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Fis, an Accessorial Factor for Transcriptional Activation of the *mar* (Multiple Antibiotic Resistance) Promoter of *Escherichia coli* in the Presence of the Activator MarA, SoxS, or Rob

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Transcription of the multiple antibiotic resistance *marRAB* operon increases when one of the sequence-related activators, MarA, SoxS, or Rob, binds to the "marbox" centered at −61.5 relative to the transcriptional start site. Previous deletion analyses showed that an adjacent upstream "accessory region" was needed to augment the marbox-dependent activation. To analyze the roles of the marbox and accessory regions on *mar* transcription, thirteen promoters, each with a different 5-bp transversion of the −96 to −32 sequence, were synthesized, fused to *lacZ*, and assayed for β-galactosidase production in single-copy lysogens with appropriate genotypes. The accessory region is shown here to be a binding site for Fis centered at −81 and to bind Fis, a small DNA-binding and -bending protein, with a K_d of ≈5 nM. The binding of MarA to the marbox and that of Fis to its site were independent of each other. MarA, SoxS, and Rob each activated the *mar* promoter 1.5- to 2-fold when it had a wild-type marbox but Fis was absent. In the presence of MarA, SoxS, or Rob, Fis further enhanced the activity of the promoter twofold provided the promoter was also capable of binding Fis. However, in the absence of MarA, SoxS, or Rob or in the absence of a wild-type marbox, Fis nonspecifically lowered the activity of the *mar* promoter about 25% whether or not a wild-type Fis site was present. Thus, Fis acts as an accessory transcriptional activator at the *mar* promoter.

The upstream region of a procaryotic promoter may contain sequences that influence its activity (6, 19, 36). UP or UAS sequences appear to interact directly with RNA polymerase, whereas other sequences bind transcriptional activator proteins that contact the polymerase. Some sequences produce an intrinsic bend in the DNA or bind proteins such as Fis, H-NS, HU, or IHF that bend the DNA. The bent DNA might enhance transcription by engendering further "backside" contacts with RNA polymerase or by creating a specific higher-order structure favorable for transcription. The end result of these interactions appears to be the enhancement of a step in transcription by RNA polymerase.

MarA is a transcriptional activator of the *mar* regulon, a dozen or more genes whose expression renders *Escherichia coli* partially resistant to various antibiotics and superoxide-generating agents (see references in reference 27) and tolerant of organic solvents (14, 45a). MarA protein is structurally and functionally similar to two other transcriptional activators of *E. coli*, SoxS and Rob, which when overproduced, generate similar resistance phenotypes and activate many of the same genes (3, 15, 22, 25, 32, 45; also see references in reference 27). The three proteins are 40 to 50% identical over a sequence of about 100 amino acids and have highly conserved helix-turn-helix DNA-binding motifs (1, 7, 12, 46). Furthermore, in vitro studies demonstrate that all three proteins bind as monomers and bend similar DNA sequences present in the promoters that they activate (9, 10, 20–22, 24, 25). They also have the ability to activate transcription of class I promoters by interacting with the C-terminal domain of the α subunit of RNA polymerase (19–22).

Transcription of the multiple antibiotic resistance *marRAB* operon (2, 7, 16, 28, 29) is regulated both positively and negatively by proteins that bind to its promoter region. The promoter is repressed by MarR (2, 7, 16), which binds to two separate operator sites in the downstream promoter region from −31 to +27 (29, 40). Derepression of the operon can be achieved by treatment with salicylate or related compounds (8, 29, 40). Salicylate has been shown to bind to MarR and to reduce the affinity of MarR for the operator sites in vitro (29). Salicylate also inactivates EmrR, a MarR-like repressor of an unrelated promoter involved in efflux of and resistance to certain hydrophobic antibacterials (26, 43). Overproduction of EmrR can inhibit the expression of the *mar* promoter (43).

Remarkably, the *mar* promoter is itself transcriptionally activated by the binding of MarA or SoxS to a "marbox" sequence at bp −69 to −54 (with respect to the RNA start site [44]) (27, 31). An adjacent "accessory marbox region" (bp −89 to −72), defined by deletion analysis, increases the extent of this transcriptional activation by an unknown mechanism (27).

To identify critical promoter sequences and host factors involved in *mar* transcriptional activation, the *mar* promoter upstream region was systematically investigated by means of synthetic transversion mutations. Basal levels of Rob (estimated at 5,000 molecules per cell [42]) were found to activate the *mar* promoter by interacting with the marbox and with RNA polymerase. Furthermore, the accessory marbox region was found to bind Fis, the small, highly versatile DNA-binding and -bending protein (for a review, see reference 11). The binding of Fis to this Fis site was independent of the binding of MarA to the marbox. However, Fis activation of transcription was totally dependent on the binding of MarA/Rob/SoxS to the marbox.

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MATERIALS AND METHODS

Materials. Purified Fis protein preparations were gifts of R. Johnson. Other materials and media were as previously described (27).

Bacterial strains and plasmids. Standard bacterial techniques were used (30). GC4468 Δ*lacU169* (3), and RA4468, a *rob*::*kan* derivative of GC4468 (3), were obtained from B. Demple; RJ1801 Δ*lacX74 fis-985*::*spec* (4) was obtained from R. Johnson; BW829 (*sox-8*::*cat*) (45) was obtained from B. Weiss; and PD218 (*emrR*::*gent*) (44) was obtained from P. Miller. N7840 is a Cam^s derivative of GC4468 *zdd-239*::Tn*9* Δ*mar* (38). The *sox*, *rob*, *fis*, and *emr* (null) mutations were introduced into strains GC4468, RA4468, or N7840 by P1*cam clr-100*-mediated cotransduction with the indicated linked antibiotic resistance genes, resulting in strains N8452 Δ*mar rob*::*kan*; N8496 Δ*mar rob*::*kan sox*::*cam emrR*::*gent*; N8921 *fis*::*spec*; N8926 Δ*mar fis*::*spec*; N8923 *rob*::*kan fis*::*spec*; and N8924 Δ*mar rob*::*kan fis*::*spec*. For simplicity, the null mutations are referred to as *mar*, *rob*, *sox*, *fis*, and *emr*, respectively.

Plasmids pRJ823 (*lacI*^{q} Tc^r p15A) and pRJ4000 (*lacP-fis* Ap^r ColE1) were obtained from R. C. Johnson (47). Plasmid p37 [*marA*(Con)] was obtained from P. F. Miller (13).

