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The Na^+-specific interaction between the LysR-type regulator, NhaR, and the *nha*A 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 *nha*A 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 *nha*A 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 *nha*A. 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 Δ*nha*R 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^{-60}. 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 *nha*A expression by undergoing a conformational change upon Na^+ binding which modifies the NhaR–*nha*A contact points.

Keywords: Na^+/H^+ antiporters/Na^+-specific transcription regulation/*nha*A-Na^+-specific footprint/NhaR

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 *nha*A, 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 *nha*A mRNA (Dover *et al.*, 1996) and study of the expression of a *nha*A′–′*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 *nha*A transcription. Furthermore, a novel regulatory gene *nha*R, which is responsible for the Na^+-specific induction of *nha*A, was identified (Rahav-Manor *et al.*, 1992; Carmel *et al.*, 1994).

*nha*R is located downstream of *nha*A and encodes a protein (NhaR) of 34.2 kDa. NhaR is a positive regulator required, in addition to *nha*A, in order to tolerate high Na^+ and Li^+ concentrations (Rahav-Manor *et al.*, 1992; Carmel *et al.*, 1994). The enhancing effect of plasmidic multicopy *nha*R on the Na^+-induced expression of *nha*A′–′*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 *nha*A.

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 *nha*A 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 *nha*A and discovered a specific effect of Na^+ on the NhaR–*nha*A 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 *nha*A, whose activity is dependent on the concentration of intracellular Na^+ (Dover *et al.*, 1996). In the present work, a direct biochemical approach

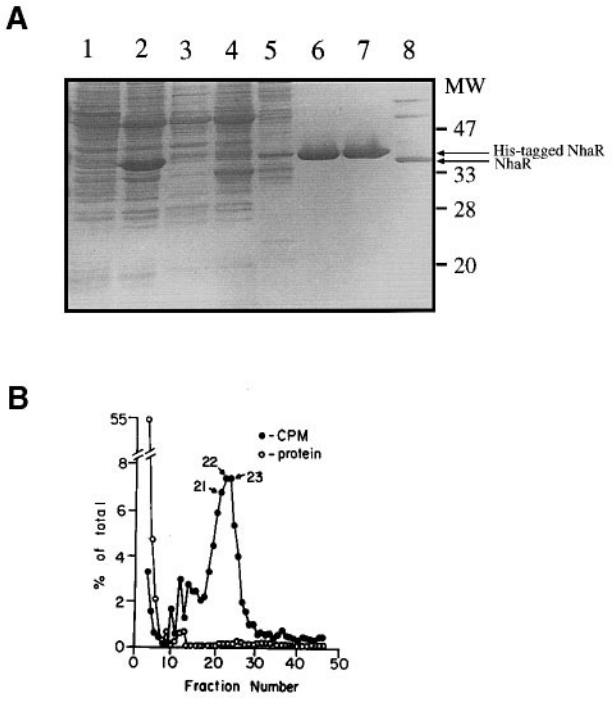

Fig. 1. Overexpression and purification of His-tagged and wild-type NhaR. (**A**) His-tagged NhaR was overexpressed and separated on a Ni^{2+}-NTA–agarose column as described in Materials and methods. Samples (30 μg of protein) from each fraction applied on or eluted from the column were run on SDS–PAGE to resolve the proteins. Lane 1, non-induced cells; lane 2, cells induced for 2 h; lane 3, void volume; lane 4, binding wash; lane 5, wash with 60 mM imidazole; lanes 6 and 7 elution with 400 mM imidazole; lane 8 shows partially purified native NhaR (20 amino acids shorter than His-tagged NhaR). (**B**) NhaR was overexpressed and specifically labeled with [^{35}S]methionine, as described in Materials and methods. A mixture of the cell-free extracts was applied to a heparin–Sepharose column and fractions collected for determination of radioactivity (●) and protein concentrations (○).

has been undertaken to study the interaction between Na^+, NhaR and the *nha*A DNA in a molecularly defined system. For the purification of the regulatory protein, we have constructed plasmid pOCRXH. In this plasmid, *nha*R is fused in-frame at its 3′ end to a sequence encoding 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 *nha*A′–′*lac*Z protein fusion. For a control, we used RK33Z cells transformed with pGM42T, a plasmid harboring wild-type *nha*R. 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 chimeric His-tagged *nha*R. These results show that the His-tagged NhaR is as active as the wild-type protein in promoting *in vivo* Na^+ induction of *nha*A.

