

Silencing of toxic gene expression by Fis

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ABSTRACT

Bacteria and bacteriophages have evolved DNA modification as a strategy to protect their genomes. Mom protein of bacteriophage Mu modifies the phage DNA, rendering it refractile to numerous restriction enzymes and in turn enabling the phage to successfully invade a variety of hosts. A strong fortification, a combined activity of the phage and host factors, prevents untimely expression of *mom* and associated toxic effects. Here, we identify the bacterial chromatin architectural protein Fis as an additional player in this crowded regulatory cascade. Both *in vivo* and *in vitro* studies described here indicate that Fis acts as a transcriptional repressor of *mom* promoter. Further, our data shows that Fis mediates its repressive effect by denying access to RNA polymerase at *mom* promoter. We propose that a combined repressive effect of Fis and previously characterized negative regulatory factors could be responsible to keep the gene silenced most of the time. We thus present a new facet of Fis function in Mu biology. In addition to bringing about overall downregulation of Mu genome, it also ensures silencing of the advantageous but potentially lethal *mom* gene.

INTRODUCTION

The temperate bacteriophage Mu infects *Escherichia coli* and several other enteric bacteria (1). One of the factors contributing to the wide host range of Mu is a novel post-replicative DNA modification function encoded by the phage gene *mom* (2–4). Sequence specific modification of adenine to N-6 acetamidoadenine by the gene product of *mom* renders the phage DNA resistant to several host encoded restriction/modification systems (5). Expression of *mom*, a late function in the lytic cycle of Mu, is cytotoxic to the host (6–8). An elaborate regulatory network has evolved to curb untimely *mom* expression (Supplementary Figure S1) (9). While Com (product of

the overlapping *com* ORF within the *mom* operon) is essential to achieve translation of *mom* mRNA (8,10,11), a complex interplay between the host proteins Dam (DNA adenine methyltransferase) and OxyR governs transcription from *mom* promoter (P_{mom}) (12–14). In addition, owing to factors like suboptimal spacing between the poor –35 and –10 elements and intrinsic curvature conferred by a T stretch, P_{mom} is far from being a classical promoter and is dependent on transcription activator C (15–17). Furthermore, activation of the activator C itself is subject to availability of Mg^{+2} (18). Moreover, in the absence of C protein, RNA polymerase (RNAP) gets recruited at a divergent overlapping promoter *momP2*, thus posing competition to the already weak *mom* promoter (17,19).

The simultaneous existence of multiple mechanisms to fine-tune a single phage function might seem excessive. For instance, despite the existence of a multi-layered transcriptional control, a further check at the translational level appears needless. However, to restrain *mom* gene from exerting its cytotoxic effect, its expression must be precisely modulated. The currently known *mom* regulatory circuit is not only elaborate but also apparently failsafe (9). In this article, we report a hitherto unidentified mechanism operating to add to the fortification. While characterizing the role of the transactivator C, we observed a P_{mom} specific DNA binding activity in crude cell extracts of *E. coli* (Figure 1A, V. Ramesh, unpublished data). This observation prompted us to identify the host factor and investigate the possibility of it regulating the expression of *mom*. Here, we have elucidated the role of this new player in *mom* regulation.

MATERIALS AND METHODS

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are as described in Supplementary Table S1.

Electrophoretic Mobility Shift Assay

End labeled P_{mom} DNA was incubated with variable amounts of cell extracts or purified protein in TMEG₁₀₀ buffer [20 mM Tris–HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM

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Purification of the host factor

Cultures of *E. coli* MG1655 were grown to log phase ($OD_{600} \sim 0.6$) and harvested. About 5g of cell pellet was resuspended in 15 ml lysis buffer [20 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM EDTA (pH 8.0), 10% glycerol, 500 mM NaCl, 200 μ g/ml lysozyme, 5 mM β -mercaptoethanol] and sonicated on ice. The extract was centrifuged at 100 000g for 2 h. An aliquot of the supernatant (crude cell extract) was used for Electrophoretic Mobility Shift Assays (EMSAs) and the rest was proceeded with as follows. Nucleic acids were precipitated using 1% (v/v) polyethyleneimine (pH 8.0). Hereafter, at each step of purification, fractions containing the host factor were selected based on their ability to bind P_{mom} in EMSA. Proteins in the extract were fractionated by a 0–65% saturated ammonium sulfate cut. The precipitated solution was spun down and the pellet was resuspended and dialyzed against TMEG₁₀₀ buffer. The dialyzed fraction was mixed overnight with 5 ml pre-equilibrated phosphocellulose matrix (Whatman). After a wash with 10 ml TMEG₁₀₀ buffer, elution was carried out with 5 ml TMEG₁₀₀₀ buffer [20 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM EDTA (pH 8.0), 10% glycerol, 1 M NaCl]. The eluate was equilibrated in TMEG₁₀₀ and passed through 1 ml SP sepharose column (GE Healthcare). Eluted fractions possessing P_{mom} specific DNA binding activity were pooled, dialyzed against 10 mM Tris-HCl (pH 7.4), lyophilized and subjected to micro LC ESI-MS (Bruker apex-ultra mass spectrometer) for whole protein molecular weight detection.

