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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 (P_{nah} and P_{sal}, 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 P_{sal} and P_{nah}. Both target sites also have very similar sequences comprised of a 4-base pair interrupted dyad containing two symmetrical guanines (−73 and −64 of P_{nah}; −71 and −62 of P_{sal}), 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 P_{nah} (−35, −45, and −58) and P_{sal} (−42 and −40) became protected from methylation, while a guanine at −52 of P_{nah} became markedly enhanced for methylation, indicating that inducer and NahR-dependent interactions with these downstream sites of each promoter are quite different. Deletion of P_{sal} 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 P_{nah}[1] or P_{sal} (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.* ClcR, 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 vivo* 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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[1] The abbreviations used are: P_{nah}, *nah* operon promoter; P_{sal}, *sal* operon promoter; bp, base pair; kb, kilobase pair.

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 these 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*.[2] 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 P_{sal} and P_{nah} 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 P_{sal} and P_{nah}. 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+%) 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. [γ^{32}-P]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* JM83 (23). Previously described mutant NahR plasmids (11) used and nature of substitutions causing their phenotype were: pMAS30 (Thr-130 → Met); pMAS258 (Gly-107 → Asp); pMAS256 (Cys-253 → Tyr); pMAS151 (Thr-204 → Ile); pMAS60 (Gly-203 → Asp); pMAS61 (Pro-35 → Ser); pMAS94 (Pro-227 → Ser); pMAS50 (His-206 → Tyr); pMG250 (Arg-21 → Stop). Before use in footprinting experiments the 3-kb *Pst*I fragments from 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.* pMAS30 → pMZ30, pMAS258 → pMZ258, etc.).

Construction of Plasmids—Plasmids used in this study are diagrammed in Fig. 1 and were constructed as follows. pMS1313: a 1.1-kb *Pst*I-*Eco*RV fragment from pSC3 was purified by electroelution, ligated with *Pst*I-*Sma*I digested pMS13 (1), transformed into *E. coli* JM107, and ampicillin-resistant transformants with pMS1313 were isolated. pMH9:*Hin*dIII-digested pMS1313 DNA was religated under dilute conditions, transformed into *E. coli* JM83, and ampicillin-resistant transformants with pMH9 were isolated. pSM10: an 800-bp *Hin*dIII-*Eco*RI fragment from pSR1 (2) was partially digested with *Ssp*I; the 140-bp fragment containing P_{sal} was isolated by electroelution, ligated into *Sma*I-*Hin*dIII digested pTZ18U, and transformed into *E. coli* JM107. A plasmid with the 140-bp insert was isolated, digested with *Sal*I and *Eco*RI, the resultant 150-bp purified fragment ligated into *Sal*I-*Eco*RI-digested pMS15 (3), and transformed into *E. coli* JM107 to give pSM10. pRSC3: *Pst*I-digested pRK415 (25) was ligated with *Pst*I-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-indoyl β-D-galactoside indicator plates. pLGS140: the 2-kb *Sal*I-*Nde*I fragment of pMG1 (16), which had been filled-in by treating with DNA polymerase I and dNTP's, was ligated with *Pvu*II-digested pLG339 (30) and transformed into *E. coli* N100; pLGS140 was obtained by selection/screening for kanamycin-resistant plasmids with 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 grown at 37 °C in L broth (26) with the appropriate antibiotics from an OD_{600nm} of 0.05 to 0.60; where indicated, the inducer salicylate was added to 0.02% (w/v) when the OD_{600nm} 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 methylation was stopped by pouring the cells into centrifuge tubes containing 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 ice-cold 10 mM Tris-HCl, pH 8, 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 Triton extraction method (28). DNA was treated with boiled 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: P_{sal}: OL8 = TTTCATGCTGTACTCGTGATGG; OL11 = TCCATGGGGCCTCGCTTGGGTT; OLRP = AACAGCTATGACCATG; P_{nah}: OL10 = TCAGCCAGAAATACATGACTA; OL9 = TGAGAAGTTCCATAAATCCGGCT. 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 μCi (10 pmol) of [γ-^{32}P] ATP and 10 units of T4 polynucleotide kinase, followed by purification on a 1-ml Sephadex G-25 spin column in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 25 mM NaCl (26).

