The Na\(^+\)-specific interaction between the LysR-type regulator, NhaR, and the *nhaA* gene encoding the Na\(^+\)/H\(^+\) antiporter of *Escherichia coli*

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We used partially purified NhaR and a highly purified His-tagged NhaR derivative to identify the cis-regulatory sequences of *nhaA* recognized by NhaR and to study the specific effect of Na\(^+\) on this interaction. Gel retardation assay with DNase I footprinting analysis showed that NhaR binds a region of *nhaA* which spans 92 bp and contains three copies of the conserved LysR-binding motif. Na\(^+\), up to 100 mM, had no effect on the binding of NhaR to *nhaA*. The dimethylsulfate methylation protection assay in *vivo* and *in vitro*, showed that bases G\(^{-}92\), G\(^{-}60\), G\(^{-}29\) and A\(^{-}24\) form direct contacts with NhaR; in the absence of added Na\(^+\) in *vivo*, these bases were protected but became exposed to methylation in a \(\Delta nhaR\) strain; accordingly, these bases were protected in *vitro* by the purified His-tagged NhaR. 100 mM Na\(^+\), but not K\(^+\), removed the protection of G\(^{-}60\) conferred by His-tagged NhaR in *vitro*. Exposure of intact cells to 100 mM Na\(^+\), but not K\(^+\), exposed G\(^{-}40\). The maximal effect of Na\(^+\) in *vitro* was observed at 20 mM and was pH dependent, vanishing below pH 7.5. In contrast to G\(^{-}60\), G\(^{-}92\) was exposed to methylation by the ion only in *vivo*, suggesting a requirement for another factor existing only in *vivo* for this interaction. We suggest that NhaR is both sensor and transducer of the Na\(^+\) signal and that it regulates *nhaA* expression by undergoing a conformational change upon Na\(^+\) binding which modifies the NhaR-*nhaA* contact points.

**Keywords:** Na\(^+\)/H\(^+\) antiporters/Na\(^+\)-specific transcription regulation/*nhaA*-Na\(^+\)-specific footprint/NhaR

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**Introduction**

Salt stress is one of the most common growth-arresting factors encountered by bacteria. This stress is multifactorial since it involves stress of osmolarity, ionic strength and desiccation, as well as a specific toxic effect of Na\(^+\) on certain essential metabolic reactions, common to all cells (reviewed in Padan and Schuldiner, 1992). Accordingly, all cells have Na\(^+\)-excreting systems to eliminate toxicity (Padan et al., 1989; Padan and Schuldiner, 1992, 1994, 1996) and an intricate regulatory network responsive to various aspects of the stress of salinity. We have discovered a specific Na\(^+\)-responsive adaptation in *Escherichia coli* (Karpel et al., 1991; Rahav-Manor et al., 1992; Carmel et al., 1994) regulating *nhaA*, the key Na\(^+\)/H\(^+\) antiporter in the tolerance of this bacterium to high Na\(^+\) and alkaline pH (in the presence of Na\(^+\)) (Padan and Schuldiner, 1994, 1996).

Northern analysis of *nhaA* mRNA (Dover et al., 1996) and study of the expression of a *nhaA*–*lac*Z translational fusion in cells (Karpel et al., 1991; Rahav-Manor et al., 1992) grown at various salt concentrations showed that Na\(^+\) and Li\(^+\) specifically induce *nhaA* transcription. Furthermore, a novel regulatory gene *nhaR*, which is responsible for the Na\(^+\)-specific induction of *nhaA*, was identified (Rahav-Manor et al., 1992; Carmel et al., 1994). *nhaR* is located downstream of *nhaA* and encodes a protein (NhaR) of 34.2 kDa. NhaR is a positive regulator required, in addition to *nhaA*, in order to tolerate high Na\(^+\) and Li\(^+\) concentrations (Rahav-Manor et al., 1992; Carmel et al., 1994). The enhancing effect of plasmidic multicopy *nhaR* on the Na\(^+\)-induced expression of *nhaA*–*lac*Z showed that NhaR works in *trans* and requires Na\(^+\) for its activity. A DNA mobility test showed that a cell-free extract from cells overexpressing NhaR contains a protein which binds to the DNA at the upstream region of *nhaA*.

