In Vivo Interactions of the NahR Transcriptional Activator with Its Target Sequences

INDUCER-MEDIATED CHANGES RESULTING IN TRANSCRIPTION ACTIVATION*

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The nahR gene from the NAH7 naphthalene degradation plasmid encodes a LysR-type transcriptional activator of the nah and sal promoters (Pnah and Psal, respectively) that responds to the inducer salicylate. In vivo methylation protection experiments with dimethyl sulfate showed that in the absence of inducer, NahR interacts in a similar manner with its target sites at Psal and Pnah. Both target sites also have several similar sequences comprised of a 4-base pair interrupted dyad containing two symmetrical guanines (−73 and −64 of Psal, −71 and −62 of Pnah), each located in adjacent major grooves on the same helical face, and both strongly protected by NahR. When inducer was present, several additional guanines of Pnah (−35, −45, and −58) and Psal (−42 and −40) became protected from methylation, while a guanine at −52 of Pnah became markedly enhanced for methylation, indicating that inducer and NahR-dependent interactions with these downstream sites of each promoter are quite different. Deletion of Pnah sequences downstream of −30 did not affect its methylation patterns suggesting that NahR alone is responsible for the altered reactivities of these nucleotides. Similar in vivo methylation analyses with inducer-insensitive or inducer-independent NahR mutants also suggested that all alterations in methylation sensitivity are directly caused by NahR. It is more probable that the salicylate-induced reactivity changes result from direct NahR-guanine contacts, which are required for, but not sufficient for transcription activation; however, they could also result from NahR-induced DNA contortions caused by upstream protein-DNA contacts.

NahR coordinately regulates expression of the two naphthalene degradation operons of plasmid NAH7. In the natural host Pseudomonas putida, or the heterologous host, Escherichia coli, NahR activates transcription from Pnah or Psal (Fig. 1) over 20-fold in response to the inducer salicylate (1−5). NahR is one of the many positive regulatory proteins in the LysR family (6, 7). LysR-type transcriptional activators regulate genes encoding a wide variety of metabolic pathways and are found in diverse prokaryotes (6−10, 14). Regulated systems include genes encoding for degradation of aromatic hydrocarbons (e.g. CleR, CatR, TfdS, and NahR), amino acid biosynthesis (e.g. IlvY, TrpI, and LysR), oxidative-stress response (OxyR), and synthesis of tetrasaccharide signal molecules involved in host-specific initiation of nitrogen fixation symbiosis of Rhizobium (NodD, SyrM) (7−10, 14). LysR family members are all very similar in size (300 ± 20 residues) and show extensive amino acid similarity in the N-terminal thirds of their polypeptides. Moreover, most of these activators are transcribed from divergent promoters that overlap with a promoter of one set of genes they regulate and show evidence of autoregulation. Biochemical and DNA sequence analysis of various types of mutants of several LysR-type activators (NahR, NodD, and OxyR (11−14)) has suggested a similar organization of the structure/function domains on these proteins. The bulk of evidence suggests that the N-terminal regions contain a DNA-binding domain partially comprised of a helix-turn-helix motif (11, 15). However, the C-terminal regions are also involved in DNA binding and transcription activation (11, 13). The central portion of the activators is involved in recognition of inducer and/or the response to the inducer that results in transcriptional activation. The region between residues 195 and 205 is particularly important, since many mutations in this region of NahR, NodD, and OxyR cause loss of response to inducer, but not DNA binding (11−14).

In vitro analysis of the interaction of several purified LysR-type activators with DNA fragments containing their respective regulated promoters also suggests that LysR-type activators may utilize very similar mechanisms of action since, in the absence of the in vitro inducer, each binds to highly conserved sequences located between −85 and −45 of each promoter (16−21). However, these sequences do not display obvious evidence of a common sequence or recognition pattern. The apparent affinity of IlvY and NahR for their respective target sites was increased only 2-fold by inducer (17, 40). This led to the proposal that the role of inducer is not to control binding of the activator to its target sites, but rather to induce a conformational change in the constitutively bound activator, which results in increased transcription from that promoter (11). Recent experiments with TrpI, another LysR-type activator, suggest an alternative possibility (9, 19). Hydroxyl radical footprinting showed that its inducer causes: 1) extension of TrpI protection of its target promoter from the −77 to −52 region (protected in the absence of inducer) down into the −32 region, 2) a 14-fold increase in target site affinity, and 3) appearance of an additional higher molecular weight species in gel retardation assays. This was interpreted to mean that inducer causes binding of an additional molecule of TrpI adjacent to the one already bound at −77 to −52 resulting in transcription activation.

All these previous studies of the interactions of LysR-type

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‡ The abbreviations used are: Pnah, nah operon promoter; Psal, sal operon promoter; bp, base pair; kb, kilobase pair.

