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Identification of the *nahR* Gene Product and Nucleotide Sequences Required for Its Activation of the *sal* Operon

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The product of the *nahR* gene, a salicylate-dependent activator of transcription of the *nah* and *sal* hydrocarbon degradation operons of the NAH7 plasmid, was identified and characterized after synthesis in *Escherichia coli* maxicells. The *nahR* gene product had a subunit molecular weight of 36,000, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, whereas gel filtration analysis of the nondenatured *nahR* protein indicated a molecular weight in excess of 250,000. However, DNase I treatment of this high-molecular-weight complex shifted the apparent molecular weight of the *nahR* protein to 40,000. Various upstream portions of the *sal* operon promoter were transcriptionally fused to the *E. coli* galactokinase gene. Fusion plasmids containing the *sal* promoter sequence from −83 to 27 (relative to the transcription start site) showed salicylate-inducible expression of galactokinase in the presence of the cloned *nahR* gene, while expression of galactokinase from a fusion plasmid containing the *sal* promoter sequence from −45 to 27 was not induced by the *nahR* gene and salicylate. Results suggest that the *nahR* gene product is a 36-kilodalton polypeptide which exerts its salicylate-dependent activation of transcription of the *sal* operon by interacting with the promoter sequence in the region of −83 to −45 base pairs before the transcription start site.

The self-transmissible NAH7 plasmid of *Pseudomonas putida* encodes the enzymes required for growth on naphthalene or salicylate as sole carbon and energy sources (2). These genes are organized in two operons. *nah* (*nahA* through *nahF*) encodes enzymes for metabolism of naphthalene to salicylate, and *sal* (*nahG* through *nahM*) encodes enzymes for metabolism of salicylate to tricarboxylic acid cycle intermediates (16). (Fig. 1). The expression of both operons is coordinately induced over 20-fold by salicylate (1, 13). In addition, the system apparently is not catabolite repressed since glucose or succinate do not affect induction of expression of the two operons (M. A. Schell, unpublished data).

Results of transposon mutagenesis experiments have shown that a regulatory locus, *nahR*, is required for induction and full expression of both the *nah* and *sal* operons (16, 17). Results of other experiments have shown that *nahR* exerts its effect in *trans* (3, 13) and is located just upstream of the *sal* operon (13, 17). The *nahR* and *sal* promoters overlap at −35 but are divergently transcribed from opposite DNA strands; *nahR* transcription is constitutive, while *sal* transcription is inducible (13a). Analysis of *nah* and *sal* mRNA levels in induced and uninduced *P. putida* (NAH7) indicated that coordinate induction by salicylate is mediated at the transcriptional level by *nahR*, which probably encodes a salicylate-dependent activator of the transcription of both operons (13). Since there is no catabolite repression and a 1.6-kilobase (kb) *nahR* DNA fragment is capable of conferring full wild-type expression and *trans*-regulation of *nah* (13), it is likely that *nahR* is the only regulatory gene product required for expression of *nah* and *sal*.

Analysis and comparison of the nucleotide sequences of both the *nah* and *sal* promoters have revealed a region of extensive sequence homology in the region between −80 and −60 base pairs (bp) before the transcription start sites of both operons (13a), in which 17 of 21 bp in the region are homologous. Since positive regulatory proteins for other operons (e.g., *araBAD*, *malT*, *lac*) have been shown to act in the −60 to −80 region (10), the homologous sequence between −60 and −80 of the *nah* and *sal* promoters is a likely location where the positive regulatory protein encoded by *nahR* could exert its salicylate-dependent activation of transcription.

The cloned *nah*, *sal*, and *nahR* genes are expressed and normally regulated in both *Escherichia coli* and *P. putida*, although the expression levels in *E. coli* are 10- to 20-fold lower (13). The use of expression in *E. coli* hosts for experimental manipulations and analysis has greatly facilitated molecular genetic analysis of regulation of NAH7 genes. We report here the use of two *E. coli*-based systems to further analyze the *nahR* gene product and its regulation of the *nah* and *sal* operons. To identify and characterize the *nahR* gene product, we subcloned an active *nahR* gene, made in vitro deletions to inactivate the *nahR* function, and compared the polypeptides synthesized from wild-type and *nahR* deletion plasmids in *E. coli* maxicells. The *nahR* protein from maxicells was found to have a subunit molecular mass of 36 kilodaltons (kDa) and to be tightly bound to DNA. In addition, we show that the *sal* promoter sequence between −45 and −83, which contains the extensive region of homology between the *nah* and *sal* promoters, is required for *nahR* gene product activation of transcription and therefore is a likely binding site for the *nahR* gene product.