NhaR is homologous to a large family of positive regulators in prokaryotes, the LysR-OxyR family (Henikoff et al., 1988; Christman et al., 1989; Rahav-Manor et al., 1992). All these proteins share, at their N-terminus, conserved sequences containing a helix–turn–helix motif, implicated in DNA binding. Interestingly, several members of this large group are proteins that are involved in the response of the organism to environmental stress (Christman et al., 1989; Storz et al., 1990; Schell, 1993). We have suggested that NhaR is a component of yet another type of stress response, essential for Li\(^+\) and Na\(^+\) tolerance, of the LysR family. Recently we have shown that the NhaR-dependent regulation of *nhaA* is affected by H-NS (Dover et al., 1996), a major DNA-binding protein and a global regulator involved in salt stress in bacteria (Owen-Hughes et al., 1992; Ussery et al., 1994).

We have purified the NhaR protein (partially) and its His-tagged derivative (to homogeneity), identified their binding sites to cis-regulatory elements of *nhaA* and discovered a specific effect of Na\(^+\) on the NhaR–*nhaA* interaction both *in vivo* and *in vitro*.

**Results**

**Construction of His-tagged NhaR and purification of both NhaR and its His-tagged derivative**

Our previous *in vivo* experiments showed that NhaR is a positive regulator of *nhaA*, whose activity is dependent on the concentration of intracellular Na\(^+\) (Dover et al., 1996). In the present work, a direct biochemical approach...
has been undertaken to study the interaction between Na⁺, NhaR and the nhaA DNA in a molecularly defined system. For the purification of the regulatory protein, we have constructed plasmid pOCRXH. In this plasmid, nhaR is fused in-frame at its 3' end to a sequence encoding the two cleavage sites of the protease factor Xa followed by six histidines. To test whether the chimeric protein (His-tagged NhaR) is active, the plasmid was transformed into RK33Z, a strain bearing a chromosomal nhaA–lacZ protein fusion. For a control, we used RK33Z cells transformed with pGM42T, a plasmid harboring wild-type nhaR. As shown previously, these cells showed marked induction of β-galactosidase activity upon addition of Na⁺ (Rahav-Manor et al., 1992). Similar Na⁺ induction was obtained with transformants of a plasmid encoding the His-tagged NhaR. These results show that the His-tagged NhaR is as active as the wild-type protein in vivo. The purified NhaR was used in some in vitro experiments as indicated.

Deletion mapping of the nhaA DNA region containing the regulatory signals recognized by NhaR

Two promoters of nhaA were mapped previously (Karpel et al., 1991 and Figure 2A). To identify the DNA region containing the cis-elements recognized by NhaR, we PCR-amplified various sequences overlapping the nhaA promoter region (Figure 2A). Each fragment was end labeled and tested for binding to the partially purified native NhaR in a DNA gel retardation assay (Figure 2B). As shown previously with a cell-free extract obtained from cells overexpressing native NhaR (Carmel et al., 1994), the partially purified NhaR binds specifically to a DNA fragment containing base pairs –424 to 130 of the upstream sequences of nhaA including the nhaA promoters (Figure 2, fragment A). Figure 2 also shows that whereas the sequences from the 5' end of this fragment down to bp 121 (fragments B and E) and sequences from the 3' end up to bp 14 (fragment D) do not bind, DNA fragments overlapping the sequences in between (fragments C, F and G) contain nhaA sequences recognized by NhaR. We have therefore concluded that the NhaR-binding sites are located between bp –120 and 14 (also indicated on the nhaA sequence in Figure 6A). In accordance with this

As expected from its longer C-terminus, His-tagged NhaR was slightly heavier (36.2 kDa) than the native NhaR (34.2 kDa) (Figure 1A, lane 8). To assess the degree of purification, the fraction eluted from the Ni²⁺ column was separated by HPLC. A single homogenous band peaking at 72.5 kDa appeared, suggesting that His-tagged NhaR is a dimer. Importantly, the activity of the His-tagged NhaR was the same, whether purified in a single step by the Ni²⁺ column or in two steps with an additional gel filtration step. With both procedures, no more than 1% of contaminants were observed by silver staining of the proteins, suggesting a very high degree of purification.

To compare the biochemical properties of His-tagged NhaR with those of the wild-type protein, we also partially purified the wild-type molecule. For this purpose, we used a mixture of cell-free extracts, one containing overexpressed NhaR and the other NhaR specifically labeled with [³⁵S]methionine. The radioactively labeled protein allowed the NhaR protein to be followed during the purification and allowed it to be optimized by determining the amount of [³⁵S]-labeled protein in each fraction. Figure 1B shows that fractions 21–23, highly enriched in the specifically radioactively labeled NhaR, were obtained by chromatography on a heparin–Sepharose column. This conclusion was supported both by silver staining of samples containing equal amounts of radioactive counts eluted in these fractions and by Western analysis using anti-NhaR antibody (Rahav-Manor et al., 1992). These results showed a prominent band at 34 kDa which cross-reacted with the antibody. Fraction 21–23 represented the highest enrichment of NhaR over other contaminating proteins, mainly of higher molecular weights. These fractions were pooled and used in some in vitro experiments as indicated. The other fractions which eluted before or after the peak (19, 20, 24 and 25) also contained a protein(s) of 34 kDa. However, since this protein did not cross-react with the anti-NhaR antibody, we assumed it to be a contaminant which co-purified with NhaR.

