MarA-mediated Transcriptional Repression of the rob Promoter*

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The Escherichia coli transcriptional regulator MarA affects functions that include antibiotic resistance, persistence, and survival. MarA functions as an activator or repressor of transcription utilizing similar degenerate DNA sequences (marboxes) with three different binding site configurations with respect to the RNA polymerase-binding sites. We demonstrate that MarA down-regulates rob transcripts both in vivo and in vitro via a MarA-binding site within the rob promoter that is positioned between the −10 and −35 hexamers. As for the hdeA and purA promoters, which are repressed by MarA, the rob marbox is also in the “backward” orientation. Protein-DNA interactions show that SoxS and Rob, like MarA, bind the same marbox in the rob promoter. Electrophoretic mobility shift analyses with a MarA-specific antibody demonstrate that MarA and RNA polymerase form a ternary complex with the rob promoter DNA. Transcription experiments in vitro and potassium permanganate footprinting analysis show that MarA affects the RNA polymerase-mediated closed to open complex formation at the rob promoter.

The Rob protein is an abundant nucleoid protein that was initially discovered bound to the right origin of replication of the Escherichia coli chromosome (1). Despite its association with the origin of replication, no experimental evidence supports the role of Rob in chromosome replication, chromatin structure, and superhelicity (2).

Rob is, however, a member of a subgroup within the AraC/XylS family of transcriptional regulators, which includes the MarA and SoxS proteins that are involved in a wide range of regulatory functions (3). The N-terminal domain of Rob shares similarity with MarA and SoxS, which is in contrast to the other members of the subgroup that share homology within the C-terminal domain (3). When induced by bile salts and dipiridyl, the C-terminal domain undergoes post-transcriptional modification that results in the conversion of Rob from a low to a high activity state in the cell (4). However, in the absence of these compounds, overexpression of either the full or C-terminal domain deleted protein is sufficient for activity and promoter binding in vitro (5, 6) and in vivo (5, 7). This observation is consistent with structural data derived from the Rob-mcdC complex, where only the N-terminal domain makes DNA-specific contacts (8). Overexpression of rob confers multidrug, organic solvent and heavy metal resistance (6, 7); in accord, some experimental data indicate that rob null mutants are hypersensitive to antibiotics, organic solvents, and heavy metals (5, 7, 9).

Transposon mutagenesis experiments aimed at defining the Rob regulon revealed eight Rob-regulated targets, namely inaA, marRAB, adIB, ybaO, mdfA, ybbD, ybiC, and galF (10), some of which are also known members of the mar and sox regulons (11). In addition, studies demonstrate that Rob induced by decanoate or bile salts results in the increased expression of acrAB in the absence of both the mar and sox loci (12).

The expression levels of rob do not vary dramatically during different growth phases, unlike other nucleoid-associated proteins (e.g. Fis) (2, 13, 14). There is, however, some evidence that increased rob expression occurs in glucose- and phosphate-limited media and in the stationary phase of cell growth, attributable to activation by the σ factor rpoS (13). Moreover, recent studies have shown that rob is subject to down-regulation by MarA and SoxS (11, 15).

It has been shown previously that all three transcriptional factors (MarA, SoxS, and Rob) regulate the expression of themselves (16, 17) and each other (15–18). This cross- and auto-regulation strongly suggest the presence of putative binding sites within the mar, sox, and rob promoters. Thus far, only the MarA/SoxS/Rob-binding site within the mar promoter has been identified and shown to be responsible for activation by all three transcription factors (17). In contrast, the repression of both soxS and rob by SoxS, Rob, or MarA occurs via a yet to be identified binding site(s) within the rob and rob promoter regions (15, 16).

Extensive genetic and crystallographic analyses have shown that the interaction of MarA/SoxS/Rob with activated promoters involves a degenerate DNA sequence known as the “marbox/soxbox” (3). These binding sites are asymmetric and exist in two possible orientations (class I and II promoters) (3) with respect to the RNA polymerase-binding sites. Generally at class I promoters, the marbox lies upstream of the −35 hexamer in the backward orientation with the exception of the zwf promoter (19), and at the class II promoters the marbox overlaps the RNA polymerase-binding site and lies in the forward orientation (19). Direct transcriptional repression by MarA has been linked more recently to a similar degenerate marbox that lies in the “backward” orientation with partial or complete overlap of the −35 hexamer (20). This configuration is unlike that of either the class I or class II marboxes necessary for activation (19).