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 $MgSO_4$, 0.05 M $MgCl_2$. To anneal the primer, the sample was heated at 50 °C for 30 min, cooled, and then 1.5 μl of 0.1 M dithiothreitol 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 M 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 nahR 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 −90 and +27 fused to the *E. coli galK* gene on a low copy number pLG339 vector (30). Transformants were plated on MacConkeys galactose agar plates containing ampicillin and kanamycin and red colonies containing mutant plasmids (about 0.1% of total transformants) picked; colonies with wild type pMS15 were white. Plasmids with mutant *nahR* alleles were isolated from red-colored colonies designated pCHn, and further analyzed by sequencing their double stranded plasmid DNAs as described previously (11). To measure *galK* expression directed by the P_{sal}-*galK* fusion gene on pLGS140 in response to mutant *nahR* alleles and salicylate, galactokinase activity produced by transformants grown in the presence and absence of salicylate was determined as described previously (16).

RESULTS

In Vivo footprinting of the nah and sal Promoters—pMS1313 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 the presence of NahR. The N7 positions of guanines intimately associated with the NahR polypeptide should be reduced in sensitivity to dimethyl sulfate and subsequent cleavage by base; fragments resulting from alkylation/cleavage at such positions should be less evident or missing from the fragmentation ladder (29). Plasmid pMS1313 contains P_{sal},

[2] M. A. Schell, unpublished data.

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. 2*A*). 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. 2*A* 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. 2*A*, 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_{sal}, which is coordinately regulated with P_{nah} and also contains the divergent *nahR* promoter is shown in Fig. 2*B*. 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_{sal} was not altered by growth in the presence of salicylate. However, analysis of cleavage fragments derived from the P_{sal} region in salicylate-induced cells showed that salicylate (and *nahR*) caused protection of two new guanines of P_{sal}: G-40T and G-42T. In contrast to P_{nah}, no guanines appeared to become enhanced in methyla-

