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The *Escherichia coli* Transcriptional Regulator MarA Directly Represses Transcription of *purA* and *hdeA**

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Thamarai Schneiders‡, Teresa M. Barbosa‡§, Laura M. McMurry‡, and Stuart B. Levy‡¶‖

From the ‡Center for Adaptation Genetics and Drug Resistance and the Departments of Molecular Biology and Microbiology and of ¶Medicine, Tufts University School of Medicine, Boston, Massachusetts 02111

The *Escherichia coli* MarA protein mediates a response to multiple environmental stresses through the activation or repression *in vivo* of a large number of chromosomal genes. Transcriptional activation for a number of these genes has been shown to occur via direct interaction of MarA with a 20-bp degenerate asymmetric "marbox" sequence. It was not known whether repression by MarA was also direct. We found that purified MarA was sufficient *in vitro* to repress transcription of both *purA* and *hdeA*. Transcription and electrophoretic mobility shift experiments *in vitro* using mutant promoters suggested that the marbox involved in the repression overlapped the −35 promoter motif and was in the "backward" orientation. This organization contrasts with that of the class II promoters activated by MarA, in which the marbox also overlaps the −35 motif but is in the "forward" orientation. We conclude that MarA, a member of the AraC/XylS family, can act directly as a repressor or an activator, depending on the position and orientation of the marbox within a promoter.

The *Escherichia coli* MarA protein, a member of the AraC/XylS family of transcriptional regulators, mediates cellular responses to stress through the differential control of a large number of chromosomal genes comprising the *mar* regulon (1–5). MarA causes decreased susceptibility to structurally unrelated antibiotics, organic solvents, household disinfectants, and oxidative stress agents (1). The MarA homologs SoxS and Rob in *E. coli* are also stress-response proteins (2).

Transcriptional activation of those *mar* regulon members that have been studied occurs when MarA binds as a monomer to the promoter region at a degenerate asymmetric 20-bp DNA sequence known as the "marbox" (6, 7). The mechanisms by which MarA activates members of the *mar* regulon and the details of MarA, SoxS, and Rob interactions with the marbox DNA have been investigated (for example see Refs. 6, 8–11). MarA was originally described as an activator. Its expression directly induced transcription of all previously studied *mar* regulon members except *ompF*, whose down-regulation resulted indirectly from MarA activation of the antisense RNA *micF*, which then inhibited the translation of *ompF* (12, 13). Repression by MarA has been shown recently *in vivo* for several dozen genes by two independent macroarray studies, although the gene overlap between the two studies was not large (4, 5). To determine whether the repression by MarA was direct or occurred by an indirect action, we focused on two genes, *purA* and *hdeA*.

In the intact cell, transcription of *purA* was decreased in cells constitutively producing MarA (4). Expression of *hdeA* was repressed by induced expression of MarA (see Ref. 5, Supplemental Material). *purA* codes for adenylosuccinate synthase, which plays an important role in the *de novo* pathway of purine nucleotide biosynthesis and is required for adenosine monophosphate synthesis from inosine −5′-monophosphate (14). An association between *purA* and virulence has also been proposed (15, 16). *hdeA* encodes a periplasmic protein involved in acid resistance (17, 18). In *Shigella flexneri* and *E. coli*, both acid-resistant organisms, *hdeA* is present, but it is absent in *Salmonella*, which is merely acid-tolerant (17).

We show here that MarA directly represses both *purA* and *hdeA*. Thus MarA is a dual regulator capable of both activation and repression, depending upon the promoter.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Growth Conditions—E. coli K12 strains and plasmids used in this study are shown in Table I. Strains were routinely grown at 37 °C in LB broth (per liter: 10 g of tryptone, 5 g of yeast extract, 5 g of NaCl).

General Molecular Biology Manipulations—Preparation of genomic and plasmid DNA and purification of PCR products and of DNA fragments from agarose gels were carried out with Qiagen products as follows: QIAamp Tissue kit, QIAprep Spin Miniprep kit, QIAquick PCR purification kit, and QIAquick gel extraction kit, respectively, following the manufacturer's instructions. Restriction enzyme digestions followed standard procedures (19). PCR amplification utilized either Taq polymerase (Invitrogen) or TripleMaster Polymerase Mix (proofreading) (Eppendorf) in a GeneAmp® PCR System 9700 thermocycler (PE Applied Biosystems, PerkinElmer Life Sciences). Oligonucleotide synthesis and DNA sequencing were performed by the Tufts University Core Facility.