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activators with their cognate recognition sites were performed in vitro with protein preparations of varying purity and relatively low specific activity. Moreover, high affinity binding of inducer to any of the protein preparations of activator proteins has not been demonstrated, in spite of the fact that concentrations as low as 0.2 μM are effective inducers in vivo. This may be due to substantial inactivation or alteration of the activators during purification, or absence of specific conditions required for biological activity (e.g. supercoiling of target DNA). To circumvent such problems we have utilized in vivo methylation protection analysis (in vivo footprinting) to explore the specific interactions that occur between the guanine residues of Pnah and Pnah and NahR, and how these interactions change in response to inducer. Our results suggest that in both the presence and absence of inducer, NahR closely contacts two guanines in a 4-bp symmetrical interrupted dyad, part of a highly conserved 16-bp sequence found in both Pnah and Pnah. In the presence of the inducer, several additional guanines between −35 and −52 become strongly enhanced or inhibited from methylation in a NahR-dependent fashion, suggesting a conformational change in the NahR-DNA complex which results in transcription activation.

MATERIALS AND METHODS

Chemicals—Piperidine (99%) and dimethyl sulfate (99+%5) were purchased from Aldrich Chemical Co. Electrophoresis chemicals were from Bio-Rad. DNA enzymes were purchased from New England BioLabs; Sequenase 2.0 was from U. S. Biochemicals. [-32P]ATP (6000 Ci/mmol) was from Du Pont-New England Nuclear. Remaining chemicals were from Sigma and were of reagent grade purity.

Bacteria and Plasmids—Bacterial strains used were E. coli JM107 (23), E. coli N100 (24), and E. coli JM38 (23). Previously described mutant NahR plasmids (11) used and nature of substitutions causing these plasmids (containing the mutant NahR alleles) were cloned into pTZ18U (22) and the resultant plasmids designated pMZn, where n refers to the allele number of the original mutant plasmid (e.g., pMS30 into pM920, pMS253 into pMZ258, etc.).

Construction of Plasmids—Plasmids used in this study are diagrammed in Fig. 1 and were constructed as follows, pMS1315: a 1.1-kb SstI-EcoRV fragment from pSC3 was purified by electrophoresis, ligated with SstI-SmaI digested pMS13 (1), transformed into E. coli JM107, and ampicillin-resistant transformants with pMS1315 were isolated. pMS9:HindIII-digested pMS1315 DNA was ligated under dialysis conditions into the HindIII-EcoRI sites of pMS13, and ampicillin-resistant transformants with pMH9 were isolated, pSM10: an 860-bp HindIII-EcoRI fragment from pSR1 (2) was partially digested with SspI; the 140-bp fragment containing Pnah was isolated by electrophoresis, ligated into SmaI-HindIII digested pTZ18U, and transformed into E. coli JM107. A plasmid with the 140-bp insert was isolated, digested with SstI and EcoRI, the resultant 150-bp purified fragment ligated into SstI-EcoRI digested pMS15 (3), and transformed into E. coli JM107 to give pSM10. pRSC3: SstI-digested pRK415 (25) was ligated with SstI-digested pSC3 (2), transformed into E. coli JM107, followed by selection for ampicillin-sensitive and tetracycline-resistant white colonies on 5-bromo-4-chloro-3-indolyl β-D-galactoside indicator plates. pLGS140: the 2-kb SalI-NdeI fragment of pMG1 (16), which had been filled-in by treating with DNA polymerase I and DNTP's, was ligated with SalI-digested pLG339 (30) and transformed into E. coli N100; pLGS140 was obtained by selection/screening for transformants containing the 2-kb insert. Concentrations of antibiotics used to maintain and select plasmids were: kanamycin, 50 μg/ml; ampicillin, 100 μg/ml; tetracycline, 25 μg/ml. Techniques used to construct plasmids were described previously (1, 3, 26).

Preparation of In Vivo Methylated Plasmid DNA—Methylation of plasmid-containing E. coli cells was performed by a modification of the method of Miller and Malamy (27): 200 ml of cells were harvested at 37 °C in L broth (26) with the appropriate antibiotics from an OD of 0.05 to 0.60; where indicated, the inducer salicylate was added to 0.02% (w/v) when the OD reached 0.2. Cells were harvested at 10,000 × g for 5 min at 25 °C, resuspended in 2 ml of 37 °C L broth and 10 μl of dimethyl sulfate mixed in; after 1 min at 37 °C, methylated plasmid DNA was isolated by processing cells into 5 g of ice and 20 ml of 0.25 M EDTA, pH 8, 0.15 M NaCl. Cells were harvested at 10,000 × g for 5 min; washed once with 10 ml of iced cold 0.1 M Tris-HCl, pH 7.5, 1 mM EDTA, 50 mM NaCl, and frozen at −20 °C. Methylated plasmid DNA was isolated from frozen cells by phenol/chloroform extraction and ethanol precipitation of a cleared lysate (35,000 × g for 45 min) prepared by a modified Trition extraction method (28). DNA was treated with RNaseA (50 μg/ml) and heat-treated T1 RNase (20 units/ml) at 37 °C for 1 h, followed by extraction with phenol/chloroform, and ethanol precipitation prior to piperidine cleavage.