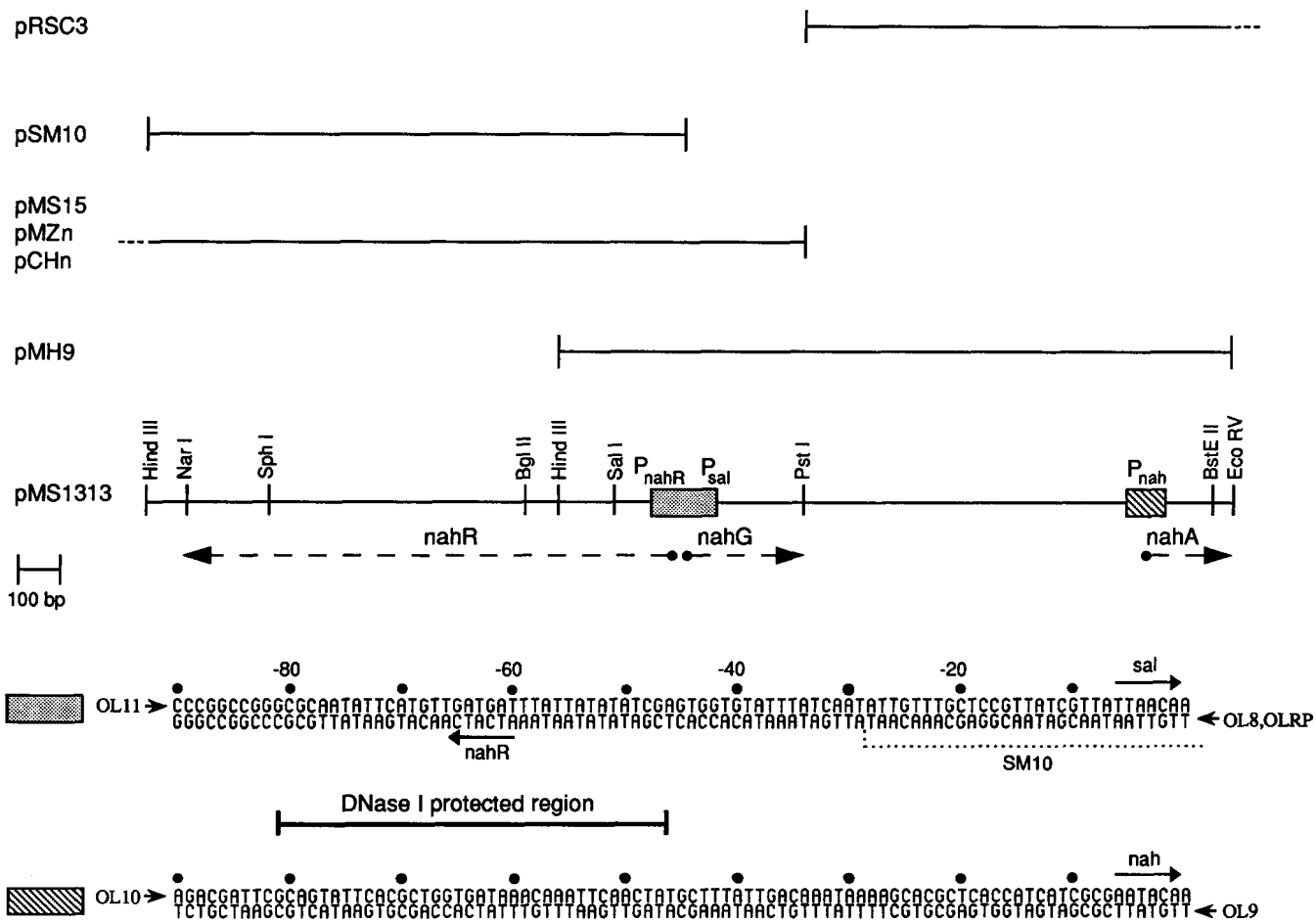

FIG. 1. **Physical and genetic maps of plasmids used in methylation protection studies.** The upper portion shows plasmids in reference to pMS1313 and its relevant restriction endonuclease cleavage sites. Only relevant portions of NAH7 DNA on pRSC3, pMS15, pMZn, and pCHn are shown. The transcripts from P_{sal} (*nahG*), P_{nah} (*nahA*), and P_{nahR} are indicated by *dashed arrows*. Nucleotide sequence (2) of the promoter regions (−90 to +1) of $P_{sal}/_{nahR}$ (*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 direction of primer extension with each oligonucleotide primer (OL8-OL11; OLRP) is designated by *solid arrows*. The region of both promoters protected *in vitro* from DNase I digestion by NahR (16) is shown. The portion of P_{sal} deleted in pSM10 is marked by a *dotted line*.