RNA Preparation and Northern Analysis—Total RNA was extracted from cells using the Qiagen RNeasy kit. Two micrograms of RNA were fractionated by electrophoresis in a denaturing formaldehyde agarose gel, which was then blotted onto a Hybond-N (Amersham Biosciences) membrane. RNA was visualized by both ethidium bromide staining of the gel and by methylene blue staining of the membrane. *purA* or *hdeA* probes were amplified by PCR using AG100 chromosomal DNA as template. For *purA*, an ~1270-bp region was amplified using the primer pair F2:P-Rev (GAAAACGATTGGCTGAAC:AAGGTGGATTCAGACCAG). For *hdeA*, an ~396-bp region was amplified using the primer pair HdeA1:HdeA2 (TTGATTCGTGACGGCTCT:ATGCAAGGAAGTACGATGT). Hybridization of membrane-bound RNA to the *purA* or *hdeA* probes was performed as described previously (4).

PCR Amplification of Promoter Regions Used in Transcription in Vitro—The promoter region of the *nfnB*-luciferase fusion in pSP-*nfnB*1 was amplified with the primer pair NFN-F1:lucR (CCCGGTACCCT-

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§ Supported in part by Subprograma Ciência e Tecnologia do 2º Quadro Comunitário de Apoio-PRAXIS XXI/BPD/22065/99, Portugal. Present address, Instituto de Tecnologia Química e Biológica, Avenida da República, Apartado 127, 2781-901 Oeiras Codex, Portugal.

‖ To whom correspondence should be addressed: Center for Adaptation Genetics and Drug Resistance, Tufts University School of Medicine, 136 Harrison Ave., Boston, MA 02111. Tel.: 617-636-6764; Fax: 617-636-0458; E-mail: stuart.levy@tufts.edu.

TABLE I
Bacterial strains and plasmids used in this study

Name	Genotype/relevant characteristics	Ref./Source
E. coli K12 strains		
AG100	*argE3 thi-1 rpsL xyl supE44* [Δ *(gal-uvrB)*?]	42; for ?, see Ref. 4
GC4468	Δ (*lac*)*U169 rpsL*	43
JHC1096	Same as GC4468 but *zdd-239*::Tn9 *del1738* (Δ 39 kb including *marRAB* locus)	26
Plasmids		
pMB102	*ori* colE1 *lacI lacZp*::*marA*, AmpR	5
pSP-nfnB1	pSP-luc+ (Promega; promoter-less luciferase gene, *ori* colE1, AmpR) containing a 177-bp fragment of *nfnB* promoter	20

TCGCGATCTGTCAACG:CTTCCAGCGGATAGAATGG), producing a 261-bp product with a 105-nt[1] transcript (20). All other PCR products were generated using AG100 chromosomal DNA. The wild type 225-bp *purA* promoter-containing template was amplified using primer pair purApF:purApR (GGAAAACGATTGGCTGAAC:CGTTCAGTCAGAAGATCG). PCR products containing mutated *purA* marboxes were made using reverse primer purApR together with a mutated forward primer. The 5′ end of a mutated forward primer corresponded to the 5′ end of the PCR product shown in Fig. 5; its arbitrary 3′ terminus was either . . . AACTCTG-3′ (for D2F and D3F) or . . . GAAAAGC-3′ (for all others). All *purA* templates yielded a transcript of 105 nt. For *hdeA*, a 193-bp PCR product of the promoter region was amplified using the primer pair HdeA1a:HdeA2a (TCTGATGCATCTGTAACTCA:GAAGCAGACCACCAAGAATA). Mutant PCR products were made with the reverse primer HdeA2a together with primers beginning at the 5′ end shown in Fig. 6. All *hdeA* transcripts were 94 nt long. The 167-bp *gnd* promoter-containing PCR product used the primers GNDF:GNDR (TCGCAACTTTGATCGAAT:TACATACTCCTGTCAGGT); the transcript was 55 nt long. All PCR products were gel-purified before being used in the transcription reactions *in vitro*.