MATERIALS AND METHODS

Chemicals. DNA enzymes were purchased from Bethesda Research Laboratories, Gaithersburg, Md.; New England BioLabs, Inc., Beverly, Mass.; or Boehringer Mannheim Biochemicals, Indianapolis, Ind. Media components were from Difco Laboratories, Detroit, Mich. Agarose and CsCl were purchased from Bethesda Research Laboratories. T4 polymerase and nucleotide triphosphates were from P-L Biochemical, Inc., Milwaukee, Wis. L-[^{35}S]cysteine (1,140 Ci/mmol) was obtained from Amersham Corp., Arlington Heights, Ill. L-[^{35}S]methionine (1,022 Ci/mmol) was from

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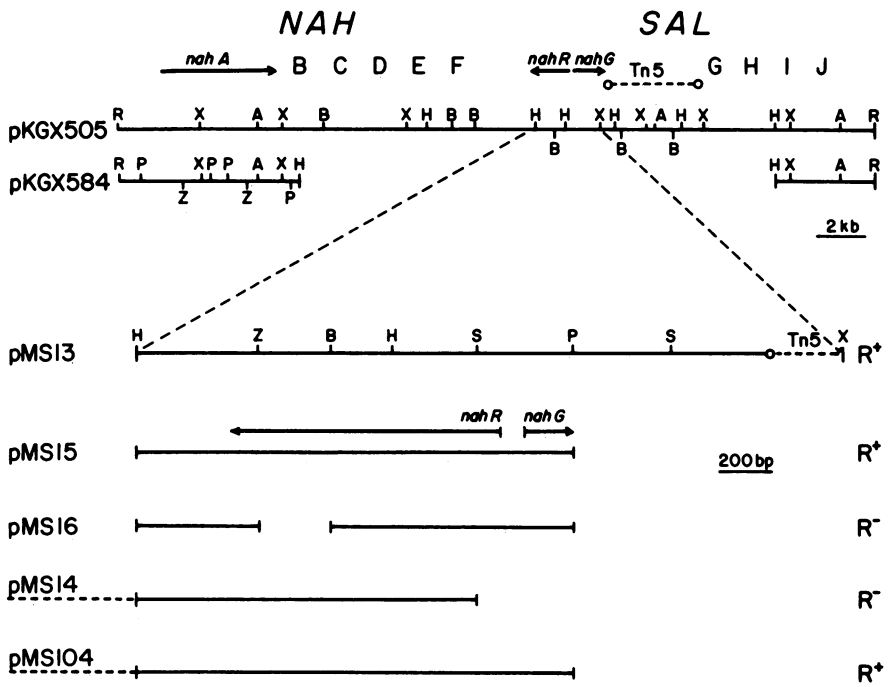

FIG. 1. Physical and genetic maps of *nahR* subclones. The upper portion shows the physical and genetic map of the *nah* and *sal* operons on pKGX505 (12). Capital letters indicate approximate locations of NAH7 catabolic genes. Lower portion shows the genetic and physical maps of subcloned NAH7 genes; pKGX584 (*nahA*$^+$), pMS15 (*nahR*$^+$), pMS16 (*nahR*), pMS14 (*nahR*), and pMS104 (*nahR*$^+$) are shown in reference to pKGX505. Restriction endonuclease cleavage sites are abbreviated as follows: X, *Xho*I; A, *Sma*I; B, *Bgl*II; P, *Pst*I; H, *Hin*dIII; Z, *Sph*I; S, *Sal*I. Plasmids pMS13 through pMS16 are on pUC8 vectors; pMS104 is on a pRK404 vector. The *Bam*HI site of pMS13 used to construct pMS15 is not shown but is located in the polylinker of the pUC8 vector near the *Xho*I site in Tn*5*. R$^+$, positive for *nahR* activation; R$^-$, negative for *nahR* activation. Only relevant portions of the cloned NAH7 DNA on plasmids pMS104 and pMS14 are shown; dotted lines represent additional DNA on pMS14 (~2.5 kb) and pMS104 (~1.5 kb) (13).