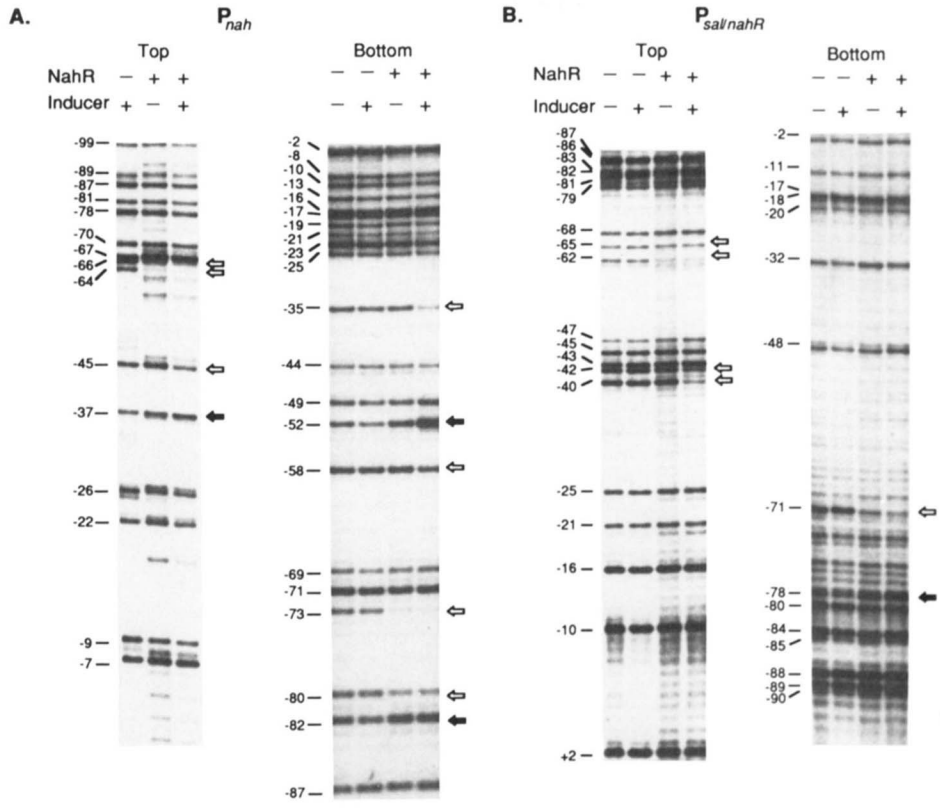

FIG. 2. **Analysis of guanines of $P_{sal/nahR}$ and P_{nah} protected from methylation *in vivo* by NahR.** *E. coli* cells containing pMS1313 (NahR$^+$) or pMH9 (NahR$^-$) were grown in the presence (inducer +) or absence (inducer −) of 0.02% salicylate, exposed to dimethyl sulfate, and plasmid DNA isolated. DNA was cleaved at methylated guanines by piperidine treatment and the resultant fragments analyzed by primer extension with ^{32}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_{nah} 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).

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, pMZ50, pMZ30, and pMZ258 (data not shown) resulted in identical levels of protection of both 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. 4*A*; pMZ50, pMZ30, pMZ94, and pMZ258: data not shown) failed to cause significant protection of G-42T and G-40T of P_{sal}. In the presence of inducer one of the mutant alleles (pMZ60) caused weak (about 4-fold less than wild type), but significant protection of G-40T, while another mutant allele (pMZ61) caused wild type levels of methylation protection of G-40T and stronger than wild type levels of protection of G-42T (Fig. 4*B*).

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

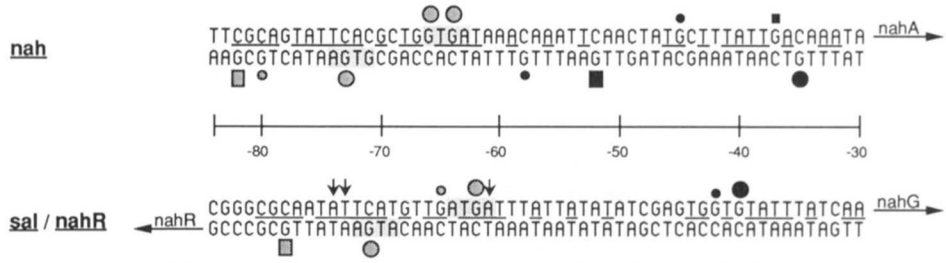

FIG. 3. **Summary of *in vivo* footprinting experiments with wild type NahR protein.** Shown are the DNA sequences (−84 to −30) of P_{nah} and $P_{sal/nahR}$. Nucleotides protected from methylation by dimethyl sulfate in both the presence and absence of the inducer salicylate are marked by *dot-filled 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 *underscored*. 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.