Transcription in Vitro—N-terminal polyhistidine-tagged MarA (His_6-MarA) protein was purified essentially as described by Jair *et al.* (21) and was a generous gift of Victoria Bartlett and Michael Alekshun (Paratek Pharmaceuticals, Boston). Single round (5 min) transcription *in vitro* was carried out essentially as described (22) in 30 μl using PCR products as DNA templates. The Reaction Buffer at pH 7.8 contained 50 mM Tris-HCl, 0.1 mM EDTA, 3 mM magnesium acetate, 0.1 mM dithiothreitol, 20 mM sodium chloride, and 250 μg/ml bovine serum albumin. Other components of the pre-incubation mixture are as follows: *E. coli* RNA polymerase holoenzyme (σ70; Epicentre), 40 nM; the RNase inhibitor SUPERase In™ (Ambion), 500 units/ml; test DNA (*purA*, *hdeA*, or *nfnB* promoter-containing DNAs); and control DNA (*gnd* promoter-containing DNA) each at 2 nM. In most experiments involving mutant templates with lower transcription levels, the concentration of each test DNA was raised to 4 or 6 nM. Purified His_6-MarA, diluted as needed in Reaction Buffer, was added to the pre-incubation mixture on ice to give 200 nM; the buffer in which His_6-MarA was prepared was similarly diluted and added to the control. Open complexes were allowed to form at 37 °C for 15 min, when a heparin/nucleotide mixture was added to initiate transcription; the final concentrations were heparin, 1.2 mg/ml, and nucleotides, 0.3 mM UTP, 0.96 mM ATP, CTP, and GTP with [α-^{32}P]UTP at 0.5 μCi/ml (PerkinElmer Life Sciences). After 5 min, an equal volume of Ambion gel loading buffer II was added to stop the reaction; the samples were boiled for 3 min, chilled, and fractionated by electrophoresis on a 7% polyacrylamide, 8 M urea gel in Tris borate EDTA, pH 8.3.

Quantification of Transcript Levels—Radioactivity was detected by exposure of membranes/dried gels to Kodak BioMax MS x-ray film; alternatively, exposure to a storage phosphor screen was followed by scanning with a Storm PhosphorImager (Amersham Biosciences). Film images for methylene blue-stained membranes showing ribosomal RNA were scanned into Adobe Photoshop, and the bands were quantified using NIH Image 1.62. Transcripts from both Northern blots and from transcription reactions *in vitro* were quantified after PhosphorImager scanning by ImageQuant (Amersham Biosciences). The amounts of the two major transcripts produced, *in vivo* by *hdeA* or *in vitro* by *purA*, were combined. The amount of each test transcript was normalized by the level of the ribosomal RNA (for Northern blots) or *gnd* transcript (for *in vitro* transcriptions).

Identification of Putative Marboxes—Putative marboxes were identified using the "search patterns" utility of Colibri (genolist.pasteur.fr/Colibri/) with the 20-bp degenerate consensus sequence A(CAT)RGCACRWWNNRYYAAA(CAT)N, where R indicates A or G; W indicates A or T; Y indicates T or C; and N indicates A, T, G, or C (7). Only marboxes between 150-bp upstream and 100-bp downstream of the transcriptional start that had 15 or more matches and the 5th C invariant were considered.

Electrophoretic Mobility Shift Assays (EMSA) for purA—Binding of purified His_6-MarA to DNA from the *purA* promoter region was measured by EMSA. The DNA used for fragments of 107–185-bp was amplified by PCR using a chromosomal AG100 template, whereas the 20–29-mers encompassing marboxes were made by annealing equimolar concentrations of complementary DNA oligomers. The forward oligomers for the 20-bp marboxes were marbox 1 (AGTGCAAAAAGTGCTGTAAC), marbox 2 (TTGAGTGCAAAAAGTGCTGT), and marbox 3 (AGGTCATTTTTGAGTGCAAA). The forward oligomer for the wild type 29-bp fragment comprising overlapping marboxes 1 and 2 was TTTTGAGTGCAAAAAGTGCTGTAACTCTG. Two mutant oligomers were designed such that all 4-bp of RE1 of either marbox 1 or marbox 2 were mutated; the mutations are shown in Fig. 5, *MB1.1F* and *MB2.1F*, respectively. In all cases, DNA was labeled at the 3′ end with digoxigenin-11-ddUTP (DIG gel shift kit, Roche Applied Science), and the EMSA reactions were performed as described previously (20).