Analysis of In Vivo Methylated DNA—Sequences (5′ → 3′) of oligonucleotides used for primer extension of cleavage products of in vivo methylated DNA were: Pnah' OLS: TTTCTAGCTGTCCTGCT-GATGG; OL1: TCCATGGGGCCTCGGTGGTT; OL2: AAACAGCTATGACGAT; Pnah': OLS: TCGAGCTATTACATTACATG; OL1: TGAAGATTTTACTATGACGAT. All were synthesized on an Applied Biosystems 380A DNA synthesizer and high performance liquid chromatography purified. Primers (10 pmol) were labeled at the 5′ ends by incubation with 50 μl of (γ-32P)ATP and 10 units of T4 polynucleotide kinase, followed by purification on a Sephadex G-25 spin column in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 25 mM NaCl (20).

Methylated plasmid DNA was cleaved by incubation in 1 M piperidine for 30 min at 90 °C followed by evaporation of piperidine in a Spin Vac concentrator. After redissolving in water, primer extension was performed by a modification of the procedure of Gralla (29): 0.5 μg of cleaved DNA was mixed with 200,000 cpm of 5′ end-labeled primer in 18 μl and 2 μl of 40 mM NaOH was added; after denaturation at 80 °C for 2 min, samples were quick-chilled on ice, and neutralized with 2.5 μl of 0.5 M Tris-HCl, pH 7.2, 0.1 M MgSO4, 0.05 mM MgCl2. To anneal the primer, the sample was heated at 50 °C for 30 min, cooled, and then 1.5 μl of 0.1 M diithiothreitol and 2 μl of 5 mM DNTP solution was added. After addition of 2 units of Sequenase and incubation at 37 °C for 10 min, 9 μl of 4 mM ammonium acetate, 20 mM EDTA was added followed by 3 volumes of ethanol and storage at −80 °C for 30 min. Labeled DNA was recovered by centrifugation and analyzed by electrophoresis on 6% polyacrylamide DNA sequencing gels (39) and autoradiography with intensifying screens.

Isolation and Analysis of Partially Inducer-independent nah Mutants—pMS15 DNA was treated with hydroxylamine and transformed into E. coli N100 containing pLGS140 as described previously (11); pLGS140 carries the sal promoter sequences between −80 and +27 fused to the E. coli galK expression directed by the P galK fusion gene on pLGS140 in the presence of NahR. The N7 positions of guanines internal to the promoter regions of the nah and sal promoters were analyzed by primer extension to identify guanines in the promoter regions which showed altered reactivity with dimethyl sulfate due to primer extension to identify guanines in the promoter regions which showed altered reactivity with dimethyl sulfate due to

RESULTS

In Vivo Footprinting of the nah and sal Promoters—pMS1315 or pMH9 DNA was isolated from E. coli cells that had been treated with dimethyl sulfate and DNA then cleaved with piperidine. The resultant fragments were analyzed by primer extension to identify guanines in the promoter regions which showed altered reactivity with dimethyl sulfate due to primer extension to identify guanines in the promoter regions which showed altered reactivity with dimethyl sulfate due to

2 M. A. Schell, unpublished data.
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P_{nah}, and nahR; pMH9 is identical except that it lacks 750 bp of the nahR coding region (Fig. 1).

Analysis of the cleavage patterns (sites) in the P_{nah} region of methylated DNA from uninduced cells with pMS1313 (nahR') or pMH9 (nahR-) clearly shows that the guanines at -64 on the noncoding (top = T) strand (G-64T) and -73 on the coding (bottom = B) strand (G-73B) are strongly inhibited from methylation, only when the functional nahR gene is present (Fig. 2A). In addition, nahR-specific protection of G-80B and G-66T from methylation, as well as an enhancement of methylation of G-82B were always detected. In Fig. 2A protection of G-66T is unclear, since it is poorly resolved from G-67T; in other gels it was very obviously protected. Other less-pronounced differences between the cleavage patterns of pMS1313 and pMH9 in the P_{nah} region can be seen in Fig. 2A, but these were not consistently observed.

