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Short sequence-paper

Molecular cloning of Na^+-ATPase cDNA from a marine alga, *Heterosigma akashiwo*

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Abstract

We cloned novel Na^+-ATPase (HANA) cDNA from marine alga *Heterosigma akashiwo*. The full-length HANA cDNA was 4467 bp long and coded for a 1330 amino acid protein with a molecular weight of 146 306. The deduced product exhibited around 40% identity in amino acids with Na^+/K^+-ATPase α-subunits. A hydrophilic sequence of 285 amino acid residues that showed no homology with any sequence listed in databases existed in the M7–M8 junction of HANA. This is the first report on the primary structure of putative Na^+-transporting ATPase from plant cells.

Keywords: Sodium pump; Na^+/K^+-ATPase; Marine alga; *Heterosigma akashiwo*

Plant cells have been believed to extrude intracellular sodium ions primarily with a Na^+/H^+-antiport system rather than Na^+-ATPase. However, cells of the marine alga *Heterosigma akashiwo* have Na^+-ATPase activity on the plasma membrane. A major feature of *H. akashiwo* Na^+-ATPase (HANA) is a close analogy with Na^+/K^+-ATPases of animal cells. The Na^+-ATPase activity was greatly stimulated in the presence of 100 mM NaCl and 10 mM KCl with 5 mM Mg^{2+} ions, and the activity was inhibited by orthovanadate, a specific inhibitor of P-type ATPases but not inhibited by ouabain [1,2]. The Na^+-ATPase formed a phosphorylated intermediate of approximately 140 kDa in the presence of Na^+ and Mg^{2+} ions [1,2], and the 140 kDa polypeptide reacted with an antibody raised against pig kidney Na^+/K^+-ATPase [3]. The plasma membrane of *H. akashiwo* has ATP-dependent Na^+-transporting activity [4]. Though the function and reaction mechanism of Na^+-ATPase seem to correspond to those of Na^+/K^+-ATPases, there were marked differences in relative molecular mass, subunit composition (140 kDa for HANA [2] vs. 110 kDa for Na^+/K^+-ATPase α-subunit plus 35 kDa for the β-subunit), and ouabain sensitivity [1]. Therefore, we have cloned Na^+-ATPase cDNA in order to obtain a better understanding of the molecule.

H. akashiwo cells were cultured at 20°C under a constant light condition. Total RNA was isolated from cells in the logarithmic phase of growth (10^9 cells)