Proteins

C protein was overproduced from pVR7 in *E. coli* BL21(DE3) and purified as described earlier (20). RNAP was purified from *E. coli* K12 MG1655 as described (21). Fis was overproduced from the IPTG inducible *lac* promoter in pUHE25-2*fis* (22) in *E. coli* W3110 (23). The protocol described by Osuna *et al.* (24) was modified to include the cation exchangers phosphocellulose, sulfopropyl sepharose and heparin sepharose to purify Fis. Protein concentration was estimated by the method of Bradford (25) as well as by analyzing on SDS-PAGE gel. Restriction enzymes and DNA modifying enzymes were obtained from New England Biolabs.

In vitro transcription

Multiple round run-off transcription reactions were carried out using the linear 327 bp *mom* or *tin7* promoter fragments (spanning the promoter from positions -136 to +79 relative to *mom* +1 start site; PCR amplified from pUW4*mom* and pUW4*tin7* (20,26), respectively, using pUC forward and reverse primers). For generating transcription templates with specific promoter regions deleted, the full-length promoter fragment (described earlier) was digested with BstUI or PvuI followed by extraction from agarose gel. Fis, at indicated concentrations was incubated on ice with 40 nM template DNA in transcription buffer [40 mM Tris-HCl (pH 8.0), 5 mM magnesium acetate,

0.1 mM EDTA, 0.1 mM DTT, 100 mM KCl, 100 μ g/ml BSA] for 5 min. C protein was included wherever indicated. RNAP (100 nM) was added to the reaction and closed complex was allowed to form for 10 min on ice. The reactions were then shifted to 37°C for 10 min to allow open complex formation. Transcription was initiated by adding 4 μ Ci [α -³²P]ATP and 0.3 mM NTP mix and the reactions were allowed to proceed at 37°C for 30 min. Reactions were terminated by the addition of 20 μ l urea loading dye (8 M urea, 0.05% bromophenol blue, 0.05% xylene cyanol), heated to 65°C for 5 min and quenched on ice. Samples were loaded on 8% urea polyacrylamide gels and analyzed by phosphorimaging. [α -³²P]ATP was purchased from Perkin-Elmer Life Sciences. NTPs and dNTPs were obtained from GE Healthcare.

Closed complex formation assays

5' ³²P labeled 78-bp P_{tin7} promoter fragments (wild-type or with mutations in Fis binding sites) extending from positions -58 to +20 relative to +1 start site (Supplementary Figure S2) were incubated with indicated concentrations of Fis in transcription buffer for 10 min on ice. RNAP (20 nM) was added and further incubated for 10 min on ice. The reactions were analyzed on 5% (w/v) native polyacrylamide gels and visualized by phosphorimaging.

DNase I footprinting

Plasmid pUW4 (0.3 pmol) was incubated on ice with indicated concentrations of Fis for 30 min in footprinting buffer [10 mM Tris-HCl (pH 7.6), 2.5 mM $MgCl_2$, 0.5 mM $CaCl_2$]. DNase I (0.05 U, NEB) was added and incubated for 30 s at 25°C. Reactions were terminated by the addition of stop buffer [100 mM Tris-HCl (pH 7.5), 25 mM EDTA, 0.5% SDS]. Samples were deproteinized by successive extractions with phenol/chloroform/isoamylalcohol (25:24:1) and chloroform/isoamylalcohol (24:1). DNA was ethanol precipitated in the presence of glycogen as a carrier and primer extension was carried out as described by Gralla (27). Briefly, DNase I treated DNA samples were used as templates for extension with Klenow polymerase using end-labeled *mom* reverse primer (5' GG AATCCGCCTTAAATAACA 3'), which anneals downstream of the +56 position with respect to the +1 transcription start site of *mom*. Samples were ethanol precipitated and resuspended in 4 μ l of formamide loading dye. After denaturation at 95°C for 5 min, samples were electrophoresed on 8% urea polyacrylamide gels alongside dideoxy sequencing reactions and analyzed by phosphorimaging.

Construction of pLW4*tin7* plasmids with mutations in Fis binding sites

The plasmid pLW4*tin7* harbors P_{tin7} extending from positions -136 to +79 (with respect to *mom* transcription start point) cloned in pNM480, resulting in a transcriptional fusion of P_{tin7} with *lacZ* (19). Mutant plasmids pLW4*tin7* FBS -49 -38, FBS -49 -38 +3 and FBS +3 were generated by the megaprimer method for site-directed mutagenesis using the plasmid pLW4*tin7*

as template. Sequences of oligonucleotides used for megaprimer generation are listed in Supplementary Table S2. The mutagenic megaprimers were then used in the Stratagene QuickChange™ side-directed mutagenesis method using Phusion high fidelity DNA polymerase (Finnzymes) to obtain mutant plasmids. Mutations introduced thus were confirmed by DNA sequencing.

β-Galactosidase assays

Plasmid borne reporter construct pLW4*tin7* (explained in the previous section) and mutant constructs were introduced into *E. coli* CSH50 (28) and CSH50 *fis::kan* (29). For assessing the effect of activator C, cells were transformed with the C encoding plasmid pVN184 (19) in addition to the reporter construct. Overnight cultures were diluted 1:50 into fresh LB media containing appropriate antibiotics and incubated at 37°C until their OD₆₀₀ readings were between 0.3 and 0.5. β-galactosidase activity was assayed as described (28). Assays were carried out in triplicate for each culture and the average values plotted.