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**Fig. 3.** DNase I protection footprint of NhaR. A DNA fragment (242 bp) end labeled with $^{32}$P at the 3' (bottom strand) in (A) or at the 5' (top strand) in (B) were incubated with His-tagged NhaR (250 and 500 ng in lanes b and c, respectively, of A and 500 ng in lane b of B) as indicated and then cleaved with DNase I as described in Materials and methods. The DNase I-protected nhaA regions are marked by the vertical lines adjacent to the sequence. Numbers indicate the position of each base relative to the first base of the initiation codon (Figure 6A).

Experiments. A reaction mixture lacking His-tagged NhaR served as a control (Figure 3A and B). As shown in Figure 3, a very long sequence on each strand of the nhaA promoter region was protected by His-tagged NhaR, extending over 92 bp [from bp –109 to –17 of the bottom strand (Figure 3A) and from –109 to –24 of the top strand (Figure 3B)]. This protected region is not continuous since it is interrupted by sites which became hypersensitive to exposure to partially purified native NhaR (even numbers), was tested in the DNA gel retardation assay. +, retardation; -, no retardation; $P_1$ and $P_2$ are nhaA promoters (Karpel et al., 1991). I, II and III are the conserved LysR motifs shown in Figure 6. Numbers in brackets refer to the transcript start site and otherwise to the first base of the initiation codon GTG.

**DNase I footprint of NhaR on a linear DNA fragment of nhaA**

The sequences of nhaA protected by either NhaR (not shown) or His-tagged NhaR (Figure 3) from a limited DNase I digestion were identical. The purified His-tagged NhaR and a linear DNA fragment (from –190 to 52 of the coding sequence, Figure 6A) were used in these experiments. A reaction mixture lacking His-tagged NhaR served as a control (Figure 3A and B). As shown in Figure 3, a very long sequence on each strand of the nhaA promoter region was protected by His-tagged NhaR, extending over 92 bp [from bp –109 to –17 of the bottom strand (Figure 3A) and from –109 to –24 of the top strand (Figure 3B)]. This protected region is not continuous since it is interrupted by sites which became hypersensitive to exposure to partially purified native NhaR (even numbers), was tested in the DNA gel retardation assay. +, retardation; -, no retardation; $P_1$ and $P_2$ are nhaA promoters (Karpel et al., 1991). I, II and III are the conserved LysR motifs shown in Figure 6. Numbers in brackets refer to the transcript start site and otherwise to the first base of the initiation codon GTG.

**DMS methylation protection assay in vitro**

Since the DNase I protection assay is limited in its resolution and DNase I attacks sequences located mainly in the minor groove of the DNA (Sasse-Dwight and Gralla, 1991), we next focused on the major groove with a more sensitive method: probing the NhaR footprint with primer extension following dimethylsulfate (DMS) methylation and subsequent breakage by piperidine of the unprotected methylated sites. DMS modifies mainly guanines and, to a lesser extent, adenines in the major groove of the DNA (Sasse-Dwight and Gralla, 1991).
Figure 4A shows that G at –92 is protected specifically by His-tagged NhaR, but addition of either KCl or NaCl (100 mM each) had no effect on the protection pattern. Similarly, the bases, A at –24 and G at –29, were protected by NhaR with no effect of either ion (Figure 4A). Strikingly, the protection of G at –60 by NhaR was affected differently by the ions (Figure 4A); it remained protected in the presence of 100 mM KCl (Figure 4A, lanes b and d) but 100 mM NaCl specifically removed the protection of G–60 by NhaR and exposed it to methylation and subsequent breakage (Figure 4A, compare lane f with lane d).

We next titrated the Na+ concentration needed to give the specific effect of Na+. Whereas at 7 mM Na+, G–60 was as protected as in 100 mM K+, 20 mM Na+ was sufficient to give the maximal exposure to methylation and subsequent cleavage (not shown), as seen in the presence of 100 mM Na+ (Figure 4A, lanes e and f). These results suggest that the Na+ concentration yielding the maximal effect is ~20 mM Na+.