In this study we have characterized the MarA-binding site within the rob promoter in relation to the RNA polymerase-binding sites and have studied the mechanism of MarA-mediated transcriptional repression. Down-regulation of rob mediated by MarA (and also SoxS and Rob) adds to the regulatory cross-talk already reported (15–17) and shows that the rob promoter and protein are controlled at both pre- and post-transcriptional levels in vivo.

MATERIALS AND METHODS

Bacterial Strains and Plasmids Used—E. coli strains (with or without plasmids) (Table 1) were grown in LB broth supplemented with either ampicillin (100 µg/ml) or kanamycin (30 µg/ml) where required.
**MarA Is a Repressor of rob**

**TABLE 1**

| Strains and plasmids used in the study | Genotype/phenotype | Reference |
|---------------------------------------|---------------------|-----------|
| AG100                                 | argE3 thi-1 rpl. ylt sapE44 | 20 |
| AG100R                                | AG100rob::kan        | See “Materials and Methods” |
| JHC1096                               | GC4468 with zdd-239::To9 | 24 |

| Plasmids                              |                     |           |
|---------------------------------------|---------------------|-----------|
| pMB101                                | ori colEl lac lacZp:marA, AmpR | 11 |
| pJPBH                                 | ori colEl lacAmpR    | 20 |

RNA Preparation and Northern Blot Analysis—Total RNA was extracted from the bacterial strains using the Qiagen RNeasy kit (Qiagen, Germany). Membranes used for the transfer of RNA were stained with methylene blue to confirm the transfer and were also used for quantitation. The rob DNA probe was generated by PCR amplification with DNA from E. coli strain AG100 (20) using the following primers: RNF, 5'-ATGATCAGGCGCGGCTA-3'; RNR, 5'-CGATCCT- GGGCTAACGC-3' (Tm = 58 °C). The subsequent 618-bp probe was random prime-labeled with [α-32P]dCTP (PerkinElmer Life Sciences). Hybridizations and subsequent ImageQuant (Amersham Biosciences) analyses were performed as described previously (20).

5'-RACE—The transcriptional start site of the **rob** gene was mapped using the 5'-RACE system (Invitrogen). Total RNA was extracted from AG100 and AG100R (constructed by P1 transduction of rob::kan from RA4468 (5) into AG100 (20)). The RNA was digested twice with DNase I to eliminate the contaminating genomic DNA prior to use in the 5'-RACE reaction. The cDNA was produced by extension with primer RR1, 5'-GCTTGGTACT- AG100 and AG100R (constructed by P1 transduction of AG100 (20) using the following primers: RobO1, 5'-GAAAAAACACTGTTGGT-3'. Moloney murine leukemia virus-reverse transcriptase (Invitrogen). The cDNA was dc-tailed and amplified by PCR with a primer hybridizing to the poly(C)-tail (Abridged 

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Transcription Studies in Vitro with **MarA**, **SoxS**, and **Rob**—Transcription experiments in vitro were carried out as described previously (20, 21). DNA fragments were gel-purified using the Qiagen Quick gel extraction kit (Qiagen, Germany). The location of the marbox was determined using DNA fragments that consisted of sequential truncations of the **rob** promoter region and/or selective mutations (see Fig. 2). The mechanism of repression was examined by preincubation experiments with either purified **MarA** (Paratek Pharmaceuticals, Boston; see Fig. 3A) or RNA polymerase (RNAP) holoenzyme (Epitector, Madison, WI). Briefly, each protein (RNAP or **MarA**) was preincubated for 5 min at 0 or 37 °C before the addition of the second protein. Following a further 10 min of incubation, the initiating nucleotide mixture with heparin was added and allowed to proceed for another 5 min. The effect of **MarA** on blocking reinitiation by RNA polymerase in vitro at the **rob** promoter was assessed by multiple-round transcription experiments. The DNA fragment (2 nM) was initially incubated for 5 min at 37 °C in the presence of RNA polymerase (40 nM) to allow for open complex formation. This was followed by the addition of 200 nM **MarA** and incubation at 37 °C for a further 5 min. The levels of transcription were assessed by adding initiating nucleotide mixture without heparin and allowed to proceed for different times (shown in Fig. 6B). The reaction was stopped by adding 30 μl of Gel Loading Buffer II (Ambion). The products were separated by electrophoresis on a 7.5% polyacrylamide, 8 M urea denaturing gel and visualized after exposure to a phosphorimaging screen. The quantification of the levels of repression was determined as described previously (20).