New England Nuclear Corp., Boston, Mass. D-[^{14}C]galactose (55 mCi/mmol) was from ICN Pharmaceuticals Inc., Irvine, Calif. Molecular weight standards were from Bio-Rad Laboratories, Richmond, Calif. DNase I was from Calbiochem-Behring, La Jolla, Calif. Antibiotics, electrophoresis chemicals, amino acids, and most other chemicals were purchased from Sigma Chemical Co., St. Louis, Mo. or were of reagent grade purity.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype	Source or reference
E. coli K-12		
N100	*galK recA pro*	K. McKenney et al. (9)
CSR603	*recA1 uvrA6 phr-1*	S. Kushner (11)
C600	*thr leu trp thi hsdR hsdM*	C. Yanofsky
JM83	*ara* Δ(*pro-lac*) *rpsL thi* Δ*lacZM15*	J. Messing
Plasmids		
pKO1M	Ap^r *galK*	S. Hollingshead (9; Ph.D. thesis)
pUGA1900	Ap^r *galK*$^+$	S. Hollingshead (Ph.D. thesis)
pKGX584	Sm^r *nahA*$^+$	M. Schell (13)
pMS13	Ap^r *nahR*$^+$	M. Schell (13)
pMS14	Ap^r *nahR*	M. Schell (13)
pMS104	Tc^r *nahR*$^+$	M. Schell (13)
pSR1	Ap^r	M. Schell (13a)

Bacterial strains. Bacterial strains and plasmids used in this study are listed in Table 1.

Construction of plasmids. Plasmid pMS15 was constructed by digestion of pMS13 (13) with *Pst*I and *Bam*HI, treatment with 1 unit of T4 DNA polymerase for 5 min at 37°C in the absence of deoxynucleoside triphosphates and then for 1.5 h in the presence of deoxynucleoside triphosphates, self-ligation, and subsequent transformation into *E. coli* JM83 (see Fig. 1). Plasmid pMS16 was obtained in an analogous manner after digestion of pMS15 with *Sph*I and *Bgl*II. pKGX584, pMS14, and pMS104 were constructed as described previously (13).

Plasmid pSR1 (13a) was digested with *Sal*I, and the 800-bp fragment containing the *sal* promoter was purified by electrophoresis on a 5% polyacrylamide gel and electroelution. The 800-bp fragment was digested with *Rsa*I, and the 202-bp *Sal*I-*Rsa*I promoter fragment was purified (see Fig. 4). The fragment was ligated with *Sma*I-digested pK01M (9; S. Hollingshead, Ph.D. thesis, University of Georgia, 1983) DNA and transformed into *E. coli* N100, and transformants were selected on MacConkey galactose agar plates containing 100 μg of ampicillin per ml. Red ampicillin resistant (Ap^r) colonies were analyzed and shown to contain a plasmid, pSU3, with the 202-bp *sal* promoter fragment fused to the *E. coli* galactokinase gene (see Fig. 4).

To construct plasmid pMS160, the 202-bp *Sal*I-*Rsa*I fragment was digested with *Hae*III, ligated into *Sma*I-digested pK01M, and transformed into *E. coli* N100. Red Ap^r transformants were analyzed by restriction endonuclease cleavage. One plasmid, pMS160, contained the 110-bp *Hae*III-*Rsa*I fragment fused to the galactokinase gene in the

TABLE 2. Levels of naphthalene dioxygenase in *E. coli* (pKGX584) containing various *nahR* subclones

Strain	Naphthalene dioxygenase activity in the following subclones[a]	
	Uninduced	Induced[b]
E. coli C600(pKGX584)	1.5	0.6
E. coli C600(pKGX584, pMS13)	1.5	25.0
E. coli C600(pKGX584, pMS15)	1.6	28.0
E. coli C600(pKGX584, pMS16)	1.7	1.0
E. coli C600(pKGX584, pMS14)	0.4	0.6

[a] Activity is in nanomoles of *cis*-D-dihydrodiol naphthalene produced per minute per milligram of protein.
[b] Induced with 0.02% sodium salicylate.

orientation shown in Fig. 4, as determined by DNA sequence analysis.