RESULTS

P_{mom}-specific DNA binding activity in *E. coli* cell extracts

The finding that a host protein binds to *P_{mom}* came across serendipitously during the course of our earlier studies investigating the role of transactivator C. A 260-bp fragment encompassing *P_{mom}* was obtained by digesting pUW4 with EcoRI and HindIII (Figure 1A, lane 1 and Supplementary Figure S2). Both the strands of the fragment were radiolabeled at their 3'-ends by Klenow end-filling. In EMSA with *E. coli* cell extracts, it was observed that a host factor bound specifically to the fragment (Figure 1A, lanes 3 and 4). Notably, binding of the factor caused only a small shift in the mobility of *P_{mom}* as compared to that caused by the 33kDa transactivator C dimer (lane 2). We next assessed the binding region of the host factor. For this purpose, the *P_{mom}* fragment was digested with PvuI, yielding three fragments of sizes 60, 18 and 182 bp (Figure 1A, lane 5 and Figure 1B). Purified activator C bound only to the larger (182 bp) fragment, consistent with the location of the C binding site therein (Figure 1A, lane 6 and Figure 1B). On the other hand, crude cell extracts bound specifically to the 60-bp fragment indicating that the host factor binds to a site upstream of the C binding site (Figure 1A, lanes 7 and 8).

Identification of the host factor that binds specifically to *P_{mom}*

In order to identify this unknown factor, we resorted to isolate it from crude extracts of *E. coli*. We observed that the host factor-*P_{mom}* complex was stable at high ionic strength and competitor DNA concentrations (Figure 1C and D). In addition, its strong affinity to cation exchange resins was an indicator of its basic nature. We employed a protocol essentially encompassing cation exchange chromatography to isolate the host factor from log phase

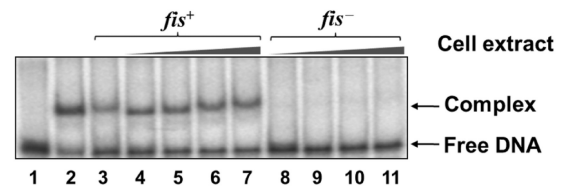


Figure 2. EMSA with cell extracts of wild-type (*fis*⁺) and *fis*⁻ strains. The 60-bp fragment (from positions -136 to -85) of *P_{mom}* (lane 1) was incubated with 5 nM Fis (lane 2), 1 µg of crude extract of *E. coli* MG1655 (lane 3), and 1, 5, 10 and 15 µg of crude extracts of *E. coli* BW25113 (lanes 4–7, respectively) and isogenic *fis*⁻ strain JW3229-1 (lanes 8–11, respectively). Specific shift as was observed with purified Fis could be seen with *fis*⁺ but not with *fis*⁻ extracts. Binding buffer for all reactions contained 400 mM NaCl.

cultures of *E. coli* MG1655 (see ‘Materials and Methods’ section). Mass spectrometric analysis of the final fraction revealed a mass of 11.25 kDa, precisely the molecular weight of the nucleoid associated protein Fis (Figure 1E). To determine if the *P_{mom}* specific DNA binding was indeed due to Fis, we examined DNA binding activity of the cell extracts from isogenic *fis*⁻ and *fis*⁺ strains JW3229-1 and BW25113, respectively (30). Specific retardation pattern, as had been observed first with *E. coli* MG1655 crude cell extracts was observed with *fis*⁺ strain but not with *fis*⁻ strain (Figure 2, lanes 3–11). To further ascertain the identity of the host factor as Fis, we compared the DNA binding activity in crude cell extracts with that of purified Fis. Crude extracts were able to retard *P_{mom}* DNA to the same extent as purified Fis, indicating that the host factor that binds specifically to *P_{mom}* was indeed Fis (Figure 2, lanes 2 and 3).

Effect of Fis on *mom* promoter activity

Fis, besides modulating chromatin architecture, is known to influence numerous promoters of *E. coli* and several other bacteria (31–33). We hypothesized that specific binding of Fis could affect the activity of *mom* promoter. To test this hypothesis, we conducted *in vitro* transcription with *mom* and *tin7* promoters in the presence or absence of Fis. Activation of *mom* promoter is dependent on transactivator C. A spontaneous mutant of *mom* promoter—*tin7* has a single base substitution at -14 position which converts it into an extended -10 promoter and thus activator independent (19). Fis repressed transcription from both *P_{mom}* and *P_{tin7}* (Figure 3A) in a concentration dependent manner (Supplementary Figure S3). In these *in vitro* reactions, Fis repressed transcription even in the presence of C (Figure 3A). However, the repressive effect of Fis was reversed upon increasing the concentration of the activator C and transcription was restored to levels observed in the absence of Fis (Figure 3B and C). From these data, it is apparent that C can override Fis-mediated repression.