A similar analysis of NahR-mediated methylation inhibition in the P_{nah} region was performed on DNA from cells grown with the inducer of transcription, salicylate. With this DNA the same extent of protection of G-73B, G-66T, and G-64T from reaction with dimethyl sulfate was again observed, indicating that the inducer did not dramatically change the interaction of NahR with these nucleotides of P_{nah}. However, the presence of inducer (and nahR) did cause several new downstream methylation protections in P_{nah}: G-58B, G-35B, and G-45T; moreover, G-52B and to a much smaller extent G-37T, showed increased methylation (cleavage) caused by the addition of salicylate.

An identical methylation protection analysis of P_{nah}, which is coordinately regulated with P_{nah} and also contains the divergent nahR promoter is shown in Fig. 2B. In the absence of inducer, strong inhibition of methylation of G-62T and G-71B are observed, as is enhanced methylation of G-78B. Much weaker, but reproducible, protection of G-65T was also observed. These altered reactivities with dimethyl sulfate are dependent on the presence of nahR, and are at positions very similar in location to those protected in P_{nah}. Likewise, the strength of these protections in P_{nah} was not altered by growth in the presence of salicylate. However, analysis of cleavage fragments derived from the P_{nah} region in salicylate-induced cells showed that salicylate (and nahR) caused protection of two new guanines of P_{nah}: G-40T and G-42T. In contrast to P_{nah}, no guanines appeared to become enhanced in methyla-

![Physical and genetic maps of plasmids used in methylation protection studies.](image-url)

**Fig. 1.** Physical and genetic maps of plasmids used in methylation protection studies. The upper portion shows plasmids in reference to pMS1313 and its relevant restriction endonuclease cleavage sites. Only relevant portions of NAH7 DNA on pRSC3, pMS15, pMZn, and pCHn are shown. The transcripts from P_{nah} (nahG), P_{nah} (nahA), and P_{nah} are indicated by dashed arrows. Nucleotide sequence (2) of the promoter regions (-90 to +1) of P_{nah} (dotted box) and P_{nah} (slashed box) as displayed on footprinting gels (Figs. 2–6) is shown below. Transcription start sites are at the extreme right; top strand of each sequence is the noncoding strand. The region of both promoters protected in vitro from DNase I digestion by NahR (16) is shown. The portion of P_{nah} deleted in pSM10 is marked by a dotted line.
tion due to the presence of salicylate and nahR. Thus, the positions of P_{sal} and P_{nah} protected from methylation by NahR in the absence of inducer are quite similar, while the positions showing altered reactivities in response to salicylate are different in location and response. The results of the methylation protection analysis of both P_{sal} and P_{nah} are summarized in Fig. 3.

In Vivo Footprinting of Activation-deficient NahR Proteins—We previously described mutant alleles of nahR (class II) encoding proteins capable of specific binding at wild type levels to P_{sal} or P_{nah} in vitro, but which were completely defective in salicylate-induced activation of their transcription in vivo (11). We proposed that these single amino acid substitution mutants were defective in either the binding of salicylate or in the transcription activation function resulting from the binding of inducer. To distinguish between these two possibilities and to gain further insight into the functional domains of NahR affected by these mutations, we examined the interaction of several class II mutant NahR proteins with P_{sal} (noncoding strand; top) and P_{nah} (coding strand; bottom) by in vivo footprinting (Fig. 4).

In the absence of inducer the in vivo interactions between the mutant NahR proteins and P_{sal} were largely the same as those observed with wild type NahR. The presence of wild type NahR (on pMS1313) or any of the mutant proteins encoded on pMZ151, pMZ256, pMZ60, pMZ61 (Fig. 4), or pMZ94, pMZ90, pMZ30, and pMZ258 (data not shown) failed to cause significant protection of G-65T and G-62T of P_{sal} from dimethyl sulfate methylation. This is consistent with previous measurements in vitro utilizing crude extracts containing NahR in a gel retardation assay (11). In contrast to wild type, when inducer was added, the mutant NahR proteins from most of the class II alleles (pMZ151, pMZ256: Fig. 4A; pMZ50, pMZ30, pMZ94, and pMZ258: data not shown) failed to cause significant protection of G-40T and G-47T of P_{sal}.

Since the positions and types of NahR/salicylate-induced alterations in dimethyl sulfate reactivities of guanines of P_{sal} and P_{nah} were so different, the interactions of the mutant NahR proteins encoded on pMZ60, pMZ61, and pMZ256 with P_{nah} were analyzed. Identical to wild type, in the absence of

![Image of Figure 2](image-url)