The His-tagged NhaR was overexpressed (compare lane 2 with lane 1 in Figure 1A) and bound readily to the Ni^{2+} column. Out of the many cytoplasmic proteins (Figure 1A, lane 2) exposed to the resin, many did not bind (Figure 1A, lane 3) or were eluted by the washes at low imidazole concentrations (≤60 mM, Figure 1A, lanes 4 and 5). At 400 mM imidazole, the His-tagged NhaR eluted as a single prominent band (Figure 1A, lanes 6 and 7). 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 Ni^{2+} 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 Ni^{2+} 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 [^{35}S]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 ^{35}S-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.

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

Two promoters of *nha*A 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 *nha*A 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 *nha*A including the *nha*A 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 *nha*A sequences recognized by NhaR. We have therefore concluded that the NhaR-binding sites are located between bp –120 and 14 (also indicated on the *nha*A sequence in Figure 6A). In accordance with this

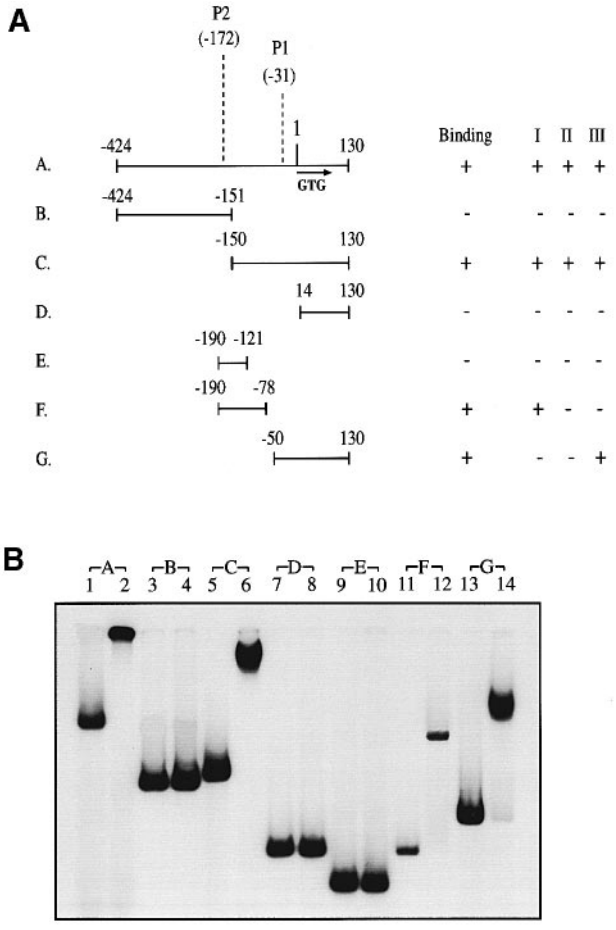

Fig. 2. Deletion mapping of the DNA region containing the *cis*-regulatory elements of *nha*A recognized by NhaR. (**A**) DNA fragments containing the *nha*A sequences marked at their ends by the number of base pairs from the first base of the initiation codon (=1) are shown (DDBJ/EMBL/GenBank accession Nos X17311, S67239 and J03897). (**B**) Each fragment on its own (odd numbers), or after 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 *nha*A 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.

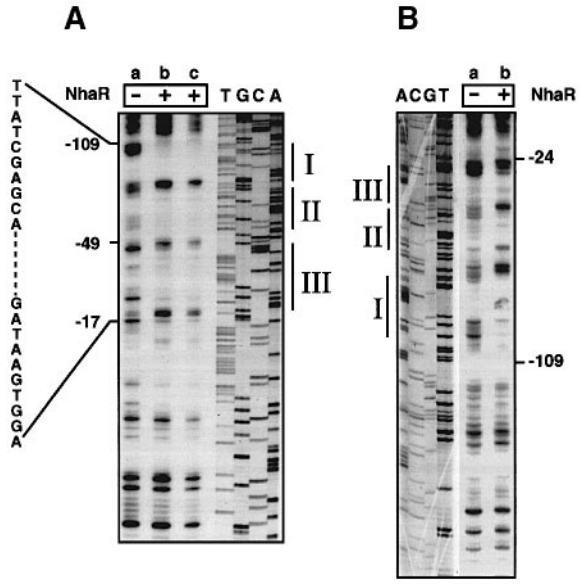

Fig. 3. DNase I protection footprint of NhaR. A DNA fragment (242 bp) end labeled with ^{32}P at the 3′ (bottom strand) in (**A**) or at the 5′ (top strand) in (**B**) were incubated with His-tagged NhaR (250 and 500 ng in lanes b and c, respectively, of A and 500 ng in lane b of B) as indicated and then cleaved with DNase I as described in Materials and methods. The DNase I-protected *nha*A 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).

conclusion, sequences between bp –424 and –191 did not bind but those between bp –424 and –78, –190 and 14, and –77 and 130 did (not shown).