The pH dependence of the Na+ effect on the methylation protection assay is summarized in Figure 5. The bases protected by NhaR which were not affected by Na+, i.e., –92, –29 and –24, were not affected by pH either. In contrast, the Na+–sensitive G–60 was affected drastically by pH; whereas at pH 6.5 it remained protected in the presence of either K+ or Na+ (100 mM each), Figure 5, lanes a–c), at pH 7.5 and pH 7.9 (Figure 5, lanes d–i), and even up to pH 9 (not shown), it was exposed to methylation in the presence of Na+ (100 mM) but not of K+ (100 mM).

Identification of the specific effect of Na+ on NhaR–nhaA interaction in vivo

The DMS protection assay was conducted in vivo in order to identify the in vivo footprint of NhaR on nhaA. Figure 4B shows that, similarly to the in vitro results, a G at position –60 is less protected when the cells are exposed to 100 mM Na+ as compared with its exposure to 100 mM K+. Strikingly, the G at –92, which did not show any response to Na+ in vitro, is dramatically exposed to methylation when the cells are exposed to Na+ (100 mM, Figure 4B, lane a) and is not affected by an exposure to K+ (100 mM, Figure 4B, lane b).

It was critical to show that these specific in vivo effects of Na+ are indeed dependent on NhaR. Support for this contention was obtained by the fact that these in vivo Na+ effects were conspicuous only in cells transformed with a multicopy plasmid bearing nhaR but not in cells having only the single chromosomal copy (not shown). Nevertheless, to prove the dependence of the Na+ effects on NhaR, we constructed a ΔnhaR strain (ORC100) and used it, either transformed or not, with plasmidic nhaR to repeat the methylation protection assay (Figure 4C). In the ΔnhaR strain, all bases at –24, –29, –60 and –92 were similarly exposed to DMS methylation when either Na+ or K+ (100 mM each) were present (Figure 4C, lanes a and b). Indeed transformation with nhaR plasmid restored protection (Figure 4C, lane c) and the specific effects shown in the presence of Na+ in vivo (not shown).
Discussion

Our previous in vivo studies suggested that as an essential part of Na\(^+\) homeostasis in E.coli, the regulation of \(nhaA\) expression by NhaR is induced specifically by a change in Na\(^+\) concentration rather than by its outcome: a change in ionic strength or osmolarity (Karpe et al., 1991). A similar role has been assigned recently to Na\(^+\) in the regulation of expression of the Na\(^+\)/ATPase of Enterococcus hirae (Murata et al., 1996). In the present study, by molecular dissection of the system in E.coli, we have proven that indeed Na\(^+\) itself is the signal for \(nhaA\) expression via NhaR, identified the regulatory cis-elements of nhaA which bind NhaR and established both in vivo and in vitro that Na\(^+\) changes the footprint of NhaR on nhaA.

Different molecular sizes were obtained in the two separation procedures of His-tagged NhaR, 36.2 kDa by SDS–PAGE and 72.5 kDa by gel filtration. The lower molecular weight value obtained under the denaturing conditions (SDS–PAGE) agrees with a monomeric form of His-tagged NhaR which, as expected, is slightly heavier than the native NhaR (34.2 kDa). The molecular weight value obtained under the non-denaturing conditions (HPLC, gel filtration) suggests that His-tagged NhaR exists as a dimer. Many of the LysR-type transcriptional regulators exist and function as dimers (Schell, 1993), although, in several cases, higher multimeric forms are also known (Toledano et al., 1994; Kullik et al., 1995).

The multimeric nature of the LysR family members is reflected in the mode of binding to their DNA target promoters; the size of their binding region is unusually long, extending over several tens of base pairs, i.e. several turns of the DNA helix. The NhaR appears to be an extreme case. It protects ~90 bp against DNase I digestion. Accordingly, the nhaA sequences binding NhaR that are revealed by the gel retardation assay (Figure 2) align with not reveal these His-tagged NhaR contacts most probably an as yet unknown NhaR–DNA stoichiometry.

A peculiarity of the LysR-type proteins is the paucity of conserved bases involved in DNA binding and the fact that they are dispersed throughout their long binding site. Recently, a detailed consensus motif was defined for the binding of OxyR (Toledano et al., 1994). It shows a 2-fold symmetry, and the spacing of the elements suggests that OxyR contacts four helical turns. This motif also fits the generic LysR family consensus sequence (T-N_11-A), which is based on a comparison of binding sites from a variety of species (Goethals et al., 1992; Schell, 1993; and see Figure 6B). Most interestingly, the deletion mapping of the NhaR binding domain on nhaA shows that each of the DNA fragments which bind NhaR contain one or more of these consensus motifs designated I, II and III (Figures 2A and 6A), which are very close to each other but yet separated by spanning sequences. Accordingly, the DNase I-protected sequences of nhaA by NhaR align with these three motifs and show that the spanning sequences separating them contain hypersensitive DNase I sites (Figures 3A and 6A). These spanning sequences separating the consensus motifs further corrobore our suggestion regarding the multimeric nature of bound NhaR.