RESULTS

Transient Induction of MarA Caused a Decrease of purA and hdeA Expression in Cells—The earlier *E. coli* DNA macroarray experiments showing that constitutively produced MarA reduced expression of *purA* (4) were confirmed subsequently using Northern assays (data not shown). To determine whether this repression was a result of MarA function, as opposed to a stress-related artifact associated with the constitutive overproduction of MarA, we controlled expression of *marA* with the IPTG-inducible *lac* promoter in plasmid pMB102 (5) in the *mar*-deleted strain JHC1096 (Table I). In the same experiment we also examined the expression of *hdeA*, which had been observed in another macroarray study to be repressed upon MarA induction in cells (5). After a 1-h induction of MarA by IPTG, a decline was seen in the levels of transcripts of *purA* (Fig. 1*A*) and *hdeA* (Fig. 1*B*). Typically, the decrease at 1 mM IPTG was ~3-fold for *purA* and 6–25-fold for *hdeA*, with less IPTG required for *hdeA*. Each of the two bands in Fig. 1*B* probably includes both genes of the *hdeAB* operon (23, 35); therefore, *hdeB* was probably also repressed. No decrease was seen in cells bearing the vector control (pJPBH) (Fig. 1, *A* and *B*). These results show that the decrease in *purA* and *hdeA* expression was caused, directly or indirectly, by transiently synthesized MarA rather than by a stress reaction due to constitutively overabundant MarA.

Purified MarA Repressed Transcription of purA and hdeA in Vitro—To determine whether repression was direct or indirect, we tested the effect of purified His_6-MarA protein on transcription of *purA* and *hdeA in vitro*. This protein, engineered to facilitate purification and hereafter referred to as MarA, had been shown previously to activate *nfnB* transcription *in vitro* (20). The DNA templates for the single round transcription reactions were PCR products bearing a promoter, its transcriptional start, and 55–105 bp downstream. The transcriptional start sites of the *purA* gene (14) and the *hdeAB* operon (23) have been defined. We mixed the promoter-containing DNA of

[1] The abbreviations used are: nt, nucleotide; EMSA, electrophoretic mobility shift assays; IPTG, isopropyl-1-thio-β-D-galactopyranoside.

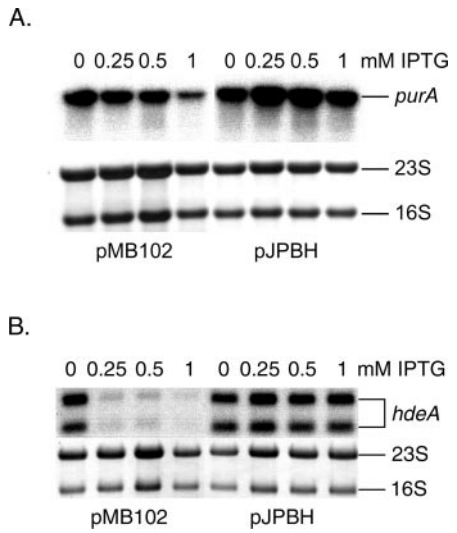

FIG. 1. **Northern blots of *purA* and *hdeA* transcripts following induction of MarA.** Total RNA was prepared from cultures of strain JHC1096 (Δ*marRAB*) bearing either pJPBH (vector) or pMB102 (*marA*) that had been induced at A_{600} = 0.5 with increasing concentrations of IPTG (0, 0.25, 0.5, and 1 mM) for 1 h. Loading of rRNA bands was visualized by methylene blue staining (shown below the Northern blots). *A*, hybridized with a ^{32}P-labeled internal fragment of the *purA* gene. The size of the band is 1.2 kb. *B,* hybridized similarly with *hdeA*, using the same RNA on a different blot. The sizes of the bands are 0.6 and 0.9 kb.

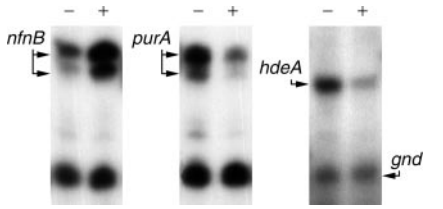

FIG. 2. **Effect of MarA on transcription *in vitro* of *purA, hdeA,* and *nfnB*.** Mixtures of test DNA templates (either *purA, hdeA,* or *nfnB*) with control template (*gnd*) were incubated for transcription *in vitro* using [^{32}P]UTP (see "Experimental Procedures") without (−) or with (+) 200 nM of purified MarA. Samples were fractionated by polyacrylamide/urea gel electrophoresis. The gel was dried and exposed to film. The larger of the two major bands for *nfnB* and *purA* represents the predicted full-length run-off transcript.

purA or *hdeA* with that of the control *gnd* (a promoter that is unresponsive to MarA) (21, 22, 24, 25). The promoter for the MarA-activated gene *nfnB* (20), also mixed with *gnd* DNA, was tested in parallel as a positive control for MarA activity. In the presence of 200 nM MarA, there was a 5–6-fold activation in *nfnB* transcription, as expected (Fig. 2). As anticipated, MarA did not affect the expression from the control *gnd* promoter. The same concentration and batch of MarA protein, on the other hand, repressed both *purA* and *hdeA* (Fig. 2). The average repression ratio was 0.44 for *purA* (see Fig. 5, *WT*) and 0.60 for *hdeA* (see Fig. 6, *WT*) relative to controls without MarA. These results showed that MarA by itself could directly repress *purA* and *hdeA* transcription.