The purified His-tagged NhaR was as active as NhaR in the gel retardation assay (not shown). Hence the purified His-tagged NhaR and the DNA fragments containing the NhaR-binding sites provide the essential tools needed for the study of the NhaR–*nha*A molecular interaction.

With the gel retardation assay, we have not found an effect of addition of Na^+ or K^+ (100 mM each) on the binding, either at pH 7 or at pH 8.5.

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

The sequences of *nha*A 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 *nha*A 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 the enzyme in the presence of NhaR (Figures 3A and B and 6A).

Addition of either Na^+ (up to 100 mM) or equimolar K^+ to the footprint reaction mixture had no effect on the footprint. Since Na^+ contaminants can be as high as 7 mM (Carmel *et al.*, 1994), it was considered that the system was already saturated with Na^+ and therefore, further addition of the ion was without effect. To exclude this possibility, the reaction mixture was purified by gel filtration, and the Na^+ concentration, as measured by atomic absorption, was reduced to 50 μM. Nevertheless, addition of Na^+ or K^+ (100 mM each) was still without effect on the footprint (not shown).

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).

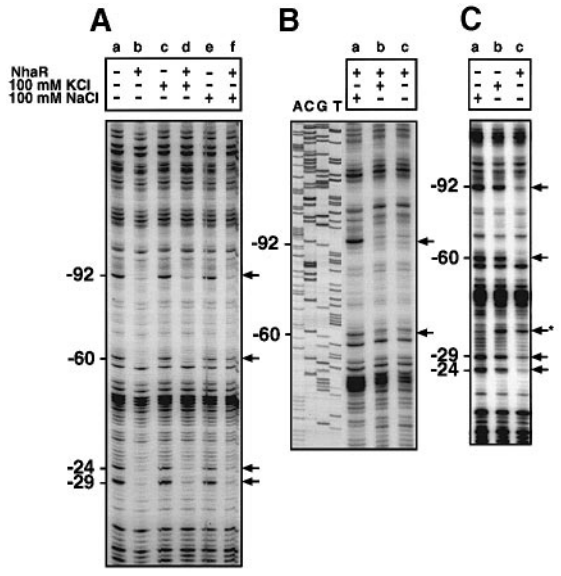

Fig. 4. DMS methylation protection by NhaR. In all panels, numbers on the left indicate the position of bases in the promoter region relative to the first base of the initiation codon (see also Figure 6A). (**A**) *In vitro*: DNA was incubated with His-tagged NhaR in the presence or absence of added KCl or NaCl as indicated in the figure, subjected to DMS methylation followed by piperidine cleavage and the products were analyzed by primer extension as described in Materials and methods. Arrows, identified bases contacting His-tagged NhaR. (**B** and **C**) *In vivo*: the cells used in (B) were HB101 transformed with pGM42T, a plasmid harboring all upstream sequences of an inactive *nha*A and wild-type *nha*R. The cells used in (C) were ORC100, a strain deleted of *nha*R and transformed with either pKR107 (lanes a and b), a plasmid harboring only the upstream sequences of *nha*A without *nha*R, or pGM42T, an *nha*R-bearing plasmid (lane c). The cells were grown in the presence of the inducer (100 mM Na^+) as indicated in the figure, exposed to DMS, plasmid DNA isolated and treated with piperidine and the resulting fragments were analyzed by primer extension as described in Materials and methods. Arrows, identified bases contacting NhaR; the starred arrow points to an unreproducible NhaR-independent modification.

