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A single amino acid substitution (Glu134 → Ala) in NhaR1 increases the inducibility by Na^+ of the product of *nha*A, a Na^+/H^+ antiporter gene in *Escherichia coli*

O.Carmel, N.Dover, O.Rahav-Manor, P.Dibrov, D.Kirsch, R.Karpel, S.Schuldiner and E.Padan

Division of Microbial and Molecular Ecology, Institute of Life Sciences, Hebrew University of Jerusalem, Jerusalem 91904, Israel

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The mutation *nha*A^{up} (*ant*up) has now been identified as a Glu134 to Ala substitution in NhaR and designated *nha*R1. This was demonstrated by sequence analysis showing that the mutant contains a wild-type *nha*A but *nha*R1 instead of *nha*R and by the finding that *nha*R1 cloned in a plasmid confers the NhaA^{up} phenotype. Na^+ (107 mM) increases by 5- to 10-fold the level of *nha*A transcripts, similar to the effect on the NhaR-mediated expression of a *nha*A′–′*lac*Z fusion. These results are in agreement with the notion that *nha*R is a positive regulator which controls Na^+-dependent transcription of *nha*A. The promoter region of *nha*R and *nha*R1 was found to reside within the *Bgl*II–*Bam*HI fragment of the C-terminal sequences of *nha*A. The mutation *nha*R1, while increasing dramatically the level of transcription, reduces the requirement for Na^+ by 3- to 5-fold both for *nha*A transcription and for the *nha*R1-mediated expression of *nha*A′–′*lac*Z fusion. NhaR1, like NhaR, binds specifically to the promoter region of *nha*A. However, at equal protein concentration NhaR1 binds more DNA and the NhaR1–DNA complex shows higher mobility than that of NhaR–DNA, suggesting the existence of two different binding complexes. Yet in this assay the DNA binding pattern of neither NhaR nor NhaR1 was affected by the addition of Na^+. The possible relevance of these two DNA-binding complexes to the Na^+-induced NhaR-mediated expression is discussed.

Key words: Escherichia coli/LysR-OxyR family/Na^+/H^+ antiporters/*nha*R1 mutation/transcriptional regulation

Introduction

All living cells maintain a Na^+ concentration gradient directed inwards and a constant intracellular pH at around neutrality (reviewed in Padan and Schuldiner, 1992). Hence all cells have Na^+ extrusion systems and homeostatic mechanisms controlling the H^+ and Na^+ circulation across the cytoplasmic membrane. Sodium/proton antiporters have been suggested to play a major role in these homeostatic mechanisms in both eukaryotes and prokaryotes (reviews in Padan and Schuldiner, 1992; Schuldiner and Padan, 1992, 1993).

We have previously shown that *Escherichia coli* has two antiporters which specifically exchange Na^+ or Li^+ for H^+ (reviewed in Schuldiner and Padan, 1992). The *nha*A (Goldberg *et al.*, 1987; Karpel *et al.*, 1988) and *nha*B (Pinner *et al.*, 1992) genes have been cloned. Deleting either *nha*A (Padan *et al.*, 1989), *nha*B or both genes together (Pinner *et al.*, 1993b) allowed the deduction of the role of these antiporters in cell physiology. *nha*A is indispensable for the adaptation to high salinity, for challenging Li^+ toxicity and for growth at alkaline pH (in the presence of Na^+) (Padan *et al.*, 1989). *nha*B by itself confers a limited Na^+ tolerance to the cells, but it becomes essential when NhaA activity limits growth (Pinner *et al.*, 1993).

The NhaA protein has been purified to homogeneity and reconstituted in a functional form in proteoliposomes (Taglicht *et al.*, 1991). Using the purified system, we have shown that NhaA is electrogenic, catalyzing an exchange of $2H^+$ per $1Na^+$ (Taglicht *et al.*, 1993). Furthermore, while the activity of NhaB is pH-independent (Padan *et al.*, 1989; Pinner *et al.*, 1993), the activity of NhaA changes more than a 1000-fold over the pH range between 7 and 8 (Taglicht *et al.*, 1991). This remarkable pH-dependence of NhaA accounts for the previously demonstrated pH-dependence of the Na^+/H^+ antiporter activity in isolated membrane vesicles (Bassilana *et al.*, 1984; Padan *et al.*, 1989). Recently, we have shown that His226 participates in the pH sensor on NhaA (Gerchman *et al.*, 1993).

Since fluxes of Na^+ and H^+ are coupled via the two antiporters, understanding the regulation of activity and expression of each of the individual elements is crucial for the understanding of both Na^+ and H^+ circulation of the cells. The regulation of expression has thus far only been studied with *nha*A. Two promoters have been identified in the upstream sequence of the gene and the corresponding start point of transcription mapped by primer extension (Karpel *et al.*, 1991). Monitoring the β-galactosidase activity of a chromosomal translation fusion of *nha*A′–′*lac*Z shows that Na^+ and Li^+ increase expression and that alkaline pH potentiates the effect of the ions. The pattern of regulation of *nha*A thus reflects its role in adaptation to high salinity and alkaline pH in *E.coli* (Padan *et al.*, 1989).

The gene *nha*R, which maps in tandem downstream of *nha*A (Mackie, 1986; Karpel *et al.*, 1988) [previously known as *ant*O (Henikoff *et al.*, 1988), ORF7 or 28 kDa protein (Mackie, 1986)], regulates the Na^+-dependent expression of *nha*A as suggested by the following findings: Δ*nha*R is more sensitive to Li^+ and Na^+ than the wild-type, even though it harbors intact *nha*A. This sensitivity is alleviated by *nha*R *in trans* (Rahav-Manor *et al.*, 1992). Na^+ induction of *nha*A′–′*lac*Z fusions is markedly increased by multicopy *nha*R plasmid (Rahav-Manor *et al.*, 1992). A DNA mobility test shows that a cell-free extract from cells overexpressing *nha*R contains a protein which specifically binds to the promoter region of *nha*A (Rahav-Manor *et al.*, 1992).

NhaR belongs to the OxyR-LysR family of positive regulators first described by Henikoff *et al.* (1988). Some

of these proteins are involved in the response of bacteria to environmental adversity, such as excessive oxidation in the case of OxyR (Christman *et al.*, 1989; Storz *et al.*, 1990). We have therefore suggested that NhaR and its effector, NhaA, are involved in a novel signal transduction pathway specific to Na^+ which is essential for halotolerance in *E.coli*.

The induction of *nha*A by Na^+ suggests that a Na^+ sensor site exists which can be identified by mutations affecting the Na^+ sensitivity of the expression system. Should such a mutation increase the affinity to Na^+ of the expression system, than at a given Na^+ concentration, it may increase the Na^+/H^+ antiporter activity in the membrane above the wild-type level. A previously isolated mutation, designated *ant*up (Niiya *et al.*, 1982; Goldberg *et al.*, 1987), increases the Na^+/H^+ antiporter activity, thereby conferring Li^+ resistance upon wild-type cells which are otherwise Li^+-sensitive (Nha^{up} phenotype). The mutation has been shown both by conjugation and transduction to be closely linked to *nha*A (Goldberg *et al.*, 1987), suggesting that it is localized either in *nha*A or in its neighboring regulatory gene *nha*R, each alternative being as feasible as the other.

In the present work we show that the nha^{up} mutation leads to a Glu134 → Ala substitution in NhaR (NhaR1) which changes the Na^+-dependent expression of wild-type *nha*A. This mutation has two effects: it modifies the specific binding of NhaR1 to the *nha*A DNA promoter region and it increases the affinity for Na^+ of the *nha*R-mediated *nha*A expression.

