATGCCTCTGAATCAGCCTTGGTAAAGTGCGTTGAACTTTGTATGTTCAATGTAACGGAGTACCGTAAGAAGAACAAGAAGGTCTGTGAGATCCCATTCCAATCCAACAAATACCAGGTTTCCATCCA	$\begin{smallmatrix}1540\\512\end{smallmatrix}$
CCGAAGGAGATGATCGTAACCTGTTGGTGATGAAGGGAGCTCCTGAACGACGATCTGAACAACTATCTTAATCAAGGGAAGGAA	1680 559
GAGCTIGGIGGIGGIGGIGGIGGIGGIGGIGGIGGIGGIGGIGG	1820 605
CATGATAGATCCTCCCCGTGCTGCTGCACCTGATGCTGTGGAAAGTGCCGCTCTGCTGGAATCAAGGTAATCATGGTGACCATCCAATCACAGCTAAGGCTATCGCTAAGGGTGTCGGCATTATTTCTGAAG M I D P P R A R V P D A V G K C R S A G I K V I M V T G D H P I T A K A I A K G V G I I S E G	1960 652
GTAACGAGACCCGTGATGACATTGCTCAACGTCTTGGAACCCCTATGAACAAGTCAACGCCAAGGATGCAAAAGCGATTGTTGTCCACGGTTCTGAGCTAAGACATAGCCCGTGAAGACCTTGGATGTTGTGTCGACG N E T R D D I A Q R L G I P I E Q <u>V N P K D A K A I V V R G S E L K D</u> I A R E D L D A I L A FERA I	2100 699
GACCATCCTGAGATCGTCTTTGCTCGTACTTCACCTAGGAGAAGTTGATCATTGTTGAAGGTTGTAGGCTGGTGGTGGTGGTGGTGGTGGTGGTGGTGATGGTGTTAATGACTCTCCAGCTCTGAAGAAGGCTGATAT D H P E I V F A R T S P Q Q K L I I V E G C Q R A <u>G A I V A V T G D G V N D S P A L K K</u> A D I FSRA II	2240 745
TGGTGTTGCTATGGGTATCGCTGGCAGTGATGTCAGCAAACAAGCAGCTGATATGATCTGCTCGATGATAACTTGCCTCCATTGTTACCGGTGTTGAAGAAGGTCGTCTGATCTTTGACAACTTGAAGAAGTCAATTG G V A M G I A G S D V S K Q A A D M I L L D D N P A S I V T G V E E G R L I F D N L K K S I A	2380 792
CATACACTOTAACCAGTAACATCCCTGAG <u>ATCAGCCCGTTCCTTATCCTCATCCGTTCGGTTGCGTTGCGATCCGGTGCGTTACGA</u> TCTGGGTACTGGTACTGGTACTGGTACCGGTACGGTACCGGTACCGGTACCGGTACCGGTACCGGTACCGGGTACCGGTACGGGTACGGGTACGGGTACGGGTACGGGTACGGGTACGGGTACGGGTACGGGTACGGGTACGGGTACGGGTACGGGTACGGGTACGGGTACGGGTACGGGTACGGGTACGGGTACGGGTACGGTACGGTACGGTACGGTACGGTACGGTACGGTACGGTACGGTACGGTACGGTACGGTACGGTACGGTACGGTACGGTACGGTACGGTACGTAC	2520 839
TATGAAGAGGCCGAGTCTGATATCATGAAACGTCGCCCCCGTGACCCTCGAGAAFGACAAGCTTGTCAACGAGCGT <u>CTCATCAGTGTATCCTATGGACAAATTGGTCTAAGCTTCAGCGGGTTTCTTTGCCTACT</u> Y E E A E S D I M K R R P R D P Q N D K L V N E R L <u>I S V S Y G Q I G M I Q A S A G F P A Y F</u>	2660 885
TGTGATCATGGGTGAGAATGGTTTCCTACCCAATGATCTGATCATGTTACGTAGCAGGTGGGACGACAAAGCCGTTCTGAATGTGGGAGGATTCCTATGGCCAACAATGGGGGTACACCCAACGTAAGCAGCTGGAA <u>TACA</u> VIMGENG FLPNDLIMLRSRWDDKAVLNVEDSYGQQWGYTQRKQLE <u>YT</u>	2800 932
$\text{CCGTGACACTGCTTTCTTTGCCTCTATTGTGGTGGTGGTACAATGGGCTGATGTCATCATTTGTAAGACACGACGTAACTCACTGATCCATCAATGAACAACTGGGTGTTGAACTTTGGTCTGTTCTTTGAGACTGCTGTCATCAATGAACTGCGAACTGCACTACTGAGACTGCGAACTGCACTACTGAGACTGCGACTGCACTGAGACTGCGACTGCACTGAGCAATGGACTACTGGTCTGTTCTTTGGAACTGCTGATCCAATGAGCAATGGACTGCGAACTGCGAATGAACAACTGGGTGTGAACTTTGGTCTGTTCTTTGGAACTGCGACTGAGCAATGGACTGAGCAATGGACTACTGGTCTTTGTAGGCCTGATGAACTACTGGACTGCAATGAACAACTGGGTGTGAACTTTGGTCTGTTCTTTGGAACTGCGAATGGAACTGCGAATGGAACTGCGAATGGACTGGACTGCGAATGGAACTGGACTGCGAATGGAACTGGTGGAACTGGACTGGACTGGACTGGACTGGACTGGACTGGACTGGACTGGACTGCGAATGGACTGGGGAAGGGAAGGGAAGGACAGGGGAAGGACGGAAGGGAAGGACGGAAGGGAAGGACGGAAGGGAAGGACGGAAGGACGGAAGGGAAGGGAAGGACGGAAGGGAAGGGAAGGACGGAAGGACGGAAGGGAAGGGAAGGACGGAAGGGAAGGACGGAAGGACGGACGGAAGGACGGACGGAAGGACGGAAGGACAGGGGAAGGACAGACGGGAAGGACGAC$	2940 979
CIGGCIGCITITCCIGICCIGCCITGGCCITGAAAAIGGICITCGGAIGGIACCCACICAGGAIGGGAIG	3080 1025
TCACAACCCTGGTGGATGGGTCGAGGTCGAGGACCTACTACTACTCGACCCCCTACACCGAGGACCACACCCCTCTCCTCACACGACCCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTC	3220 1038
CTTCATCATCCAGCCATCCCCTCAACTTCCACCCCTTACACGGCCTAGCCACGGCCCTGCCACGCCCTGCCACATCCCCCAACGCTATCCTCAAATCTCTCCCCCTCCTGAGCTCCTAACCCCT	3360
Alaccagatettccttctcgtccatcaaggaggategattgaalatgccagcagcccaagctctcattctcaagttcgctgataacctgtgcaagcaa	3500
CGGAACTCTTGTGTTTTTAAGATACCCAAGCTTCATCACGTTATGAATTTTTCAAACTAAACTCAAATTTTTATTCATGATACATTTGATTTTATTCATGTCATGGTACTATTTTAATAATTGAGATTCTATTGTAAACGAA	3640
TGAAAATAGAAACTTATTGAAAAGTATTCTATTAGAAACTGGATTGAATCCAAACATTCTATTAAACACTTATTGATGTTTAAGTATGCAGFTATATTGTATAGTATTACAGATGACAATTTTGGTGTAAAATGAAA	3780
tatattgttgatgatgatgatgatgatgatgatgatgatg	3920
cattreatataagaaaatgeaattgeatteatteatteagagteattteaaactgaggtacaggataceagattetttgggtaagattatettttggetaagattatettttggtgaattgtttggaagtaaga	4060
a A T T T T G A G G G C G A G G G G G A G G G G G G G G	4200
${\tt GCCCCCAAGCAAAAATATCAGAATTTTTTTTTAACGAACAACTGTTCTAGGGCAGTTGATTTAATAAACATTTAAGAACTTGTGATTAAAAAATTAATAAGGTGTTCTGGTTGTCCTTGAATAAAAATTGTT$	4340
TTTAATGTTCCCCCCGAAARAAAAAAAAAAAAAAAAAAAA	4401