Plasmid pMS24 was constructed by digestion of the 202-bp *Sal*I-*Rsa*I fragment with *Taq*I, treatment with the Klenow fragment of *E. coli* DNA polymerase I in the presence of dCTP and dGTP, and ligation with *Sma*I-digested pK01M DNA. Red Ap^r transformants were analyzed for the presence of plasmids containing the 72-bp *Taq*I-*Rsa*I promoter fragment. One of the plasmids, pMS24, contained the fragment in the orientation shown in Fig. 4, as determined by DNA sequence analysis (8).

Maxicell analysis. After transformation of *E. coli* CSR603 with plasmid DNA, maxicells were prepared by a modification of the method of Sancar et al. (11), utilizing [^{35}S]methionine or [^{35}S]cysteine. Cell extracts of the cells were prepared in 20 mM sodium phosphate buffer (pH 7.5) by sonication of washed maxicells and subsequent centrifugation at 10,000 × *g* for 10 min. To purify the radiolabeled *nahR* protein from other labeled polypeptides in maxicells, 1-ml fractions of the cell extract of maxicells were chromatographed on a Sephadex G-100 column (23 by 1.5 cm), and the void volume fractions (V_e = 13 to 16 ml) were pooled. Analysis of ^{35}S-labeled plasmid-encoded proteins was performed on 12% sodium dodecyl sulfate (SDS)-polyacrylamide gels by the method of Laemmli (6), and autoradiography of the dried gels was performed.

Gel filtration analysis. The native (i.e., nondenatured) molecular weight of the partially purified *nahR* protein after various treatments was determined by chromatography on a Bio-Gel A 1.5m column (by 25 cm by 1.0 cm). Samples of 0.3 ml were loaded and run in 25 mM Tris (pH 7.5)–0.1 M NaCl–10 mM $MgCl_2$–1 mM dithiothreitol; 1.0-ml fractions were collected and counted in scintillation fluid. The column was calibrated with bovine catalase (250 kDa) and bovine serum albumin (67 kDa).

Galactokinase assays. Cells were grown at 37°C in L broth in the presence or absence of inducer (0.02% sodium salicylate) from A_{600} = 0.1 to 0.8. Cells were then harvested, and extracts were prepared by sonication in kinase buffer and by centrifugation as described above. Galactokinase assays and protein determinations were performed as described previously (15).

Assay of *nahA* expression. Cells were grown in L broth at 30°C in the presence or absence of inducer (0.02% sodium salicylate) to late log phase (A_{600} = 0.8) and analyzed for expression of *nahA* by measuring levels of naphthalene dioxygenase activity as described previously (13).

Recombinant DNA methods. Methods used for restriction digestion, ligation, transformation, and restriction endonuclease analysis have been described previously (7, 14).

RESULTS

Subcloning and deletion mapping of the *nahR* gene. The *nahR* gene is located just upstream of the *sal* operon (13, 17) and is required for coordinate induction of both the *nah* and *sal* operons in response to salicylate (13, 16, 17). The cloned *nahR* gene is contained on a 3.0-kb *Hin*dIII-*Xho*I fragment (Fig. 1, pMS13) and produces a salicylate-dependent *trans* activator of expression of the *nah* operon. The *nahR* activator function was measured in *E. coli* by analyzing the ability of cloned DNA fragments to activate expression of the *nahA* gene (13), the first gene of the *nah* operon (which encodes naphthalene dioxygenase and is totally contained on plasmid pKGX584) (Table 2, pKGX584 and pKGX584, pMS13). A smaller 1.8-kb *Hin*dIII-*Pst*I DNA fragment was subcloned from pMS13 to produce plasmid pMS15 (Fig. 1) which still contained an active *nahR* gene, since salicylate-dependent *trans* activation of expression of *nahA* in *E. coli* cells containing the cloned *nahA* gene (pKGX584) and pMS15 was observed (Table 2, pKGX584, pMS15). However, deletion of a 300-bp *Sph*I-*Bgl*II fragment from pMS15 generated a plasmid, pMS16, which was no longer capable of salicylate-dependent activation of the cloned *nahA* gene in *E. coli* cells containing both plasmids (Table 2, pKGX582, pMS16). Similarly, deletion of 70 bp from the 5′ end of the *nahR* gene located between the *Sal*I and *Pst*I sites of pMS15 to produce plasmid pMS14 resulted in the loss of the ability to *trans* activate *nahA* expression. Since approximately 400 bp of the cloned DNA fragment of pMS15 is part of the *nahG* gene (13a), the maximum size of the *nahR* gene can be only 1.4 kb, and therefore the estimated maximum subunit molecular mass of the transcription activator product is 50 kDa.