DNA Sequences Involved in Binding by MarA, EMSA—Activation of transcription by MarA involves binding of this protein as a monomer to a degenerate 20-bp "marbox" located upstream from or overlapping the −35 motif within the promoter (6, 10). It seemed likely that for repression, a marbox in the promoter region would also be involved, although perhaps with a different location or orientation.

The *purA* promoter region contained five consensus marboxes (see "Experimental Procedures" for criteria); all were

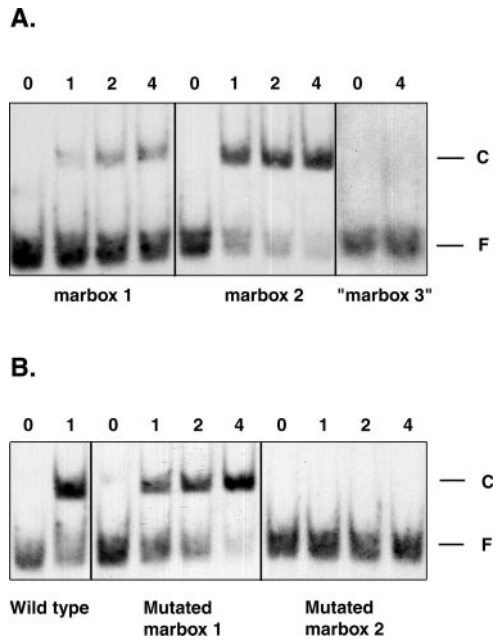

FIG. 3. **Electrophoretic mobility shift of *purA* promoter regions by MarA.** The concentrations of MarA used were 0, 100, 200, or 400 nM (*lanes 0*, *1*, *2*, and *4*, respectively). *A*, wild type DNA. The DNA fragments were 20-bp duplexes, each comprising marbox 1, marbox 2, or the less conserved control marbox 3. *B*, mutations in marbox 1 or marbox 2. The DNA fragments were 29-bp duplexes comprising both marboxes 1 and 2. An unmutated fragment (*wild type*) was the control, whereas the other two fragments were mutated at all 4 bp of the RE1 of either marbox 1 or marbox 2 (these mutations are shown in Fig. 5 in MB1.1F and MB2.1F, respectively). *C*, complex of MarA/DNA; *F*, free DNA.

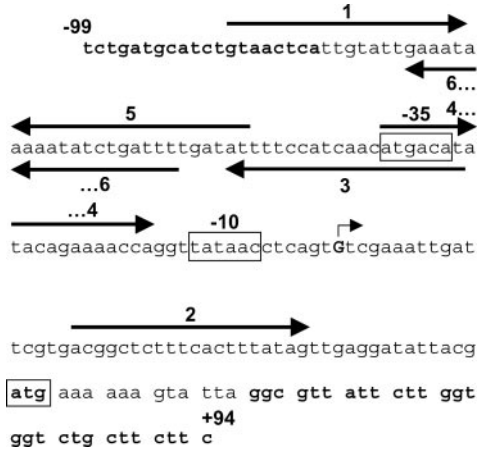

FIG. 4. **Putative marboxes within the *hdeA* promoter.** Six putative marboxes were found using the criteria described under "Experimental Procedures." The direction of the *arrows* depicts the orientation of the marboxes. The sequences in *boldface* indicate the forward and reverse primers used in the amplification of the wild type PCR fragment. RNA polymerase recognition sequences are *boxed.* The *G* in *boldface* with the *bent arrow* indicates the transcriptional start site (23). The translational start site is *boxed* and in *boldface.*

upstream of the translational start site. By EMSA using PCR products covering various regions of the *purA* promoter, we showed that MarA bound with similar affinity to fragments spanning bp −140 to +45, −121 to −14, and −96 to +45, relative to the transcriptional start (data not shown). Therefore, binding occurred between bp −96 and −14, eliminating three of the marboxes from consideration. The remaining were marbox 1 (forward, bp −49 to −30, 15/20 consensus bp) and the overlapping marbox 2 (backward, bp −33 to −52, 15/20 consensus bp) (see Fig. 5). Further mobility shift assays with 20-bp