Electrophoretic Mobility Assays (EMSA)—EMSA experiments were performed with the wild type, truncated, and mutated **rob** promoter fragments. To define the marbox sequence, annealed oligomers representing four sequential nonoverlapping promoter sequences of 20–21 bps each were incubated with **MarA**, **SoxS**, or Rob (Paratek Pharmaceuticals, Boston; see Fig. 3A). The promoter fragments (only the coding strands of the oligomer sequences are shown here) were used as follows: RobO1, 5'-CTAAAAACATCTCTACTAAG-3'; RobO2, 5'-GAAAAAACACTGTTGGT-3'; RobO3, 5'-AACAGCAGGATTCCTCAA-3'; RobO4, 5'-CCAAATTACCCTGATGTCCAGGT-3' (see Fig. 2). The orientation of the marbox was examined using amplified PCR fragments harboring mutations within recognition elements (RE1 and RE2) of the **MarA**-binding site in both the forward (RobO3F6) and backward (RobF7M6, RobF7M5) marboxes (see Fig. 3 legend for mutation descriptions). The annealed oligomers and DNA fragments were end-labeled using T4 polynucleotide kinase and [γ-32P]ATP (PerkinElmer Life Sciences). All binding reactions were performed with 32P-end-labeled DNA oligomers (2 nM), purified **MarA** (200–400 nM), and competitor DNA (100 mg/ml, poly[d(I-C)]). The binding reactions were performed at 25 °C for 15 min before the addition of loading buffer (0.25× TBE, 80% glycerol, 40%). The samples were subjected to electrophoresis at room temperature for 2 h on a 6% polyacrylamide gel. The formation of closed complexes was determined as described by Nechaev and Severinov (22). Briefly, binding reactions with **MarA** (200–1000 nM) and the **rob** promoter fragment (pRobF4) were performed at 0 °C for 10 min prior to the addition of RNAP (40 nM). Both proteins were incubated on ice for a further 10 min. For the supershift experiments, 1 μl of neat penta-**His** antibody (Qiagen, Germany) was added directly to the reactions, which were performed as described above. Following the addition of loading buffer, products were loaded immediately onto a 3.5% native polyacrylamide gel. The samples were subjected to electrophoresis at 4 °C for 4 h. The gel was then dried and exposed for PhosphorImager analysis.

Potassium Permanganate Footprinting—The footprinting experiments were performed as described previously (23). Briefly, the promoter fragment (pRobF4 at 1 × 10^6^ dpm) and **MarA** (400 nM) or RNAP (40 nM) only or **MarA** and RNAP together were incubated in 5× binding buffer (25% glycerol, 0.5 M NaCl, 25 mM MgCl2, 0.5 mM EDTA, 5 mM dithiothreitol, 0.25 mg/ml bovine serum albumin, 100 mM Tris-HCl, pH 8) for 15 min at 37 °C. One microliter of 200 mM potassium permanganate was added to each reaction, mixed, and incubated for a further 4 min. The reaction was stopped with the addition of 50 μl of Stop solution (3 M ammonium acetate, 0.1 mM EDTA, 1.5 M 2-mercaptoethanol). After extraction with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1), the aqueous phase was precipitated with ethanol at −80 °C for
15 min. The pellet was then resuspended in 10% piperidine and incubated at 90 °C for 30 min. The reaction was stopped with the addition of 50 μl of 1 m LiCl and cold absolute ethanol. The pellet was washed in ethanol before resuspension in 5 μl of sequencing gel loading buffer (Epicenter, Madison, WI). The resulting products were then analyzed by electrophoresis in an 8% denaturing urea gel.