Maxicell analysis of polypeptides of *nahR* clones. Plasmids containing a functional *nahR* gene (pMS13 and pMS15) and those containing a nonfunctional, partially deleted *nahR* gene (pMS14 and pMS16) were transformed into *E. coli* CSR603, and analysis of plasmid-encoded ^{35}S-labeled polypeptides synthesized by each strain was performed by the maxicell technique (11) and SDS-polyacrylamide gel electrophoresis. Maxicells containing pMS15 synthesized three major ^{35}S-labeled polypeptides (36, 32, and 28 kDa; (Fig. 2, lane 3), with the 36-kDa polypeptide accounting for greater than 70% of the total protein, as estimated from

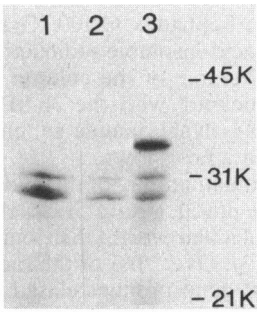

FIG. 2. Maxicell analysis of plasmid-encoded proteins of *nahR* subclones. Autoradiograph of a 12% SDS-polyacrylamide gel analysis of cell extracts of various plasmid-containing maxicells. Lane 1, 200 μg of CSR603(pMS16) maxicell extract; lane 2, 200 μg of CSR603(pUC8) maxicell extract; lane 3, 200 μg of CSR603(pMS15) maxicell extract. The migration of molecular weight standards are indicated at the right, in thousands (K).

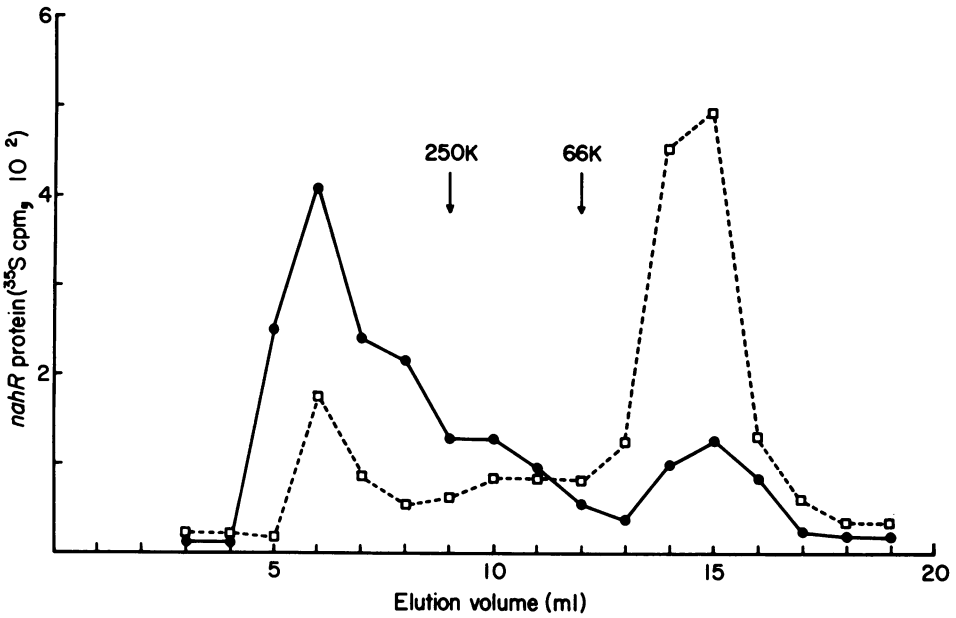

FIG. 3. Gel filtration analysis of the *nahR* gene product in the presence and absence of DNase I. The figure shows the elution profile of the radiochemically pure *nahR* gene product (2,000 cpm; 0.2 mg protein in 250 μl) chromatographed on a Bio-Gel A 1.5-m column (1 by 25 cm; void volume 6.0 ml) (●). The elution profile of an identical sample treated with 200 μg of DNase I per ml for 2 h at 37°C is also shown (□). The elution volume of bovine catalase (250,000 [250K]) and bovine serum albumin (66,000 [66K]) used as molecular weight markers is shown.