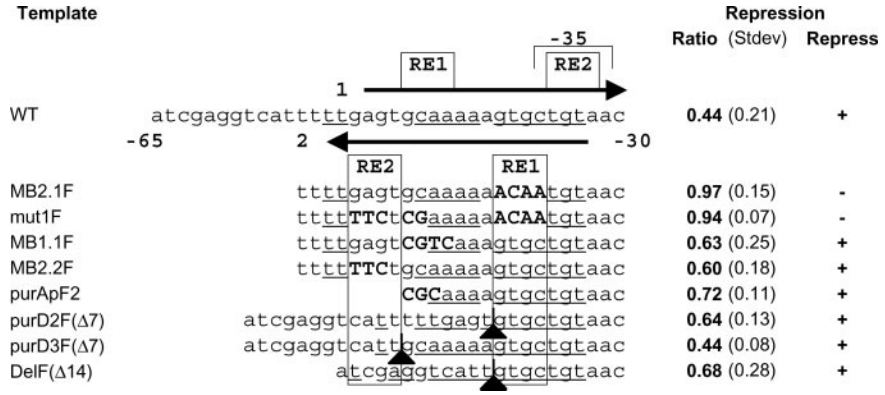

FIG. 5. **Effect of mutations in the *purA* promoter upon repression *in vitro* by MarA.** Residues are numbered with respect to the transcriptional start site, which is +1. The wild type sequence (*WT*) is shown at the *top*, with overlapping, oppositely oriented marboxes 1 (*forward arrow*) and 2 (*reverse arrow*); the −35 promoter hexamer is *below a bracket*. Each succeeding line represents a mutant template. All templates began at the *left-most* residue shown except for the wild type template, which began at bp −120. All templates ended at bp +105. Mutations are shown by *boldface uppercase*. Deletions are located by a *vertical tick*/*arrowhead*, with the number of bp deleted shown beside the template name. Residues conforming to the marbox consensus (29) for marbox 2 are *underlined*. The effect of MarA is expressed as the ratio of *purA* transcription in the presence of MarA divided by *purA* transcription in its absence, normalized for any differences in the transcription of *gnd*. The lower the ratio, the more the repression. Values represent an average of 2–12 experiments. *Stdev*, standard deviation; *RE*, marbox recognition element; +, repressed; −, not repressed.

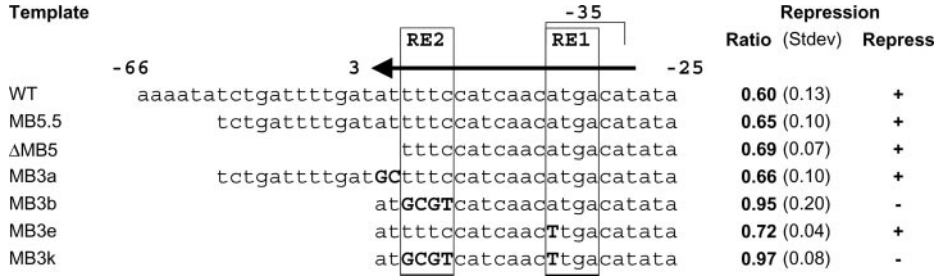

FIG. 6. **Effect of mutations in the *hdeA* promoter upon repression *in vitro* by MarA.** See the legend of Fig. 5 for format. The *arrow* indicates marbox 3. The wild type template began at bp −99. All the other templates had 5′ start sites as shown. All templates ended at bp +94. Values represent an average of 4–12 experiments.

DNA fragments comprising the individual marboxes showed that MarA did not bind to a "control" poor consensus backward "marbox 3" (bp −42 to −61; only 12/20 consensus bp) but did bind to marboxes 1 and 2. The affinity for marbox 2 ($K_d < 100$ nM) was greater than that for marbox 1 ($K_d > 400$ nM) (Fig. 3*A*), suggesting that marbox 2 was preferred.