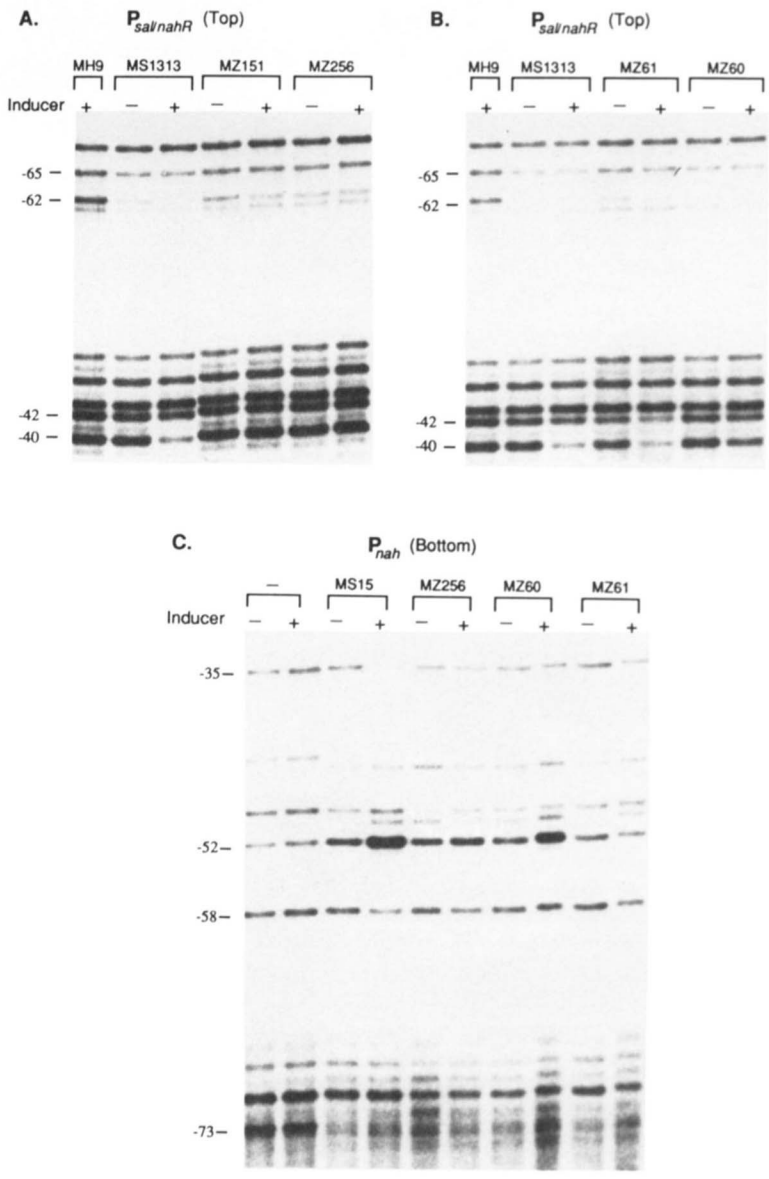

FIG. 4. **Analysis of guanines of $P_{sal/nahR}$ and P_{nah} protected from methylation *in vivo* by activation-deficient (class II) NahR mutants.** *E. coli* cells containing pMS1313 ($NahR^+$), pMS15 ($NahR^+$), pMH9 ($NahR^-$), or a class II mutant (pMZ151, pMZ256, pMZ60, or pMZ61) were grown in the 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_{nah} 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_{nah} was protected from *in vivo* methylation by the presence of all class II *nahR* mutants tested (Fig. 4*C*). Addition of salicylate to cells containing pMZ256 or pMZ61 did not cause any of the major changes in reactivity to dimethyl sulfate observed with the wild type allele; pMZ60-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_{nah} (Fig. 4*C*) it was necessary to utilize a binary plasmid system where the mutant *nahR* alleles were in high copy number, while the P_{nah} target site was at lower copy number (pRSC3 using a pRK404 vector); in all other analyses *nahR* 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 NahR mutants were ineffective in making the strong salicylate-induced promoter interactions observed with wild type NahR, while their ability to make the uninduced interactions was unaffected.

Construction and Analysis of Inducer-independent NahR Mutants— Experiments described above established a strong correlation between the presence of the inducer-dependent methylation protections or enhancements at G-35B, G-52B, and G-58B for P_{nah} and G-40T, and G-42T for P_{sal}, and transcription activation caused by NahR and salicylate. All *nahR* 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 NahR proteins with P_{sal} and P_{nah}. These *nahR* mutants could activate transcription from P_{sal} by 10- to 20-fold independent of any added inducer, as indicated by the increase in expression of *galK* (galactokinase) when placed in *E. coli* cells containing of P_{sal}-*galK* fusion plasmid pLGS140; addition of inducer caused an additional 3- to 6-fold increase in transcription from P_{sal} (Table 1). These NahR 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 NahR reading frame (Table I).