**Fig. 2.** Analysis of guanines of P_{sal/nahR} and P_{nah} protected from methylation *in vivo* by NahR. E. coli cells containing pMS1313 (NahR') or pMH9 (NahR) were grown in the presence (inducer +) or absence (inducer −) of 0.02% salicylate, exposed to dimethyl sulfate, and plasmid DNA isolated. DNA was cleaved at methylated guanines by piperidine treatment and the resultant fragments analyzed by primer extension with P_labeled oligonucleotides and separation on 6% denaturing polyacrylamide gels and autoradiography. Hollow arrows mark guanines protected from dimethyl sulfate methylation by NahR. Solid arrows identify guanines enhanced in dimethyl sulfate methylation by NahR. A, fragments derived from cleavage of guanines in the −99 to −2 region of P_{sal} were analyzed using OL9 for the top (noncoding) strand and OL10 for the bottom (coding) strand. B, fragments derived from cleavage of guanines in the −90 to +2 region of P_{sal/nahR} were analyzed using OL8 for the top strand and OL11 for the bottom strand. Numbers at the left indicate position of each guanine in the promoter regions (Fig. 1). In vitro methylation of pMH9 resulted in cleavage patterns identical to the in vivo pattern of pMH9 (not shown).
**Protein-DNA Interactions of NahR**

**FIG. 3.** Summary of in vivo footprinting experiments with wild type NahR protein. Shown are the DNA sequences (−84 to −30) of P_nah and P_sal/nahR. Nucleotides protected from methylation by dimethyl sulfate in both the presence and absence of the inducer salicylate are marked by dot-filled circles. Nucleotides protected by NahR only in the presence of inducer are marked by solid circles. Nucleotides enhanced in dimethyl sulfate methylation in both the presence and absence of inducer are marked by dotted squares. Those enhanced for methylation only in the presence of the inducer are marked by solid squares. More pronounced protections/enhancements are denoted by larger symbols. Homologous base pairs of the two promoters are underlined. Regions containing sequences with dyad symmetry are shaded. Position of mutations affecting transcription activation of P_sal (16) are marked by arrows. For both nah and sal, the top strands are the noncoding sequence. For nahR, the bottom strand of P_sal/nahR is the noncoding sequence.

**FIG. 4.** Analysis of guanines of P_sal/nahR and P_sal protected from methylation in vivo by activation-deficient (class II) NahR mutants. E. coli cells containing pMS1313 (NahR+), pMS15 (NahR+), pMH9 (NahR+), or a class II mutant (pMZ151, pMZ256, pMZ60, or pMZ61) were grown in the presence (inducer +) or absence (inducer −) of 0.02% salicylate. Plasmid DNA was isolated, cleaved, and analyzed by primer extension (OL8 for A and B; OL10 for C) as in Fig. 2. Cells used for panel C additionally contained P_sal on the compatible plasmid pRSC3. Cells used for the first and second lanes in panel C contained only pRSC3 and no NahR containing plasmid.
inducer, G-73B of P\textsubscript{nah} was protected from \textit{in vivo} methylation by the presence of all class II nah\textsubscript{R} mutants tested (Fig. 4C). Addition of salicylate to cells containing pMZ256 or pMZ21 did not cause any of the major changes in reactivity to dimethyl sulfate observed with the wild type allele; pMZ260-containing cells also showed no salicylate-induced protections, but did show a small (<20% of wild type) enhancement of G-52B. For this analysis of P\textsubscript{nah} (Fig. 4C) it was necessary to utilize a binary plasmid system where the mutant nah\textsubscript{R} alleles were in high copy number, while the P\textsubscript{nah} target site was at lower copy number (pRSC3 using a pRK404 vector); in all other analyses nah\textsubscript{R} and its target sites were on the same plasmids. This difference should be considered in quantitative comparisons of these data. Nonetheless, with the exception of pMZ61 and pMZ60, nearly all the class II Nah\textsubscript{R} mutants were ineffective in making the strong salicylate-induced promoter interactions observed with wild type Nah\textsubscript{R}, while their ability to make the uninduced interactions was unaffected.

Construction and Analysis of Inducer-independent Nah\textsubscript{R} Mutants—Experiments described above established a strong correlation between the presence of the inducer-dependent methylation protections or enhancements at G-55B, G-52B, and G-58B for P\textsubscript{nah} and G-40T, and G-42T for P\textsubscript{nah}, and transcription activation caused by Nah\textsubscript{R} and salicylate. All nah\textsubscript{R} alleles which did not produce these protections were similarly defective in salicylate-induced transcription activation. Positive correlation of these altered reactivities with salicylate-induced transcription activation was provided by methylation protection analysis of the interaction of partially inducer-independent Nah\textsubscript{R} mutants with P\textsubscript{nah} and P\textsubscript{nah}\textsubscript{a}. These nah\textsubscript{R} mutants could activate transcription from P\textsubscript{nah} by 10- to 20-fold independent of any added inducer, as indicated by the increase in expression of galK (galactokinase) when placed in \textit{E. coli} cells containing of P\textsubscript{nah}-galK fusion plasmid pLGS140; addition of inducer caused an additional 3- to 6-fold increase in transcription from P\textsubscript{nah} (Table 1). These Nah\textsubscript{R} mutants were isolated as described under “Materials and Methods”; DNA sequence analysis showed that each mutant allele contained a single nucleotide change resulting in a single amino acid substitution in the Nah\textsubscript{R} reading frame (Table 1).