Results

The mutant W3133-2S carries a mutation in nhaR (nhaR1; E134A)

Since the mutation which causes the $NhaA^{up}$ phenotype in *E.coli* strain W3133-2S enhances the Na^+/H^+ antiporter activity and is tightly linked to *nha*A, we tested the possibilities that the mutation resides either in *nha*A or in *nha*R. A DNA segment containing both *nha*A, its upstream sequences and the entire *nha*R was amplified by PCR using DNA from either the wild-type (TA15) or the mutant (W3133-2S). A unique product of the expected size (2.65 kb) was formed in each case and direct sequencing of the amplified fragments showed that whereas both fragments bear an unmodified *nha*A, *nha*R of W3133-2S is mutated (Figure 1). Its 134 codon (GAA) has been changed to (GCA) leading to a substitution of Glu134 of NhaR with Ala (E134A). This mutation was designated *nha*R1.

Cloning of nhaR1

Cloning of *nha*R1 from the mutated strain was then undertaken to test whether the E134A mutation confers the $NhaA^{up}$ phenotype. For yet unknown reasons, attempts to clone the PCR-amplified fragment directly into plasmid pBR322 failed, hence another cloning strategy was adopted. NM81 *rec*$^-$ bears the Δ*nha*A1 mutation (Padan *et al.*, 1989) which renders the cells highly sensitive to Li^+ (100 mM) and Na^+ (0.7 M, pH 7.5). This mutant becomes resistant to the ions upon transformation with multicopy plasmids bearing *nha*A. Whereas the plasmid-restored resistance to Na^+ is similar to that of the wild-type, the resistance conferred for Li^+ is even higher than that of the

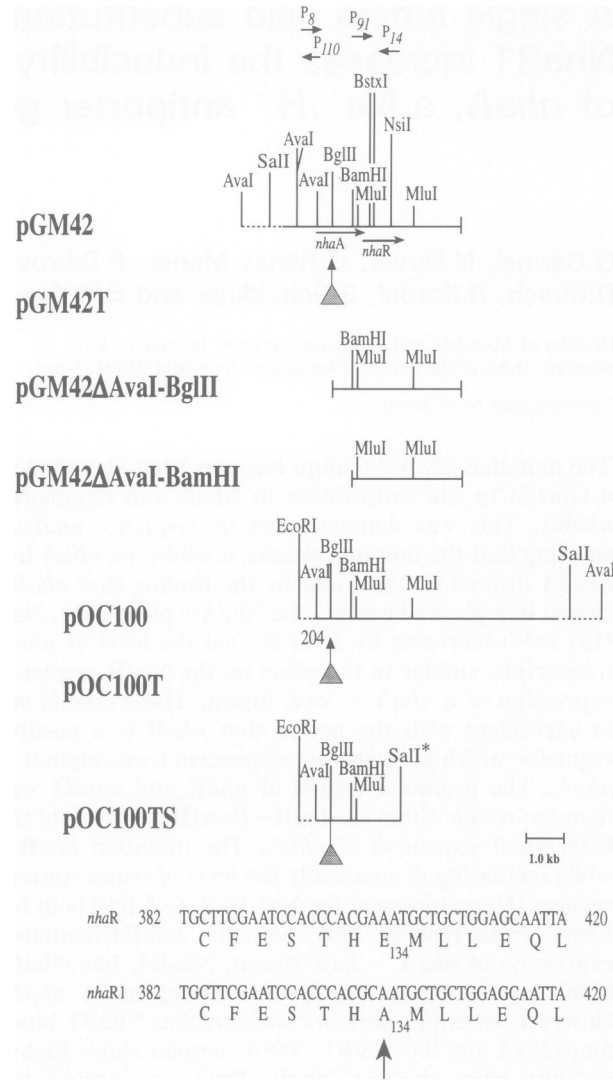

Fig. 1. Inserts of plasmids used. ▲, Universal translation terminator (Karpel *et al.*, 1988). The base pair or amino acid numbers are according to the GenBank *nha*A or *nha*R accession numbers (J03879, S67239 and L24072). P_{91}, P_{14} and P_8 are oligonucleotide primers described in Materials and methods. The *nha*R1 (E134A) mutation cloned in pOC100 is also shown. Solid line, insert sequences; dotted line, pBR322 sequences. *Sal*I is an introduced site.

wild-type. Therefore, Δ*nha*A *rec*$^-$ serves as a very powerful tool to clone inserts carrying antiporter genes by functional complementation to Na^+ or Li^+ resistance. Since *nha*R is localized downstream of, and in tandem to, *nha*A, it was assumed that a DNA library prepared from W3133-2S would include a plasmid bearing a DNA insert overlapping both *nha*A and *nha*R. If expressing NhaA, such a plasmid would yield transformants resistant to NaCl upon transformation to Δ*nha*A *rec*$^-$. Indeed with this protocol, a recombinant plasmid pOC100 was obtained carrying a 6.5 kb insert (Figure 1). As opposed to Δ*nha*A *rec*$^-$, which is highly sensitive to 0.7 M NaCl or 100 mM LiCl, Δ*nha*A *rec*$^-$/pOC100 grows unhindered in the presence of these ion concentrations. Furthermore, membrane vesicles isolated from Δ*nha*A *rec*$^-$/pOC100 show a marked increase in the Na^+/H^+ antiporter activity (Figure 2).

Restriction analysis and sequencing of pOC100 insert show that at one end, fused to base pair 375 of pBR322, the inserts

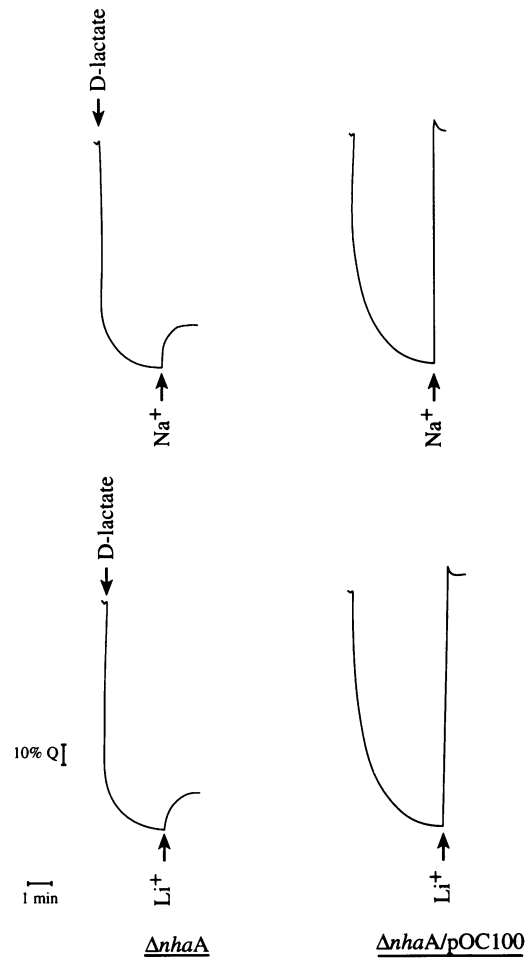

Fig. 2. The antiporter activity of everted membrane vesicles prepared from *E.coli* strain NM81 or this strain transformed with pOC100. Membrane vesicles were prepared from cells growing in melibiose-containing minimal medium. ΔpH was monitored with acridine orange in medium containing 140 mM potassium chloride, 10 mM tricine (pH 8), 5 mM $MgCl_2$, acridine orange (0.5 μM) and membrane vesicles (50 μg of protein). At the onset of the experiment Tris-D-lactate (5 mM) was added and the fluorescence quenching (Q) was recorded. Either NaCl or LiCl (10 mM, each) was then added and the new steady state of fluorescence obtained (dequenching) after each addition was monitored.