Site-directed mutagenesis. Site-directed mutagenesis was accomplished by a modification (23) of the overlapping PCR (17) method with overlapping mutant-sequence primers for sequences within the *mar* promoter and primers corresponding to sequences outside the *mar* promoter present in plasmid pRS551 (41). The template DNA was from pRGM351 (27), which contains the wild-type *mar* promoter from bp −106 to +36 attached by linkers to pRS551. The two fragments were mixed and amplified with only the primers corresponding to the pRS551 sequences. The product was digested with *Bam*HI and ligated to pRS551 pretreated with *Bam*HI and alkaline phosphatase (41). The resulting *marR*::*lacZ* (for simplicity, referred to as *mar*::*lacZ*) transcriptional fusions, fused after position +17 of *mar*, were identified as previously described (27). The plasmids all carry, in addition to the transversion or insertion mutation indicated in Fig. 3B and Table 3, substitutions of C for T at position +25 and C for A at position +27 (nucleotides 1441 and 1443, respectively, of Cohen et al. [7]), generating a *Pml* site between +25 and +30. The same is true for five mutants which carry insertions of 5 bp (ACCGA), 10 bp (TTTGA ACCGA), 15 bp (TTTAG CAAGA ACCGA), or 20 bp (TTTAG CAAAA CGTGA ACCGA) between positions −71 and −70.

Single λ lysogens were obtained (41) by selection for kanamycin resistance in N7840 after growth in N7840 (27). The sequences of both strands of the mutants were verified in the pRS551 derivatives, and that of one strand was verified in the single λ lysogens after PCR amplification with Circumvent DNA sequencing kits (New England Biolabs). The single-copy λ lysogens of N7840 Δ*mar* containing wild type or transversion mutation fusions 1 to 13 are named N8695 and N8711 to N8723, respectively. Spontaneously released phage from these lysogens were used to lysogenize strains GC4468, N8452, and N8496 to generate the corresponding sets of strains N8707 and N8741 to N8753, N8795 and N8801 to N8813, and N8739 and N8771 to N8783, respectively. Phage carrying wild type and transversion mutations 2, 3, 7, and 8 were also used to lysogenize strain N8924, yielding strains N8900 and N9396 to N9399. These in turn were transformed with the two plasmids pRJ823 and pRJ4000 by selection for ampicillin and tetracycline resistances, yielding Fis-inducible strains N9390 to N9394, or with p37 (MarA constitutive) to yield strains N9414 to N9418. Phage carrying wild type and transversion mutations 3, 7, and 8 were also used to lysogenize strains RA4468 and N8926, yielding strains N8890 and N8892 to N8894 and N9006 and N9008 to N9010. The latter four in turn were transformed with plasmids pRJ823 and pRJ4000, yielding strains N9511, N9508, N9512, and N9510, respectively, or with p37 (MarA constitutive) to yield strains N9410 to N9413. Strains N8695, N8713, N8717, N8718, N8795, N8802, N8803, N8807, and N8808 similarly transformed with p37 yielded strains N9400 to N9408, respectively. *mar* promoter DNA with a 5-bp insertion (ACCGA) between −71 and −70 (5bpA), which also contained substitutions of A for T at bp −70 and G for C at bp −48, and the 5-(5bpB), 10-, 15-, and 20-bp insertions between −71 and −70 indicated above were used to construct single-copy λ lysogens of N8924, N8452, N8926, and N7840 named N9424 to N9428, N9429 to N9433, N9419 to N9423 and N8697, and N8886 to N8889, respectively. N9424 to N9426, N9428, N9419 to N9421, and N9423 were transformed with the two plasmids pRJ823 and pRJ4000 by selection for ampicillin and tetracycline resistance, yielding strains N9439 to N9442 and N9434 to N9438, respectively. Strains N8795 and N8807 were transformed with *mar* constitutive plasmids pRGM185 and p37 to give strains N8831 to N8834, respectively.

Assays. DNase I footprinting and gel mobility shift assays have been described previously (27) as have other standard molecular methods (39). β-Galactosidase assays, including induction by 50 μM paraquat or 5 mM sodium salicylate, followed the previously described standard protocol at 32°C (30, 38). Induction of Fis in strains harboring pRJ823 and pRJ4000 was effected by growth overnight at 32°C in medium containing 100 μM isopropyl-β-D-thiogalactopyranoside (IPTG), followed by cell dilution and growth for 2 h at 32° in broth with 100 μM IPTG. For paraquat induction, the cells were further diluted twofold in medium with 100 μM IPTG and grown for 1 h with or without 50 μM paraquat. All assays were performed at least twice in duplicate and had standard deviations of <10%.

RESULTS

Binding of Fis protein to the marbox accessory region. On the basis of deletion analyses, we previously reported that full transcriptional activation of the *mar* promoter required the sequence between positions −89 and −71 (27). Noting that bp −88 to −74 contained a perfect match to the degenerate 15-bp core consensus sequence for the small DNA-binding protein, Fis (18), we assayed for DNA-binding by gel mobility (Fig. 1A). A 135-bp *mar* promoter fragment from position −112 to +23 formed a complex with Fis (K_d, ≈5 nM).

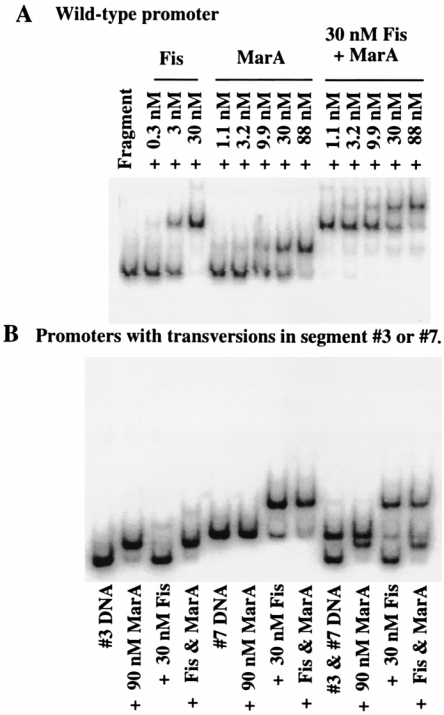

FIG. 1. Binding of *mar* promoter DNA to MarA and Fis. (A) 5′ end-labelled (^{32}P) *mar* DNA fragments (bp −112 to +23) prepared by PCR of the wild-type promoter were incubated for 15 min at room temperature with the indicated concentrations of purified MarA or Fis and then subjected to polyacrylamide gel electrophoresis on a 6% gel. (B) The 5′ end-labelled (^{32}P) *mar* DNA fragments of Fis site transversion mutant 3 (bp −112 to +19 plus 54 bp corresponding to the sequence downstream of the *Bam*HI site in plasmid pRS551) and marbox mutant 7 (base pairs are as those for marbox mutant 3 but with an additional 44 bp corresponding to the sequence upstream of the *Bam*HI site in plasmid pRS551) treated as in panel A were analyzed on a 4.5% gel.