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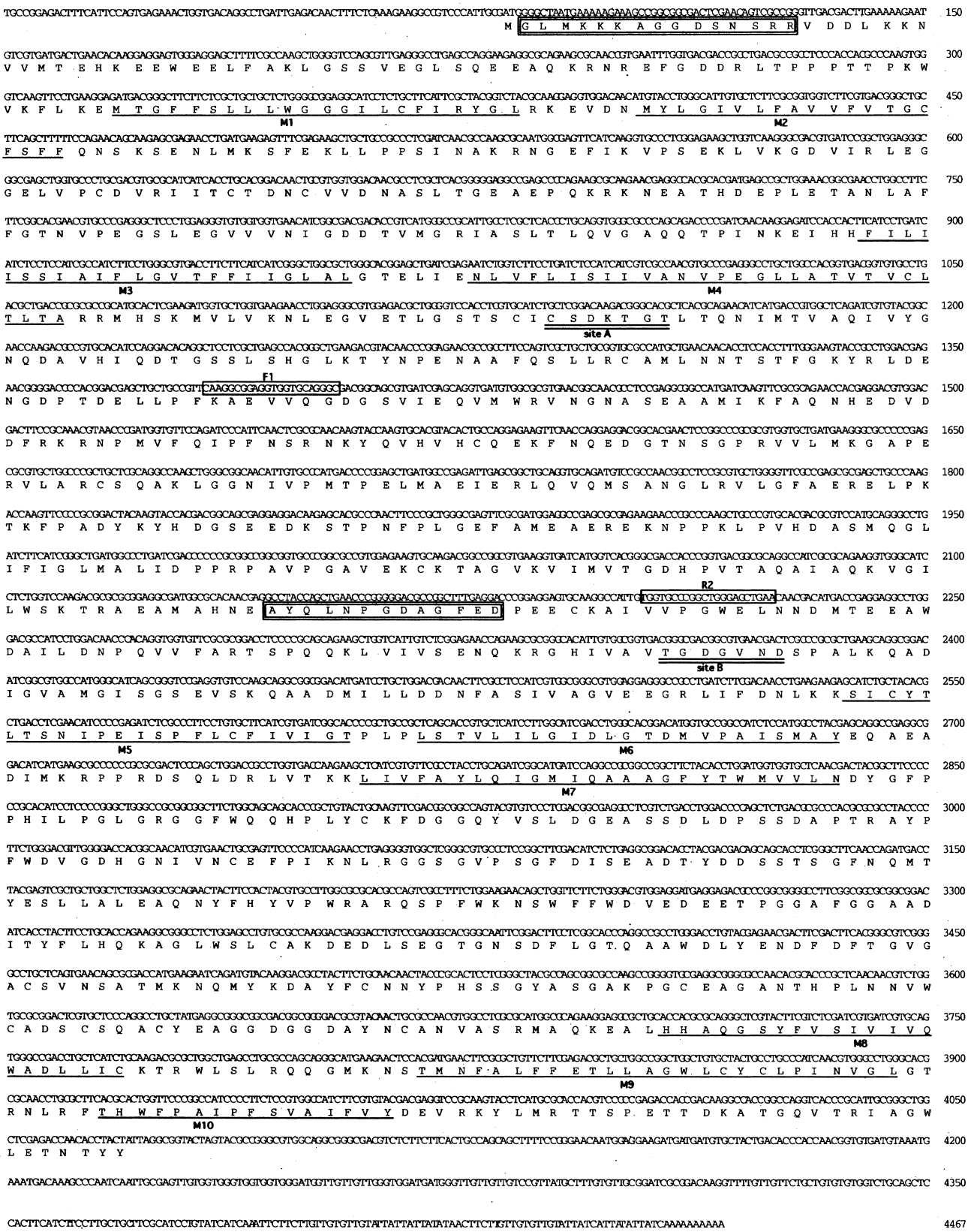
M1
M2
M3
M4
site A
F1
R2
site B
M5
M6
M7
M8
M9
M10

Fig. 1. Nucleotide sequence of HANA and deduced amino acid sequence. The 10 putative transmembrane regions, which were predicted by the TMpred program based on the procedure of Hofmann and Stoffel [21], are underlined (M1–M10). Parts underlined twice indicate site A and site B, those were used for designing the forward and reverse primers for PCR amplification. The boxes indicate the F1 and R2 regions, those were used as the primers for the 3'-RACE and 5'-RACE methods, respectively. The double lined boxes indicate the amino acid sequences detected by protein analysis of 140 kDa Na^+-ATPase of *H. akashiwo*.
←

by the method of Okayama et al. [5]. Degenerate oligodeoxyribonucleotides corresponding to the two highly conserved regions of P-type ATPases were used as PCR primers. The 20-mer oligodeoxyribonucleotides have the following sequences: TG(T,C)TCIGA(T,C)AA(A,G)ACIGGIAC from the fully conserved sequence, CSDKTGT, in the phosphorylation site (site A), and TC(G,A)TTIACICC(G,A)TCICCIGT from a conserved sequence, TGDGVND, in the ATP binding site (site B) of P-type ATPases. A DNA fragment of 1.2 kb was amplified from *H. akashiwo* mRNAs. Using the fragment, clones of 2.2 kb and 3.0 kb were obtained by means of the 3'-RACE and 5'-RACE methods, respectively. The two clones contain a 742 bp overlapped region, and reveal a 3990 bp open reading frame with 81 bp 5'-untranslated and 396 bp 3'-untranslated sequences. A protein of 1330 amino acid residues with a molecular mass of 146 kDa (Fig. 1) was encoded. We named this gene *HANA*, based on ***H. a****kashiwo* **N**a^+-**A**TPase.