scans of autoradiographs; a similar result was obtained for pMS13 (data not shown). Maxicells containing the pUC8 vector alone showed synthesis of only the two smaller polypeptides of 31 and 28 kDa (Fig. 2, lane 2). This suggests that the 36-kDa polypeptide is the *nahR* gene product and that it is highly expressed in *E. coli* relative to the β-lactamase (31 kDa) gene of the pUC8 vector. Analysis of polypeptides produced by the *nahR* deletion plasmid pMS16 which has no *nahR* gene product activity (Table 2, pKGX584, pMS16) showed no synthesis of the 36-kDa polypeptide (Fig. 2, lane 1); similar results were obtained with the other *nahR* deletion plasmid pMS14 (data not shown). Apparently, the 36-kDa polypeptide is the *nahR* gene product, a result which is consistent with the predicted maximum size of the *nahR* gene product as determined by the subcloning experiments described above.

Characterization of the *nahR* gene product. Extracts of ^{35}S-labeled polypeptides synthesized by CSR603(pMS15) maxicells were prepared and fractionated by gel-filtration chromatography on Sephadex G-100. Greater than 70% of the trichloroacetic acid-insoluble radioactivity in the extract eluted in the void volume of the column, of which greater than 95% was associated with the 36-kDa polypeptide as determined by SDS-polyacrylamide gel electrophoresis and autoradiography (data not shown).

Gel-filtration chromatography of this radiochemically pure 36-kDa *nahR* gene product on a Bio-Gel A 1.5m column showed a broad molecular weight distribution of the labeled *nahR* protein (Fig. 3). Over 70% of the radioactivity associated with the *nahR* gene product eluted with a molecular mass greater than 250 kDa, while approximately 20% eluted near the volume expected for a subunit with a molecular mass of 40 kDa. Treatment of the *nahR* protein sample with DNase I before gel-filtration chromatography caused the elution profile of the *nahR* gene product to dramatically shift from the large molecular mass value to the smaller molecular mass value of 40 kDa (Fig. 3). After treatment with DNase I, only 15% of the *nahR* radioactivity eluted with a molecular mass greater than 250 kDa, while 70% eluted at about 40 kDa. The presence of the inducer, salicylate, or 1 M NaCl during chromatography did not significantly alter the elution profile. The results indicate a strong association of the *nahR* protein with DNA.

Location of *nahR* control sequence of *sal* promoter. To determine the DNA sequences required for the activation of the *nah* and *sal* operons by the *nahR* gene product, the *sal* operon promoter was transcriptionally fused to the *E. coli* galactokinase gene (Fig. 4). This was accomplished by cloning a 202-bp *Sal*I-*Rsa*I fragment of pSR1 (13a), containing the 175-bp DNA sequence preceding the transcription start site of *sal* promoter, into a derivative of pK01 to produce plasmid pSU3 (Fig. 4). The expression of the galactokinase gene from this transcriptional fusion plasmid was analyzed in *E. coli* in the presence and absence of the inducer salicylate and the cloned *nahR* gene (Table 3). Plasmid pSU3 directed synthesis of low levels of galactokinase; however, in the presence of salicylate and the cloned *nahR* gene on plasmid pMS104, nearly a 100-fold increase in galactokinase synthesis directed by pSU3 was observed (Table 3, pSU3, pMS104). In the presence of the cloned *nahR* gene but in the absence of the inducer salicylate, only low levels of galactokinase synthesis were detected. Thus, in the presence of the cloned *nahR* gene the fusion plasmid pSU3 shows salicylate-inducible expression that is characteristic of the *nah* and *sal* operons and must therefore contain the DNA sequences required for *nahR* gene product regulation of the *sal* operon.

A *galK* fusion plasmid with a smaller promoter fragment of the *sal* operon and containing only 83 bp preceding the transcription start site of the *sal* operon was constructed (pMS160). This plasmid contained the *sal* operon promoter DNA sequences from position −83 to 27 (relative to the transcription start site) fused to the *E. coli* galactokinase gene (Fig. 4). This fusion plasmid directed low-level synthe-