The location of the Fis-binding site was examined by DNase I footprinting (Fig. 2). The *mar* promoter exhibited hypersensitive sites characteristic of Fis sites (11): 3′ to bp −85 on the *mar* nontemplate strand (Fig. 2A) and 3′ to bp −76 on the template strand (Fig. 2B). In addition, the Fis footprint showed protection from DNase I digestion at bp −78 to −86 and hypersensitivity at position −88 on the template strand and protection from positions −86 to −89 on the nontemplate strand.

Proximity of the bound MarA and Fis proteins is suggested by the disappearance of a MarA hypersensitive site 3′ to bp −75 on the template strand when the promoter fragment was digested in the presence of both MarA and Fis (Fig. 2B), consistent with some interaction of Fis with the sequences flanking the binding site (34).

Hydroxyl-radical footprinting with MarA revealed a hypersensitive site 3′ to position −64 of the template strand when MarA was bound to the promoter both in the presence and absence of Fis (data not presented). Fis binding had no significant effect on hydroxyl-radical footprints. These results are

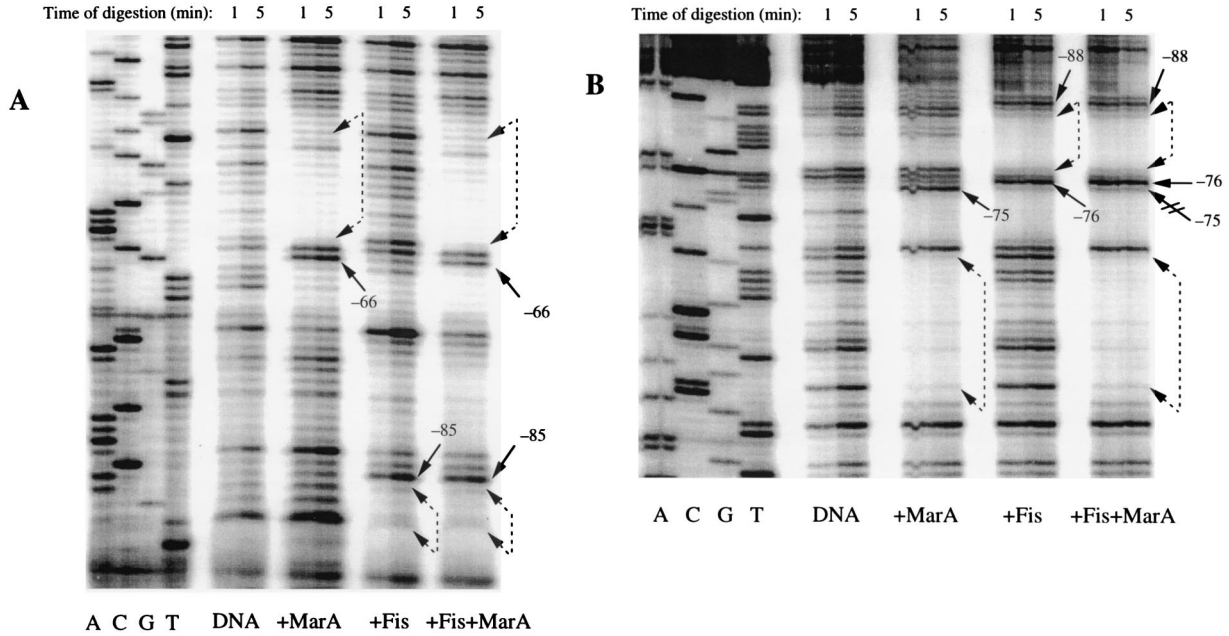

FIG. 2. DNase I footprint of the *mar* promoter region in the absence or presence of 50 nM Fis and/or 100 nM MarA. *mar* DNA fragments prepared by PCR with 5′ end-labelled (^{32}P) primers labelled at position −132 (A) or +23 (B) were digested with DNase I for the indicated times. The bands within the dotted lines represent regions protected from DNase I digestion, and the solid arrows indicate hypersensitive regions. The solid arrow with the crosses indicates the hypersensitive band found upon digestion of the MarA complex which does not show hypersensitivity when the MarA plus Fis complex is digested.

summarized in Fig. 3C. We conclude that the marbox accessory region contains a Fis-binding site.

Isolation and characterization of Fis site and marbox transversion mutants. To determine the functional relationships between the Fis site, the marbox, and transcriptional activity, 13 mutant promoters (Fig. 3A and B), each with a different 5-bp transversion from position −96 to position −32, were synthesized. The Fis- and MarA-binding properties of the mutant promoters were tested and corresponded to those expected on the basis of the footprint analyses (Fig. 4), i.e., Fis site mutants 2 through 5 were significantly reduced in Fis binding but bound MarA normally, whereas marbox mutants 6 through 9 were reduced for MarA binding but bound Fis normally.

The mutant promoters were fused to *lacZ* after the ninth nucleotide of *marR* (bp +36) and integrated in the chromosome at *attP* with a λ vector (41). The β-galactosidase activities of these *mar*::*lacZ* transcriptional fusions are taken as a measure of their transcriptional activities.