An in-frame stop codon is located 36 bp upstream from the first methionine codon and the sequence around it conforms to the Kozak consensus sequence [6]. The first methionine was followed by GLMKKKAGGDSNSRR. This sequence coincides with the N-terminal sequence of HANA, which was obtained by protein sequencing. Another partial sequence of HANA protein was also encoded in the same reading frame as shown in Fig. 1 (double line boxed). Northern blotting analysis revealed a transcript of 4.8 kb (data not shown) and immunoblotting analysis detected an approximately 146 kDa polypeptide at almost the same abundance in all batches of cells cultured for 1 week at various concentrations of NaCl from 0.3 to 0.5 M (Fig. 2).

The amino acid sequence of HANA was compared with those of P-type ATPases, including human Na^+/K^+-ATPase α1-subunit (ATN1-h) [7], human H^+/K^+-ATPase α1-subunit (ATHA-h) [8], yeast Na^+-ATPase (ATN2-y) [9], yeast Ca^{2+}-ATPase (ATC1-y) [10] and *Arabidopsis* H^+-ATPase (PMA1-a) [11]. These ATPases have several conserved sequences, include the phosphorylation site CSDKTGT (site A) and ATP binding site TGDGVND (site B). HANA showed remarkable homology with human Na^+/K^+-ATPase α-subunits with 41% identity. HANA was also homologous with other cation pumps, showing 36% identity to ATHA-h, 23% to ATN2-y, 22% to ATC1-y, and 20% to PMA1-a. As shown in Fig. 3, hydropathy analysis revealed that these ATPases have 10 putative transmembrane domains and their hydropathy profiles are quite similar. Thus, the overall structures of the ATPases should be almost the same. HANA exhibited high similarity with ATN1-h at predicted transmembrane domains (average similarity 65%). Particularly, at the sixth transmembrane (M6), HANA showed 83% identity to ATN1-h and only 48% identity to ATHA-h.

Though HANA is strikingly similar to ATN1-h, its alignment reveals a major difference. Between the

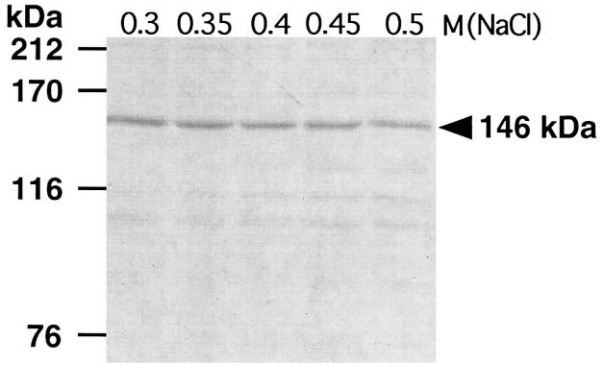

Fig. 2. Immunoblotting analysis of HANA. *H. akashiwo* cells were cultured for 5 days at 20°C under 16 h of light and 8 h of darkness condition in ASP-7 medium, which contained various concentrations of NaCl from 0.3 M to 0.5 M. The cells were lysed in a buffer containing 1% SDS and 30 mM Tris–HCl (pH 6.8) and the lysate was centrifuged at 10 000 × *g* for 5 min. The extract was analyzed with 7% SDS–PAGE and subsequent immunoblotting using an anti-HANA antibody, that was raised against pGEX-6P-HANA (929–1261 aa) fusion proteins.