Fis-mediated *in vivo* repression

We next investigated the *in vivo* scenario. In order to assess the impact of Fis on transcription from *P_{mom}*, it was necessary to obviate the requirement for the positive

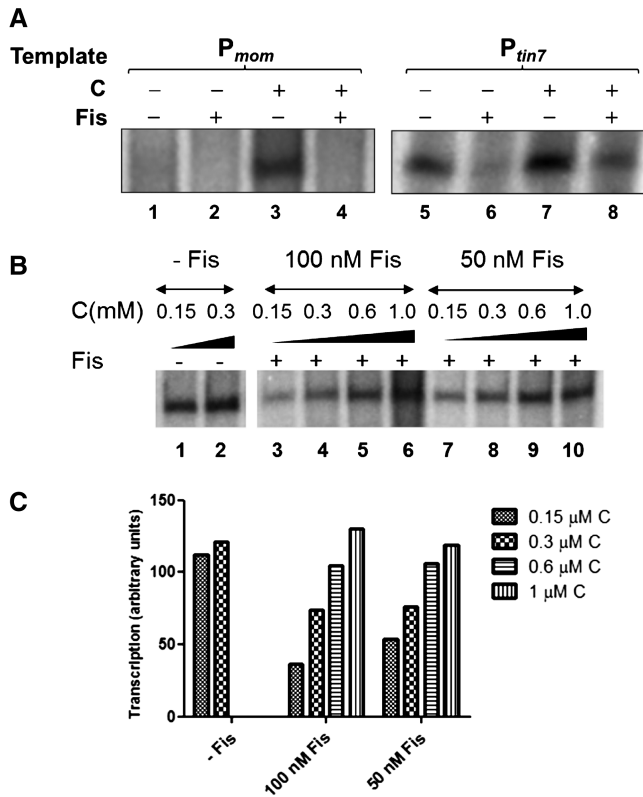


Figure 3. Effect of Fis on *mom* promoter activity. (A) Effect of Fis on *in vitro* transcription from P_{mom} and P_{tin7} . Multiple round run-off transcription reactions were carried out using linear promoter fragments (-136 to +79) P_{mom} (lanes 1-4) and P_{tin7} (lanes 5-8) in the presence or absence of 200 nM Fis. C (300 nM) was included as indicated. Transcription reactions were proceeded as described in 'Materials and Methods' section. At P_{tin7} , transcription is observed even in the absence of C. (B) Effect of C on Fis-mediated transcriptional repression. *In vitro* transcription reactions using P_{tin7} template were carried out with increasing concentrations of C (as indicated) in the absence (lanes 1 and 2) or presence of Fis (100 nM in lanes 3-6 and 50 nM in lanes 7-10). (C) Densitometric analysis of the transcript bands from (B) was carried out and the values plotted.

regulator C. We therefore took advantage of the C-independent P_{tin7} promoter. Plasmid borne reporter construct pLW4*tin7* containing *tin7-lacZ* fusion was transformed into *E. coli* CSH50 wild-type and *fis*⁻ cells and the β -galactosidase activity was measured. β -galactosidase levels in *fis*⁻ cells were found to be significantly higher than those in the wild-type (*fis*⁺) strain (Figure 4A), consistent with the Fis-mediated transcriptional repression observed *in vitro*.

To address the opposing effects of Fis and C *in vivo* and to see if C could overcome the repression imparted by Fis, wild-type and *fis*⁻ cells were transformed with the C encoding plasmid and promoter activity assayed. No significant difference in the β -galactosidase levels was observed between the two strains (Figure 4B) in contrast to the scenario in the absence of C, shown in Figure 4A. This finding is in agreement with the *in vitro* results indicating that C can counter the repressive effect of Fis to activate transcription.

DNase I footprinting on P_{mom} shows multiple Fis binding sites

DNase I footprinting was employed to map the Fis binding sites located in P_{mom} (Figure 5). Fis protected a region between positions -7 to +12 in the immediate vicinity of the *mom* transcription start site as well as an extended region from -60 to -22. Further upstream, Fis protected two other regions between positions -107 to -84 and -133 to -115. Besides, addition of Fis also led to DNase I hypersensitivity around certain positions in the promoter, consistent with the properties of Fis to induce conformational changes in DNA (34,35). Inside the limits of each of the protected regions, a sequence similar to the consensus Fis binding site was detected (36-40). In the protected region spanning positions -60 to -22, two closely spaced sequences matching the Fis consensus site were detected. Totally, we identified five Fis binding sites in P_{mom} centered at positions -123, -98, -49, -38 and +3, respectively. EMSAs with the promoter fragments and

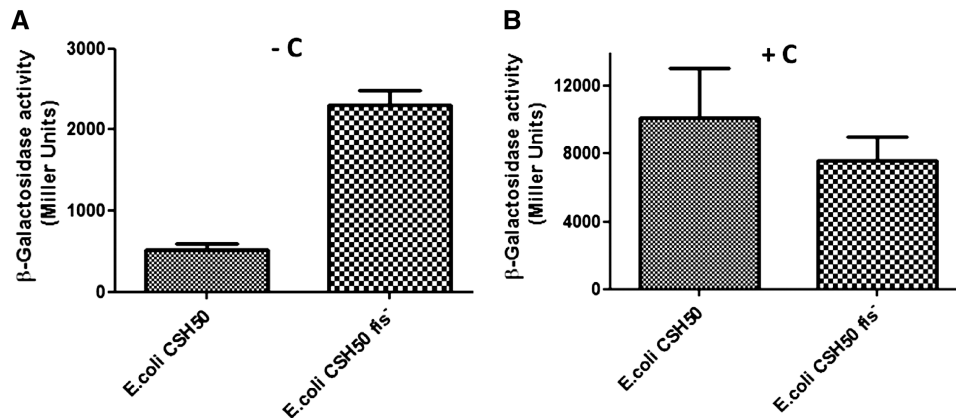


Figure 4. Influence of Fis on promoter activity *in vivo*. (A) β -Galactosidase expression was measured in *E. coli* CSH50 wild-type and *fis*⁻ strains containing $P_{tin7-lacZ}$ fusion reporter construct. β -Galactosidase activity in wild-type cells was 4- to 5-fold lower than in the *fis*⁻ strain. (B) β -Galactosidase activity measured in CSH50 wild-type and *fis*⁻ strains containing the C encoding plasmid in addition to the plasmid borne $P_{tin7-lacZ}$ fusion reporter construct.