In vivo methylation protection analysis of the interactions of the partially inducer-independent NahR proteins encoded on pCH5 and pCH10 with P_{sal} showed that in the absence of salicylate they caused protection of G-65T and G-62T to the same extent as wild type *nahR* (Fig. 5*A*). However, under the same noninducing conditions these mutant NahR proteins also caused protection of G-42T and G-40T, to nearly the same extent observed with wild type *nahR* only when inducer was present. Addition of inducer caused these protections (G-42T and G-40T) to increase to wild type induced levels. Three other 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 pCH5 or pCH10 (data not shown). Analysis of the effect of several of these alleles (pCH5, Fig. 5B; pCH4 and pCH10, not shown) on methylation of P_{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 *nahR* 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 NahR proteins which partially activate transcription in the absence of inducer all cause protection or enhancement of the same N7's in P_{sal} and P_{nah} that are affected by wild type NahR only when inducer was present, providing further correlation between the interaction of NahR with G-40T and G-42T of P_{sal} and G-35B and G-58B of P_{nah}, and its ability to activate transcription.

TABLE I
Analysis of inducer-independent NahR alleles

Plasmid	Amino acid change	Salicylate activation of GalK[a]	
		NI	I
pMS15	Wild type	0.12	8.2
pMG250	Arg-21 → Stop	0.10	0.13
pCH2	Ala-201 → Val	0.92	7.6
pCH4	Leu-154 → Phe	2.72	14.0
pCH5	Met-116 → Ile	1.45	4.9
pCH6	Ala-231 → Val	1.45	5.1
pCH10	Cys-252 → Tyr	1.20	7.2

[a] Specific activity ((nanomole of galactose phosphorylated min^{-1} mg^{-1} protein) × 10^{-2}) of galactokinase in *E. coli* N100 (pLGS140) cells containing the indicated plasmids grown with (I) or without (NI) the inducer salicylate.

Evidence that NahR Is Directly Responsible for Inducer-dependent Protections— The salicylate-inducible protections were absolutely dependent on NahR and thus it is likely that they are caused by actual contact or interactions of NahR with these nucleotides. However, we cannot *a priori* rule out the possibility that the salicylate-induced protections (especially at G-40T of P_{sal} and G-35B of P_{nah}) 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 NahR contact comes from methylation protection analysis of pSM10, which is identical to pMS15 (Fig. 1), but lacks the P_{sal} sequences between −28 and +27. When this promoter was fused to the *E. coli galK* gene no salicylate-induced activation of expression was observed when *nahR* was placed in trans, indicating that the deleted promoter does not undergo increased transcription (binding) by RNA polymerase caused by NahR and salicylate. The *in vivo* methylation protection pattern of this deleted promoter, however, was nearly identical to that of wild type P_{sal} (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 NahR with these nucleotides rather than RNA polymerase interactions.

DISCUSSION

Analysis of methylation of the guanines of P_{sal} and P_{nah}, as affected by NahR and salicylate, was used to probe the *in vivo* changes of the interactions of this transcriptional activator with its target sites in response to inducer. This analysis and previous *in vitro* studies show that in the absence of inducer, NahR 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 NahR 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