start at base pair 204 (GenBank acccession number S67239) of the wild-type *nha*A DNA sequences (Figure 1). It contains the entire unmodified open reading frame of *nha*A, additional 28 bases upstream, but none of its promoters. The two sites of initiation of transcription of *nha*A were localized by primer extension at −31 and −177 away from the initiation codon (Karpel *et al.*, 1991). In tandem to *nha*A the insert contains *nha*R1, the mutated *nha*R bearing the E134A substitution. In addition, the insert contains 2.5 kb of the DNA sequences downstream of *nha*R1 (Figure 1).

nhaR1 confers the Nha^{up} phenotype

Since pOC100 contains *nha*A, it was necessary to assess whether the Na^+/Li^+ resistance phenotype conferred by the plasmid is due to multicopy *nha*A, *nha*R1 and/or to the sequences downstream to *nha*R1 carried by pOC100. To differentiate between these alternatives, plasmid pOC100TS was constructed from pOC100, in which all sequences

Table I. Growth rates

Strain	Growth rates (1/h)	
	NaCl (100 mM)	LiCl (100 mM)
TA15	0.75	0.39
TA15/pGM42T	0.8	0.36
TA15/pGM42Δ*Ava*I−*Bgl*II	0.74	0.36
TA15/pOC100T	0.8	0.64
TA15/pOC100TS	0.86	0.67
W3133-2S	0.85	0.7

Cells were grown in melibiose-containing minimal medium at pH 7 containing either 100 mM NaCl or 100 mM LiCl as indicated.

downstream of *nha*R were deleted and *nha*A was inactivated by introducing translation terminator sequences into its open reading frame (Figure 1). For a control, we used similar plasmids bearing inactivated *nha*A but a wild-type *nha*R [pGM42T (Rahav-Manor *et al.*, 1992) or pGM42Δ*Ava*I−*Bgl*II, Figure 1]. All the plasmids—pGM42T, pGM42Δ-*Ava*I−*Bgl*II and pOC100TS—were transformed into wild-type cells (TA15) possessing a single copy of *nha*A, and the growth phenotype of the transformants was determined (Table I). Whereas all the transformants show a similar capacity to grow on NaCl, the growth rate of TA15/pOC100TS is double that of TA15/pGM42T or TA15/pGM42Δ*Ava*I−*Bgl*II when grown on LiCl. Assuming that the copy number of all plasmids is identical, it is suggested that the mutation identified in *nha*R confers the NhaA^{up} phenotype. Indeed, in the presence of Li^+ the growth rate of TA15/pOC100TS is very similar to that of W3133-2S, which carries a single copy *nha*Rl (Table I). Thus the phenotype conferred by a single copy or multicopy *nha*R1 is identical. Plasmid pOC100T, which is similar to pOC100TS but contains additional downstream sequences (Figure 1), confers upon TA15 a phenotype identical to that of pOC100TS (Table I), implying that the extra sequences downstream of *nha*R1 cloned in pOC100 are not involved in the NhaR1 phenotype.

To test whether the *nha*R1 phenotype involves *nha*A, both plasmids pGM42T and pOC100TS were transformed into NM81, which has *nha*A deletion. These transformants remained both Na^+- and Li^+-sensitive. Hence *nha*R1 is effective only in the presence of *nha*A, as expected for a mutation in a regulatory protein acting *in trans* (Rahav-Manor *et al.*, 1992).

The promoter region of nhaR

In the chromosome, *nha*A is located in tandem upstream of *nha*R. Since there are no conspicuous consensus sequences of promoters in the non-coding DNA sequences separating the two genes, we have previously suggested that *nha*R forms an operon with *nha*A (Padan and Schuldiner, 1994). However, the absence of *nha*A promoters in pOC100 implies that either plasmidic or insert sequences upstream of *nha*R serve as *nha*R promoters in pOC100.

To locate the DNA region bearing the *nha*R promoters we constructed plasmids, pGM42T derivatives, lacking varying regions upstream of *nha*R overlapping parts or all of *nha*A. These plasmids were used to transform bacteria (RK33Z) which contain instead of the genomic *nha*A, a *nha*A′−′*lac*Z protein fusion (Karpel *et al.*, 1991). Following

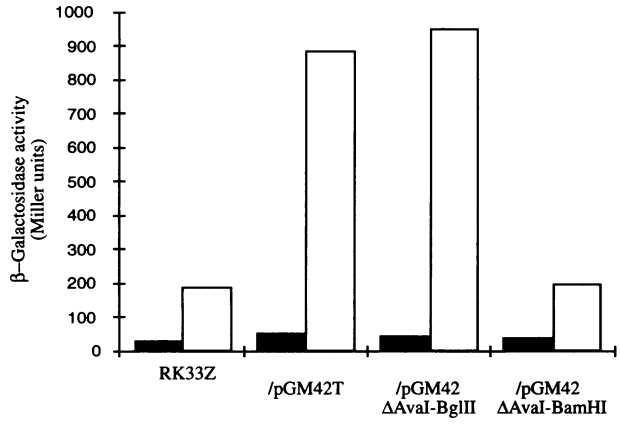

Fig. 3. The promoter region of *nha*R. Plasmid pGM42T and its derivatives, with various lengths of the upstream sequences of *nha*R, pGM42Δ*Ava*I−*Bgl*II and pGM42Δ*Ava*I−*Bam*HI deleted (Figure 1), were transformed into RK33Z (*nha*A′−′*lac*Z). The transformants were grown in LBK containing 60 mM BTP (pH 7.5) and 7 mM Na^+ (dark bars) or induced for 70 min by the addition of Na^+ to 107 mM (light bars). β-Galactosidase activity was determined as described by Karpel *et al.* (1991). Extracellular Na^+ concentrations were measured by atomic absorption as described in Materials and methods.

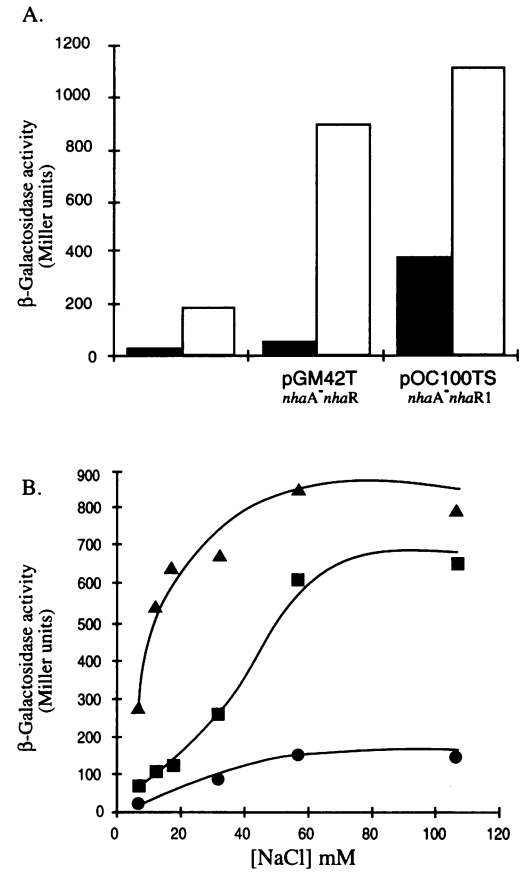

Fig. 4. *nha*R1 changes the dependence on Na^+ of the *nha*A′−′*lac*Z expression. RK33Z (*nha*A′−′*lac*Z) (●) was transformed with pGM42T (*nha*A⁻*nha*R) (■) or pOC100TS (*nha*A⁻*nha*R1) (▲) as indicated. The cells were grown in LBK containing 60 mM BTP (pH 7.5) and 7 mM NaCl and induced for 70 min by the addition of Na^+ to reach final concentrations of 107 mM Na^+ (**A**) or various Na^+ concentrations (**B**). β-Galactosidase activity was determined as described by Karpel *et al.* (1991). Extracellular Na^+ concentrations were measured by atomic absorption; dark bars, 7 mM Na^+; light bars, 107 mM Na^+.

the expression of this *nha*A′−′*lac*Z protein fusion, we have previously shown that wild-type *nha*R exerts its *trans* effect on *nha*A expression in a Na^+-dependent fashion. At pH 7.5, the multicopy wild-type *nha*R (pGM42T) increase 4- to 5-fold the Na^+-dependent induction of *nha*A but hardly affects the low level of the Na^+-independent expression of the gene [(Rahav-Manor *et al.*, 1992) and Figure 3].