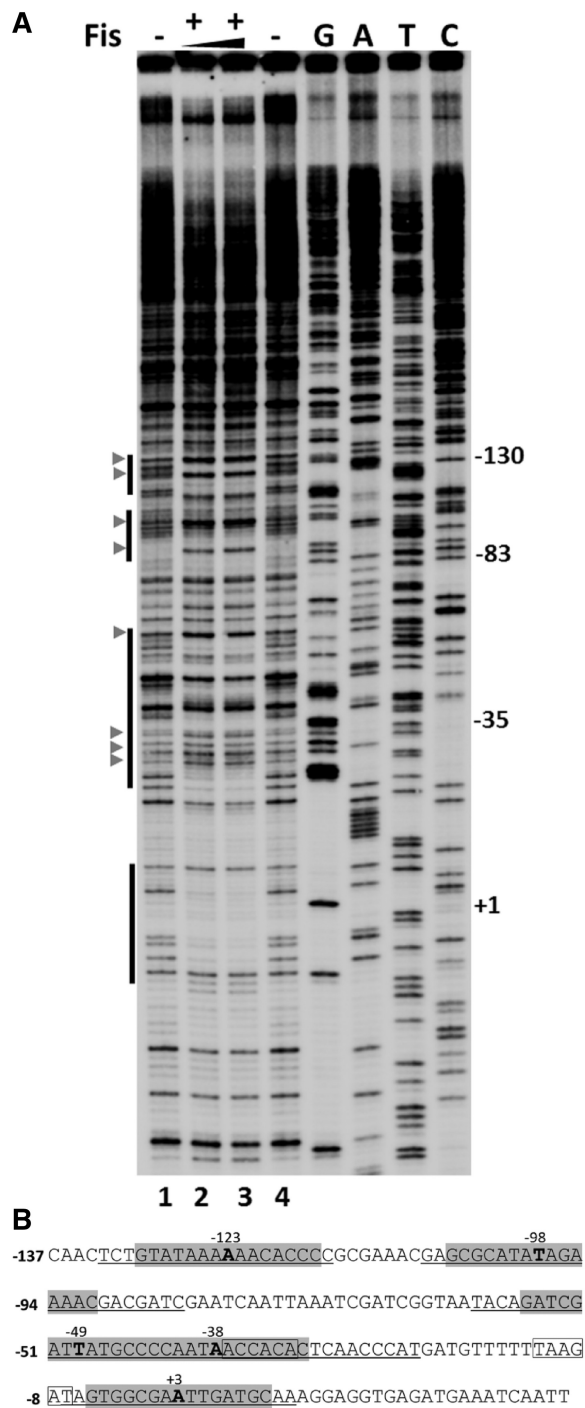


Figure 5. Footprinting analysis of Fis binding at P_{mom} . (A) DNase I footprinting analysis of Fis binding sites in P_{mom} . Plasmid pUW4 was incubated with Fis (288 and 384 nM in lanes 2 and 3, respectively) prior to digestion with DNase I. Primer extension was carried out with Klenow polymerase using *mom* reverse primer. The vertical bars on the left side indicate regions protected from DNase I in the presence of Fis. G, A, T and C refer to Sanger's dideoxy sequencing ladders of P_{mom} obtained using *mom* reverse primer. Numbers on the right denote nucleotide positions with respect to the +1 start site of P_{mom} . DNase I hypersensitive sites are depicted by arrowheads. (B) Sequence of *mom* promoter. Regions protected by Fis are underlined and sequences matching with the consensus Fis binding site are highlighted with the central nucleotide emboldened and its position indicated. The -35 and -10 promoter elements are boxed.

purified Fis exhibited a ladder-like appearance, indicative of multiple Fis binding sites in P_{mom} , corroborating the footprinting data (Supplementary Figure S4 and Figure 6A, lanes 1–6).

Fis excludes RNAP binding *in vitro*

The occurrence of multiple Fis binding sites spread over the *mom* regulatory region together with the observed inhibition of transcription suggested that indeed Fis binding may interfere with interaction of RNAP with the promoter. It was important at this point to examine if the transcriptional repression imparted by Fis diminished upon deletion/mutation of Fis binding sites. The Fis binding site at -123 (the site involved in Fis binding in cell extracts, see Figure 1) was targeted first for deletion. The 327-bp transcription template (encompassing -136 to +79 region of *mom* promoter) was digested with BstUI, removing the Fis binding site at -123 (Supplementary Figure S2). When this shortened promoter was used as a template for *in vitro* transcription, surprisingly, Fis continued to repress transcription (Supplementary Figure S5). A similar observation was made upon removal of the further downstream Fis binding site centered around -98 using PvuI, thus removing both the Fis binding sites located at -123 and -98 (Supplementary Figures S2 and S5). From these analyses, it appeared that the remaining promoter proximal Fis binding sites (centered at -49, -38 and +3) might be involved in transcriptional regulation of the promoter.