In a wild-type bacterial host (GC4468), transversions in seg-

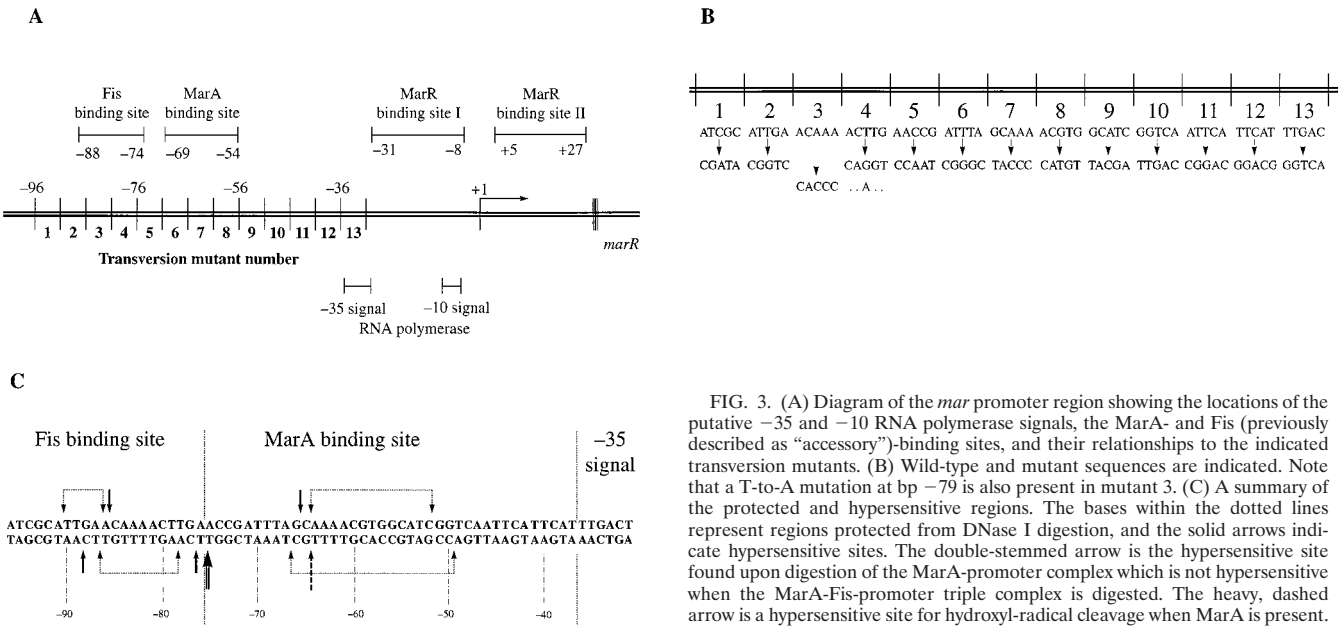

FIG. 3. (A) Diagram of the *mar* promoter region showing the locations of the putative −35 and −10 RNA polymerase signals, the MarA- and Fis (previously described as "accessory")-binding sites, and their relationships to the indicated transversion mutants. (B) Wild-type and mutant sequences are indicated. Note that a T-to-A mutation at bp −79 is also present in mutant 3. (C) A summary of the protected and hypersensitive regions. The bases within the dotted lines represent regions protected from DNase I digestion, and the solid arrows indicate hypersensitive sites. The double-stemmed arrow is the hypersensitive site found upon digestion of the MarA-promoter complex which is not hypersensitive when the MarA-Fis-promoter triple complex is digested. The heavy, dashed arrow is a hypersensitive site for hydroxyl-radical cleavage when MarA is present.

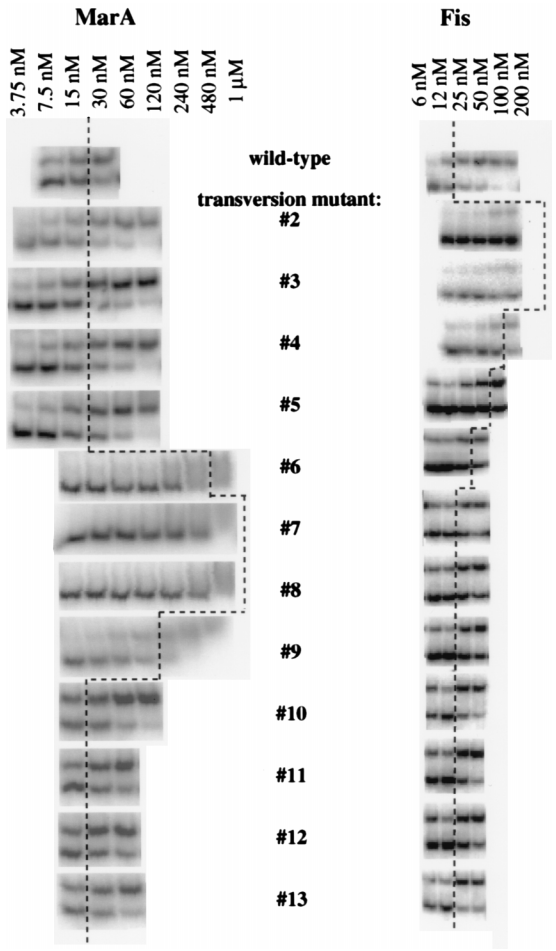

FIG. 4. Binding of *mar* promoter mutant DNAs to MarA or Fis. 5′ end-labelled (^{32}P) *mar* DNA fragments prepared by PCR from wild type and the transversion mutants were incubated for 15 min at room temperature with the indicated concentrations of purified MarA or Fis. The samples were then subjected to polyacrylamide gel electrophoresis on a 4.5% gel. The dotted lines indicate the protein concentrations at which the ratio of unretarded to retarded DNA fragments changes from >1 to <1. The fact that the preparation of Fis used for this experiment had been stored for several years at −70°C may account for its reduced binding affinity.

ments 1, 10, 11, and 12 did not significantly alter the levels of β-galactosidase, indicating that specific sequences from bp −96 to −92 and −51 to −37 are not critical for *mar* promoter activity (Fig. 5A). In contrast, the very low activity due to transversions in segment 13 supports the prediction that bases in the segment −36 to −32 bp are part of the −35 RNA polymerase signal (7).

Transversions in the marbox region (segments 6 to 9) decreased transcriptional activity to 20% of wild type (Fig. 5A). Since comparable reductions in β-galactosidase activity were also found for cells derepressed by treatment with 5 mM salicylate, the defect in transcription exhibited by these transversion mutants does not appear to be related to the levels of active repressor, MarR.

Transversions in the Fis site region (segments 2 to 5, bp −91 to −72) diminished transcriptional activity but to a lesser extent than those in the marbox. Similar reductions in *lacZ* fusion activities were seen previously for the deletions (Δ*marO280* and Δ*marO288*) which originally defined the marbox accessory region (27). Fis site transversions (2 to 5) also had diminished activity in an isogenic host that was deleted for the *mar* operon, N7840 (Fig. 5B).

Notably, Fis did not appear to activate transcription by itself. In mutant hosts lacking MarA and Rob, the same transcriptional activity was seen for the wild-type promoter as for all of the mutant promoters (except for mutant 13) whether or not they were capable of binding Fis (Fig. 5C and D). Thus, activation by Fis is dependent on the presence of MarA or Rob (or induced levels of SoxS; see below). Further elucidation of the role of Fis in activation of the *mar* promoter first required clarification of the role of Rob.

The roles of Rob in *mar* promoter activity. If MarA and SoxS were the only proteins capable of activating the *mar* promoter via the marbox, no difference in activity between wild-type and defective marbox promoters should be seen in strains lacking MarA and uninduced for SoxS (27, 38). (Unless induced, SoxS activity is negligible.)

In eliminating MarA to test this hypothesis, we utilized a *mar* deletion that also eliminated the repressor, MarR. Therefore, in the *marRAB* deletion strain the loss of repression resulted in a four- to sixfold increase in β-galactosidase activity for all of the promoters compared with the same promoters in the wild-type (*mar*$^+$) strain (Fig. 5B). However, the relative activities of the various promoters were similar to those seen in the wild-type host, i.e., the fusions containing marbox transversions (6 to 9) had between 28 and 40% of the activity of the wild-type promoter fusion.