Fig. 2. Nucleotide sequence of cDNA for sea urchin Na⁺, K⁺-ATPase α-subunit and its deduced amino acid sequence. The nucleotide sequence is a combination of SUA5, SUA51 and R4. The number of the nucleotide residues is given at the right end of each line. The deduced amino-acid sequence is shown below the nucleotide sequence. Eight major hydrophobic regions (H1-H8), likely to be transmembrane domains, are boxed. Catalytic sites in the cytoplasmic region were identified by homology with diverse ATPases shown by underlines. P: phosphorylation site, FITC: fluorescein isothiocyanate (FITC) binding region, FSBAI, II: 5'-(*p*-fluorosulfonyl)-benzoyladenosine binding regions.

human (Kawakami *et al.*, 1986; Ovchinnikov *et al.*, 1988; Shull *et al.*, 1989). Two kinds of isoforms were also reported in *Artemia* (Baxter-Lowe *et al.*, 1989; Macías *et al.*, 1991). However, only a single gene for α -subunit was found in *Drosophila melanogaster* (Lebovitz *et al.*, 1989). A region of significantly low homology between the isoforms of vertebrate was reported to be locating 11 amino acid upstream from the lysine residue in the FITC-binding site (Lingrel *et al.*, 1990). This region deduced from the sea urchin cDNA resembles that of α 3.

