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A novel P-type ATPase from yeast involved in sodium transport

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The gene *ENA1* was cloned by its ability to complement the Li^+ sensitivity of a low Li^+-efflux strain The nucleotide sequence of the cloned DNA fragment showed that there are two almost identical genes in tandem, and predicts that they encode P-ATPases Disruption of both genes originated a strain defective in Na^+ and Li^+ effluxes, and sensitive to Na^+, to Li^+ and to alkaline pH By transformation with *ENA1* the defective effluxes and tolerances were repaired

Sodium pump, Sodium ATPase, Sodium efflux, Lithium efflux, *Saccharomyces cerevisiae*

1. INTRODUCTION

All living cells exclude Na^+, and create a Na^+- concentration gradient across the membrane. In the naked cells of animals, this gradient is generated by the Na^+-pump, (Na^+, K^+)-ATPase [1], and plays a central role in their physiology, as most uphill transports are Na^+ coupled [2], and a major H^+ extruding system is a Na^+/H^+ antiport [3] In contrast, in eucaryotic cells with walls, the membrane potential is generated by a H^+-pump ATPase [4], secondary transports are coupled to H^+ [5], and Na^+ efflux has never been shown to be a primary process Here we present evidence that P-ATPases mediate Na^+, Li^+, and probably K^+ effluxes in yeast

2 METHODS

2 1 *Media, strains, and cation analyses*

Standard media and routine genetic methods have been described previously [6] Arginine-phosphate medium, free of ammonium and alkali cations, has also been described [7] The yeast strains used in this work were derived from the Li^+-tolerant strains (growth at 40 mM Li^+, 1 mM K^+) DBY746 (*Matα ura3–52 leu2–3 leu2–112 his3–Δ1 trp1–289*) and DBY747 (*Mata*, isogenic with DB746), and the Li^+-sensitive strain (no growth at 5 mM Li^+, 1 mM K^+) GF36 (*Matα*) *Escherichia coli* DH5α [8] was used as the bacterial host for plasmids

Li^+ and Na^+ effluxes from the cells were determined by chemical analyses, as described previously [9,10], in 10 mM 3-{[2-hydroxy-1,1-bis(hydroxymethyl) ethyl]-amino}-1-propanesulphonic acid adjusted to pH 8 0 with $Ca(OH)_2$ containing 0 1 mM $MgCl_2$, 10 mM KCl and 2% glucose

2 2 *Recombinant DNA techniques and DNA sequencing*

Standard protocols [8] and manufacturer's instructions were followed for plasmid preparations, restriction enzyme digestion, phosphatase treatment, ligation, transformation, and agarose gel electrophoresis DNA sequencing was performed by the dideoxy-chain termination method of Sanger et al [11] as modified for use with Sequenase (U S BiochemicalCorp Cleveland, OH)

2 3 *Disruption of ENA1 and ENA2*

The 3 9-kb *Bam*HI fragment of pGH1 1 (Fig 1) was deleted and replaced with the 3 0-kb *Bgl*II fragment of plasmid YEp13 [12] containing the *LEU2* gene Then, the *Xba*I–*Pst*I fragment of this construction was used for single-step gene disruption [13] in strain DBY746 DNA samples from Leu^+ transformants were digested with *Xba*I, then the restriction fragments were separated by electrophoresis in 0 7% agarose gels, transferred to nylon membranes (Hybond-N, Amersham), and hybridized to a ^{32}P-labeled probe derived from the 2 3-kb *Xba*I fragment of pGH6 (Fig 1) The probe was radiolabeled by the random primer method, and hybridization was carried out at 42°C in 50% formamide following standard methods [8]

3 RESULTS

After failure to obtain Na^+-sensitive yeast mutants, the gene encoding a Na^+ efflux system in yeast was cloned by complementation of Li^+ sensitivity in a low Li^+-efflux strain. This strategy was chosen because such strains are common in yeast [14] and Li^+ is an analogue of Na^+ in many transport systems. The selected strain (GF36) was repeatedly backcrossed with DBY747 to obtain the more suitable Li^+-sensitive strain RH2, which carried appropriate auxotrophic markers and had good transformation efficiency This strain did not grow in the presence of 5 mM Li^+, at 1 mM K^+, and Li^+ efflux was almost an order of magnitude lower than in the original DBY747 It was also moderately sensitive to Na^+, and Na^+ efflux was lower than in DBY747