The results summarized in Figure 3 show that plasmid pGM42Δ*Ava*I−*Bgl*II deleted of the upstream sequences of *nha*A and its N-terminal part from the *Bgl*II site (Figure 1) increased the Na^+-dependent expression of *nha*A′−′*lac*Z to the same extent as pGM42T (Figure 3). However, a larger deletion reaching the *Bam*HI site of *nha*A (Figure 1, pGM42Δ*Ava*I−*Bam*HI) abolished the effect of the plasmid (Figure 3). We therefore suggest that the promoter region of *nha*R resides within *nha*A, in the *Bgl*II−*Bam*HI fragment.

The orientation of the pOC100TS insert with respect to the vector sequences is opposed to that of pGM42T (Figure 1). Furthermore, as will be described below, the effect on the expression of *nha*A′−′*lac*Z of *nha*R1 in pOC100TS differs from that of *nha*R in pGM42T in being stronger and requiring lower concentrations of Na^+ (Figure 4). Nevertheless, similar deletions which inactivate *nha*R in pGM42T inactivate *nha*R1 in pOC100TS as observed by their effect on the expression of the *nha*A′−′*lac*Z fusion. A deletion of *nha*R1 upstream sequences from *Bgl*II of *nha*A up to *Eco*RI in the vector had no effect on the activity of pOC100TS whereas an extended deletion reaching the *Bam*HI site of *nha*A inactivates pOC100TS (not shown). These results strengthen our suggestion that *nha*R promoters reside in the *Bgl*II−*Bam*HI fragment of *nha*A upstream of *nha*R.

nhaR1 changes the Na^+-dependence of the nhaA′−′lacZ expression

The dependence on Na^+ of *nha*A′−′*lac*Z expression in the presence of multicopy *nha*R1 (strain *nha*A′−′*lac*Z/pOC100TS) and multicopy *nha*R (strain *nha*A′−′*lac*Z/pGM42T) is shown in Figure 4A and B. The results show that, as compared with the multicopy wild-type *nha*R gene, the multicopy of the mutation *nha*R1 has two effects on the expression of the *nha*A′−′*lac*Z fusion: it slightly increases the maximal *nha*A′−′*lac*Z expression (observed in the presence of 60−100 mM NaCl) and, most importantly, although like NhaR it is Na^+-dependent it requires a much lower Na^+ concentration to exert its phenotype. Thus, while in the presence of multicopy wild-type *nha*R and contaminating Na^+ (7 mM NaCl), the expression of *nha*A′−′*lac*Z [strain *nha*A′−′*lac*Z/*nha*A⁻*nha*R (Karpel *et al.*, 1991; Figures 4A and B)] is on average 8% of the maximum, at least 32% of full expression of *nha*A is observed in the presence of *nha*R1 (strain *nha*A′−′*lac*Z/*nha*A⁻*nha*R1) under similar conditions (Figures 4A and B). Accordingly, the *nha*R1-mediated induction is half-maximal at a quarter of the Na^+ concentration (12 mM NaCl) than does that of NhaR (45 mM NaCl). We therefore surmise that the *nha*R1 mutation increases the apparent affinity of the *nha*A′−′*lac*Z expression system for Na^+.

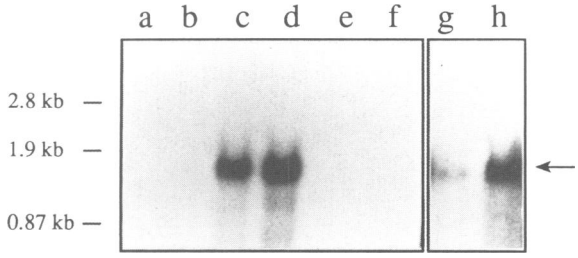

Fig. 5. Na$^+$ increases the level of *nha*A transcripts. Northern analysis of *nha*A transcripts isolated from TA15 (lanes a and b), W31332S (lanes c and d) and RK20 (lanes e and f). With each strain the first sample was obtained from non-induced cells (grown in LBK in the presence of 7 mM) and the second sample from cells grown in the presence of 107 mM NaCl. Lanes g and h are duplicates of lanes a and b, but whereas the exposure time of the autoradiogram of lanes a–f was 70 h, that of lanes g and h was 216 h. In lanes a and f there was no signal even after the long time of exposure (not shown). Molecular weight markers are shown as well as the position of *nha*A transcript (arrow).

nhaR1 increases the transcription of nhaA and increases the apparent affinity of the expression system for Na$^+$

The *nha*A′–′*lac*Z fusion used is a protein fusion which does not allow the discrimination between an effect of Na$^+$ on transcription and/or translation. We therefore compared the effect of Na$^+$ on the *in vivo* level of *nha*A transcripts in the wild-type (TA15) bearing chromosomal single copy of each wild-type *nha*A and *nha*R and W3133-2S which contains *nha*R1 rather than *nha*R, but is otherwise isogenic with TA15 (Goldberg *et al.*, 1987). As a control, we used RK20, which lacks the entire *nha*A gene. The Northern analysis of *nha*A transcripts summarized in Figure 5 shows that in the presence of 7 mM NaCl the levels of *nha*A transcripts in the wild-type is very low, detected only following 216 h exposure of the autoradiogram (compare lanes a and g of Figure 5). Addition of 100 mM Na$^+$ increases this low level of the wild-type by ~5- to 10-fold (compare lanes h and g of Figure 5). Hence Na$^+$ induces *nha*A transcription. Accordingly, the effect of Na$^+$ on the level of the *nha*A transcripts parallels its effect on *nha*A′–′*lac*Z expression (Figure 4 and Karpel *et al.*, 1991).

Furthermore, the level of *nha*A transcripts in a strain lacking *nha*R (OR100, Rahav-Manor *et al.*, 1992) was extremely low (not shown) and similar to that observed in wild-type cells grown with no added Na$^+$ (Figure 5). However, whereas addition of Na$^+$ (100 mM) induced transcription in the latter strain (Figure 5), an increase of Na$^+$ concentrations up to 300 mM had no effect on Δ*nha*R (not shown). This result further substantiates our inference that *nha*R is the positive regulator of the Na$^+$-dependent expression of *nha*A (Rahav-Manor *et al.*, 1992).

As compared with wild-type *nha*R, the overall transcription of *nha*A is greatly increased in the *nha*R1 mutant; an RNA band of the appropriate size is already observed after short exposure (compare lanes c and a of Figure 5). Furthermore, the addition of 100 mM Na$^+$ to the 7 mM already existing in the LBK medium increases the level of transcripts by at most 2- to 3-fold (compare lanes c and d of Figure 5). We therefore suggest that the *nha*A transcription in the presence of *nha*R1 shows much larger apparent affinity for Na$^+$ than in the presence of *nha*R.

To determine the effect of Na$^+$ on the expression of *nha*R we produced antibodies directed against NhaR. These antibodies detected NhaR and NhaR1 only in strains overexpressing the respective genes from a foreign promoter (T7) (Figure 7B). We could not detect either protein when expressed from a single copy of chromosomal gene or from multicopy plasmidic genes whether or not the cells were grown in the presence of high Na$^+$ concentrations (300 mM). Accordingly, Northern analysis do not detect *nha*R or *nha*R1 transcripts. Hence these results show that the level of *nha*R or *nha*R1 expression is very low; as yet we do not know whether Na$^+$ affects it.