This result suggested that the elevated activity of the wild-type promoter in the *mar* strains might result from activation by some other transcriptional activator, possibly the structurally related Rob protein. Indeed, in the absence of both MarA and Rob, the activity of the wild-type promoter was reduced to about 33% (Fig. 5C) of that found in the *mar rob*$^+$ background and equalled that of the marbox mutant promoters. Thus, basal cellular levels of Rob are responsible for about 65% of *mar* promoter transcription in the absence of MarR and MarA.

Similar comparisons of promoter activities between *rob*$^+$ *mar*$^+$ and *rob mar*$^+$ strains at first seemed inconsistent with

TABLE 1. Effects of *mar* and *rob* mutations and of induction by salicylate and paraquat on transcriptional activities of *mar*::*lacZ* fusions[a]

Strain no.	Host genotype	*mar*::*lacZ* mutation	β-Galactosidase fold stimulation		
			Miller units	Sal[b]	PQ[b]
N8707	Wild type	Wild type	180	7.1	
N8743		Fis site 3	76	9.2	
N8747		Marbox 7	29	4.8	
N8748		Marbox 8	42	4.9	
N8890	*rob*	Wild type	140	8.8	
N8892		Fis site 3	100	8.6	
N8893		Marbox 7	69	3.8	
N8894		Marbox 8	92	4.0	
N8695	*mar*	Wild type	750		1.8
N8713		Fis site 3	380		2.4
N8717		Marbox 7	200		1.1
N8718		Mutant 8	240		1.1
N8795	*rob mar*	Wild type	280		3.1
N8803		Fis site 3	260		3.3
N8807		Marbox 7	230		1.1
N8808		Marbox 8	290		1.1

[a] Bacteria were grown and assayed for β-galactosidase as described previously (38).

[b] Ratio of activities for cells treated with 5 mM sodium salicylate (Sal) or 50 μM paraquat (PQ) for 1 h compared to the same untreated cells.

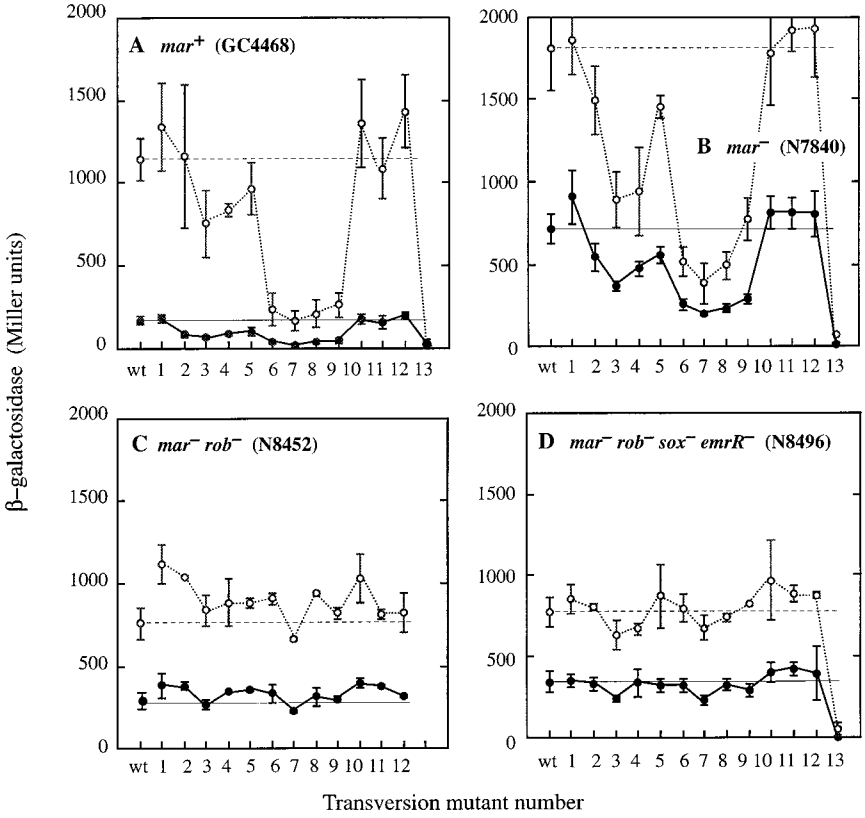

FIG. 5. Promoter activities of the wild-type (wt) and mutant *mar*::*lacZ* fusions in the indicated genetic backgrounds. The filled circles and the open circles (with standard deviations indicated) represent the control and salicylate-induced activities of the strains (connected by thick, solid lines and dotted lines), respectively. The horizontal lines are for comparison with the control (thin, solid lines) and salicylate-induced (dashed lines) wild-type cultures, respectively. Each point is the average of at least four independent assays carried out in duplicate.

this conclusion. The transcriptional activity of the wild-type *mar*::*lacZ* promoter in the *rob mar*$^+$ strain was diminished by only 22% compared to the *rob*$^+$ *mar*$^+$ strain (from 180 to 140 U of β-galactosidase activity [Table 1]). Furthermore, the activities of the mutant promoters 3, 7, and 8 were lower in the *rob*$^+$ *mar*$^+$ strains (76, 29, and 42 U) than in the *rob mar*$^+$ strains (100, 69, and 92 U, respectively). However, these results can be understood by considering the effects of Rob on the levels of MarR. In a strain with a wild-type chromosomal *marRAB* operon, Rob activates the promoter, and therefore the *rob*$^+$ *mar*$^+$ strain produces more MarR repressor than the *rob mar*$^+$ strain. Consequently, while Rob increases the transcriptional activity of the *mar*::*lacZ* promoter, it also increases repression in the *mar*$^+$ strains. For the marbox transversion mutants (no. 7 and 8) which are insensitive to stimulation by Rob, the presence of Rob increases the transcription of the chromosomal *marRAB* promoter and therefore only increases the repression of the mutant fusions. For these reasons, the effects of Rob on the *mar*::*lacZ* promoter are more directly measured in *mar* strains than in *mar*$^+$ strains.

To assess the effect of excess MarA on *mar* promoter activity in the absence of MarR and Rob, MarA was overexpressed from a plasmid in a *rob mar* strain and the β-galactosidase synthesized from the wild-type or marbox transversion mutant 7 was measured (Table 2). Wild-type *mar*::*lacZ* promoter expression increased fourfold when MarA was overexpressed from either of two plasmids. As expected, MarA overexpression did not affect the activity of the marbox mutant 7. In a similar experiment with a *rob*$^+$ *mar* strain, excess MarA stimulated *mar* transcription of a wild-type *mar*::*lacZ* fusion by only 1.6-fold (27). Thus, the contributions of MarA and Rob to the activity of the *mar* promoter are essentially independent.