By transformation of RH2 with plasmids of a yeast genomic library constructed in the shuttle vector YCp50 [13] (prepared in the laboratory of A. Jimenez from the Li^+-tolerant strain 483), and screening at 40 mM Li^+, 1 mM K^+, we isolated plasmid pGH1.1 (Fig. 1) The 5.8-kb *Bam*HI–*Bgl*II internal fragment of the insert of

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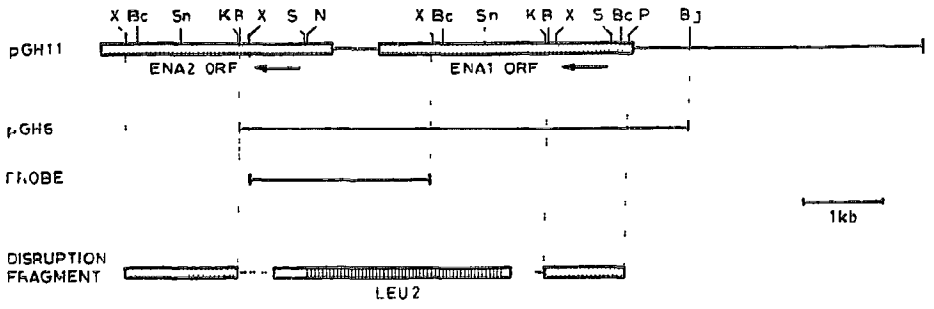

Fig 1 Restriction map of inserts in pGH1 1 and pGH6, and DNA fragments used for disruption of *ENA1* and *ENA2*, and to probe the disruption (see text) The direction of transcriptions are indicated by the arrows The position of the restriction sides are indicated for *Bam*HI (B), *Bcl*I (Bc), *Bgl*II (Bg), *Kpn*I (K), *Nco*I (N), *Pst*I (P) *Sac*I (S), *Sno*I (Sn), *Xba*I (X)

pGH1 1 subcloned in YCp50 (pGH6) was sufficient to confer Li^+ tolerance to strain RH2, and to increase Li^+ and Na^+ effluxes of RH2 Therefore, it was assumed to contain a gene for Na^+ and Li^+ effluxes, which we called *ENA1* (for *exitus natrii*)

The sequence of the 5.8-kb *Bam*HI–*Bgl*II DNA fragment of pGH1-1 revealed an open-reading frame of 3273 b which could encode a polypeptide of 1091 amino acids, M_r 120 371. This sequence was coincident with that of gene PMR2 reported previously to encode a putative Ca^{2+}-ATPase [15], exclusively based on sequence homology

Downstream of *ENA1* in pGH1-1, at 610 b of the TGA triplet, we found a second open reading frame of 2903 b, which is not closed in pGH1 1 The nucleotide sequence of this second open-reading frame was almost identical to that of *ENA1*, and was designated *ENA2*.

To study the function of *ENA1*, we disrupted both *ENA1* and *ENA2* by a single-step gene disruption [13], as described in section 2. However, because of the homologies between *ENA1* and *ENA2*, integration of the disruption fragment (Fig. 1) in the chromosome of DB746 could occur in three different forms, disrupting *ENA1*, *ENA2*, or both By Southern blot analysis of several Leu^+ transformants we selected strain RH16.6, in which the 2.3-kb *Xba*I fragment, overlapping *ENA1* and *ENA2*, was missing (Fig 2). This strain presented a very low Li^+ tolerance and an almost zero Li^+ efflux The tolerance to Na^+ was also very low, specially at alkaline pH values. At acidic pH values, Na^+ was less toxic, and the strain presented a slight but significant efflux

Transformants of RH16 6 (*ena1 ena2*) with plasmid pGH6 (*ENA1*) tolerated much higher concentrations of Li^+ than strain RH16 6, at any pH, and also much higher concentrations of Na^+ when growth was tested at pH 8 0 (Fig 3) Consistent with the increase in Li^+ and Na^+ tolerances, transformants of RH16 6 with pGH6 recovered Li^+ and Na^+ effluxes (Fig 4). None of these effects were observed with plasmids which did not contain *ENA1*

In addition to the Na^+ and Li^+ sensitivity, strain RH16 6 did not grow well at alkaline pH values, and the defect was enhanced by external K^+ At low K^+ (15 mM), RH16 6 did not grow at pH 8 4, but at 500 mM