RESULTS

Analysis of rob Expression Under Different Conditions—Microarray analyses have demonstrated that either salicylate induction of MarA or overexpression of MarA or SoxS results in decreased expression of rob (11). To confirm that the decrease in rob expression was a result of MarA expression, we analyzed the levels of the rob transcript by Northern blot analysis and reverse transcription-PCR under different conditions. Levels of the rob transcript were determined in a strain deleted for the marRAB locus (JHC1096 (24)), but which was transformed with an IPTG-inducible plasmid either with (pMB102) or without (pJPBH) marA. As expected, the levels of rob remained unaffected in the vector only control (JHC1096/pJPBH), but a decrease was observed under IPTG induction of MarA (JHC1096/pMB102) (Fig. 1). The levels of repression at 0.25, 0.5, and 1 mM IPTG were 0.43, 0.28, and 0.21, respectively (all ratios were normalized to the amounts of rRNA present on the membrane; see Fig. 1). Using both Northern blot and reverse transcription-PCR analyses, the salicylate induction of marA decreased levels of rob expression in AG100 to 0.67 relative to the uninduced sample (data not shown). Thus both pLAMP-mediated expression and salicylate induction of MarA, directly or indirectly, reduced the levels of rob expression 2–4-fold in vivo.

Identification of the Transcriptional Start Site of rob—It was necessary to map the transcriptional start site of the rob gene to determine the positions of the RNA polymerase-binding sites and subsequently the location of the marA-binding site (marbox) relative to these signals. PCR analysis using the anchor primer (see "Materials and Methods") and a rob gene-specific primer yielded a 180-bp product that was subsequently cloned into the pGEMT vector. Four clones were sequenced, and found to harbor the same rob and dC-tailed primer junction sequence. The transcriptional start site was mapped to 43 nucleotides upstream of the open reading frame with the 5′-RACE method (Fig. 2). The experimentally defined position of the transcriptional start site is consistent with the size of the transcripts obtained in both the Northern blot and in vitro transcription experiments (see below). Based on the position of the transcriptional start site, potential −10 and −35 sequences within the rob promoter sequence could also be deduced (Fig. 2).
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FIGURE 3. SDS-PAGE analysis of purified proteins and binding of MarA, SoxS, and Rob to the rob promoter. A, composite SDS-polyacrylamide gel of purified His$_6$-MarA, Rob, and SoxS (provided by Michael Alekshun and Victoria Bartlett, Paratek Pharmaceuticals, Boston). Lane M denotes the marker where the molecular weights are indicated; lane 1, purified Rob protein; lane 2, purified SoxS protein; lane 3, purified MarA protein. B, annealed $^{32}$P-labeled 20–21-bp oligomers sequentially spanning the protein; C, complex of MarA; F, free DNA. D, binding of Rob and SoxS proteins to Rob03 and Rob04 (spanning the putative marbox) in the rob promoter. C1, complex with Rob protein; C2, complex with SoxS protein; F, free DNA. E, MarA (200 nM) binding to fragments bearing mutations in recognition elements. RBO3F6 is mutated in recognition element 1 of the forward marbox to acaATGgaaagttctatctat; RBF7M6 is mutated in recognition element 2 of the backward marbox to acaATGgaaagttctatctat; and RBF7M5 is mutated in recognition element 1 in the backward marbox acacagaaaaatctatc. C, complex of MarA, F, free DNA. A–D represent different gels. The gel shift reactions were examined by electrophoresis on 6% native gels for approximately 2 h in a noncoiling gel system. The temperature increase within the gel matrix combined with a longer electrophoresis time may account for the variations in migration patterns.

Further truncations of the rob promoter were restricted by the presence of the putative −35 hexamer (atgcta). In an attempt to confirm whether the −35 hexamer was indeed where predicted and important for transcription, we introduced point mutations within the putative region (Fig. 4A). First, a C (atgcta) to G (atgGta) change in the −35 region resulted in the loss of gene transcription completely (Fig. 4A). In addition, the change of the potential −35 hexamer (atgcta) in the rob promoter to the E. coli consensus sequence (ttgcc) did not alter transcription, dramatically contrasting the effect noted previously at the hdeA promoter (20). This finding could be attributed to the original −35 sequence already being a constitutive promoter given the relative abundance of Rob molecules within the cell (1, 14). Thus the effects on gene transcription observed in vitro support our hypothetical −10 and −35 predictions within the rob gene promoter. Unexpectedly, the consensus promoter appeared much less responsive to MarA repression (ratio, 0.8) in comparison to the original sequence (ratio, 0.29), especially because changes introduced were not within the marbox sequence. The introduction of the consensus E. coli −35 hexamer may have altered RNAP binding at the rob promoter making it less responsive to MarA-mediated repression. In vitro transcription experiments involving the full-length promoter fragment and purified Rob protein also resulted in repression (ratio of 0.33 compared with no Rob protein control) (Fig. 4B). Therefore, both MarA and Rob are able to repress the rob promoter directly in vitro, and this repression occurs at the level of transcription.