In parallel, we investigated the possibility of Fis directly interfering with RNAP binding. Indeed, one of the various ways by which Fis represses transcription is by competing with RNAP for binding to the promoter (41–44). Besides, the occurrence of three Fis binding sites (centered at -49, -38 and +3, respectively) overlapping the RNAP binding region substantiated the notion that Fis binding could pose steric hindrance to the polymerase and thus interfere with transcription initiation. To pursue this line of investigation further, we analyzed the effect of Fis on formation of RNAP closed complex on *mom* promoter, employing P_{tim7} , as it is amenable to closed complex analysis in the absence of C. Remarkably, at a 78-bp promoter fragment spanning positions -58 to +20 with respect to +1 start site, containing Fis binding sites at -49, -38 and +3, we observed that increasing concentrations of Fis progressively inhibited the binding of RNAP to the promoter, as evident from a diminishing closed complex (Figure 6A, lanes 7–12). Thus, Fis appears to downregulate the promoter activity by denying access to RNAP at the promoter. To test this further, we disrupted the above-mentioned Fis binding sites (singly and in combination) and examined the ability of Fis to compete with RNAP at the respective mutant promoter variants. At promoter fragments carrying mutations in Fis binding sites, Fis could no longer compete with RNAP, as evident by a stable closed complex even in the presence of high concentrations of Fis (Figure 6B–D). From these results, it appears that Fis binding at multiple sites in the promoter region is necessary for efficient repression.

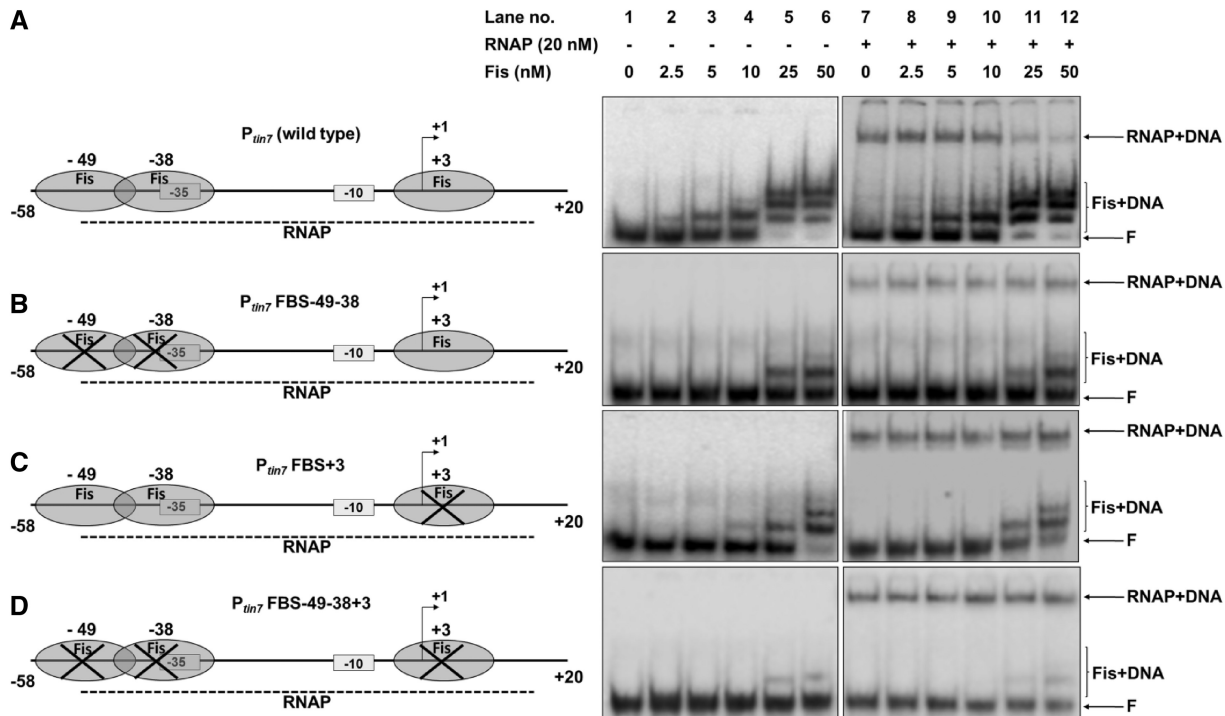


Figure 6. Effect of Fis on closed complex formation on various promoter fragments. (A) P_{tin7} (–58 to +20 relative to +1 start point) (lane 1) was incubated with increasing concentrations of Fis as indicated (lanes 2–6). Subsequently, samples were incubated with 20 nM RNAP to allow closed complex formation (lanes 7–12). Mutant promoter fragments namely P_{tin7} FBS-49-38 (Fis binding sites at –49 and –38 mutated) (B), P_{tin7} FBS+3 (Fis binding site at +3 mutated) (C), P_{tin7} FBS-49-38+3 (all three Fis binding sites at –49, –38 and +3 mutated) (D) were also examined for the effect of Fis on closed complex formation at the respective mutant promoters. On the left are schematic depictions of occurrence or disruption of Fis binding sites in the respective fragments. F denotes free promoter DNA. DNA–protein complexes are indicated.