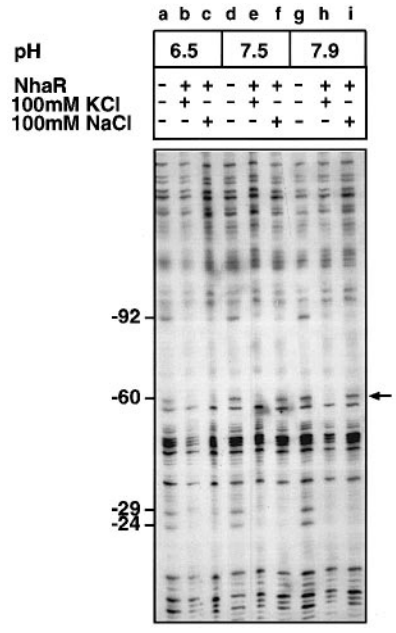

Fig. 5. Effect of pH on the *in vitro* methylation protection pattern. The DNA was incubated with His-tagged NhaR at the indicated pH obtained by titration of the binding buffer with HCl, otherwise the experimental system was as in Figure 4A.

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 absence or 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^+. There was no effect of Na^+ on the methylation reaction in the absence of NhaR (Figure 4A).

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 *nha*A. 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 *nha*R 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 Δ*nha*R strain (ORC100) and used it, either transformed or not, with plasmidic *nha*R to repeat the methylation protection assay (Figure 4C). In the Δ*nha*R 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 *nha*R 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 *nha*A expression by NhaR is induced specifically by a change in Na^+ concentration rather than by its outcome: a change in ionic strength or osmolarity (Karpel *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 *nha*A expression via NhaR, identified the regulatory *cis*-elements of *nha*A which bind NhaR and established both *in vivo* and *in vitro* that Na^+ changes the footprint of NhaR on *nha*A.

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 *nha*A sequences binding NhaR that are revealed by the gel retardation assay (Figure 2) align with the DNase I-protected sequences (Figures 3 and 6A).

Since the LysR regulatory proteins including NhaR each have only one helix–turn–helix motif in their N-terminus, through which binding to DNA is mediated, a single molecule is unlikely to span more than one helix turn. Hence, we suggest that similarly to other members of the LysR family, the His-tagged NhaR binds as a multimer in 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 *nha*A 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 *nha*A by NhaR align with these three motifs and show that the spanning sequences