Defining the MarA-Mediated Mechanism of Repression—Because the marbox in the rob promoter lies between the −10 and −35 hexamers, it is possible that MarA mediates repression by excluding the access of RNA polymerase to the promoter. To test this hypothesis, we performed gel shift analyses of the rob promoter with MarA preincubation at 0°C prior to the addition of RNAP. MarA binding to the rob promoter produced, as expected, a shifted complex (Fig. 5, A1); RNAP binding produced three complexes (Fig. 5, R1–R3). The preincubation of increasing concentrations of MarA (200–1000 nm) with the rob promoter followed by the addition of RNAP (40 nm) produced complexes (Fig. 5, see complexes in lanes 6, 8, and 10) similar to those formed by RNAP alone (Fig. 5, R1–R3, lane 4). Because MarA binds as a monomer (28) and has a molecular mass of 15 versus 450 kDa for RNA polymerase, the differences in shifts of a heavier ternary complex (MarA-RNAP-DNA), for the complexes (Fig. 5, R4), would not be easy to differentiate from complexes formed with RNAP-DNA alone (Fig. 5, R1–R3). However, the heavier complexes (RA) formed in Fig. 5, lanes 12 and 14, suggest a ternary complex of DNA-RNAP-MarA.

To confirm that MarA was indeed a part of the RA complexes (Fig. 5, lanes 6, 8, 10, 12, and 14), supershift assays with a penta-his tag antibody destroyed MarA binding. These results suggest that the repressed rob promoter marbox also lies in a backward orientation, as was reported previously for the hdeA and parA promoters (20).

Transcription Studies in Vitro—To determine whether the binding of MarA/SoxS and Rob to the rob promoter reflected direct repression of the rob promoter, we performed transcription in vitro experiments. As described previously (20), the test transcripts (regulated by MarA) in in vitro studies were compared against a control (gnd) transcript (not regulated by MarA) after background subtraction. The average ratios among these transcripts in the absence and presence of MarA represented the level of repression. (We find that this approach corrects for the inter- and intra-experimental variability of both the test and control transcripts.) Initially a large fragment of the rob promoter region (−234 to +110; see Fig. 2), amplified with the primers reported by Michan et al. (15), was used. With this full-length rob promoter fragment, MarA (200 nm) reduced transcription to 0.29 ± 0.024 (Fig. 4A). The promoter regions upstream of the −35 hexamer (see Fig. 2) were sequentially removed to define the region of the marbox involved in MarA repression. Transcription from all the 5′-deleted promoter fragments tested was repressed by MarA (data not shown for all fragments except for representative truncated fragments, pRobF5 and pRobF4; see Fig. 4 and Fig. 6A, respectively).
promoters, although the levels of activation were greatest with MarA (Fig. 6A, lane 1). As expected, when RNA polymerase was preincubated with the roβ promoter at 37 °C for 5 min, no repression was observed with the addition of MarA (200 nM) (Fig. 6B); only higher MarA concentrations (400 and 800 nM) was some repression noted under the 37 °C reaction conditions (Fig. 6B). The transcription in vitro results together with the gel shift data indicate that MarA-mediated repression at the roβ promoter occurs before open complex formation but subsequent to RNA polymerase binding; once the open complexes were formed, MarA had little effect.

To determine whether MarA can repress the roβ promoter after transcription elongation, thereby interfering with reinitiation by RNA polymerase, we performed multiple-round transcription experiments. The promoter (pRobF4) was preincubated with RNA polymerase for 5 min at 37 °C (previously shown to be sufficient for open complex formation at the roβ promoter) before the addition of MarA followed by initiating nucleotides. The multiple-round transcription experiments demonstrated that in the absence of MarA, the mRNA transcripts for both the roβ and gnd promoters accumulated during the course of the assay (Fig. 6C). However, in the presence of MarA the roβ transcript levels decreased over time, but as expected the levels of the gnd transcript (the control) remained unaffected (Fig. 6C). Thus MarA is able to repress the roβ promoter after the preformed open complex clears the promoter.