In \textit{vivo} methylation protection analysis of the interactions of the partially inducer-independent Nah\textsubscript{R} mutants encoded on pCH5 and pCH10 with P\textsubscript{nah} showed that in the absence of salicylate they caused protection of G-65T and G-62T to the same extent as wild type nah\textsubscript{R} (Fig. 5A). However, under the same noninducing conditions these mutant Nah\textsubscript{R} proteins also caused protection of G-42T and G-40T, to nearly the same extent observed with wild type nah\textsubscript{R} only when inducer was present. Addition of inducer caused these protections (G-42T and G-40T) to increase to wild type induced levels. Three partially inducer-independent alleles (pCH4, pCH2, and pCH6) also caused protection of G-40T and G-42T in the absence of inducer, but to a lesser extent than with P\textsubscript{nah} (data not shown). Analysis of the effect of several of these alleles (pCH5, Fig. 5B; pCH4 and pCH10, not shown) or methylation of P\textsubscript{nah} again showed that even without inducer these mutants caused the same protections (G-35B and G-58B) and enhancements (G-52B) observed with wild type nah\textsubscript{R} only when inducer was present. As expected, G-73B was protected by both wild type and mutants in both the presence and absence of inducer. Thus the Nah\textsubscript{R} proteins which partially activate transcription in the absence of inducer all cause protection or enhancement of the same N7’s in P\textsubscript{nah} and P\textsubscript{nah}\textsubscript{a} that are affected by wild type Nah\textsubscript{R} only when inducer was present, providing further correlation between the interaction of Nah\textsubscript{R} with G-40T and G-42T of P\textsubscript{nah} and G-35B and G-58B of P\textsubscript{nah}\textsubscript{a} and its ability to activate transcription.

**Evidence that Nah\textsubscript{R} Is Directly Responsible for Inducer-dependent Protections**—The salicylate-inducible protections were absolutely dependent on Nah\textsubscript{R} and thus it is likely that they are caused by actual contact or interactions of Nah\textsubscript{R} with these nucleotides. However, we cannot a priori rule out the possibility that the salicylate-induced protections (especially at G-40T of P\textsubscript{nah} and G-35B of P\textsubscript{nah}\textsubscript{a}) result from induced binding of RNA polymerase. This is less likely since addition of rifampicin prior to dimethyl sulfate treatment did not qualitatively or quantitatively alter the methylation protection patterns in any way (data not shown). Furthermore, the protections expected to result from RNA polymerase binding, especially near -10 (31), were not observed.

Further support for the conclusion that the salicylate-dependent protections result directly from Nah\textsubscript{R} contact comes from methylation protection analysis of pSM10, which is identical to pMS15 (Fig. 1), but lacks the P\textsubscript{nah} sequences between -28 and +27. When this promoter was fused to the \textit{E. coli} galK gene no salicylate-induced activation of expression was observed when nah\textsubscript{R} was placed in trans, indicating that the deleted promoter does not undergo increased transcription (binding) by RNA polymerase caused by Nah\textsubscript{R} and salicylate. The \textit{in vivo} methylation protection pattern of this deleted promoter, however, was nearly identical to that of wild type P\textsubscript{nah} (Fig. 6). Most importantly, the salicylate-induced protections of G-40T and G-42T are clearly observed in spite of the apparent lack of increased RNA polymerase binding or interactions. Thus, these protections occur in the absence of transcription activation and probably result from intimate association of Nah\textsubscript{R} with these nucleotides rather than RNA polymerase interactions.

**DISCUSSION**

Analysis of methylation of the guanines of P\textsubscript{nah} and P\textsubscript{nah}\textsubscript{a} as affected by Nah\textsubscript{R} and salicylate, was used to probe the \textit{in vivo} changes of the interactions of this transcriptional activator with its target sites in response to inducer. This analysis and previous \textit{in vitro} studies show that in the absence of inducer, Nah\textsubscript{R} binds to and protects a homologous interrupted dyadic sequence ([TTCAnnnnnnTGAT] found near -73 to -60 of both its target promoters (Fig. 3). The results of methylation protection studies strongly suggest that Nah\textsubscript{R} protein directly interacts with (contacts) the N7 positions of two symmetrically located guanines at positions 3 and 12 of this dyad (underlined above), and lying in adjacent major grooves on