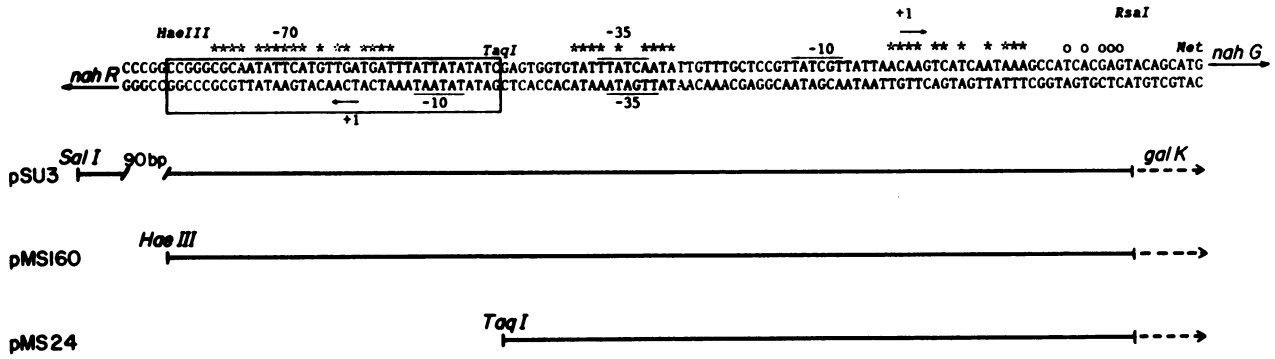

FIG. 4. Nucleotide sequences of promoter regions of *sal* operon-*galK* fusion plasmids. The figure shows the various portions of the nucleotide sequence of the *sal* operon promoter (13a) that were fused to the *E. coli* galactokinase gene (*galK*) to produce each promoter fusion plasmid; pSU3 (−175 to 27), pMS160 (−83 to 27), pMS24 (−45 to 27). Asterisks indicate the location of homologous bases which are identical in both the *nah* and the *sal* promoters; the arrow at the top right designated +1 shows the transcription start site of the *sal* operon; open circles indicate the putative ribosome binding site. The −35 and −10 promoter sequences of *sal* are overlined. nahG underscored with an arrow indicates the putative translation initiation codon of the *nahG* gene. The boxed-in sequence indicates the region shown to be involved in transcription activation by the *nahR* gene product. The arrow at the lower left designated +1 indicates the transcription start site of *nahR*. The −35 and −10 promoter sequences of *nahR* are underlined.

sis of *E. coli* galactokinase which was similar to the levels produced from pSU3; but in the presence of the cloned *nahR* gene and salicylate the expression was increased over 60-fold. (Table 3, pMS160, pMS104). The pMS160 fusion plasmid must still contain the *sal* operon promoter sequences necessary for salicylate-dependent *nahR* gene product activation of transcription.

Cloning of a smaller, 72-bp, *Taq*I-*Rsa*I fragment from the *sal* promoter produced plasmid pMS24 which contained the DNA sequence from −45 to 27 of the *sal* promoter fused to galactokinase in the orientation shown in Fig. 4. This fusion plasmid directed synthesis of low levels of galactokinase which were nearly identical to those produced by other fusion plasmids grown in the absence of salicylate (Table 3, pMS160 versus pMS24). However, in contrast to pMS160, galactokinase levels produced by pMS24 were unaffected by the presence or absence of the cloned *nahR* gene and salicylate (Table 3, pMS160, pMS104 versus pMS24, pMS104). Thus, plasmid pMS24 apparently only lost the ability to be *trans* activated by the *nahR* gene product and salicylate and thus must have lost a portion of the DNA sequences necessary for *nahR* gene product-mediated induction. Thus, the sequence between −45 and −83 is required for *nahR*-mediated activation of transcription of the *sal* operon and is probably the location of its binding site on the *sal* promoter.

DISCUSSION

The identification of the transcription start sites and the promoters of the coordinately regulated *nah* and *sal* operons along with subsequent alignment and comparison of the promoter sequences revealed three regions of significant homology (13a). By far the most extensive region of homology (85%) was found in the region between −60 and −80 bp preceding the transcription start sites, in which 17 of 21 bases were identical for both the *nah* and *sal* promoters. Since other positive regulatory proteins have been shown to exert their effects at about position −60 (10), it was proposed that the homologous region of *nah* and *sal* centered around −70 is the site at which the *nahR* gene product in the presence of salicylate exerts its activation of transcription (13a). Evidence presented in this report strongly supports this hypothesis. Galactokinase fusion plasmids containing the *sal* promoter sequence, including the homologous sequence at position −70, are inducible by salicylate in the presence of the *nahR* gene, while promoter-fusion plasmids lacking the sequence at −70 are not inducible. In the absence of salicylate or *nahR*, expression from the *sal* promoter is not affected by the presence or absence of the sequence at −70. Therefore, the 38-bp sequence (−83 to −45) seems to be required for *nahR* gene product activation of transcription. Furthermore, it is possible that the *nahR* gene product physically interacts with this region (possibly the 17-bp homologous sequence) to activate the *nah* and *sal* promoters in the presence of salicylate. However, the exact mechanism by which it activates transcription of *nah* and *sal*, the role of RNA polymerase, and the critical nucleotides involved in its actual binding to the *nah* and *sal* promoters at this site remain to be elucidated.