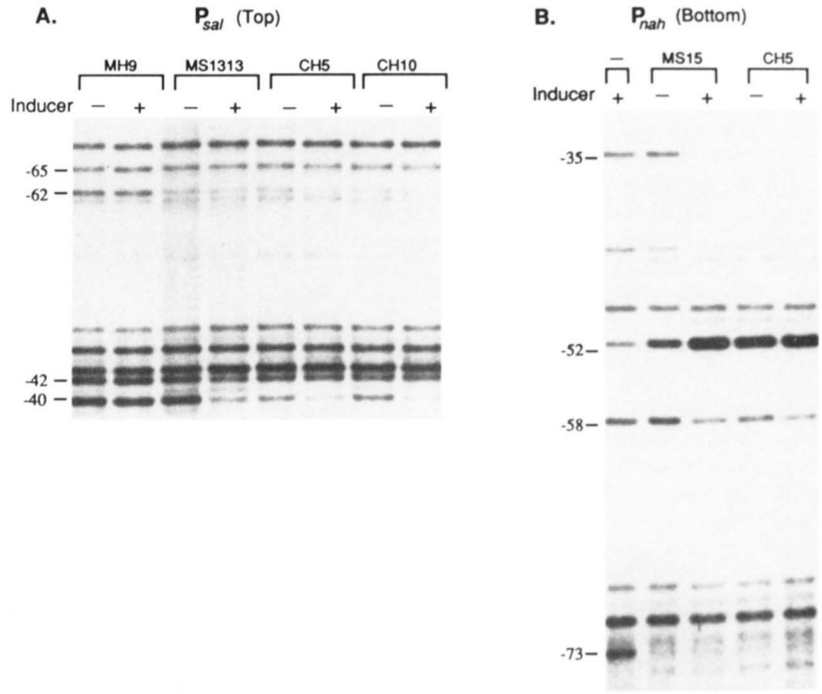

FIG. 5. **Analysis of guanines of P_{sal} and P_{nah} protected from methylation *in vivo* by inducer-independent NahR mutants.** *E. coli* cells containing pMH9 ($NahR^-$), pMS1313 ($NahR^+$), pMS15 ($NahR^+$), or a NahR mutant (pCH5, pCH10) were grown in the presence (inducer +) or absence (inducer −) of salicylate, treated with dimethyl sulfate, and analyzed as described in the legends to Figs. 2 and 4. For *A* OL 8 was used; for *B* OL10 was used and cells additionally contained P_{nah} on pRSC3.

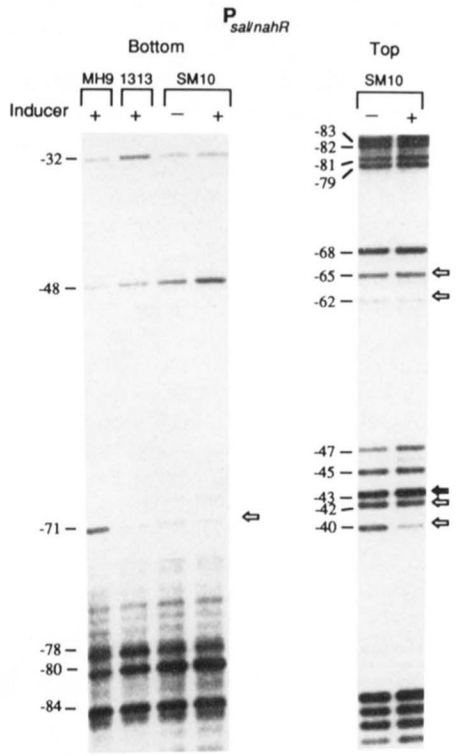

FIG. 6. **Methylation protection of partially deleted *sal* promoter.** *E. coli* cells containing the indicated plasmids were grown with (inducer +) or without (inducer −) salicylate, treated with dimethyl sulfate, and the methylation pattern analyzed as described in the legend to Fig. 2. OLRP was used 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_{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 symmetrical guanines are also found in the upstream regions of nearly all promoters regulated by NodD (CATnnnnnnnATG) (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-35B of P_{nah} and G-40T of P_{sal} (Fig. 3) without altering the upstream contacts occurring in the absence of inducer; weaker NahR-dependent protections caused by salicylate occurred in P_{sal} (G-42T) and P_{nah} (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_{sal} sequences between −28 and +27, which completely eliminated salicylate-NahR-stimulated RNA polymerase interactions at P_{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_{nah} or P_{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 P_{nah} and P_{sal} are very different. In P_{sal} only two nearly-adjacent guanines (G-42T and G-40T) showed inducer-dependent changes in dimethyl sulfate reactivity, whereas in P_{nah} 5 nucleotides extending over a 24-bp region (G-58B 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 P_{nah} and P_{sal} may be caused by the presence of the divergent *nahR* promoter overlapping Psal; simultaneous transcription and autoregulation of P_{nahR} may constrain or affect interactions of NahR with this region.

One particularly striking difference is the absence from P_{sal} of the strong inducer-dependent enhancement of G-52B of P_{nah}. This region of P_{sal} 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 P_{sal}. The increased reactivity of G-52B of P_{nah} 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 P_{nah} and P_{sal} 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 irregardless of inducer. The pMZ60 mutant allele caused weak but significant salicylate-induced alterations of both P_{nah} and P_{sal}, yet was completely deficient in causing transcription activation; while the pMZ61 allele caused salicylate-induced changes only at P_{sal}. 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 P_{sal} and P_{nah} may be directly caused by NahR-guanine contacts. However, it is also possible that they result from DNA contortions 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 the contacts result in activation of transcription by RNA polymerase, and whether other LysR-type activators employ similar mechanisms.

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