Table II. Intracellular Na$^+$ concentrations at which *nha*A′–′*lac*Z expression occurs in the presence of either *nha*R1 or *nha*R.

Strain (relevant genotype)	*nha*A′–′*lac*Z induction (%)	Na$^+$ concentration (mM)	
		extracellular	intracellular
*nha*A′–′*lac*Z/*nha*R	10–15	12	0.15
	50–55	45	3.20
*nha*A′–′*lac*Z/*nha*R1	50–55	12	0.19
	95–100	45	2.35

Cells of strains RK33Z/PGM42T (*nha*A′–′*lac*Z/*nha*R) and RK33Z/POC100TS (*nha*A′–′*lac*Z/*nha*R1) were grown in LBK in the presence of glucose and the indicated $[Na^+]_{out}$ under conditions used to measure *nha*A′–′*lac*Z expression. $[Na^+]_{out}$ and $[Na^+]_{in}$ were determined as described in Materials and methods.

Lower intracellular Na$^+$ concentrations are required for the effect of nhaR1 on nhaA expression than that of nhaR

Previous results suggested that intracellular rather than extracellular Na$^+$ is the signal for *nha*R-dependent *nha*A expression (Padan and Schuldiner, 1992). Thus the effectiveness of extracellular Na$^+$ ($[Na^+]_{out}$) in inducing *nha*A increases under conditions at which the intracellular concentration ($[Na^+]_{in}$) rises: at alkaline pH (Pan and Macnab, 1990; Karpel *et al.*, 1991) and in antiporter-deficient strains (Padan and Schuldiner, 1992). Accordingly, a reduction in $[Na^+]_{in}$ caused by multicopy *nha*A or *nha*B inhibits expression even at high Na$^+$ concentrations (Padan and Schuldiner, 1992). We therefore determined the $[Na^+]_{in}$ at the points at which the $[Na^+]_{out}$ induced half-maximal *nha*R1- or *nha*R-mediated *nha*A expression (12 or 45 mM Na$^+$ respectively). It is evident from the results summarized in Table II that the $[Na^+]_{in}$, 0.19 mM, at which half-maximal *nha*R1 effect is observed is ~17-fold lower than that of cells exhibiting *nha*R-mediated *nha*A′–′*lac*Z expression (3.2 mM).

Given that, albeit at different critical concentrations, intracellular Na$^+$ is the signal for *nha*R1-mediated expression as it is for *nha*R-mediated expression (Padan and Schuldiner, 1992) it is expected that changing the $[Na^+]_{in}$ (without changing the $[Na^+]_{out}$) at around the critical concentrations affecting the low affinity system (*nha*R) will be less effective on the high affinity system (*nha*R1). Indeed, while as shown previously enhanced Na$^+$ secretion caused by either active multicopy *nha*A or *nha*B almost totally reduces the *nha*R-mediated *nha*A′–′*lac*Z induction (Padan *et al.*, 1992; Figure 6), similar conditions have a smaller effect on the *nha*R1-mediated induction of *nha*A′–′*lac*Z (Figure 6).

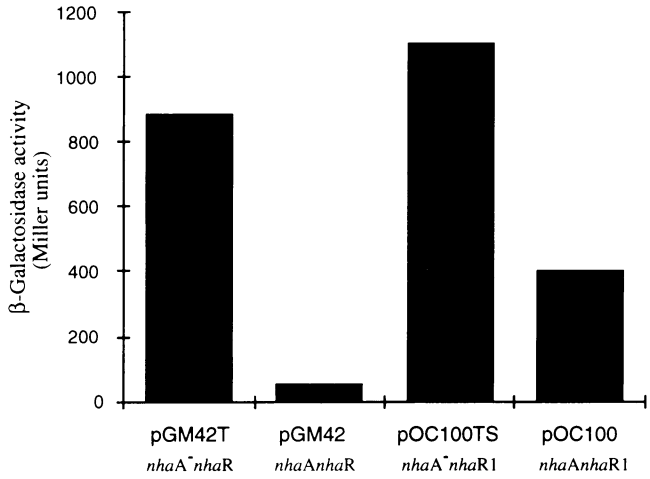

Fig. 6. Low $[Na^+]_{in}$ which do not induce the *nha*R-mediated expression of *nha*A still induce the *nha*R1-mediated expression. RK33Z (*nhaA'−'lacZ*) was transformed with various plasmids: pGM42T (*nhaA*⁻*nha*R), pGM42 (*nhaAnha*R), pOC100TS (*nhaA*⁻*nha*R1) or pOC100 (*nhaAnha*R1) as indicated. The cells were induced at pH 7.5 with 107 mM NaCl and the β-galactosidase activity was determined as described in the legend to Figure 3.

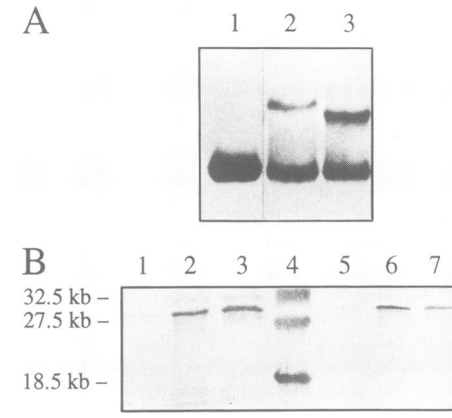

Fig. 7. The pattern of the DNA binding of NhaR1. (**A**) The DNA probe (3 ng) was incubated for 1 h at 25°C in the absence (lane 1) or presence of cell-free extract prepared from strains overexpressing *nha*R (TA15/pGP1-2/pDT2, lane 2) or *nha*R1 (TA15/pGP1-2/pOCR1, lane 3). The total protein concentration in each sample was 100 ng containing equal levels of NhaR or NhaR1 as determined immunologically (panel B). The reaction mixtures were then subjected to polyacrylamide gel electrophoresis, and the dried gels were visualized by autoradiography. (**B**) Western analysis of the cell-free extacts obtained from TA15/pGP1-2/pDT2 (5, 10 and 20 μg protein in lanes 1, 2 and 3 respectively) and TA15/pGP1-2/pOCR1 (5, 10 and 20 μg protein in lanes 5, 6 and 7 respectively) used in panel A utilizing the anti-NhaR antibody as described in Materials and methods. The molecular weight markers are shown in lane 4.

NhaR1 shows different pattern of binding to nhaA promoter region as compared with NhaR

We have previously shown that NhaR specifically binds to a DNA fragment containing the *nha*A promoter region and changes its electrophoretic mobility in a gel retardation assay (Rahav-Manor *et al.*, 1992). In a similar assay we compared the effect on the DNA mobility of a cell-free extract obtained from a strain overexpressing *nha*R with that overexpressing *nha*R1 (Figure 7). The results show that at equal concentrations of the respective proteins determined immunologically, NhaR1 binds more DNA. Furthermore, the mobility shift caused by a crude extract prepared from the mutant strain was smaller than that caused by an extract prepared similarly from the wild-type strain. Similar results were obtained with partially purified preparation of proteins (not shown). This result suggests that the NhaR1−DNA complex differs from that of NhaR−DNA. As yet we have not been able to show an effect of Na^+ on the DNA binding of either strains.

Discussion

In this work the mutation *nha*A^{up} was identified as a Glu134 → Ala substitution in *nha*R and designated *nha*R1. The mutated codon 134 (GCA) was found in a PCR-amplified DNA fragment bearing otherwise unchanged *nha*R and *nha*A and the cloned *nha*R1 conferred the NhaA^{up} phenotype; it increased Li^+ resistance and Na^+/H^+ antiporter activity of the wild-type strain.