It is remarkable that within the three consecutive consensus motifs, I, II and III, in the binding domain of NhaR, we identified by the DMS methylation protection assay, but not by the DNase I assay, four single bases which form direct contacts with NhaR: \(G^\sim 92\) in I, \(G^\sim 60\) in II and \(G^\sim 29\) and \(A^\sim 29\) in III. In the absence of Na\(^+\) both in vivo and in vitro, these bases were protected by NhaR or His-tagged NhaR respectively and exposed to methylation in the absence of the regulator (Figure 4A and C). The fact that the DNase I protection assay did not reveal these His-tagged NhaR contacts most probably stems from the difference in the sensitivity and mechanism
of these assays. DNase I digests the DNA in unprotected sites which reside mainly in the minor groove of the DNA (Saase-Dwight and Gralla, 1991). DMS methylates mainly the N-7 position of guanine residues in the major groove of the DNA. Hence, we suggest that each contact site is located in different consecutive major grooves separated from each other by two turns of the helix (20 bp, Figure 6A). It is conceivable that additional binding bases exist which cannot be identified by the DMS methylation protection assay.

Na⁺ had no effect on the binding of NhaR to nhaA as measured by the gel retardation assay. This result suggests that whether Na⁺ is present or not, NhaR is constantly bound to the nhaA DNA. This behavior is characteristic of many members of the LysR family; these regulators remain bound to their target DNA, with no change in affinity even in the absence of the specific inducer. It is only the footprint which is changed upon addition of the inducer (Storz et al., 1990; Schell, 1993; Toledano et al., 1994). Indeed, while Na⁺ had no effect on the footprint assayed by DNase I protection, the footprint discovered by the DMS methylation protection assay showed an effect of Na⁺. The binding of the His-tagged NhaR to two guanines was changed dramatically upon addition of Na⁺; G–60 was exposed specifically to DMS methylation by Na⁺ (100 mM) since in the absence of the ion or in the presence of K⁺ (100 mM) it was protected by His-tagged NhaR. The specific Na⁺ effect on G–60 was found both in vitro and in vivo with both linear and supercoiled plasmidic DNA. On the other hand, G–92 was exposed to methylation by the ion only in vivo. We therefore suggest that Na⁺ directly affects the interaction of NhaR with G–60 of nhaA but indirectly affects the interaction with G–92. The latter most probably requires either a particular topology of the DNA or another factor existing only in vivo.

In this respect, we recently have established a connection between the Na⁺-specific, NhaR-dependent regulation of nhaA and H-NS, a DNA-binding protein and a global regulator (Dover et al., 1996). Although the mechanism of regulation mediated by H-NS is not known, it has been suggested to involve a change in the topology of the DNA (Tupp et al., 1994).

Similarly to other members of the LysR family, the long footprint of NhaR on nhaA as revealed by the DNase I protection assay overlaps with P₁, one of the two promoters of nhaA. The other, P₂, maps further upstream. Interestingly, we have found recently that P₁, but not P₂, is involved in the Na⁺ induction of nhaA (N.Dover, O.Carmel and E.Padan, unpublished results).

Na⁺ is a very common ion encountered by cells. Its intracellular concentration, although always actively maintained lower than the extracellular concentration, can reach the millimolar range. In E.coli growing in the presence of 100 mM Na⁺, intracellular Na⁺ is ~10 mM (Harel-Bronstein et al., 1995). Above this concentration, the growth rate is inhibited. Most interestingly, it is within this range, 10–20 mM, that Na⁺ exerts its specific effect in vitro on the nhaA footprint while KCl up to 100 mM has no effect.

Monitoring the expression of an nhaA′−lacZ fusion, we previously have found that the Na⁺-specific NhaR-dependent induction of nhaA is enhanced ~10-fold by a pH shift from 7 to 8.5 (Karpel et al., 1991). Since we have found previously that intracellular Na⁺ is the signal for induction (Dover et al., 1996), these results were explained by the previously observed increase in intracellular Na⁺ with pH (Pan and Macnab, 1990). Nevertheless, the present results show directly, in vitro, that the Na⁺-specific interaction between His-tagged NhaR and G–60 of nhaA is pH dependent, within the same range affecting expression in vivo (Figure 5), suggesting a direct competition between Na⁺ and H⁺.