To determine whether basal levels of SoxS or EmrR affect the basal expression of *mar* transcription, the activities of the various *mar*::*lacZ* fusions were measured in an isogenic *mar rob sox emr* strain (Fig. 5D). The results were comparable to those obtained with the *rob mar* strain, including the effect of treatment with 5 mM salicylate. Thus, as previously observed, neither the basal levels of SoxS (27) nor those of EmrRAB (43) are sufficient to affect *mar* promoter activity significantly. However, induction of SoxS synthesis with 50 μM paraquat stimulated transcription of the wild-type *mar* promoter but not of the marbox promoter mutants 7 or 8, whether they were in *rob*$^+$ *mar* or in *rob mar* strains (Table 1). This result again demonstrates that SoxS activation of the *mar* promoter is dependent on a functional marbox (27).

Despite the absence of functional MarA, MarR, SoxS, Rob, and EmrRAB, salicylate stimulated the wild-type and mutant promoter activities twofold (Fig. 5D). Thus, this unexplained effect of salicylate is not mediated by these proteins or by the specific sequences between bp −96 and −37.

Transcriptional activator-RNA polymerase interaction. We previously demonstrated that in vitro transcriptional activation by MarA at the *zwf* promoter requires interaction with the carboxy-terminal domain of the α subunit of RNA polymerase (21) and inferred that MarA activates the *mar* promoter by enhancing the binding of RNA polymerase (27). To test whether a specific spatial relationship between RNA polymer-

TABLE 2. Activation of *mar* transcription by MarA provided in *trans*[a]

Strain no.	*mar*::*lacZ* promoter	Plasmid[b]	β-Galactosidase (Miller units)	Fold stimulation by MarA
N8795	Wild type	None	280	
N8831		pRGM185	1,150	4.1
N8832		p37	1,120	4.0
N8807	Marbox 7	None	190	
N8833		pRGM185	160	0.84
N8834		p37	220	1.2

[a] *rob mar* cells were grown to early log phase and assayed for β-galactosidase as previously described (38).
[b] Plasmid pRGM185 carries a *marR*-deleted *marRAB* operon and expresses *marA* at high levels (28). Plasmid p37 (31) contains *marA* under the control of a *tet* promoter.

ase and activator is required at the *mar* promoter, the phasing between the marbox and the −35 hexamer was varied by means of DNA insertions. To minimize sequence-specific effects that might be associated with the insertions, two different oligonucleotides were inserted at two sites whose sequence is not critical for transcriptional activity. In a *mar* set of strains (Table 3), inserting 5 bp between the marbox and the −35 hexamer reduced the basal expression of the *mar* operon to 30% of the normal level, the value obtained when transcriptional activation was totally eliminated. In contrast, almost double this activity (i.e., ~42% recovery of stimulatory activity) was found when 10 bp were inserted at the same sites. This result suggests that the spatial relationship between the marbox and the −35 signal is important for activation by Rob. Similar results were obtained when SoxS was induced by paraquat or MarA was provided from a plasmid (data not presented). All this is consistent with the hypothesis that MarA, SoxS, and Rob make protein-protein contacts with RNA polymerase at the *mar* promoter.

Effects of Fis in vivo. To assess the role of Fis in transcriptional activation in vivo, we constructed *fis* strains that were also *mar* and either *rob*$^+$ or *rob*. Since the level of Fis expression is strongly regulated by cell growth (4, 33), we also constructed a set of *fis* strains that contain plasmids which permit the induction of Fis by IPTG (47). We examined *mar*::*lacZ* fusions with the wild-type promoter, Fis site transversion mutations 2 and 3, or marbox transversions 7 and 8.

Transcription of the wild type and all of the mutant promoters tested was reduced approximately 25% by the presence of Fis in the absence of the transcriptional activators MarA, SoxS, and Rob (Table 4, no marbox activator). This was true whether isogenic *fis* and *fis*$^+$ strains or uninduced and induced activities in the *fis*-inducible strains were compared. Whatever the mechanism for this apparent reduction in *mar* transcription by Fis, it cannot be the result of the specific repression of the *mar* operon, since the same reduction is observed whether or not the promoter is capable of binding Fis.

Since Fis is known to both induce and repress a plethora of genes, including those for ribosomal RNA (4, 47), it is reasonable to suppose that this 25% reduction of transcription of the *mar* operon represents no change in the rate of *mar* transcription but, rather, results from an overall increase in growth rate. In support of this possibility, we find that all of our *fis* strains grow more slowly than their *fis*$^+$ counterparts. Furthermore, when the rate of synthesis of *mar*::*lacZ* β-galactosidase is normalized for time of growth rather than cell density, strains N8795 (*fis*$^+$ *rob mar*) and N8900 (*fis rob mar*) have identical values (data not presented).

The intrinsic expression of Rob in a *fis rob*$^+$ *mar* strain was sufficient to induce transcription of the *mar* promoter approximately 1.7-fold relative to that in the corresponding *fis rob mar* strain. This required a functional marbox but not a functional Fis site (Table 4, Rob, and summarized in Table 5).

Notably, in the presence of Rob, Fis had a positive effect on *mar* transcription, unlike the situation in its absence, in which Fis weakly reduced transcription (Table 4 and summarized in Table 6). Fis stimulated transcription an additional 1.5-fold (or 2-fold if normalized for the reduction normally elicited in *mar* transcription by Fis) in *rob*$^+$ *mar* strains whether the comparison was between isogenic *fis rob*$^+$ *mar* and *fis*$^+$ *rob*$^+$ *mar* strains or between a *rob*$^+$ *mar* strain in which Fis was not expressed or was overexpressed by induction. In each case, the activation by Fis required both a functional marbox and a Fis site. The dependence on Rob for the effect of Fis explains why the transcription of the wild-type promoter is approximately 2.7-fold higher in a *fis*$^+$ *rob*$^+$ *mar* strain than in a *fis*$^+$ *rob mar* strain.

Fundamentally the same results were found for the induction by Fis of the wild-type *mar* promoter in the presence of the transcriptional activators SoxS (Table 4, SoxS) or MarA (Table 4). Both SoxS and MarA activated the promoter by greater than twofold (Table 5) in the absence of Fis (requiring only a functional marbox to do so), and the effects of both were

TABLE 3. Effects of insertions on transcriptional activities of *mar*::*lacZ* fusions[a]

Strain	Site of insertion[b]	Size (bp)	Sequence inserted	β-Galactosidase (Miller units)[c]	% Wild-type stimulation[d]
N8695	None			750 (1.0)	100
N8718	None (defective marbox)			193 (0.26)	0
N8700	−52/−51	5	GCATC	40 (0.32)	8
N8866	−44/−43	5	GAATT[e]	220 (0.29)	5
N8702	−52/−51	10	ACGTG GCATC	420 (0.56)	41
N8867	−44/−43	10	GCGCC TAATT[e]	430 (0.57)	43

[a] *rob*$^+$ *mar* bacteria were grown and assayed for β-galactosidase as described previously (38).
[b] Location is given relative to the transcription start site.
[c] Values normalized to the wild-type promoter activity are shown in parentheses.
[d] Calculated with the formula

$$X = 100 \times \frac{(\text{insertion mutant activity} - \text{defective marbox mutant activity})}{(\text{wild-type activity} - \text{defective marbox mutant activity})}$$

[e] For unrelated reasons, these strains have substitutions of A for T at bp −70 and G for C at bp −48 which have no significant effects on their activities.