The resistance to Na^+ was unchanged both in the mutant W3133-2S and in the wild-type bearing plasmidic *nha*R1, suggesting that a rate limiting step independent of *nha*R determines the upper limit of Na^+ tolerance in the wild-type cells. This limiting step may act in the adaptation to high osmolarity.

It has previously been shown that *nha*R is a positive regulator of *nha*A, which acts *in trans* and requires Na^+ for its activity (Rahav-Manor *et al.*, 1992). Accordingly *nha*R1, like *nha*R has no phenotype in a strain deleted of *nha*A and multicopy of both genes increase the Na^+-dependent expression of a *nha*A'−'*lac*Z protein fusion. However, the effect on the *nha*A expression of the mutated gene differs from that of the wild-type *nha*R: it requires a much lower concentration of Na^+; in the presence of plasmidic *nha*R, the very low expression of *nha*A'−'*lac*Z, observed with no addition of Na^+, is increased 10-fold upon the additon of the ion (half maximum at 45 mM NaCl); in cells bearing *nha*R1 the maximal expression is slightly higher, and is observed at 3- to 5-fold lower $[Na^+]_{out}$ (half maximum at 12 mM NaCl).

Since the *nha*A'−'*lac*Z fusion is a protein fusion, these and previous experiments (Karpel *et al.*, 1991; Rahav-Manor *et al.*, 1992) could not determine whether the effect of *nha*R is on *nha*A transcription and/or translation. The Northern analysis accomplished in this study demonstrates that the effect of both the wild-type *nha*R and its mutant *nha*R1 is on *nha*A transcription. *nha*A transcripts are barely detected in the wild-type and increase ~5- to 10-fold upon the addition of Na^+; this Na^+-induced transcription is positively regulated by *nha*R since in a Δ*nha*R strain the level of *nha*A transcripts is as low as in the non-induced wild-type strain but is not increased by Na^+. In the *nha*R1 mutant the maximal level of *nha*A transcripts is ~10-fold higher than that in *nha*R and it occurs at much lower Na^+ concentrations. We thus suggest that *nha*R controls *nha*A transcription in a Na^+-dependent fashion, and the mutation *nha*R1 at the same time increases the level of *nha*A transcription and reduces the level of the Na^+ concentrations required for its induction.

The very low level of *nha*A transcripts detected in the Δ*nha*R strain suggests the existence of a low constitutive level of expression of *nha*A. This constitutive expression accounts for the limited but higher Na^+ and Li^+ resistance

previously observed in the Δ*nha*R strain (OR100) as compared with a Δ*nha*A strain (NM81, Rahav-Manor *et al.*, 1992).

It should be noted that these results do not exclude the possible existence of translational control of *nha*A. In fact, the change caused by *nha*R1 in maximal *nha*A transcription (Figure 5) is much higher than that in maximal *nha*A′−′*lac*Z expression (Figure 4), observed in both cases in the presence of ~100 mM Na^+. A basis for a translation control of *nha*A might be the extensive putative secondary structure in the RNA predicted in the 5′ end of the gene (Karpel *et al.*, 1988), and its first codon, GTG rather than ATG (Taglicht *et al.*, 1991). GTG has been found to mediate the initiation of translation in ~8% of the documented *E.coli* proteins (Gold and Stromo, 1987) and it has been suggested that it may be used in mRNAs that are poorly translated. Also, the codon usage in *nha*A is typical of poorly expressed proteins (Pinner *et al.*, 1992a). We have estimated that under the growth conditions which are standard in our laboratory (LBK, or minimal salt medium to which Na^+ is not added) *nha*A is a minor component of the membrane (<0.2%, or the equivalent of <500 copies per cell (Taglicht *et al.*, 1991). Accordingly, the expression with an exogenous promoter (*tac*) is much higher when the regulatory sequences of *nha*A are deleted (Taglicht *et al.*, 1991), implying that, at least under some conditions, the upstream region has an inhibitory effect on expression of *nha*A.

*nha*R is a regulatory protein which binds DNA (Rahav-Manor *et al.*, 1992) and exhibits homology with the LysR-OxyR family of positive regulators (Christman *et al.*, 1989). In this family, all of these regulators are between 30 and 35 kDa in size and, while being positive regulators of expression of their target genes, they often negatively regulate their own transcription. In many cases the promoters of the effector and regulator genes overlap and transcription proceeds divergently in opposite directions (Christman *et al.*, 1989; Storz *et al.*, 1990). They are only present in minute quantities in the cell and a change in their expression does not play a crucial role in the mechanism of signal transduction mediated by these proteins.

In its transcription direction and localization of promoters with respect to its target gene *nha*A, *nha*R differs from the LysR-OxyR family. The direction of transcription of *nha*R parallels that of *nha*A which is localized in tandem upstream of it (Rahav-Manor *et al.*, 1992). Using plasmids carrying *nha*R and various length deletions in its upstream region and studying their effect on the Na^+ induced expression of the *nha*A′−′*lac*Z fusion protein we have localized the promoter region of *nha*R in the C-terminal end of *nha*A. However, although as yet we do not know the pattern of regulation of *nha*R, our results suggest that like the other LysR-OxyR members, the level of NhaR in the wild-type cells is very low. Using antibodies, site-directed against a specific peptide of NhaR, we were able to detect and quantify NhaR or NhaR1 only in a strain overexpressing the genes using a foreign strong promoter (T7) for overexpression (Figure 7). These antibodies did not detect either NhaR or NhaR1 expressed from a single copy gene in the chromosome, or multicopy plasmidic genes, whether or not the cells had been grown in the presence of NaCl (up to 300 mM). Accordingly, we were unable to detect *nha*R or *nha*R1 transcripts by Northern analysis. Hence as yet we cannot exclude the possibility that Na^+ affects *nha*R expression.

However, the results presented in this work suggest that the *nha*R1-mediated Na^+-induction of *nha*A does not involve a change in the level of *nha*R. The mutation *nha*R1 is a missense mutation (Glu134 to Ala) in the protein which does not map in the *nha*R1 promoters, and the growth phenotype conferred by a single copy *nha*R1 is similar to that of multicopy *nha*R1. Furthermore, the effect conferred by the latter is qualitatively different from that conferred by the multicopy wild-type *nha*R. *nha*R1 reduces the Na^+ concentration required for the expression of NhaA and as described below, changes the pattern of binding of NhaR to the DNA fragment bearing the *nha*A promoters.

Like NhaR, NhaR1 binds specifically to the promoter region of *nha*A. However, at equal protein concentrations as determined immunologically, NhaR1 binds more DNA than NhaR. It is possible that the affinity of binding of NhaR1 is higher than that of NhaR and/or the stoichiometry of binding is different. Purified proteins are required to determine these parameters accurately.

Furthermore, the *nha*R1 mutation decreases the DNA mobility shift caused by NhaR, suggesting that the DNA−NhaR and the DNA−NhaR1 complexes are different. The possibility that this difference is caused by a chemical modification of NhaR1 cannot be ruled out; however, Western analysis did not reveal any difference in the electrophoretic mobility of the proteins on SDS−PAGE (Figure 7). Other possibilities that are considered are a change in the conformation of the complex and/or the stoichiometry of binding. Interestingly, a single mutation in the Lrp regulatory protein was shown to change the mobility of the respective protein−DNA complex, its modification by leucine and thereby its regulatory effect on the expression of the *ilu*IH operon (Ricca *et al.*, 1989; Willins *et al.*, 1991).