A possible evolutionary relationship of Na⁺, K⁺-ATPase α -subunit is proposed in Fig. 5. The tree suggests that the sea urchin Na⁺, K⁺-ATPase α -subunit has evolved from a common ancestor of vertebrates and has diverged before the three vertebrate isoforms (α 1, 2 and 3) appeared. Iwabe *et al.* (1996) also reported a phylogenic tree of ion transport ATPases, and also proposed that evolution of these tissue-specific isoforms occurred after the separation of invertebrate and vertebrate. Two isoforms of *Artemia* were suggested to occur after vertebrate-invertebrate divergence (Macías *et al.*,



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 existance of two types of ouabain-sensitive ATPase (Canfield *et al.*, 1992). Analysis of the isoforms in sea urchin embryos is now under investigation.

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SU : HD : DM : C1 : C2 : C3 : SP :	MITMAARVEKSGEGRGESYRVAVQTEVPDDGRNAAGKPKSKKKEKKRDLEELKKEMEFDDHKISLEDLVARLDSNITTGL MADP·DLESRGKADSYSVAEKKS·P·KI···NAN·AK·D···L·MTE·SMK·S·LSMYETSLEK·· MAKG·QK·GK·N····LDI·F···PI·ECYQ··G··PE··· MALRSDYEHGRADSY·VATVIATDDDNRTA··QYKSRRKMPA·VN·EN·DD·Q·LDI·F···P·EMYQ·FQTHPEN·· MGKG·ASEKYQPAATSENA·NSK·S·S·TT·D····VSL···LN·DE·HQKYGTDL·Q·· MGKGAGRDKYEPTATSEHGTK···A·E··MD····ISM····L·DE·HRKYGTDLSR·· MDGREYSPAATTSENGGGRR·Q·EKE·D····VNL···L·DE·GRKYQVDLSR·· MGCKGVGRDKYEPAAVSEHGDK··AK·E··MD····VSM···L·DE·HRKYGTDLNR··	80 69 43 81 63 61 58 50 61
SU : HD : AR : DM : C1 : C2 : C3 : SP :	TVAQAAHVLARDGPNSLTPPPK-TPEWIKFCQQLFGGFATLLWMGSILCFLAYTIEAATKDE-PNSDNLYLGIVLASVVII SENIV·RN·E···L·A····Q···V··K·M···SM··I·A···F·FG·R·VRDTN-··M·E····SV···· N···RSNIE····L·A····Q···V··K·M···SM··I·A···F·FG·R·VRDTN-···M·E····SV···· SH·R·KEN·E····LTP·-··Q···V··ED···VAM-···I·A····V·S·Q·S·SE···AD·····SA···V P·R·KEI·····A····T···V··R····SI···L···G·Q···SE····AD···········ST······ SN·R·E···Q············V··R····SI···L···G·Q···SE···························· SN·R·E···Q·······························	159 148 123 159 142 140 137 129
SU : HD : DM : C1 : C2 : C3 : SP :	TGCF YQEAKSSK IMESFKNMVPQEALVLRAGEWHS INAVNVNT DIVEVKGGDR IPAD IRVVESKSFKVDNSSLTGESE ····S·····K·I····D··KKIT···EQC·V··V·F·F I····C·GL····· ·····S·N·····F·T·I·E··KP·LR·EDL·L·VL·LEF··L·LVY·II·ARD····· ······L·IISAC·C····· ······D···C···· Q···I·D··KS···EQ·V··L···· L·IISAHGC····· ····· D····· Q····· L·IISAHGC····· ····· Q····· I····· V···· ····· Q····· I····· V···· ····· Q····· L·IISAHGC····· ····· ····· Q····· L····· V···· L·IISAHGC····· ····· Q····· Q····· L····· V···· L·IISAHGC····· ····· Q····· Q····· L····· L·IISAHGC····· L·IISAHGC·····	240 229 204 223 221 218 210 221
SU : HD : DM : C1 : C2 : C3 : SP :	PQSRSPEFTSDNPLETKNIAFFSTNASEGTCRGIVISTGDNTVMGRIAGLASGLDVGDTPIAKEIEHFIHIITAVAVFLGV ···AVD··HE··I··L··V·AT··VRI····N··GS·K··L···V·G···V·G···V·G···V·G····V·G····V·G····V·G····V·G····V·G····V·G·····V·G······	321 310 285 320 304 302 299 291 302
SU : HD : DM : C1 : C2 C3 SP :	SFFFLSFPUGYTWLEACVFLIGIIVANVPEGLLATVTVCLTLTAKRMAHKNCLVKNLEAVETLGSTSTICSDKTGTLTQNR · LII·LAM·H····II······························	402 391 366 401 385 383 380 372 383
SU : HD : AR : DM : C1 : C2 : C3 : SP :	H4 P MTVAHMWFDNTIVEADTTEDQSGGQFDKSSPTWMALARIACLCNRSEFKAGQENVPILKRDTTGDASESALVKCVELCMFN ····································	483 472 447 482 466 464 453 464
SU :: HD :: DM :: C1 :: C2 :: SP ::	VTEYRKKNKKVCEIPFNSTNKYQVSIHETEGD-D-RNLLVMKGAPERILDRCTTILIKGKEVDMNDEMQTAFNNAYLELGG 'MDI·AT	562 551 528 563 546 545 534 534 545