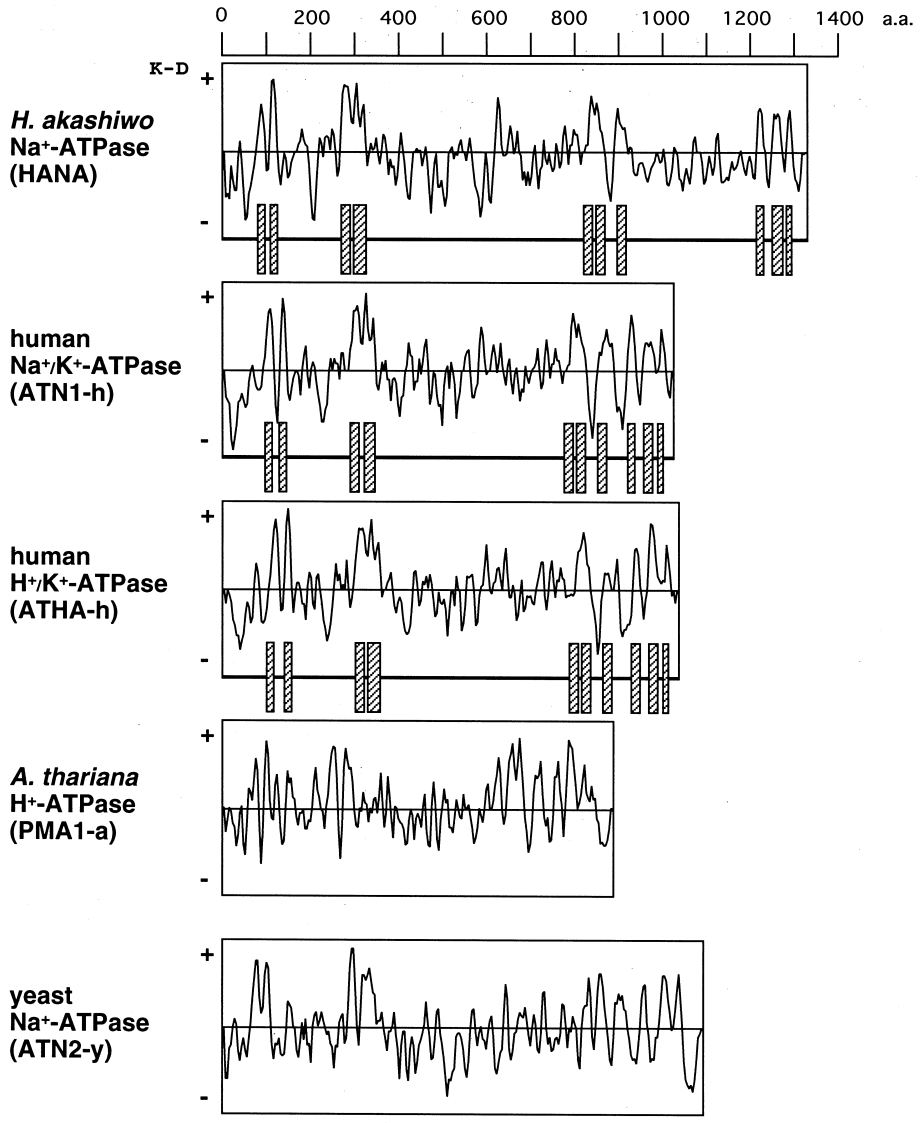

Fig. 3. Hydropathy plots of HANA, human Na^+/K^+-ATPase α1-subunit (ATN1-h), human H^+/K^+-ATPase α1-subunit (ATHA-h), H^+-ATPase of *Arabidopsis* (PMA1-a), and Na^+-ATPase of yeast (ATN2-y). The shadowed boxes indicate putative transmembrane regions. The hydropathy program is based on the procedure of Kyte and Doolittle [22].

seventh and eighth transmembrane domains (M7–M8 junction), HANA has a hydrophilic sequence of 285 amino acids which shows no homology with any sequence listed in the databases.

Lemas et al. [12] found that a 26 amino acid sequence (NDVEDSYGQQWTFEQRKIVEFTCHTA) in the M7–M8 junction of Na^+/K^+-ATPase α1 is important for the α–β-subunit interaction. Wang et