TABLE 4. Effects of Fis on transcriptional activities of *mar*::*lacZ* fusions[a]

Marbox activator[b]	Promoter mutation[c]	Strain no.			β-Galactosidase (Miller units)		
		Fis−	Fis+[d]	Fis±	Fis−	Fis+	Ratio of Fis+ to Fis−
None							
	Wild type	8900	8795		355	304	0.86
	2 (Fis site)	9396	8802		414	292	0.71
	3 (Fis site)	9397	8803		373	230	0.62
	7 (Marbox)	9398	8807		340	259	0.76
	8 (Marbox)	9399	8808		378	295	0.78
	Wild type			9390	335	263	0.79
	2 (Fis site)			9391	379	261	0.69
	3 (Fis site)			9392	274	178	0.65
	7 (Marbox)			9393	222	149	0.67
	8 (Marbox)			9394	228	157	0.69
Total					330 ± 67		0.72 ± 0.073
Rob							
	Wild type	9006	8695		537	839	1.56
	3 (Fis site)	9008	8713		601	369	0.62
	7 (Marbox)	9009	8717		337	212	0.63
	8 (Marbox)	9010	8718		346	174	0.50
	Wild type			9511	570	784	1.37
	3 (Fis site)			9508	525	470	0.89
	7 (Marbox)			9512	295	215	0.73
	8 (Marbox)			9510	320	238	0.74
SoxS							
	Wild type	8900	8795		761	987	1.30
	2 (Fis site)	9396	8802		908	967	1.06
	3 (Fis site)	9397	8803		738	720	0.98
	7 (Marbox)	9398	8807		319	223	0.70
	8 (Marbox)	9399	8808		324	256	0.79
	Wild type			9390	590	842	1.43
	2 (Fis site)			9391	767	803	1.05
	3 (Fis site)			9392	555	506	0.91
	7 (Marbox)			9393	266	185	0.70
	8 (Marbox)			9394	257	198	0.77
SoxS + Rob							
	Wild type	9006	8695		811	1,494	1.84
	3 (Fis site)	9008	8713		904	872	0.96
	7 (Marbox)	9009	8717		367	226	0.62
	8 (Marbox)	9010	8718		363	266	0.73
	Wild type			9511	622	879	1.41
	3 (Fis site)			9508	560	683	1.22
	7 (Marbox)			9512	226	173	0.77
	8 (Marbox)			9510	238	164	0.69
MarA							
	Wild type	9414	9404		833	1,371	1.65
	2 (Fis site)	9415	9405		1,041	986	0.95
	3 (Fis site)	9416	9406		855	882	1.03
	7 (Marbox)	9417	9407		242	178	0.73
	8 (Marbox)	9418	9408		257	284	1.11
MarA + Rob							
	Wild type	9410	9400		773	1,226	1.59
	3 (Fis site)	9411	9401		874	927	1.06
	7 (Marbox)	9412	9402		276	177	0.64
	8 (Marbox)	9413	9403		278	230	0.83

[a] Unless otherwise indicated, *mar*-deleted *fis rob* bacteria were grown, induced if indicated (see Materials and Methods) and assayed for β-galactosidase (38). Fis−, without Fis; Fis+, with Fis.

[b] Rob presence was due to chromosomal wild-type *rob* expression, SoxS presence was due to paraquat induction of the otherwise quiescent *soxRS*, and MarA presence was due to plasmid p37.

[c] The *mar* promoter mutation in the *mar*::*lacZ* fusion and, in parentheses, the site it affects are indicated.

[d] Fis production was due either to chromosomal wild-type *fis* expression (first group of strains in each set) or to IPTG induction of *fis* plasmid pRJ4000 (second group of strains).

TABLE 5. Average stimulation by activators Rob, SoxS, and MarA of *mar*::*lacZ* in *fis* mutant strains[a]

Promoter	Activator		
	Rob	SoxS	MarA
Wild type	1.7	2.4	2.5
Fis site mutants	1.7	2.6	2.9
Marbox mutants	1.0	1.0	0.8

[a]The β-galactosidase values from *fis* strains with either wild-type, Fis site, or marbox promoter mutations (e.g., marbox mutants N9009 and N9010) and uninduced for Fis expression (e.g., N9512 and N9510) were combined and averaged. This number was then divided by 330, the average value for all the uninduced *fis* strains in the first section of Table 4, to give the effect of each activator in the absence of Fis.

further enhanced ~1.5-fold by Fis (Table 6), if and only if the promoter contained both a functional Fis site and a marbox. We conclude that transcriptional activation by Fis is dependent on the presence of MarA, SoxS, or Rob at the *mar* promoter.

Spacing between Fis site and marbox and the mechanism of Fis activation. To characterize further the relationship of Fis bound to the Fis site with the marbox activators bound to the marbox, the spatial relationship between the Fis site and the marbox was probed with DNA insertions. MarA and Fis bound with normal affinity to the insertions containing 5 or 10 bp between positions −70 and −71. However, unlike the insertions between the −35 polymerase signal and the marbox, where activity was lost with a 5-bp insertion and partially recovered by a 10-bp insertion, all Fis stimulatory activity was lost with 5-, 10-, 15-, and 20-bp insertions (data not shown).

We have considered three mechanisms for the activation by Fis of the *mar* promoter. The first is that Fis acts primarily as a recruitment signal for MarA. In this case the binding coefficient of MarA to a DNA fragment should be altered in the presence of Fis. However, no cooperative or inhibitory effect of Fis on MarA binding was observed (Fig. 1A).

Second, we considered the possibility that Fis and MarA might form a protein-protein complex (in addition to their DNA-binding and/or RNA polymerase binding capacities). While we have not ruled out this possibility, we have been unable to find any evidence for a triple complex of MarA, Fis, and DNA when the DNA was mutant in either the marbox or the Fis site (Fig. 1B). This leaves a third possibility that the binding of both Fis and MarA to their respective sites facilitates the folding of the promoter into a more active configuration and/or allows Fis to make a favorable contact with RNA polymerase.