Taken together, these results suggest that NhaR is both the sensor and the transducer of the Na⁺ signal which regulates expression of nhaA, and undergoes a conformational change upon Na⁺ binding. This change is expressed directly in a decrease in NhaR binding to G–60 in a pH-dependent fashion. This is also manifested in the binding of NhaR to G–92: Observed only in vivo, the G–92–NhaR interaction suggests an involvement of yet another factor in vivo.

Materials and methods

Bacterial strains and culture conditions

Most of the bacterial strains used in this study are E.coli K-12 derivatives. TA15 is mbrBLid nhaA′ nhaB′ nhaC′ Z (Goldberg et al., 1987). OR100 contains ΔnhaR2::kan (NhaR, Kan) and is otherwise isogenic to TA15 (Rahav-Manor, 1992). RK33Z is ΔnhaA3::kan Φ (nhaA: lacZ)1(Hyb) thr-1 and otherwise isogenic to TA15 (Karpel et al., 1991). HB101 is F′λrg7-protA362 leuB6 supE44 araB14 galK2 lacY1 (xmrC–mer) rpsL20(Str) sly-5 mit-1 recA13 hadS280(tg)1. BL21 is an E.coli B F′ dcm ompT hsdS(rK27) gal LE392 is el4 (McrA−) rpsL51 supE44 supF58 lacY1 or Δ(lacZ)M15 galK2 galT2 Δmbl1 trpR55. OR100 is a LE392 derivative containing ΔnhaR2::kan. This nhaR deletion was constructed by P1 transduction using OR100 as a donor and LE392 as an acceptor, selecting for Kan⁺ colonies. One of these transductants was isolated, designated OR100 and verified to contain the respective mutation by colony PCR using the appropriate primers. ORC100 cells were also tested phenotypically and shown to be Na⁺ sensitive on an agar plate assay (Carmel et al., 1994).

Growth in rich or minimal medium and test for resistance to Na⁺ and Li⁺ on agar plates were as described (Carmel et al., 1994).

Plasmids

Plasmid pGM42 is pBR322 derivative bearing wild-type nhaA and nhaR (Karpel et al., 1988). pGM42T is a derivative of pGM42 inactivated in nhaA (Rahav-Manor, 1992). pET2D is a plasmid in which nhaR lacks its own promoter but is placed under control of the T7 RNA polymerase promoter (Karpel et al., 1988). pGP1-2 encodes the T7 RNA polymerase (Tabor and Richardson, 1985). pGM36 carries wild-type nhaA (Goldberg et al., 1987). Plasmids encoding His-tagged NhaR derivatives are pET20b(+) (Novagen, USA) derivatives as described below. pKR107 carries the upstream sequences of nhaA (Karpel, 1990). It was constructed by cloning the 1.4 kb BamHI–BglII fragment of pGM36 into the BamHI site of pPS3-ML (Glaser et al., 1983).

Construction of His-tagged NhaR plasmid, pOCRXH

A DNA fragment (52 bp) encoding two factor Xa cleavage sites in its intracellular DNA fragment was then cloned between these restriction sites of the polylinker in pET20b(+). The sequence of the cloned fragment in the recombinant plasmid designated pET20b(X) was verified by DNA sequencing through the ligation sites. For construction of pOCRXH, a DNA fragment (1206 bp) bearing nhaR was produced by PCR amplification using pGM42 as a template and primers 91 and 51 (Table I) which exchange the stop codon TAA for a serine codon followed by a Nol restriction site. The fragment was digested with BamHI, end filled and then digested with Nol. It was then ligated with the 3584 bp fragment of pET20b(X), produced by digestion with XhoI, end filling and subsequent digestion with Nol. In this recombinant plasmid designated pOCRXH, nhaR is placed under control of the T7 RNA polymerase promoter and fused in...
Overexpression and purification of His-tagged NhaR