These competition experiments also showed that disruption of single Fis binding site markedly reduces the ability of Fis to prevent closed complex formation.

Disruption of Fis binding sites alleviates repression *in vivo*

Having observed *in vitro* that disrupting Fis binding sites renders Fis incapable of competing with RNAP, we next tested the effect of disruption of these sites on the promoter activity *in vivo*. Complete alleviation of Fis-mediated repression is observed when all the three Fis binding sites were mutated whereas disruption of the site at +3 alone or those at –49 and –38 resulted in partial reduction of repression (Figure 7 and Supplementary Table S3). Thus, although both *in vitro* and *in vivo* lines of evidence highlight the importance of multiple Fis binding for maximal repression of transcription, the effect of disruption of the +3 site singly or those at –49 and –38 is not very pronounced *in vivo*. Perhaps this difference could be attributed to the fact that in addition to the repressive action of Fis, *mom* is subjected to a complex array of regulation with other factors also influencing the promoter activity (Supplementary Figure S1).

DISCUSSION

Fis was first discovered for its role in stimulating Gin catalyzed inversion of the G segment of phage Mu genome (45). Three Fis binding sites within the coding

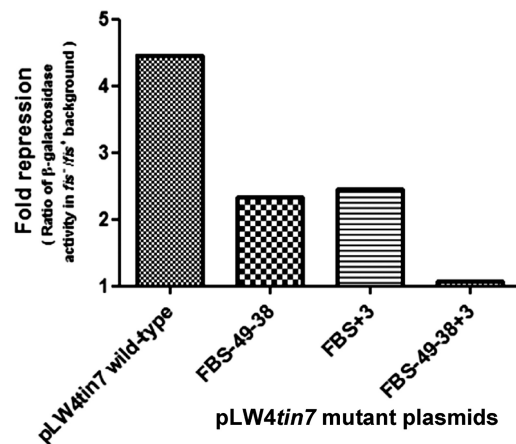


Figure 7. Effect of disrupting Fis binding sites on Fis-mediated repression. pLW4tin7 and variants containing mutations in Fis binding sites were introduced into isogenic strains CSH50 fis^- and CSH50 wild-type (fis^+) and β -galactosidase activity was measured. Fold repression (the ratio of the β -galactosidase activity in fis^- strain to that in fis^+ strain) was plotted.

region of *gin*, the upstream neighbor of *mom* were identified in the course of the above investigation (46–48). These sites are located ~550 bp upstream of the *mom* transcription start site and are distinct from those which we report in this study to be involved in regulating

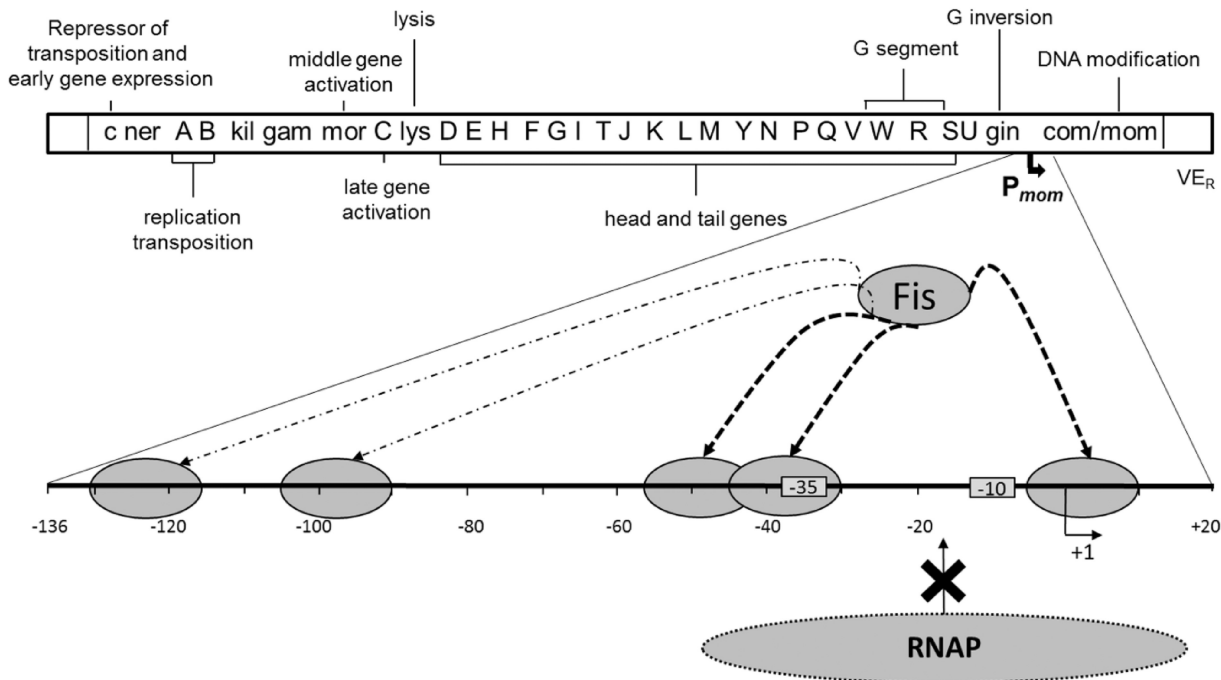


Figure 8. Model showing Fis-mediated silencing of *mom*. The rightmost gene in the Mu genome is *mom*. P_{mom} , the regulatory region of *mom* has been enlarged. The -35 and -10 promoter elements of P_{mom} are boxed. The dashed arrows indicate binding of Fis (small oval) to multiple sites in the promoter (as shown by DNase I footprinting). RNAP (large oval) cannot bind to P_{mom} because Fis is occluding the RNAP binding region. The Fis binding sites implicated in interfering with RNAP binding and thus with transcription are indicated by thickened arrows. Effect of Fis on other regions of the genome (see ‘Discussion’ section) is not shown.