It was not straightforward to further distinguish the relative importance of marboxes 1 and 2 because they overlapped at 17 of their 20 bp (see Fig. 5). We therefore took advantage of the two highly conserved marbox "recognition elements," RE1 and RE2, of 4 bp each which are particularly critical for activation by SoxS (27) and presumably by MarA. The crystal structure of MarA bound to marbox DNA shows the two recognition helices of the protein in contact with the recognition elements of the marbox (10). We mutated all 4 bp of RE1 of either marbox 1 or marbox 2 (see "Experimental Procedures" and Fig. 5) in 29-bp duplexes containing both *purA* marboxes 1 and 2. The mutations in the RE1 of one marbox did not affect the RE1 of the other. We found that MarA still bound to mutated *purA* marbox 1 but did not bind to mutated marbox 2 (Fig. 3*B*). This result confirmed the importance of marbox 2.

In the case of *hdeA*, there were six consensus marboxes (Fig. 4; see "Experimental Procedures" for criteria). However, EMSA experiments were not conclusive because a shift in mobility was seen for only a very small fraction of *hdeA* promoter DNA, even with MarA at 750 nM.

DNA Sequences Involved in Repression by MarA, Transcription Assays in Vitro—For *purA*, the EMSA analyses had identified marbox 2 as more important for MarA binding but did not prove its involvement in MarA-mediated transcriptional repression. Therefore, we repeated the *in vitro* transcription reactions using promoters with mutations in marboxes 1 and 2 to look for loss of repression. Both deletions and point mutations were used. Some of these mutations caused a reduction in transcription itself (see "Discussion"), so a doubling or tripling in the amount of template DNA (including with wild type controls) was used to enhance the amount of transcript (see "Experimental Procedures").

For *purA*, changing 3 bp in RE2 of marbox 2 did not prevent repression (Fig. 5, *MB2.2F*) nor did deletions of 7 or 14 bp in marboxes 1 and 2 (Fig. 5, *purD2F, purD3F,* and *DelF*). Truncation of the final 6 bp of marbox 2 decreased but did not eliminate repression (Fig. 5, *purApF2*). The unexpected repression still seen despite marbox deletions and truncation is discussed below. Nevertheless, repression was clearly prevented by the alteration of all 4 bp of RE1 in marbox 2 (Fig. 5, *MB2.1F*), whereas no effect was seen for mutations in RE1 of marbox 1 (Fig. 5, *MB1.1F* and *mut1F*). These latter results again pointed to marbox 2, and not marbox 1, as critical for repression of *purA*.

For *hdeA*, a template missing marbox 1, the last 13 bp of marbox 6 and the last 6 bp of marbox 5 still showed repression (Fig. 6, *MB5.5*), as did a template missing all of marbox 5 (and consequently the last 2 bp of marbox 3) (Fig. 6, Δ*MB5*). Therefore, it appeared that marboxes 1, 5, and 6 were not needed for repression. Not surprisingly, mutations of the last 2 bp of marbox 3 did not block repression (Fig. 6, *MB3a*). However, alteration of all 4 bp of RE2 of marbox 3 did block repression

(Fig. 6, *MB3b*). Therefore, marbox 3 was necessary for repression of *hdeA*. To investigate the role of RE1 of marbox 3, two of its 4 bp (within the −35 hexamer) were changed, but this reduced basal levels of transcription such that quantification was not possible. When the −35 hexamer (atgaca) was mutated to the consensus hexamer (ttgaca), transcription increased notably; repression by MarA still occurred (Fig. 6, *MB3e*), even though the mutation also affected 1 bp of RE1 of marbox 3. When this single mutation in the −35 hexamer was then combined with the RE2 mutations of MB3b, transcription remained high, and repression was again lost (Fig. 6, *MB3k*). These results together indicated that marbox 3, and particularly its RE2, was critical for repression of *hdeA* by MarA. No further mutations were attempted in RE1 as it completely overlapped the −35 hexamer.

DISCUSSION

By using transcription analysis *in vitro,* we have demonstrated that purified MarA protein was sufficient to down-regulate expression of *purA* and *hdeA* via the promoter region of these genes. How the same protein is able both to activate and to repress transcription probably relates to the identity and location of DNA sequence motifs that are recognized by MarA. For our studies here, we presumed that marboxes within the promoter region would be involved. By a combination of EMSA and transcription experiments *in vitro* using wild type and mutant marboxes, we found that the marbox most likely to contribute to repression of both *purA* and *hdeA* overlapped the −35 promoter motif and was oriented in the "backward" direction. "Class II" promoters activated by MarA also have marboxes overlapping the −35 motif, but these marboxes are in the forward direction (6). Presumably the two different marbox orientations result in two types of interactions between MarA, RNA polymerase, and/or DNA, one leading to activation and the other to repression.