Nevertheless, as yet we have not found an effect of Na^+, whether present during cell growth and/or in the assay, on the mobility of either DNA−NhaR or DNA−NhaR1. It is therefore possible that the effect of NhaR1 on the mobility of the *nha*A promoter−DNA fragment is irrelevant to its effect on the Na^+ affinity of the *nha*A expression system. On the other hand, it is also possible that the gel retardation assay only reveals the stable conformations and the unstable ones conferred upon NhaR by Na^+ are not detected by this assay. In this respect it should be noted that many of the OxyR-LysR regulatory proteins change their site of binding 'footprint' on the DNA upon interaction with their respective inducers while exhibiting no modification in a gel retardation assay (Storz *et al.*, 1990). Purification of NhaR and NhaR1 is under way to determine the exact binding site of these proteins to the DNA and accurately determine the effect of Na^+ with minimized effect of the contaminating ion.

Homology exists among proteins of the LysR-OxyR family; this homology is highest in the N-terminus where a helix−turn−helix motif is found (Hennikof *et al.*, 1988; Christman *et al.*, 1989; Rahav-Manor *et al.*, 1992). The homology is decreased downstream towards the C-terminus, where the specific inducer-responsive domain has been implicated (Christman *et al.*, 1989). A mutation causing the constitutive phenotype of *oxy*R maps within this region. An inducer-independent *nod*D was also found to map in the C-terminal region of NodD (Burn *et al.*, 1987; Christman *et al.*, 1989). As shown here the *nha*R1 mutation also maps to the C-terminal half of NhaR and changes the affinity for Na^+ of the *nha*R1-mediated *nha*A expression. Since as yet

we do not know whether the effect of Na^+ on either NhaR or NhaR1 is direct, we suggest that either Glu134 bears a negative charge and is part of the Na^+ binding site in NhaR or that Glu134 interacts with an additional protein which serves as the 'Na^+ sensor'.

Materials and methods

Bacterial strains and culture conditions

The bacterial strains used in this study are K12 derivatives. W3133-2S is *mel*BLid, Δ*lac*ZY and contains the mutation causing the NhaA^{up} phenotype which is tightly linked to *nha*A (Niiya *et al.*, 1982; Goldberg *et al.*, 1987). TA15 is *mel*BLid, *nha*A$^+$, Δ*lac*ZY (Goldberg *et al.*, 1987). NM81 is *mel*BLid, Δ*nha*A1, *kan*$^+$, Δ*lac*ZY, *thr*1 (Padan *et al.*, 1989). RK20 is *mel*BLid, Δ*nha*A2, *kan*$^+$, Δ*lac*ZY, *thr*1 (Karpel, 1990). As opposed to NM81 in which two-thirds of *nha*A is deleted, RK20 lacks the entire open reading frame of *nha*A. NM81 *recA306*::Tn*10* was constructed by P1 transduction of NM81 using as a donor JC15503 [*recA306*::Tn*10* (306 Δ*srl-rec*A), which was kindly obtained from A.Cohen of Hadassah Medical School, Hebrew University]. RK33Z is *mel*BLid, Δ*nha*A3, *kan*^{R}, Δ*lac*ZY, φ(*nha*A′−′*lac*Z) l(hyb) (Karpel *et al.*, 1991). Cells were grown either in minimal medium A (Davies and Mingioli, 1950) without sodium citrate, but supplemented with thiamine (2.5 μg/ml) or in modified L broth (LBK) of which added NaCl was replaced by KCl (87 mM, pH 7.5). The contaminating Na^+ in LBK in this work was 7 mM NaCl as measured by atomic absorption. When indicated, the carbon source was 10 mM melibiose and when required threonine (0.1 mg/ml) was added. 1.6% agar was used for the plates. The antibiotics were 100 μg/ml ampicillin and/or 50 μg/ml kanamycin. The growth test of NhaA^{up} phenotype included: (i) growth on minimal medium A containing melibiose as the carbon source and 100 mM LiCl as compared with growth on a similar medium containing instead of LiCl 100 mM NaCl and (ii) growth on L broth containing 0.7 M NaCl.

Plasmids

All plasmids used in this work which are pBR322 derivatives are described in Figure 1. Both pGM42T (Rahav-Manor *et al.*, 1992) and pOC100T (this work) were obtained by introducing a universal translation terminator [described by Karpel *et al.* (1988)] into the *Bgl*II site of pGM42 (Karpel *et al.*, 1988) or pOC100 (described in the Results section) respectively. In order to obtain plasmid pGM42Δ*Ava*I−*Bgl*II, pGM42 was digested with *Ava*I and *Bgl*II and the 6.4 kb fragment, bearing *nha*R and the 3′ end of *nha*A, was end-filled and self-ligated. pGM42Δ*Ava*I−*Bam*HI was obtained by digestion of pGM42 with *Ava*I and *Bam*HI and ligating the 5.8 kb fragment. For the construction of pOC100TS, a 1.28 kb fragment was amplified by the polymerase chain reaction (PCR) using chromosomal DNA isolated (Gillen *et al.*, 1981) from W3133-2S and oligonucleotide primers AACTGGCGCGTCTGCCTG and GGGCAAATAATGAG**G**T**C**GA**C**GC-AAAA (P_{91} and P_{14} respectively, Figure 1). As depicted the latter oligonucleotide was constructed with two mutations to introduce the *Sal*I site (in bold). Therefore, the amplified fragment containing the chromosomal *Bam*HI site and the new *Sal*I site was digested with these enzymes and the *Bam*HI−*Sal*I fragment was ligated in to *Bam*HI (in insert)−*Sal*I (in pBR322) fragment (6 kb) obtained from pOC100T (Figure 1).

Deletions similar to those introduced upstream of *nha*R (Figure 1) were formed upstream of *nha*R1 using pOC100, the respective restriction sites in *nha*A (*Bgl*II or *Bam*HI) and *Eco*RI of the vector (Figure 1). To obtain plasmid pOCR1, pOC100TS was digested with *Bam*HI and *Sal*I. The small fragment, 1.4 kb, was ligated to plasmid pT7-5 (Tabor and Richardson, 1985) digested with the same enzymes so that *nha*R1 is fused to the T7 promoter. pDT2 was described by Karpel *et al.* (1988), pGP1-2 by Tabor and Richardson (1985), and pGM36 by Goldberg *et al.* (1987).

Amplification of bacterial chromosomal DNA by the polymerase chain reaction

Chromosomal DNA was prepared by the method of Gillen *et al.* (1981). The oligonucleotide primers used to amplify the DNA fragment overlapping *nha*A and *nha*R were P_8 (ATCGCTCTCTTTAACCCA) and P_{14} (see above and Figure 1). The PCR mixture (100 μl final volume) contained 1 μg of chromosomal DNA, 50 mM KCl, 10 mM Tris−Cl (pH 9, at 25°C), 1.5 mM magnesium chloride, 0.1% gelatin (w/v), 1% Triton X-100, 200 μM of each of the deoxynucleoside triphosphates, 100 pmol oligomeric primers and 2.5 U of *Thermus aquaticus* DNA polymerase (Promega). The reaction mixture was overlaid with 100 μl of light mineral oil (Sigma) and subjected to 35 cycles of denaturation (1 min, 94°C), annealing (45 s, 50°C) and extension (3 min, 72°C) using a DNA Programmable Thermal Controller (M.J. Research Inc. model PTC 100). The amplification products were analyzed on 0.7% agarose gels.

DNA sequencing

The dideoxyoligonucleotide-based technique (Sanger *et al.*, 1977) with the modification of Del Sal *et al.* (1988) was applied to the plasmid DNA. When the PCR-amplified DNA fragment served as a template, it was first purified using a Geneclean kit (Bio 101, La Jolla, CA). The denaturation reaction mixture (8 μl) contained 10 pmol of the oligomeric primers and 350−500 ng of the DNA fragment, and the test tubes were sealed with Parafilm. Denaturation was at 94°C for 3 min, followed by rapid cooling in liquid nitrogen. After a quick spin, the mixture was placed on ice. For annealing, 1 μl containing 10 pmol of the oligomeric primer, 2 μl of 5 × the sequencing reaction buffer (USB) and 1 μl of single-stranded DNA binding protein (0.5 μg/μl, USB) were added and incubation continued for additional 15−20 min. Sequencing then proceeded as described (Sequenase version 2.0 kit, USB). The wild-type DNA sequence of *nha*A and *nha*R are in the GenBank (accession numbers: *nha*A, J03879, S67239; *nha*R, L24072).