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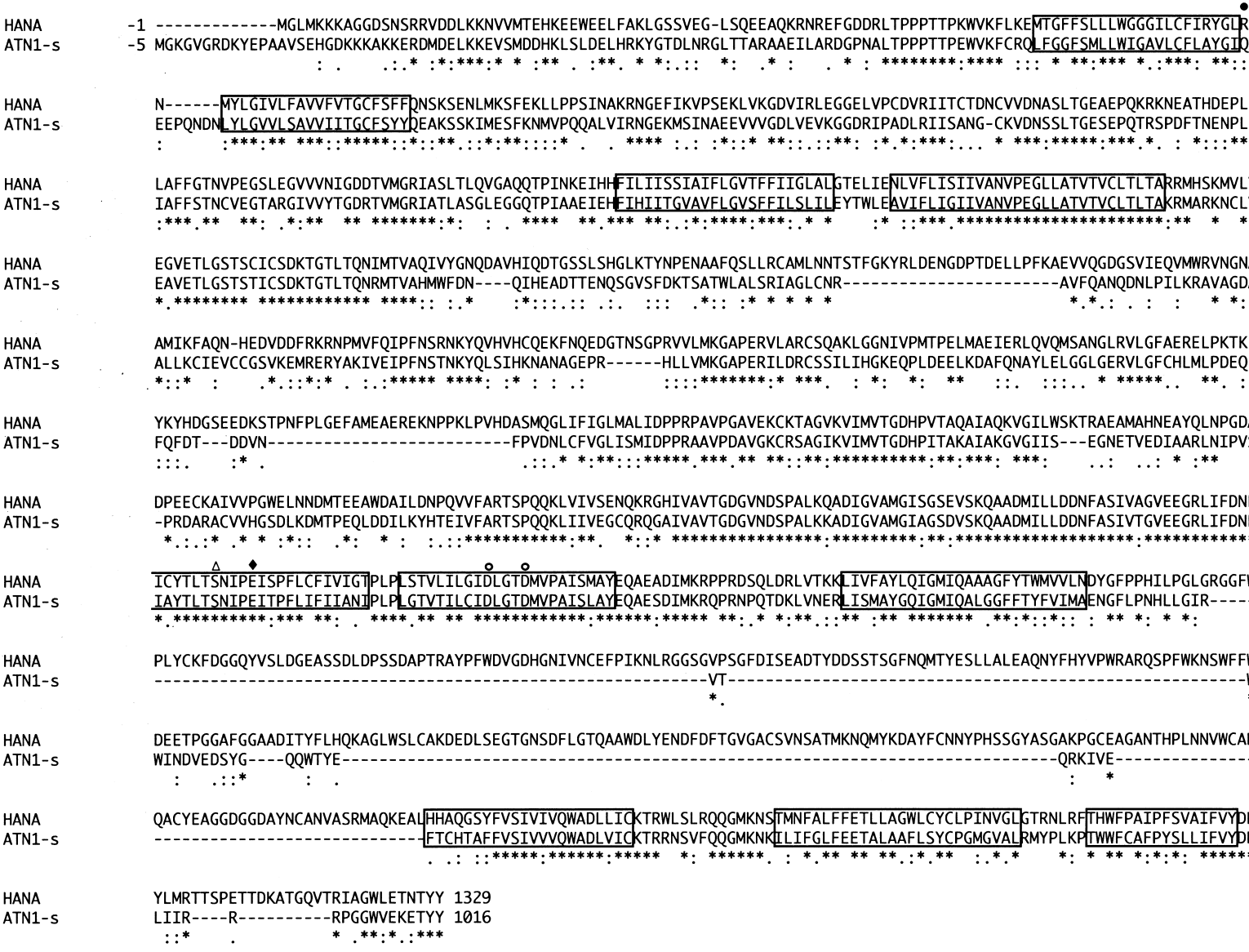
Fig. 4. Alignment of the deduced amino acid sequences with HANA and the sheep Na^+/K^+-ATPase α1-subunit (ATN1-s) [23]. Asterisks indicate identical amino acids, colons indicate amino acids that have high similarity, periods indicate amino acids that have low similarity, and dashes indicate gaps. Solid circle indicates Arg^{101}. Open triangle indicates Ser^{825} and solid diamond indicates Glu^{829}. Open circles indicate Asp^{854} and Asp^{858}. The putative transmembrane regions are boxed. The alignment program is Clustal W (1.8), based on the procedure of Thompson et al. [24].