**Table 1**

| Plasmid | Amino acid change | Salicylate activation of GalK* |
|---------|-------------------|-------------------------------|
| pMS15   | Wild type         | 0.12 8.2                      |
| pMC250  | Arg-21 Stop       | 0.10 0.13                     |
| pCH2    | Ala-231 \rightarrow Val | 0.92 7.6                   |
| pCH4    | Leu-154 Phe       | 2.72 14.0                     |
| pCH5    | Met-116 Ile       | 1.45 4.9                      |
| pCH6    | Ala-231 \rightarrow Val | 1.45 5.1                   |
| pCH10   | Cys-252 Tyr       | 1.20 7.2                      |

*Specific activity (nanomole of galactose phosphorylated min⁻¹ mg⁻¹ protein) × 10⁻⁶ of galactokinase in \textit{E. coli} N100 (pLGS140) cells containing the indicated plasmids grown with (I) or without (NI) the inducer salicylate.
Analysis of guanines of \( P_{\text{sal}} \) and \( P_{\text{nadh}} \) protected from methylation in vivo by inducer-independent NahR mutants. E. coli cells containing pMH9 (NahR⁺), pMS1313 (NahR⁺), pMS15 (NahR⁺), or a NahR mutant (pCH5, pCH10) were grown in the presence (inducer +) or absence (inducer −) of salicylate, treated with dimethyl sulfate, and analyzed as described in the legends to Figs. 2 and 4. For A OL8 was used; for B OL10 was used and cells additionally contained \( P_{\text{nadh}} \) on pRSC3.

**Fig. 5.** Protein-DNA Interactions of NahR

A. \( P_{\text{sal}} \) (Top)  

| Inducer | MS1313 | CH5  | CH10 |  
|---------|--------|------|------|  
| +       | -      | -    | -    |  
| -       | +      | +    | +    |  

Bottom

| Inducer | MS1313 | CH5  |  
|---------|--------|------|  
| +       | -      |      |  
| -       | +      |      |  

B. \( P_{\text{nadh}} \) (Bottom)  

| Inducer | MS15 | CH5  |  
|---------|------|------|  
| +       | -    |      |  
| -       | +    |      |  

**Fig. 6.** Methylation protection of partially deleted sal promoter. E. coli cells containing the indicated plasmids were grown with (inducer +) or without (inducer −) salicylate, treated with dimethyl sulfate, and the methylation pattern analyzed as described in the legend to Fig. 2. OL10 was used for the top strand; OL11 was used for the bottom strand. Solid arrows: enhanced methylation; hollow arrows: decreased methylation.

the same helical face. It is likely that this sequence is the primary recognition/binding site of NahR. This hypothesis is supported by the observation that substitution mutations at positions −73 or −74 of \( P_{\text{sal}} \) (Fig. 3) result in loss of NahR binding (16). Consistent with previous in vitro experiments with NahR (16, 38), as well as other LysR-type activators (14, 17, 19, 21, 33), in vivo binding/recognition at this site occurs independently of inducer.

The binding sites of several other LysR activators show a similar organization and methylation protection pattern to that of NahR. The IlvY target site (−71 to −60: (TGCAnnnnnTGCA) also contains an interrupted dyad with symmetrically located guanines in adjacent major grooves on the same helical face that are also strongly protected from dimethyl sulfate methylation in vitro by IlvY (17). Other interrupted dyads with symmetrically guanines are also found in the upstream regions of nearly all promoters regulated by NodD (CATnnnnnnATG) (18) or AmpR (GTTnnnnnAAC) (34), two other LysR-type activators. While these sequences are located at nearly identical positions (−73 to −62) and are protected from DNase I by their activators, methylation protection studies of these promoters have not yet been reported. Thus, if such sequences represent a common binding and recognition motif of LysR-type activators remains to be seen.

Addition of inducer produced a very strong new protection of the N7 of G-35R of \( P_{\text{nadh}} \) and G40T of \( P_{\text{sal}} \) (Fig. 3) without altering the upstream contacts occurring in the absence of inducer; weaker NahR-dependent protections caused by salicylate occurred in \( P_{\text{sal}} \) (G-42T) and \( P_{\text{nadh}} \) (G-45T and G-58B). It is likely that these protections result directly from new interactions (possibly contacts) between NahR protein and the N7 of these guanines and not from RNA polymerase, since rifampicin (37) did not affect any methylation patterns, and no changes in dimethyl sulfate reactivities of nucleotides in the −30 to +27 RNA polymerase-binding region were observed (31). Moreover deletion of the \( P_{\text{sal}} \) sequences between −28 and +27, which completely eliminated salicylate-NahR-stimulated RNA polymerase interactions at \( P_{\text{sal}} \), as evidenced by loss of its transcription activation, had no effect on the methylation protection patterns. While dimethyl sulfate protection is suggestive of direct protein-DNA contacts, without crystallographic data indirect effects (e.g. contortions) from a distant site cannot be ruled out.