Potassium Permanganate Footprinting—Potassium permanganate has been shown to preferentially modify single-stranded thymines (T) and cytosines (C) to some extent, which permits the identification of melted DNA in RNA-polymerase-mediated open complexes (29). Preincubation with either MarA or RNA polymerase resulted in cleavage at the following sites: -11 (T), -10 (T), -4 (A), -3 (A), -2 (T), -1 (T) (Fig. 7). Preincubation of the roβ promoter with MarA resulted in a dramatic decrease in cleavage, and thus of open complex formation after RNA polymerase addition (Fig. 7). In contrast, preincubation with RNA polymerase and the subsequent open complex formation were not affected by MarA. Moreover, the same cleavage patterns were observed in the presence or absence of MarA (Fig. 7). This finding suggests that MarA

\[ \text{MarA Is a Repressor of rob} \]

![Figure 6. Effect of MarA on single and multiple-round transcription experiments from the roβ promoter.](image)

A. Single round transcription with MarA or RNA polymerase preincubation. Single round in vitro transcription experiments of the roβ promoter fragment (pRobF4) spanning -75 to +110 at 37 °C. The upward arrowheads indicate that MarA (200 nM) was preincubated with the promoter fragment for 5 min prior to the addition of 40 nM RNA polymerase (40 nM) was preincubated with the promoter fragment for 5 min before the addition of MarA (200 nM) (lane 4). Each reaction had an RNA polymerase-only control (lanes 1 and 3). B. Transcription following transition from closed to open complex formation at the roβ promoter. The roβ promoter fragment (pRobF4) was incubated with RNA polymerase (40 nM) at either 0 °C (lanes 1–4) or 37 °C (lanes 5–8) for 5 min before the addition of increasing concentrations of MarA (100–800 nM) and transfer to 37 °C for a further 10 min before the addition of initiating nucleotides. C. Multiple-round run-off transcription: MarA/RNA polymerase interaction at the roβ promoter. The DNA fragment (pRobF4) was incubated for 5 min with RNA polymerase (40 nM) at 37 °C before the addition (+) of MarA (200 nM). The transcription mix was added at time 0, and the reaction was terminated after the different times indicated above the figure.
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FIGURE 7. Potassium permanganate footprinting of the rob promoter. The upward and downward arrowheads depict the order of addition of MarA and RNAP as in Fig. 6A. The DNA (top strand) in the presence of either MarA/RNAP or no protein was modified with potassium permanganate (10 mM) and cleaved with piperidine. The numbers (−10, −11, −1 to −4) indicate the cleavage sites.

does not alter the RNAP-DNA conformation after open complex formation. The potassium permanganate findings support the results obtained in the in vitro transcription experiments.

DISCUSSION

This study demonstrates that MarA can repress the expression of the rob gene both in vivo and in vitro via a marbox sequence located within its promoter. Like the previously characterized repression marboxes for hdeA and purA (20), the rob marbox is also in the backward orientation, but lies between the −10 and −35 hexamers. This region has been called the “exclusive zone of repression” (30), as transcription factor-binding sites that lie within this position almost always lead to repression, e.g., IclR at the iclR promoter (31), Fur at the aerobactin promoter (32), and the 434 repressor at 434 bacteriophage P, promoter (33). Exceptions to this rule do exist such as positive regulation by MerR at the merTPAD promoter (34), Arc at the Pm variant promoter of bacteriophage P22 (35), and SoxS at the soxS promoter (36).

The two marboxes (type I and type II) (see Fig. 8) involved in repression utilize the same degenerate MarA-binding sequence (this work and see Ref. 20) as those involved in activation (19). In addition the DNA and MarA contacts found to be crucial in activation (19, 26, 28, 37) are also important for repression (20) as mutations within RE1 in both the purA and rob marboxes were sufficient to abolish MarA binding and, in the case of purA, to eliminate MarA-mediated repression in vitro (20).