HANA -1 ----------MGLMKKKAGGDSNSRRVDDLKKNVVMTEHKEEWEELFAKLGSSVEG-LSQEEAQKRNREFGDDRLTPPPTTPKWVKFLKEMTGFFSLLLWGGGILCFIRYGLRKEVD 105
ATN1-s -5 MGKGVGRDKYEPAAVSEHGDKKKAKKERDMDELKKEVSMDDHKLSLDELHRKYGTDLNRGLTTARAAEILARDGPNALTPPPTTPEWVKFCRQLFGGFSMLLWIGAVLCFLAYGIQAATE 115
HANA N------MYLGIVLFAVVFVTGCFSFFQNSKSENLMKSFEKLLPPSINAKRNGEFIKVPSEKLVKGDVIRLEGGELVPCDVRIITCTDNCVVDNASLTGEAEPQKRKNEATHDEPLETAN 219
ATN1-s EEPQNDNLYLGVVLSAVVIITGCFSYYQEAKSSKIMESFKNMVPQQALVIRNGEKMSINAEEVVVGDLVEVKGGDRIPADLRIISANG-CKVDNSSLTGESEPQTRSPDFTNENPLETRN 234
HANA LAFFGTNVPEGSLEGVVVNIGDDTVMGRIASLTLQVGAQQTPINKEIHHFILIISSIAIFLGVTFFIIGLALGTELIENLVFLISIIVANVPEGLLATVTVCLTLTARRMHSKMVLVKNL 339
ATN1-s IAFFSTNCVEGTARGIVVYTGDRTVMGRIATLASGLEGGQTPIAAEIEHFIHIITGVAVFLGVSFFILSLILEYTWLEAVIFLIGIIVANVPEGLLATVTVCLTLTAKRMARKNCLVKNL 354
HANA EGVETLGSTSCICSDKTGTLTQNIMTVAQIVYGNQDAVHIQDTGSSLSHGLKTYNPENAAFQSLLRCAMLNNTSTFGKYRLDENGDPTDELLPFKAEVVQGDGSVIEQVMWRVNGNASEA 459
ATN1-s EAVETLGSTSTICSDKTGTLTQNRMTVAHMWFDN----QIHEADTTENQSGVSFDKTSATWLALSRIAGLCNR----------AVFQANQDNLPILKRAVAGDASES 447
HANA AMIKFAQN-HEDVDDFRKRNPMVFQIPFNSRNKYQVHVHCQEKFNQEDGTNSGPRVVLMKGAPERVLARCSQAKLGGNIVPMTPELMAEIERLQVQMSANGLRVLGFAERELPKTKFPAD 578
ATN1-s ALLKCIEVCCGSVKEMRERYAKIVEIPFNSTNKYQLSIHKNANAGEPR------HLLVMKGAPERILDRCSSILIHGKEQPLDEELKDAFQNAYLELGGLGERVLGFCHLMLPDEQFPEG 561
HANA YKYHDGSEEDKSTPNFPLGEFAMEAEREKNPPKLPVHDASMQGLIFIGLMALIDPPRPAVPGAVEKCKTAGVKVIMVTGDHPVTAQAIAQKVGILWSKTRAEAMAHNEAYQLNPGDAGFE 698
ATN1-s FQFDT---DDVN-------------------------FPVDNLCFVGLISMIDPPRAAVPDAVGKCRSAGIKVIMVTGDHPITAKAIAKGVGIIS---EGNETVEDIAARLNIPVSQVN 649
HANA DPEECKAIVVPGWELNNDMTEEAWDAILDNPQVVFARTSPQQKLVIVSENQKRGHIVAVTGDGVNDSPALKQADIGVAMGISGSEVSKQAADMILLDDNFASIVAGVEEGRLIFDNLKKS 818
ATN1-s -PRDARACVVHGSDLKDMTPEQLDDILKYHTEIVFARTSPQQKLIIVEGCQRQGAIVAVTGDGVNDSPALKKADIGVAMGIAGSDVSKQAADMILLDDNFASIVTGVEEGRLIFDNLKKS 768
HANA ICYTLTSNIPEISPFLCFIVIGTPLPLGTVLILGIDLGTDMVPAISMAYEQAEADIMKRPPRDSQLDRLVTKKLIVFAYLQIGMIQAAAGFYTWMVVLNDYGFPPHILPGLGRGGFWQQH 938
ATN1-s IAYTLTSNIPEITPFLIFIIANIPLPLGTVTILCIDLGTDMVPAISLAYEQAESDIMKRQPRNPQTDKLVNERLISMAYGQIGMIQALGGFFTYFVIMAENGFLPNHLLGIR-------- 880
HANA PLYCKFDGGQYVSLDGEASSDLDPSSDAPTRAYPFWDVGDHGNIVNCEFPIKNLRGGSGVPSGFDISEADTYDDSSTSGFNQMTYESLLALEAQNYFHYVPWRARQSPFWKNSWFFWDVE 1058
ATN1-s ----------VT----------WDDR 886
HANA DEETPGGAFGGAADITYFLHQKAGLWSLCAKDEDLSEGTGNSDFLGTQAAWDLYENDFDFTGVGACSVNSATMKNQMYKDAYFCNNYPHSSGYASGAKPGCEAGANTHPLNNVWCADSCS 1178
ATN1-s WINDVEDSYG----QQWTYE----------QRKIVE---------- 908
HANA QACYEAGGDGGDAYNCANVASRMAQKEALHHAQGSYFVSIVIVQWADLLICKTRWLSLRQQGMKNSTMNFALFFETLLAGWLCYCLPINVGLGTRNLRFTHWFPAIPFSVAIFVYDEVRK 1298
ATN1-s ----------FTCHTAFFVSIVVVQWADLVICKTRRNSVFQQGMKNKILIFGLFEETALAAFLSYCPGMGVALRMYPLKPTWWFCAFPYSLLIFVYDEVRK 999
HANA YLMRTTSPETTDKATGQVTRIAGWLETNTYY 1329
ATN1-s LIIR----R----------RPGGWVEKETYY 1016