In the case of *purA*, marbox 2 was defined as critical by its affinity for MarA in EMSA and by the fact that point mutations in RE1 of marbox 2 prevented both the mobility shift and the repression by MarA *in vitro*. Although deletions within this marbox did not prevent repression, this result could readily be explained (for the 7-bp deletions) by the formation of new backward marboxes from the resultant joined sequences, with consensus bp of 15/20 (D2F) or 16/20 (D3F) (see Fig. 5). In the case of the 14-bp deletion (DelF), the newly created marbox was 1 bp too short, but it had 14 consensus bp. However, mutant template purApF2, lacking the terminal 6 bp of the marbox, was still somewhat repressed (Fig. 5). That finding suggested that only the first part of this marbox was needed for repression. Transcription using the templates with deletions was considerably reduced, possibly due to destruction of a potential "UP element" (28) comprising bp −41 to −57 in the *purA* promoter.

For *hdeA*, even though repression of transcription was seen *in vitro*, EMSA experiments showed minimal binding of MarA to the *hdeA* promoter region. Discrepancies between DNA binding as measured by EMSA and by other means have also been observed for other promoters, for example *ybjC* (29) and *inaA* (6). We therefore depended upon the transcription assays *in vitro* to define the particular marbox critical for repression of *hdeA*. Specifically, upstream marboxes 1, 5, and 6 were not required. Marbox 3, overlapping the −35 promoter motif, was critical for repression, because there was no repression if mutations were placed in all 4 bp of its RE2 (Fig. 6). The role of RE1 of marbox 3 remained unclear because RE1 completely overlapped the −35 hexamer and could not be extensively mutagenized. The last 2 bp of marbox 3 could be deleted without eliminating repression *in vitro* (ΔMB5), suggesting that, as with *purA*, an entire 20-bp marbox may not be needed for repression (Fig. 6). Of note, RE2 was important for repression of *hdeA* but not *purA*.

Two relatives of MarA were reported to directly repress transcription of other promoters, but the marbox involved was not determined. IscR, a newly recognized member of the AraC family of transcriptional regulators and a "distant" MarA relative (20% identity, 40% amino acid similarity), was able to function *in vitro* as a transcriptional repressor of the *iscRSUA* operon (30). Intriguingly, we had observed down-regulation of the same promoter by constitutive expression of MarA (4) (*iscS* = b2530). Also, the MarA homolog SoxS repressed its own expression in whole cells and bound its own promoter *in vitro* (31); no further studies of this observation have been reported.

The physiological significance of the repression of *purA* and *hdeA* by MarA is not known. Other proteins also repress *purA* and *hdeA*. The *purA* promoter is repressed by PurR in the presence of purines (32), and the promoter of *hdeA* is repressed by HN-S (33). The *hdeA* promoter can be recognized by both σ70 and RpoS (23), and MarA can activate both σ70- and RpoS-dependent promoters (34). Interestingly, GadX binds to a region in the *hdeA* promoter (35) which overlaps the MarA-binding site, marbox 3. The repression of *purA* by MarA was quantitatively similar *in vivo* and *in vitro,* whereas that of *hdeA* was greater *in vivo* (~6–25-fold) than *in vitro* (~1.7-fold). Possibly repression *in vivo* of *hdeA* by MarA is aided by a cellular product that is constitutive or is induced by MarA.

From our results we postulate that MarA represses by binding to a region of the promoter that overlaps the RNA polymerase −35 binding motif. Certain other repressors also have binding sites that overlap or lie between the RNA polymerase-binding motifs; they may provide models for MarA action (36, 37). LexA sterically blocks the binding of RNA polymerase to the *uvrA* promoter (38). MerR bends the −10 motif of the *merT* promoter away from RNA polymerase, causing a reduction in the rate of open complex formation (39, 40). Arc protein at the P_{ant} promoter of bacteriophage P22 slows the rate of open complex formation (41).

Although our sample of genes directly repressed by MarA is small, the location and orientation of the marboxes in the two repressed genes studied here are unlike that in either of the two classes of the MarA-activated genes. We have postulated that it is the backward marbox overlapping the −35 motif that uniquely gives rise to repression. Other cases in which proteins activate some promoters and repress others have been described (37). However, we have found no previous example in which the change between activation and repression relies on the orientation of the binding site.

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