mom expression. Our study demonstrates that Fis represses transcription from *mom* promoter. However, it would be too presumptuous to attribute a primary regulatory role to Fis, given that *mom* expression is subject to a multitude of regulatory checkpoints. In addition, *mom* promoter presents a classic case of a weak, activator dependent promoter (15). Upstream of *mom* transcription start site is a busy area containing one or more binding sites for proteins Dam, OxyR, C, RNAP and Fis, subjecting the gene to a complex regulatory system (Supplementary Figure S1). Occlusion of *mom* promoter by Fis appears to prevent RNAP recruitment and thus transcription from P_{mom} . The present study provides evidence that Fis, a host nucleoid associated protein has been employed by the phage to achieve silencing of its potentially lethal but advantageous function. Deployment of nucleoid associated proteins as transcriptional silencers could be an effective strategy to maintain toxic genes like *mom* in the phage genome.

It is noteworthy that nutrient availability and growth phase of the bacterial culture have a pronounced effect on the intracellular concentrations of Fis. Fis levels markedly fluctuate from over 50 000–100 000 copies per cell in the early exponential phase or following a nutrient upshift to less than 100 copies per cell during the stationary phase and starvation (49). Earlier work has appreciated the role of Fis in Mu lysogeny. Studies employing the temperature sensitive mutant repressor *cts62* have demonstrated that Fis aids the repressor in downregulating both early gene expression and transposition of phage Mu, thus

contributing to maintain the phage in a lysogenic state (50). Furthermore, earlier observations have indicated that conditions of limited growth such as starvation and stationary phase (when Fis levels are low) could favor spontaneous induction of phage Mu (51,52). In such Fis depleted situations when the lytic cycle sets in, Fis-mediated repression of *mom* can be anticipated to diminish. In the lysogenic phase, however, there is a requirement to keep the genome silenced and abundance of Fis could effectively ensure silencing of *mom*. In other words, regulation of *mom* expression by Fis is likely to be mediated by a change in the protein abundance. Fis is known to suppress transcription from *guaB*, *crp*, *fis*, *gyrA* promoters by directly competing with RNAP (41–44). Pre-binding of Fis at these promoters impedes RNAP recruitment on account of steric hindrance. We suggest that a similar phenomenon of promoter occlusion by Fis could be responsible for silencing *mom* expression (Figure 8). Although it appears that binding of Fis at multiple sites in *mom* promoter has a cumulative repressive effect, each site on its own can also exert a partial negative effect on transcription from this promoter (Figure 7).

An orchestrated cascade operating at transcriptional and translational levels regulates *mom* expression (Supplementary Figure S1). Most importantly, transcription of *mom* is contingent on C protein, a well-established activator of P_{mom} that is expressed only in the middle phase of the lytic cycle (53). C facilitates RNAP recruitment by realigning the out of phase -35 and -10

promoter elements (54). At a further step, C enhances promoter clearance and overcomes the influence of negative factors responsible for silencing of *mom* (55). Multistep activation of P_{mom} by C ensures *mom* expression at the late lytic phase. This irreversible genetic switch would have to overcome Fis-mediated repression. Since the binding region of C and two of the Fis sites overlap around the -35 region of the promoter, it is likely that under conditions of Fis abundance and in the lysogenic state, Fis would predominantly occupy the region. During the lytic cycle, low concentrations of C might not be sufficient to displace Fis bound to the promoter (Figure 3A). However, higher concentrations of the activator effectively promote RNAP recruitment and activity, evident from the significant stimulation of transcription brought about by increased concentrations of C even in the presence of inhibitory concentrations of Fis (Figure 3B and C). RNAP recruited at the promoter would also compete out Fis bound to the downstream site. Thus, the activator and the polymerase seem to join hands to overcome the repression imparted by Fis.

Silencing of P_{mom} by Fis is important in yet another context. Promoter-up mutations are not uncommon in nature. Spontaneous emergence of promoter-up mutations in P_{mom} during lysogeny would result in premature *mom* expression and death of the host. The role of Fis in silencing *mom* becomes especially important during the lysogenic state to avoid the lethal effects of untimely expression arising due to promoter mutations. The ability of Fis to repress transcription from P_{tin7} , an activator independent and spontaneous promoter-up mutant of P_{mom} emphasizes its importance as a silencer of *mom*, capable of eliminating any leaky expression from the promoter. Thus, Fis appears to play a dual role in Mu lysogeny; not only does it contribute to overall silencing of gene expression and transposition but also represses a toxic gene of Mu. We propose that Fis-mediated repression adds an additional layer to the already elaborate *mom* regulatory circuit. After all, the selective advantage imparted by the anti-restriction function of *mom* can outweigh the risk associated with its untimely expression by silencing of this cytotoxic gene till the late lytic phase.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Tables 1–3 and Supplementary Figures 1–5.

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