al. [13] showed that Tyr^{898}, Val^{904}, and Cys^{908} (Tyr^{901}, Val^{907}, and Cys^{911}, respectively in Fig. 4) in the 26 amino acid sequence are crucial. HANA does not have a homologous sequence to this in the M7–M8 region, indicating that HANA may not bind a β-subunit. This seems consistent with our previous finding [2] that Na^+-ATPase in *H. akashiwo* does not have any accessory subunit, based on SDS–PAGE analysis. Given that finding, we considered the possibility that the hydrophilic sequence in the M7–M8 region of HANA might have a similar function to the β-subunit of Na^+/K^+-ATPase. However, we could find no homology in this region to the β-subunit of Na^+/K^+-ATPase or to any polypeptide registered in SWISS-PROT.

In recent years, using chemical modification and site-directed mutagenesis, amino acids were identified that were responsible for determining ouabain sensitivity and cation binding in Na^+/K^+-ATPases. Price and Lingrel [14] and Price et al. [15] showed that Arg^{111} and Asp^{122}, which are located between the first and second transmembrane segments (M1–M2 junction) of the rat Na^+/K^+-ATPase α1-subunit, are involved in ouabain resistance. Jaisser et al. [16] suggested substitution of Gln^{111} of the sheep Na^+/K^+-ATPase α1-subunit to Arg is important for ouabain resistance. Na^+-ATPase in *H. akashiwo* showed an ouabain resistance phenotype [1], and substitution (Arg^{101} for Gln^{111}) and deletion of amino acids found at the corresponding region (M1–M2 junction) of HANA is in agreement with their results (Fig. 4). Ser^{825} in HANA corresponds to Ser^{775} in sheep Na^+/K^+-ATPase, which was suggested to play a role in K^+ binding and K^+ transport (Argüelllo and Lingrel