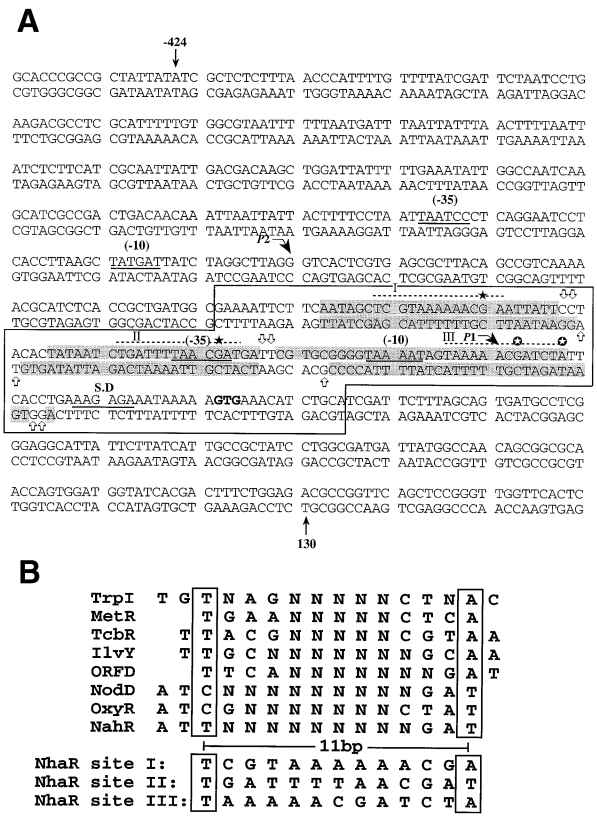

Fig. 6. The *nha*A sequence bound by NhaR is modified by Na^+. (**A**) The upstream DNA sequences (see Table I for accession No.) containing the *cis*-regulatory sequences of *nha*A are shown. The shortest fragment (bp –120 to 14) binding His-tagged NhaR in the gel retardation assay (Figure 2) is delimited. The shaded sequences show the His-tagged NhaR domain protected from DNase I digestion (Figure 3). G^{-92} and G^{-60} specifically affected by Na^+ in the DMS methylation assay, *in vivo* or both *in vivo* and *in vitro* respectively (Figure 4), are marked by dark stars. G^{-24} and A^{-29} protected by NhaR but not affected by Na^+ in the DMS methylation protection assay are indicated by open stars. Open vertical arrows show the DNase I-hypersensitive sites. Three sequential consensus motifs of the lysR family (Schell, 1993) designated I, II and III are shown by interrupted lines above the *nha*A sequence (see also B). Numbers in parentheses relate to the indicated promoters P_1 and P_2 of *nha*A. Other numbers relate to the first base (=1) of the initiation codon GTG, in bold, while its upstream neighboring base = –1. (**B**) The generic consensus sequence of the LysR family according to (Schell, 1993). The consensus sequences recognized by NhaR which appear sequentially three times in the NhaR-binding domain and are designated I, II and III (Figure 6A) are also shown.

separating them contain hypersensitive DNase I sites (Figures 3A and 6A). These spanning sequences separating the consensus motifs further corroborate 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^{-92} in I, G^{-60} in II and G^{-29} and A^{-24} 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 *nha*A as measured by the gel retardation assay. This result suggests that whether Na^+ is present or not, NhaR is constantly bound to the *nha*A 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 vivo* and *in vitro* 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 *nha*A 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 *nha*A 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 (Tupper *et al.*, 1994).

Similarly to other members of the LysR family, the long footprint of NhaR on *nha*A as revealed by the DNase I protection assay overlaps with P_1, one of the two promoters of *nha*A. The other, P_2, maps further upstream. Interestingly, we have found recently that P_1, but not P_2, is involved in the Na^+ induction of *nha*A (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 *nha*A footprint while KCl up to 100 mM has no effect.

Monitoring the expression of an *nha*A′–′*lac*Z fusion, we previously have found that the Na^+-specific NhaR-dependent induction of *nha*A 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 *nha*A 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 *nha*A, 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 *mel*BLid *nha*A^+ *nha*B^+ Δ*lac*ZY (Goldberg *et al.*, 1987). OR100 contains Δ*nha*R2::*kan* ($NhaR^-$, Kan^R) but is otherwise isogenic to TA15 (Rahav-Manor, 1992). RK33Z is Δ*nha*A3::*kan* Φ (*nha*A::*lac*Z)1(Hyb) thr-1 and otherwise isogenic to TA15 (Karpel *et al.*, 1991). HB101 is F^-Δ(*gpt-pro*A)62 *leu*B6 *sup*E44 *ara*14 *gal*K2 *lac*Y1 Δ(*mcr*C-*mrr*) *rps*L20(Str^r) *xyl*-5 *mtl*-1 *rec*A13 *hsd*S20($r_B^-m_B^-$). BL21 is an *E.coli* B F^-*dcm ompT hsd*S($r_B^-m_B^-$)*gal*. LE392 is e14$^-$(McrA$^-$)*hsd*R514 *sup*E44 *sup*F58 *lac*Y1 or Δ(*lac*IZY)6 *gal*K2 *gal*T22 *met*B1 *trp*R55. ORC100 is a LE392 derivative containing Δ*nha*R2::*kan*. This *nha*R deletion was constructed by P1 transduction using OR100 as a donor and LE392 as an acceptor, selecting for Kan^R colonies. One of these transductants was isolated, designated ORC100 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 *nha*A and *nha*R (Karpel *et al.*, 1988). pGM42T is a derivative of pGM42 inactivated in *nha*A (Rahav-Manor, 1992). pDT2 is a plasmid in which *nha*R 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 *nha*A (Goldberg *et al.*, 1987). Plasmids encoding His-tagged NhaR derivatives are pET20b(+) (Novagen, USA) derivatives as described below. pKR107 carries the upstream sequences of *nha*A (Karpel, 1990). It was constructed by cloning the 1.4 kb *Bam*HI–*Bgl*II fragment of pGM36 into the *Bam*HI 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 tandem flanked at the 5′ and 3′ ends by *Not*I and *Xho*I restriction sites respectively was generated by annealing two complementary single-stranded DNA primers 49 and 50 (Table I). The *Not*I–*Xho*I fragment was then cloned between these restriction sites of the polylinker in pET20b(+). The sequence of the cloned fragment in the recombinant plasmid designated pET20Xb(+) was verified by DNA sequencing through the ligation sites. For construction of pOCRXH, a DNA fragment (1206 bp) bearing *nha*R 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 *Not*I restriction site. The fragment was digested with *Bam*HI, end filled and then digested with *Not*I. It was then ligated with the 3584 bp fragment of pET20Xb(+), produced by digestion with *Xba*I, end filling and subsequent digestion with *Not*I. In this recombinant plasmid designated pOCRXH, *nha*R is placed under control of the T7 RNA polymerase promoter and fused in-