FITC

SU : LGERVLGFCHCYLPADQFPLGFAFDADEV-NFPLEKLCFVGLMSMIDPPRAAVPDAVGKCRSAGIKVIMVTGDHPITAKAI HD : ·····V··V··V··K··L·KTE·EQ···G··L··L··S AR : ·····DYL··L·KY·H···N··DA-···TG·R·A···A DM : ·····DFM··S·KY·N··K·NT·DI-···IDN·R···A TC : ·····LK·STSK··E·YP··VE·P-··ITD C1 : ·····LA··D···E··Q··T····V C2 : ·····P·K··R··R···TSD C3 : ·····LM··DE···E··Q··T·D·-···VDN SP : ·····LM··DE···E··Q··T·D·-···VDN	642 632 608 643 625 625 621 614 625
SU: AKGVGIISEGNETRDDIAQRLGIPIEQVNPKDAKAIVVHGSELKDIAREDLDAILADHPEIVFARTSPQQKLIIVEGC HD: ·····CEE··L·N··L·DLSEDQK·S··C·I·AK··KN·E·K·C·T AR: ·S·····VE··A··N·VSE··R··A··G·R··TPDA·E·RH· DM: ·S·····VE··A··N·VSE··RE··A··A·R·VSSDQ·E·RY·T TC: ·····VE··A··N·VS··R··C···TD··LSH·N·D·HY·T C1: ·····D··VE··A··N·VS··R··C···D··MTS·Q·D·LH·T C2: ······N·VS···RE···C···D··MTA·Q·E·RN·T C3: ········VE···A··N·VS···R···C···D··MSS·QI·E··QN·T SP: ·······S·····S···R···C·····MTP·Q·D··KY·T	720 713 686 721 704 703 699 692 703
SU QRAGA I VAVTGDGVNDSPALKKAD I GVAMG I AGSDVSKQAADM I LLDDNFAS I VTGVEEGRL I FDNLKKS I AYTL TSN I PE HD ··Q AR ·Q OM ··M TC ·Q C1 ·Q C2 ·Q SP ·Q	801 794 767 802 785 784 780 773 784 780
SU: ISPFLIFILASTPLPLGVVTILCTDLGTDLVPAISLAYEEAESDIMKRRPRDPQNDKLVNERLISVSYGQIGMIQASAGFF HD: ····································	882 875 848 865 865 865 865
HS H6 SU: AYFVIMGENGFLPNDLIMLRSRWDDKAVLNVEDSYGQQWGYTQRKQLEYTCHTAFFASIVVVQWADVIICKTRRNSLIHQG HD: T····A· SV: G: W: Y-FG: Q: MSNN·LL: F: F: AR: V····A·C W: FG: KK: FG: KK: FG: KK: FG: KK: S: NDLT: F: TC: S: LA: I: G: FK: E: V: S: V: M: V: A: I: A: V: F: V: H: V: I: V: I: I: G: S: I: I:	963 956 929 964 947 946 942 935 946
SU : MNNWVLNFGLFFETALAAFLSYCPGLENGURMYPLRIGWWFVAFPFSLLIFVYDECRRFILRHNPGGWVELETYY HD : T. F	1038 1031 1004 1039 1022 1021 1017 1010 1021

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; α1, α2 and α3 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, FSBAI, 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 α2850 and pArATNa136 (Baxter-Lowe *et al.*, 1989; Macías *et al.*, 1991), *Drosophila melanogaster* (Levobitz *et al.*, 1990), electric ray (Kawakami *et al.*, 1985), white sucker (Schönrock *et al.*, 1991), *Xenopus laevis* (Verrey *et al.*, 1989), giant toad (Jaisser *et al.*, 1992), chicken α1-3 (Takeyasu *et al.*, 1988; 1990), rat α1-3 (Shull *et al.*, 1986), horse α1 (Kano *et al.*, 1989), pig (Ovchinnikov *et al.*, 1986), sheep (Shull *et al.*, 1985) and human α1-3 (Kawakami *et al.*, 1986; Shull *et al.*, 1989; Ovchinnikov *et al.*, 1988). The horizontal bar represents 1 amino acid replacements per 100 sites.
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