Transcription initiation is a multistep process and is represented by the formation of several intermediary transcription factor-RNAP-DNA complexes (38). Given the location of the rob marbox, several possible scenarios for the mechanism of MarA-mediated repression emerge. First, MarA could simply sterically hinder the access of RNAP to the rob promoter. Second, MarA could inhibit the transition from closed to open complex formation or from open to initiated complex formation. Finally, MarA could hinder promoter cleavage.

Preincubation of the rob promoter with saturating concentrations of MarA before the addition of RNAP did not prevent the formation of RNAP-DNA complexes (see Fig. 5). The prior presence of either MarA or RNAP did not inhibit binding of the other protein to the rob promoter. When RNAP was added to the rob promoter at 0 °C prior to MarA addition and then transfer to 37 °C, MarA inhibited rob transcription (see Fig. 6B). These results argue against steric hindrance as the MarA-mediated mechanism of repression at the rob promoter. Instead MarA, RNAP, and DNA form a ternary complex as demonstrated by the supershift assays performed with penta-his antibody (directed against the purified MarA).

At the rob promoter, MarA represses by inhibiting the transition from the closed to open complex (see Fig. 6B), but it has no effect after the formation of open complexes (see Fig. 6, A and B, and Fig. 4A). These findings are further supported by the potassium permanganate footprinting results that show that MarA preincubation decreases the formation of open complexes at the rob promoter; once formed, the open complexes are not affected by MarA (see Fig. 7). Because open complex formation is a transient stage in vivo, the multiple-round transcription experiments showed that MarA was able to repress once the bound RNAP had cleared the rob promoter (see Fig. 6C).

Residues 265 and 294 within the α-CTD of RNAP contact MarA when interacting at the activated promoters (39). Based on helical spacing experiments between the marbox and the RNAP binding signals at the activated genes, it is predicted that MarA binds the same face of the DNA relative to RNAP (3, 19). Our data demonstrate that MarA and RNAP co-bind the rob promoter (19), and however, the conformational aspects of this ternary complex (MarA-rob-RNAP) are unclear. The crystal structures of MarA with the mar promoter (28) and Thermus aquaticus RNAP holoenzyme with a fork junction promoter DNA (40) suggest that both MarA and RNAP might bind the same face of the DNA. However, this predictive model does not take into account the DNA bends introduced by each protein. Therefore, other conformations may exist at the rob promoter. Because helical dependence is a critical factor in activation by MarA (19), the transcriptional outcome, repression versus activation, may be dependent on which DNA face the protein binds as is the case for GalR (41). The phage 434 protein represses the P rob promoter via a binding site that lies between the −10 and −35 hexamers (33, 42). Ethylation interference experiments at this promoter demonstrate that repression occurs via the binding of the 434 repressor and RNAP on opposite faces of the promoter (33). Alternatively, specific interactions between MarA and the rob promoter may be different from that described for the activated promoters (28) and occur through only one of the helix-turn-helix motifs as predicted in the Rob-
micF crystal structure, thereby allowing both proteins to bind the same face of the DNA (8).

The interaction of MarA via the α-CTD of RNAP in the absence of DNA strengthens the hypothesis that a MarA–RNAP complex scans the chromosome for cognate binding sites for both proteins (43, 44). At the activated promoters, the relative positions of both the MarA and RNAP-binding sites suggest that the disassociation of the MarA–RNAP complex may not be necessary before the complex contacts DNA (39). The position of the rob promoter marbox does not exclude the possibility of the MarA–RNAP complex binding opposite faces of the helix simultaneously but in a nonproductive conformation, or that RNAP or MarA dissociate from the complex and bind the promoter independently. At other promoters with repressor binding sites similar to that in the classic promoter, MarA-repressed genes (45), and at the early A2c promoter, RNAP has been shown to recruit the repressor p4 (46).

This study identifies a uniquely positioned marbox, unlike those described previously (19, 20), and demonstrates the mechanism of MarA-mediated repression at the rob promoter. Examples of MarA-activated genes involve marboxes that can be in the “forward” or backward orientation and in different positions relative to the RNAP-binding sites (19) (see Fig. 8). In contrast, in all three known examples of MarA-repressed genes (ideA, purA, and rob), the marbox is in the backward orientation and is proximal to or overlapping the RNAP-binding site (see Fig. 8).

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