Table I. DNA primers used in this study

Primer	Sequence	Location
8	ATCGCTCTCTTTAACCCA	(–424)–(–407)[a]
28	CATCGCCGACTGATCACAAATTAAT	(–260)–(–237)[a]
46	ATGATTATCTAGGCTTAGGGTCACT	(–190)–(–166)[b]
57	CCGTCAAAAACGCATCTCACCGCTG	(–150)–(–125)[b]
58	TATAATCTGATTTTAACGATGATT	(–77)–(–54)[b]
60	GCGGGGTAAAATAGTAAAAACGATCTATTCACCTG	(–50)–(–16)[b]
53	AGCTTAAGGTGAGGATTCCTGAGGG	(–191)–(–215)[b]
47	CTGTAAGCGCTCACGAGTGACCCTA	(–151)–(–175)[b]
59	GCCATCAGCGGTGAGATGCGT	(–121)–(–141)[b]
7	GTGTAGGAATAATTCG	(–78)–(–93)[b]
42	GAATAATGCCTCCCGAG	(52)–(36)[b]
110	TCTCCAGAAAGTCGTGATACCATC	(130)–(107)[c]
91	AACTGGCGCGTCTGCCTG	(941)–(958) [c]
51	TTTTCCTTTTGCGGCCGCTGA[898]ACGCACCGCTGGACTAAAAAGCG[875]	(898)–(875)[d]
49	AAGGAAAAAAGCGGCCGCAATCGAAGGGCGTATCGAAGGTCGTCTCGAGCGG	
50	CCGCTCGAGACGACCTTCGATACGCCCTTCGATTGCGGCCGCTTTTTTCCTT	

DDBJ/EMBL/GenBank accession numbers are: [a]X17311, [b]S67239, [c]J03897 for *nha*A and [d]L24072 for *nha*R. Location numbers are relative to the first GTG codon (*nha*A) or the first ATG codon (*nha*R).

frame downstream with a sequence encoding two factor Xa cleavage sites followed by six histidines.

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.*, 1994) to OD_{600} = 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 on a Ni^{2+}-NTA–agarose column (Qiagen, Hilden, Germany and Figure 1A). The frozen cells, resuspended in 15 ml of binding buffer containing 4 mM imidazole (pH 7.9), 500 mM KCl, 20 mM Tris–HCl (pH 7.9) and 5 mM β-mercaptoethanol (BME) were lysed by three passages through a French pressure cell (20 000 p.s.i., Model SLM-Aminco FA-078, 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), 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 KCl, 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

RK33Z 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 (Karpel *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 pGP1-2 in TA15 cells (1L) and cell-free and membrane-free extract prepared as described (Carmel *et al.*, 1994). For specific labeling of NhaR with [^{35}S]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 $MgCl_2$. To follow NhaR during the purification steps, the cytoplasmic fraction containing the overexpressed unlabeled NhaR was mixed with the ^{35}S-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 pGM36 as a template and various *nha*A 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 pg of ^{32}P-end-labeled DNA probe.

DNase I footprinting

The *nha*A 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 *nha*A coding for the N-terminus of NhaA as well as 190 bp upstream of *nha*A. For the DNase footprinting, a ^{32}P-end-labeled probe was incubated with 0.5 µg of purified protein and then digested with DNase I as described (Tartaglia *et al.*, 1989).

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 ^{32}P-end-labeled primer (0.3 pmol).

For the *in vitro* footprinting by methylation protection assay, DNA (300 ng linear or supercoiled) was incubated for 30 min at 37°C with 5 µg of His-tagged NhaR in 50 µl of the binding buffer used in the gel retardation assay. DMS (10 mM) was added, incubation continued for an additional 5 min at 37°C and the reaction stopped by adding 100 µl of stop buffer containing 3 M ammonium acetate, 100 mM BME, 20 mM EDTA (pH 8.0) and 1 µg/ml yeast tRNA. Methylated DNA was ethanol precipitated, dried, resuspended in 100 µl of 1 M piperidine and processed further as for *in vivo* footprinting.

Quantitation of proteins and [Na^+]

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

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