TABLE 6. Average stimulation by Fis of *mar*::*lacZ* in the absence or presence of activators[a]

Promoter	Activator			
	None	Rob	SoxS	MarA
Wild type	0.8	1.5	1.5	1.6
Fis site mutants	0.7	0.8	1.0	1.0
Marbox mutants	0.7	0.7	0.7	0.8

[a] The Fis^+-to-Fis^- β-galactosidase ratios in the presence of the indicated marbox activator for each of the three promoter types were averaged and divided by 0.72, the average Fis+-to-Fis− ratio for all the strains in the first section of Table 4.

DISCUSSION

***mar* activation sites.** The *mar* promoter may be divided into two functional segments with respect to the putative −35 RNA polymerase binding element (Fig. 3), an upstream transcriptional activation region (27) and a downstream repressor-binding region (29). To analyze systematically the upstream region (from −96 to −32), we prepared 13 promoters, each bearing a 5-bp sequence in which transversions (A↔C and G↔T) have been generated. The losses of function due to these mutations help define three elements of the promoter: (i) the severely reduced activity of mutant 13 coincides with the predicted (7) −35 transcription signal; (ii) the loss of transcriptional activation in mutants 6 to 9 coincides with the marbox, previously identified by footprinting and deletion studies (27); and (iii) the partial loss of transcriptional activation in mutants 2 to 5 coincides with the Fis site, previously identified in deletion studies (27) as an accessory marbox region.

Marbox function. The marbox was originally identified as a sequence whose sensitivity to DNase I was altered by the presence of MarA (27). Transversions of this sequence are present in mutants 6 to 9 and result in reduced promoter activity in vivo and reduced affinity for MarA in vitro. While the adjacent base pairs (from −51 through −37) appear to play no sequence-specific role in transcriptional activation, 5-bp insertions in this region (between bp −52 and −51 or between −44 and −43) abolished transcriptional activation, whereas 10-bp insertions had lesser effects. Thus, an appropriate alignment of the marbox with the −35 RNA polymerase binding site appears to be essential for activation, presumably to allow RNA polymerase and MarA (or SoxS or Rob) to interact.

Activators of the marbox. In addition to MarA and SoxS, this report shows that Rob is a transcriptional activator of the *mar* promoter via interaction with the marbox. Genetic elimination of both MarA and Rob resulted in complete loss of activation, equal to that seen in marbox transversion mutants (Fig. 5C). About 65% of *mar* transcription in vivo results from activation by basal levels of Rob in chromosomally *mar* strains. In the presence of wild-type *mar*, it is difficult to estimate the transcriptional activation by Rob on a *mar*::*lacZ* fusion due to Rob's activation of the chromosomal locus, resulting in *marR* expression and the repression of *mar*. In contrast to MarA and Rob, basal expression of SoxS does not contribute significantly to *mar* activation (compare Fig. 5C and D) (27). Nevertheless, when induced by paraquat, the activity of SoxS was not dependent on either MarA or Rob (Table 1). Thus, basal levels of MarA and Rob and induced levels of SoxS independently contribute to transcriptional activation of *mar* and require a functional marbox for activity.

This is the first demonstration of a function for basal levels of Rob in vivo. Recent experiments show that basal levels of MarA and Rob each contribute about 50% to the activity of another *mar* regulon promoter, *inaA* (37). Previously, overexpression of Rob was shown to induce multiple antibiotic and superoxide resistance phenotypes (3), to activate expression of *fumC*, *inaA*, and *sodA* in vivo (3), and to activate the transcription of six *mar*/*soxRS* regulon promoters in vitro by mechanisms that are strikingly similar to that of MarA and SoxS (22). Since it is estimated that *E. coli* contains 5,000 molecules of Rob under normal conditions (42), why this is sufficient to activate *mar* and *inaA* but not other regulon promoters is unclear.

Identification of a Fis site in the *mar* promoter. Deletion analysis of the marbox region led to the identification of an "accessory" region (−89 to −72) which was involved in transcriptional activation but did not itself bind MarA (27). The

finer analysis afforded by the transversion mutations presented here verifies that this region stimulates *mar* transcription twofold but does not affect the binding of MarA. Instead, this region (present in segments 2 to 6) is shown here to contain a sequence-specific binding site for the Fis protein (Fig. 1 to 3). The Fis consensus sequence (GNNPyPuNNA/TNNPyPuNNC [18]) is perfectly matched by the *mar* sequence from −88 to −74 which lies in segments 2 to 5. DNase I footprinting studies (Fig. 2) revealed characteristic hypersensitive sites on one strand at −85 and on the other at −76 due to binding and presumably bending of the DNA by Fis.

Curiously, in the absence of transcriptional activators, Fis reduced *mar* transcription approximately 25%. This reduction occurred whether or not the promoter was capable of binding Fis. Thus, Fis itself is neither a transcriptional activator of the *mar* promoter nor a repressor. Nevertheless, Fis stimulated the transcriptional activation of the promoter by Rob, SoxS, and MarA. Each of these activators, by binding to the marbox region, was capable of stimulating transcription 1.5- to 2-fold in a *fis* mutant. In *fis*$^+$ strains the induction increased by an additional twofold, provided the promoter contained both a functional marbox and a Fis site. Fis is therefore acting as an accessory transcriptional activator of the *mar* promoter (5, 11). This is in contrast to other promoters in which Fis activation is independent of other known activators (5).

Although the stimulation of transcription by MarA (and SoxS and Rob) appears to result from protein-protein interaction with RNA polymerase, there is little to suggest that protein-protein interactions between Fis and the activators mediate the effect of Fis on *mar*. Fis does not appear to interact with MarA, at least not in vitro, since there was evidence neither of cooperativity between Fis and MarA for binding to *mar* promoter DNA (Fig. 1A) nor of the formation of MarA-Fis-DNA triple complexes in the absence of either a functional marbox or a Fis site (Fig. 1B). However, we have not ruled out the possibility of weak protein-protein interactions between the activators and Fis when both are bound to adjacent sequences. In the latter case it appears that such interactions cannot take place when the marbox and the Fis sites are separated by 5 or more bp (data not shown).

Another explanation for activation by Fis is that it requires the formation of a DNA configuration induced in part by the transcriptional activator. It has recently been proposed that another DNA-binding and -bending protein, integration host factor, promotes open complex formation at the *ilvPG* promoter by locally deforming the DNA (35).

MarR-independent effects of salicylate. The *mar* promoter is regulated by two independent mechanisms, transcriptional activation, depending on Fis and the MarA/Rob/SoxS activators, and repression due to MarR. Depending on the levels of activators and MarR, promoter activity can vary over about a 40-fold range. Surprisingly, salicylate induced a twofold increase in promoter activity from the *lacZ* fusions that was independent of sequences from −91 to −32, of MarA, Rob, and SoxS activators and of MarR and EmrR repressors (Fig. 5D). Whether this is due to the interaction of salicylate with an unknown repressor or with the transcription complex itself remains to be determined.

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