For overexpression of His-tagged NhaR, BL21 cells (250 ml) transformed with pOCRXH were grown at 37°C in LBK medium (Carmel et al., 1991) to OD600 = 0.6. To induce overexpression, 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added and growth continued for an additional 2 h. The cells were centrifuged and the pellet was stored at −70°C. The His.Bind™ protocol (Novagen, Madison, WI) was used to affinity purify the His-tagged NhaR from the soluble fraction of the cells of E.coli HB101 transformed with either plasmid pGM42T or pPCR107 was used. For the preparation of methylated DNA, 10 ml of cells were grown overnight at 37°C in L broth in the presence of either 100 mM NaCl or KCl. Methylation was started by adding DMS to a final concentration of 10 mM and proceeded for 5 min at 37°C. The cells were harvested by centrifugation (12 000 g, 5 min), the methylated DNA, primer extension was performed using PCR (amplification, 40 cycles; denaturation, 1.5 min at 94°C; annealing, 5 min at 58°C and elongation, 2 min at 72°C) in the presence of plasmid pGM42T or pKR107 was used. For the preparation of methylated DNA, 10 ml of cells were grown overnight at 37°C in L broth in the presence of either 100 mM NaCl or KCl. Methylation was started by adding DMS to a final concentration of 10 mM and proceeded for 5 min at 37°C. The cells were harvested by centrifugation (12 000 g, 5 min), the methylated plasmid DNA isolated (Qiagen) and dialyzed overnight at 4°C in a buffer containing 50 mM KCl, 10 mM BME and 10% glycerol. The protein was frozen in liquid nitrogen and stored at −70°C. For analysis of the in vivo labeling of NhaR with [35S]methionine, the same expression system was used (10 ml of cells) as described (Karpel et al., 1989). (Pharmacia) at a flow rate of 1–3 ml/min. The column was washed with the binding buffer but contained 400 mM imidazole and 10 mM β-mercaptoethanol (BME). The NhaR-containing fraction was dialyzed overnight at 4°C against 20 mM Tris–HCl (pH 7.9) and 5 mM β-mercaptoethanol (BME) (BME) were lysed by three passages through a French pressure cell (20 000 p.s.i., Model SLM-Aminco FA-10, SLM Instruments, Inc., Urbana, IL). The elution buffer was equal to the binding buffer but contained 400 mM imidazole and 10 mM BME. The NhaR-containing fraction was dialyzed overnight at 4°C against 20 mM Tris–HCl (pH 7.9), 100 mM KCl, 1 mM EDTA (pH 8.0), 15 mM BME and 10% glycerol. The protein was frozen in liquid nitrogen and stored at −70°C. This procedure yielded 6–7 mg of purified His-tagged NhaR. The protein (9 mg) was fractionated further by HPLC on a Superdex 75 HiLoad 16/60 column (Pharmacia) pre-equilibrated with a buffer containing 100 mM Tris–HCl, 20 mM Tris–HCl (pH 7.9), 1 mM EDTA, 1 mM dithiothreitol (DTT) and 0.02% NaCN. Fractions containing the peak concentration of His-tagged NhaR were pooled, glycerol added to 10% and aliquots (100 µl) stored at −70°C.

Induction of nhaA–lacZ

RK337 cells transformed with various plasmids as indicated were induced at pH 7.5 by the addition of Na+ (100 mM). The β-galactosidase activity of the cells was determined as described (Kerpel et al., 1991; Rahav-Manor et al., 1992).

Partial purification of native NhaR

NhaR was overexpressed from the T7 promoter of plasmid pDT2 in the presence of plasmid pGPI-2 in TA15 cells (1L) and cell-free and membrane-free extract prepared as described (Carmel et al., 1994). For specific labeling of NhaR with [35S]methionine, the same expression system was used (10 ml of cells) as described (Karpel et al., 1988). The soluble fraction from the labeled cells was prepared (Carmel et al., 1994) and dialyzed overnight at 4°C in a buffer containing 50 mM KCl, 50 mM HEPES (pH 7.6), 0.1 mM EDTA, 0.5 mM DTT and 2 mM MgCl2. To follow NhaR during the purification steps, the cytoplasmic fraction containing the overexpressed unlabeled NhaR was mixed with the [35S]-labeled protein (500 000 c.p.m.) and the mixture (7 ml) was applied to a heparin column (3.7 g heparin-Sepharose CL-6B (Pharmacia)) at a flow rate of 1–3 ml/min. The column was washed with 120 ml of the latter buffer containing 0.1 M KCl. Protein was eluted with a 70 ml linear gradient of the buffer containing 0.1–0.5 M KCl (Figure 1B). The fractions 21–23 containing the maximal radioactivity eluted at 0.25–0.28 M KCl were pooled, frozen in liquid nitrogen after addition of glycerol (10%) and stored at −70°C.