Cloning of nhaR1 from W3133-2S in plasmid pOC100

Chromosomal DNA of W3133-2S was prepared essentially as described in (Gillen *et al.*, 1981). A genomic library was prepared with this DNA partially digested with *Sau*3AI, size selected (2−6 kb) and ligated to pBR322 digested with *Bam*HI. The NM81 *rec*$^-$ was transformed with the ligation mixture and plated on LBK plates containing ampicillin. The transformants were collected by flooding the plates with LBK and replated on selective plates for Na^+-resistant cells (LB, 0.7 M NaCl, pH 7.5). Plasmids of these transformants, one of which is pOC100, were isolated and retransformed into NM81 *rec*$^-$ to ensure that the phenotype would be encoded by the plasmids.

Everted membrane vesicles and measurement of Na^+/H^+ antiporter activity

Everted membrane vesicles were prepared (Goldberg *et al.*, 1987) essentially as described by Rosen (1986). The Na^+/H^+ antiporter activity in everted membrane vesicles was estimated based on its ability to collapse a transmembrane pH gradient. Acridine orange fluorescence was monitored to estimate ΔpH as previously described (Goldberg *et al.*, 1987). The fluorescence was monitored in a Perkin−Elmer fluorometer (Luminescence Spectrometer, LS-5). The exciting light was 490 nm and the emission light was measured at 530 nm.

Protein concentration in cells and everted membrane vesicles was determined as described (Bradford, 1976).

Northern blot analysis

RNA was isolated from cells according to Furano and Wilell (1976). The RNA (20 μg) was separated by electrophoresis on 1% agarose gel and then transferred to a nylon filter (Amersham, Hybond-N) according to Sambrook *et al.* (1989). To ensure that the amount of RNA blotted on the filter was equal in each sample, the blot was stained with methylene blue (Sambrook *et al.*, 1989) and the amount of RNA was assessed using a densitometer. The DNA probe was obtained by PCR amplification using oligonucleotide primers P_{10} (GATGGTATCGCGACTTTCT) and P_{51} (CCCCGCC-AGAGTTGCGCGAACCCCC) and DNA isolated from pGM42 (Figure 1 and Karpel *et al.*, 1988). The resulting DNA fragment (0.6 kb) contained two-thirds of *nha*A from its N-terminus. The probes were end-labeled and hybridization to the blot was performed under high-stringency conditions (0.2 × SSC at 68°C), followed by washes and autoradiography (Sambrook *et al.*, 1989). For comparison, the density of each band was measured with a densitometer.

Induction of nhaA′−′lacZ

RK33Z cells transformed with various plasmids as indicated, were induced (if not otherwise stated) by the addition of Na^+ (100 mM) or Li^+ (100 mM), and the β-galactosidase activity of the cells was determined as described by Karpel *et al.* (1991).

Determination of intracellular and extracellular Na^+

Cells were grown (to an OD_{600} of 0.5) in LBK supplemented with 50 mM glucose, 50 mM 2-[*bis*(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol (BTP) (pH 7.5) and the indicated $[Na^+]_{out}$ were determined by atomic absorption (Perkin−Elmer, Model 403). Aliquots (0.4 ml) of the cell suspension were incubated in 24-well cell culture dishes (well diameter 16 mm, Costar Corporation, Cambridge, MA) under agitation at 37° for 1 h in the presence of 0.5 μCi carrier-free ^{22}Na. Cells were then filtered through a membrane filter (Schleicher and Schuell, 0.45 μm

diameter), washed with 4 ml of prewarmed fresh growth medium, and the radioactivity measured with a γ-counter model GAMMA matic (Kontron). Toluenized cells were used to subtract non-specific absorption of the isotope. Internal Na^+ concentrations in each case were calculated from values obtained in parallel experiments in which equilibrium was obtained by incubation of the cells with 50 μM carbonyl cyanide *m*-chlorophenylhydrazone under anaerobic conditions [covered with 0.4 ml light mineral oil (Sigma)]. Protein of intact cells were quantified as described (Lowry *et al.*, 1951).

Extracts of cells overexpressing nhaR or nhaR1

Overexpression of *nha*R or *nha*R1 was obtained with pDT2 or pOCR1 respectively, transformed into TA15 containing plasmid pGP1-2. Overexpression from the T7 promoter was obtained essentially as previously described by Tabor and Richardson (1985) and Rahav-Manor *et al.* (1992). Cells carrying the plasmids were grown at 30°C in LBK to and OD_{600} of 1−1.5 and shifted for 25 min to 42°C, rifampicin (100 μg/ml) was added and the cells were shifted back to 37°C for another 90 min. Cells were harvested by centrifugation, resuspended in 1 ml of buffer containing 20 mM Tris−HCl (pH 7.4), 20 mM NaCl (if not otherwise indicated), 1 mM EDTA, and 10% (w/v) glycerol and sonicated (Heat systems-Ultrasonics, W-385, Farmingdale, NY) 10 times for 10 s with 10 s cooling intervals. Intact cells were separated by centrifugation (10 000 *g*) for 10 min at 4°C and the cell-free extract was stored at −70°C.

DNA binding

The DNA probe was obtained by PCR amplification using plasmid pGM36 and the *nha*A primers, P_8 and P_{110} (TCTCCAGAAAGTCGTGATACCATC) (Figure 1). The latter primer was 5′ end-labeled with phage T4 polynucleotide kinase (New England Biolabs) in the presence of 20 μCi of [γ-^{32}P]ATP (3000 Ci/mmol, Amersham) and purified on a 5% (w/v) acrylamide gel in 1 × TBE buffer, according to Maniatis *et al.* (1982). The labeled PCR product was 0.55 kb long.

DNA binding assay was conducted according to Nachaliel *et al.* (1989). About 3 ng of the DNA probe were incubated for 50 min at 25°C with 0.1−0.12 μg of protein from the crude extracts in a 10 μl total volume of a reaction mixture containing 10 mM HEPES (pH 8), 60 mM KCl, 0.5 mM $(NH_4)_2SO_4$, 1 mM dithiothreitol and 5% polyethylene glycol (8000). The reaction was stopped by adding 4 μl of stopping solution containing 90% formamide, 10 mM NaOH, 0.2% xylene cyanol and 0.2% bromophenol blue. The DNA−protein complex was resolved on 5% acrylamide gel cotaining 0.5% bisacrylamide, 22.5 mM Tris−borate and 0.5 mM EDTA. The gel was dried and autoradiographed.

Preparation of site-specific antibodies and quantification of NhaR

Rabbits were immunized with the peptide Lys-Arg-Lys-Gly-Thr-Trp-Ser-Arg-Thr-Gln-Arg-Ala-Gly-Glu-Leu-Val-Tyr corresponding to amino acids 54−70 of NhaR, coupled to thyroglobulin and boosted with the same peptide coupled to bovine serum albumin. Antibodies were purified from total serum by an affinity column prepared by coupling the peptide to Affi-Gel (Bio-Rad). For Western blots, proteins were resolved by SDS−PAGE (Laemmli, 1970) in gels containing 20% acrylamide and 0.5% bisacrylamide, and transferred to nitrocelluse membranes (Hybond-C extra, Amersham), prior to reaction with the antibody and detection using alkaline phosphatase coupled to anti-rabbit IgG (King *et al.*, 1985).

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