The upstream NahR recognition sequences in \( P_{\text{nadh}} \) or \( P_{\text{sal}} \) and the location of NahR-contacted nucleotides within each site are quite similar suggesting that in the absence of inducer
the interactions of NahR with both recognition sequences are largely the same. However, the locations of nucleotides and types of salicylate-induced changes in dimethyl sulfate reactivity at Pnah and Psal are very different. In Psal, only two nearly-adjacent guanines (G-42T and G-40T) showed inducer-dependent changes in dimethyl sulfate reactivity, whereas in Pnah, 5 nucleotides extending over a 24-bp region (G-55B to G-35B) showed altered reactivity in response to salicylate. Nonetheless, the presence of these salicylate-dependent contacts always correlated with the levels of transcription activation. The differences between Pnah and Psal may be caused by the presence of the divergent nahR promoter overlapping Psal; simultaneous transcription and autoregulation of Pnah may constrain or affect interactions of NahR with this region.

One particularly striking difference is the absence from Psal of the strong inducer-dependent enhancement of G-52B of Pnah. This region of Psal contains only adenine and thymine; however, even under conditions designed to enhance detection of altered reactivity of adenines to dimethyl sulfate (37, 39), we did not see any methylation changes near or at −52 of Psal. The increased reactivity of G-52B of Pnah could signify a conformational change in the DNA caused by NahR + salicylate or RNA polymerase binding. Alternatively, it could be caused by a salicylate-induced conformational change in NahR resulting in localized concentration of dimethyl sulfate (32) near G-52B. Increased reactivity of a similarly located guanine in an IlvY-regulated promoter was also observed, and as with NahR, only under conditions of transcription activation (17). NodD has also been reported to cause DNase I hypersensitivity at the −56 position of one of its regulated promoters; the precise cause and meaning of these enhanced reactivities produced by LysR-type activators awaits further clarification.

Analysis of activation-deficient (inducer-insensitive) and inducer-independent NahR mutants showed that in the absence of inducer all these mutant proteins interacted with nucleotides in the upstream recognition sites of Pnah and Psal in a manner identical to wild type NahR. However, interactions with the downstream regions (−35 to −52) usually correlated with their ability to activate transcription. Activation deficient NahRs failed to make these contacts, even in the presence of inducer, while inducer-independent NahRs made the contacts irrespective of inducer. The pMZ60 mutant allele caused weak but significant salicylate-induced alterations of both Pnah and Psal, yet was completely deficient in causing transcription activation; while the pMZ61 allele caused salicylate-induced changes only at Psal. This suggests that the indicated structural changes in the promoter (which with wild type occur only in the presence of inducer) do not result from transcription activation, but rather may be a prerequisite for it.

Results with the pMZ60-encoded NahR suggested that it could bind and partially respond to salicylate by weakly making some of the promoter alterations required for transcription activation (Fig. 4), implying that this mutation may effect a region of NahR directly involved in transcription activation rather than inducer binding. The mutation causes a Gly-203 → Asp substitution in a region of NahR that is a hotspot for mutations affecting transcription activation but not DNA binding (at residues 201, 204, and 206; Ref. 11 and Table I). Substitution mutations resulting in loss of transcription activation have also been found in the exact same region of the NodD (13) and OxyR (14, 20) activators, further suggesting that this is a very important functional domain of LysR-type transcription activators. It is likely that all of our activation-deficient NahR mutants (except pMZ60 and pMZ61) have amino acid substitutions which prevent the salicylate-induced conformational change required to make contacts required for activation, while partially inducer-independent NahRs are probably caused by substitutions which change NahR in such a way as to mimic the effect of inducer binding.

While it is clear that inducer does not control binding of NahR to its recognition site, and that occupancy of this site alone does not cause transcription activation, inducer does control formation of the NahR contacts near the −35 region which are apparently required for transcription activation. However, whether these induced contacts result from binding of an additional molecule(s) of NahR adjacent to the one bound at the upstream recognition site or from a salicylate-induced conformational change in the already bound NahR causing extension of these contacts into the downstream region cannot be ascertained from this data. It has been proposed that two other activators, AraC (35, 36) and TrpI (9, 19), use the former type of mechanism (i.e. binding of an additional molecule downstream). The fact that the sequences of the upstream and downstream contact sites for NahR are so different favors the conformational changes hypothesis, as does the absence of multiple retarded species in gel shift assays with NahR and its promoter fragments. The salicylate-induced altered reactivities in the downstream regions of Pnah and Psal may be directly caused by NahR-guanine contacts. However, it is also possible that they result from DNA conformations induced by NahR-protein-DNA contacts upstream. Further biochemical analysis is needed to define the mechanism by which salicylate causes NahR to make new contacts, how these contacts result in activation of transcription by RNA polymerase, and whether other LysR-type activators employ similar mechanisms.

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