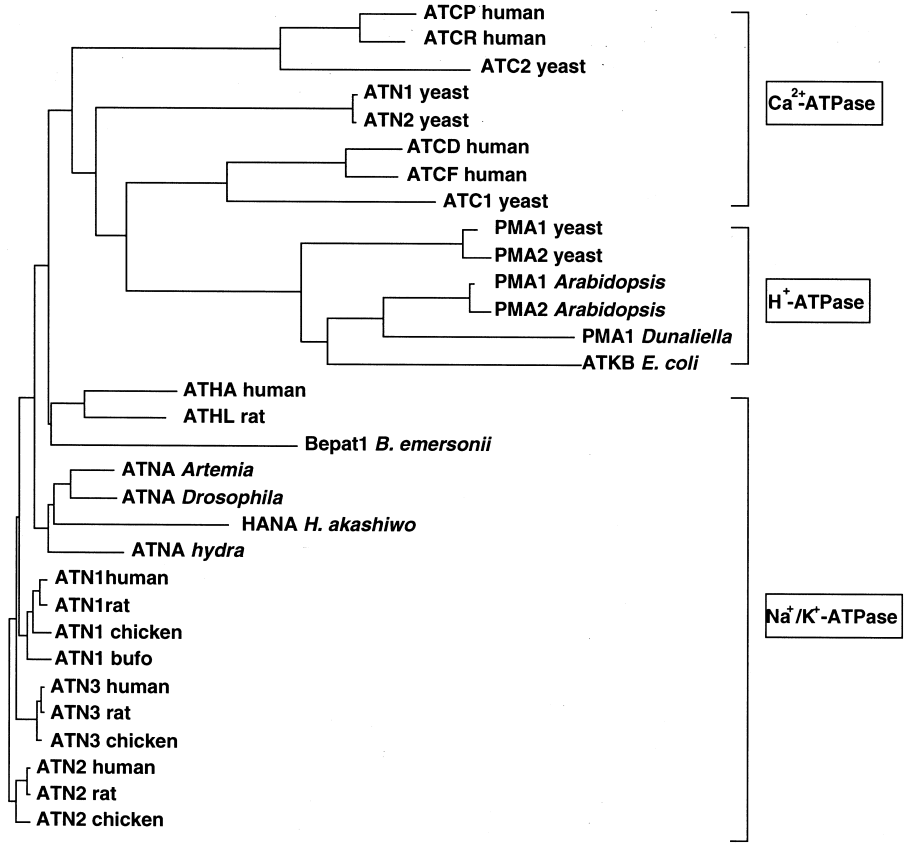


Fig. 5. Phylogenetic tree of P-type ATPases, constructed using the neighbor-joining method in GrowTree Phylogram of Wisconsin GCG DNA sequence analysis software. Sequences of ATPases were obtained through the GenBank, EMBL, and SWISS-PROT databases. From the top to bottom of the figure, the accession numbers for these sequences are as follows: P20020, P23634, P38929, P13587, Q01896, P16614, Q93084, P13586, P05030, P19657, P20649, P19456, P54211, P03960, P20648, P54708, AJ001045, P28774, P13607, AB017481, P35317, P05023, P06685, P09572, P30714, P13637, P06687, P24798, P50993, P06686, P24797.

[17]). The Glu^{781} of rat Na^+/K^+-ATPase (corresponds to Glu^{779} in Fig. 4), proposed to be an important coordinate of cation selectivity and activation (Koster et al. [18]), corresponds to Glu^{829} of HANA. Kuntzweiler et al. [19] showed that both Asp^{804} and Asp^{808} of the sheep Na^+/K^+-ATPase are required for normal Na^+ and K^+ interaction with the protein, possibly coordinating these cations during transport. HANA protein also has Asp residues at both corresponding positions (Fig. 4), but Ca^{2+}-ATPase (ATC1-y) or human gastric H^+/K^+-ATPase (ATHA-h) has an Asp residue at one of the corresponding regions. Two Asp residues in this particular region might be one of the important features for the transport of both Na^+ and K^+ ions.

A phylogenetic tree of a P-type ATPase family is shown in Fig. 5. The tree comprises three major clusters, mainly correlating with cation specificity, as follows: Ca^{2+}-ATPases, H^+-ATPases, and Na^+/K^+-ATPases. HANA is included in the cluster of Na^+/K^+-ATPases, being especially close to the Na^+/K^+-ATPases in invertebrates such as *Artemia*, *Drosophila*, or *Hydra*. However, yeast Na^+-ATPases (ATN1-y and ATN2-y) are classified into the cluster of Ca^{2+}-ATPases.

The principal function of Na^+/K^+-ATPases in animal cells is related to the maintenance of cellular ion homeostasis, e.g. high intracellular K^+ and low Na^+ [20]. *H. akashiwo* is a wall-less unicellular marine alga. This alga is always bathed in high concentrations of Na^+ ions, like animal cells, and may well have an ion-transporting ATPase on its plasma membrane to exclude cytoplasmic Na^+ ions. Comparison of HANA with Na^+/K^+-ATPases and further mutational analysis of the cloned gene could reveal the function of some important amino acid residues and especially the function of the β-subunit of Na^+/K^+-ATPases.

The nucleotide sequence data of HANA appear under the accession number AB017481 in the DDBJ/EMBL/GenBank databases.

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