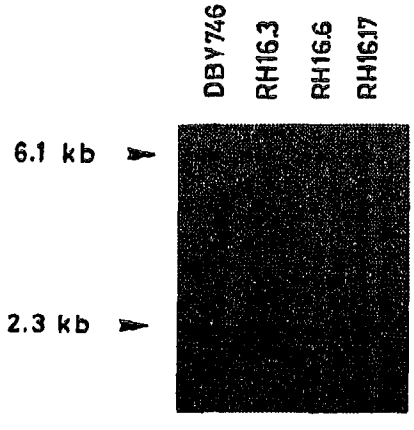

Fig 2 Southern blot analysis of three typical Leu^+ clones obtained after transformation of DBY746 for single-step gene disruption of *ENA1* and *ENA2* In RH16 6 the 2.3-kb *Xba*I fragment was missing indicating that the *Bam*HI fragment of the chromosome had been replaced by the *LEU2* gene as a result of two homologous recombinations, one in *ENA1* and the other in *ENA2* In the other two clones only *ENA1* or *ENA2* was disrupted

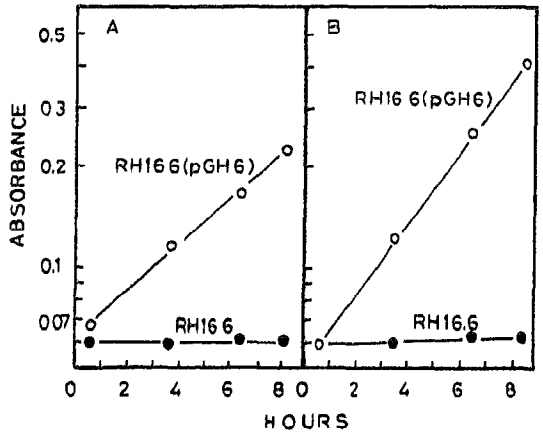

Fig 3 Growth of strains RH16 6 and RH16 6(pGH6) in arginine-phosphate medium with 5 mM Li^+, 1 mM K^+, pH 6 5 (A), and with 25 mM Na^+, 1 mM K^+, pH 8 0 (B)

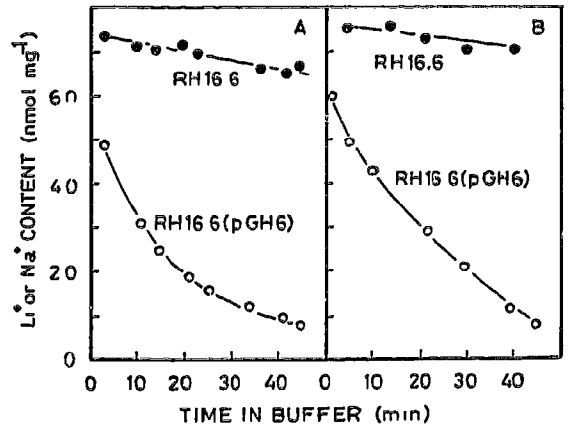

Fig 4 Li^+ (A) and Na^+ (B) losses from cells loaded with these cations Cells were grown overnight in arginine-phosphate medium containing 5 mM Li^+, 10 mM K^+, or 3 mM Na^+, 1 mM K^+ in RH16 6, and 5 mM Li^+, 1 mM K^+, or 10 mM Na^+, 1 mM K^+ in Rh16 6(pGH6) Then the cells were transferred to Li^+- and Na^+-free buffer, pH 8 0, and analyzed as described in text

K^+ the maximum pH tolerated was 7 2 RH16 6 (pGH6) grew up to pH 9.1 with 15 mM K^+ and up to pH 8.8 with 500 mM K^+. These results suggest that the product of *ENA1* may also be involved in K^+ efflux

4 DISCUSSION

Present results show that the product of *ENA1* is required for Li^+, Na^+ and probably K^+ effluxes, although at acidic pH values Na^+ and K^+ may have efflux systems independent of this product, possibly H^+/cation antiporters [9,10]. The homology of the predicted proteins encoded by *ENA1* and *ENA2* with P-ATPases [15] leaves little doubt that these proteins are cation pumps, whose function is probably to pump Li^+, Na^+ and K^+ out of the cell The rapid stop of Li^+ efflux when the cells are ATP depleted [9], and the absence of significant defects in RH16.6 (*ena1 ena2*), except for the functions related to Li^+, Na^+ and K^+ effluxes, make it unlikely that the product of *ENA1* pumps another cation whose concentration regulates the activity of the actual transport system for Li^+, Na^+ and K^+. Therefore, the possibility that *ENA1* (*PMR2*) encodes a Ca^{2+} pump [15] has not actually physiological support.

The main function of the product of *ENA1* may be to pump Na^+ Li^+ would be pumped because of its analogy with Na^+, and K^+ might be pumped when there is no Na^+ in the cytoplasm This hypothesis of a Na^+-pump ATPase may apply not only to yeast but also to plants as suggested previously [16]

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