The *nahR* promoter apparently overlaps with the *sal* operon promoter at position −35 (13a); however *nahR* and *sal* are divergently transcribed from opposite DNA strands. Therefore, it should be noted that the *sal* promoter sequence between −83 and −45 that is involved in transcriptional activation of *sal* by the *nahR* gene product also contains the transcription start site of *nahR*. This may indicate some

TABLE 3. Galactokinase levels in *E. coli* strains carrying *sal* promoter fusion plasmids and *nahR*

Strain	Galactokinase activity in the following subclones[a]:	
	Uninduced	Induced[b]
E. coli N100(pSU3)	1.1	0.8
E. coli N100(pSU3, pMS104)	1.0	98.0
E. coli N100(pMS160)	1.2	1.4
E. coli N100(pMS160, pMS104)	0.8	65.0
E. coli N100(pMS24)	1.2	1.6
E. coli N100(pMS24, pMS104)	1.3	1.6
E. coli N100	0.01	0.01
E. coli N100(pKO1M)	0.11	0.12
E. coli C600	0.21	1.86[c]
E. coli N100(pMS104)	0.02	0.03

[a] Activity is in nanomoles of galactose phosphorylated per minute per milligram of Protein × 10^{-2}.
[b] Induced with 0.02% salicylate.
[c] Induced with 0.1% galactose.

autoregulation of *nahR* expression by its own gene product. Furthermore, it also suggests that the *nahR* gene product may simultaneously control expression of two transcription units via the overlapping promoter structure.

The homologous sequence at about position −70 shows no significant homology or similarity to analogous sequences of *araBAD*, *malT*, *xylABC*, or *xylDEFG* which are also regulated by positive regulatory genes (4, 10). The operons *xylABC* and *xylDEFG* are found on the aromatic hydrocarbon degradation plasmid TOL and are regulated by the *xylR* and *xylS* genes, which appear to act in a manner similar to that of *nahR* (5). However, consistent with this lack of promoter sequence homology is the observation that *xylR* or *xylS* or both cannot *trans* activate *nahA* expression (M. Schell and T. Nakazawa, unpublished data). There is apparently only superficial similarity between the regulatory systems of the NAH7 and TOL hydrocarbon degradation systems.

Identification of the *nahR* gene product as a 36-kDa polypeptide, along with subsequent analysis of its molecular properties, has given further insight into *nahR* control of the *nah* and *sal* operons. Since the maximum size of the *nahR* regulatory locus, as indicated by subcloning and deletion analysis, is 1.4 kb and a 36-kDa gene product is synthesized from this gene, it is likely that the *nahR* gene product and salicylate alone are sufficient for transcription activation of *nah* and *sal*. However, there could be an additional 14-kDa polypeptide encoded by the *nahR* region that is also involved in regulation. The fact that the *nahR* gene product binds tightly to DNA is additional strong circumstantial evidence of its role as a regulator of transcription of the *nah* and *sal* operons. Although the apparent lack of effect of salicylate on the DNA-binding activity of the *nahR* gene product suggested that this interaction may be nonspecific, recent DNA-binding experiments have confirmed that the *nahR* gene product specifically binds to the *nah* and *sal* promoters in both the presence and absence of salicylate (M. Schell and E. Faris, manuscript in preparation). This suggests that in vivo the *nahR* gene product may always be bound to DNA and undergo an alteration in protein-DNA interaction in response to the inducer salicylate. Further purification of the *nahR* gene product, followed by a complete analysis of DNA sequences to which it binds and of its effects on in vitro transcription of *nah*, *sal*, and *nahR*, will clarify its specific interactions with the DNA of the *nah* and *sal* promoters.

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