DNA gel retardation assay

The DNA probes were obtained by PCR amplification using plasmid pGm360 as a template and various nhaA primers (Table I and Figure 2). The DNA gel retardation assay (Rahav-Manor et al., 1992) with partially purified NhaR or purified His-tagged NhaR (0.2 µg each) was carried out (20 min, 25°C) in a buffer (10 µl) containing 50 mM KCl, 20 mM Tris–HCl (pH 7.9), 1 mM DTT, 10% glycerol, 125 µg/ml bovine serum albumin and 0.5 µl of [32P]-end-labeled DNA probe.

DNase I footprinting

The nhaA DNA fragment used for footprinting (Galas and Schmitz, 1978) was generated by PCR (primers 46 and 42 in Table I) and contained 52 bp of nhaA and 4 L24072 for nhaR. Location numbers are relative to the first GTG codon (nhaA) or the first ATG codon (nhaR).

Footprinting by methylation protection assay

For the in vivo methylation protection assay (Sasse-Dwight and Gralla, 1991), E.coli HB101 transformed with either plasmid pGM42T or pKR107 was used. For the preparation of methylated DNA, 10 ml of cells were grown overnight at 37°C in L broth in the presence of either 100 mM NaCl or KCl. Methylation was started by adding DMS to a final concentration of 10 mM and proceeded for 5 min at 37°C. The cells were harvested by centrifugation (12 000 g, 5 min), the methylated plasmid DNA isolated (Qiagen) resuspended in 100 µl of 1 M piperidine and cleaved by incubation for 30 min at 90°C followed by purification on a 1 ml Sephadex spin column (G-50, fine, Sigma) in water.

For analysis of the in vivo methylated DNA, primer extension was performed using PCR (amplification, 40 cycles; denaturation, 1.5 min at 94°C; annealing, 5 min at 58°C and elongation, 2 min at 72°C) in 35 µl containing 500–600 ng of cleaved DNA and 2P-labeled primer (0.3 pmol).

Quantitation of proteins and [Na+]i

Western analysis of NhaR was performed as described in Carmel et al. (1994). Proteins were determined according to Lowry et al. (1951). [Na+]i concentration was determined by atomic absorption (Perkin-Elmer, Model 403).

### Table I. DNA primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Location</th>
</tr>
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<tbody>
<tr>
<td>8</td>
<td>ATTCGCTTCTTTAACCAG</td>
<td>(–424)–(–407)</td>
</tr>
<tr>
<td>28</td>
<td>CACCCGACCTGACACAAAATAT</td>
<td>(–376)–(–359)</td>
</tr>
<tr>
<td>46</td>
<td>ATATATCTGAGGCTGTTAAG</td>
<td>(–353)–(–329)</td>
</tr>
<tr>
<td>57</td>
<td>CCGTCAAAACCGCATCTCCAGCCTG</td>
<td>(–193)–(–167)</td>
</tr>
<tr>
<td>58</td>
<td>TATAATCTGTTTAAACGATCATTCCTCAGCTG</td>
<td>(–108)–(–80)</td>
</tr>
<tr>
<td>60</td>
<td>GCCGGTTAATTAGTAAAACGACCCATTCACGGCTG</td>
<td>(–61)–(–33)</td>
</tr>
<tr>
<td>53</td>
<td>AGCTTAAGGATGAGTTCTCGGAGCGG</td>
<td>(–191)–(–153)</td>
</tr>
<tr>
<td>47</td>
<td>CTTGAAGCCTCAAGCTGAGTCCTCAG</td>
<td>(–151)–(–125)</td>
</tr>
<tr>
<td>49</td>
<td>GCAATCGCCGCTTAGACGCGG</td>
<td>(–121)–(–141)</td>
</tr>
<tr>
<td>42</td>
<td>GAAATATTCTCCCGAG</td>
<td>(–78)–(–96)</td>
</tr>
<tr>
<td>110</td>
<td>TCTCCAGGAGCCTGTTGACACCAC</td>
<td>(130)–(107)</td>
</tr>
<tr>
<td>91</td>
<td>AAATGGC CGCGTCTGCCG</td>
<td>(941)–(958)</td>
</tr>
<tr>
<td>51</td>
<td>TTATGTTCCTTTGGCGCCGCCGGAACCCACCGCTCGTACTAAGAACCCGCGTGCGTCGG</td>
<td>(898)–(875)</td>
</tr>
<tr>
<td>49</td>
<td>AAGGAAAAGAAGGGGCACATCGAAGGCGCTATCGAAGGTGCTTCGAGGCGCTGAGAAAC</td>
<td>(228)–(197)</td>
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<tr>
<td>50</td>
<td>CGCCTGAGAAGCACCTTCTGCAAGCCTCCTGAGTCAG</td>
<td>(228)–(197)</td>
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</tbody>
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References


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