Contributions of UP Elements and the Transcription Factor FIS to Expression from the Seven *rrn* P1 Promoters in *Escherichia coli*

CHRISTINE A. HIRVONEN, WILMA ROSS, CHRISTOPHER E. WOZNIAK, ERIN MARASCO,
JENNIFER R. ANTHONY, SARAH E. AIYAR, VANESSA H. NEWBURN,
AND RICHARD L. GOURSE*

Department of Bacteriology, University of Wisconsin—Madison, Madison, Wisconsin 53706

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The high activity of the *rrnB* P1 promoter in *Escherichia coli* results from a *cis*-acting DNA sequence, the UP element, and a *trans*-acting transcription factor, FIS. In this study, we examine the effects of FIS and the UP element at the other six *rrn* P1 promoters. We find that UP elements are present at all of the *rrn* P1 promoters, but they make different relative contributions to promoter activity. Similarly, FIS binds upstream of, and activates, all seven *rrn* P1 promoters but to different extents. The total number of FIS binding sites, as well as their positions relative to the transcription start site, differ at each *rrn* P1 promoter. Surprisingly, the FIS sites upstream of site I play a much larger role in transcription from most *rrn* P1 promoters compared to *rrnB* P1. Our studies indicate that the overall activities of the seven *rrn* P1 promoters are similar, and the same contributors are responsible for these high activities, but these inputs make different relative contributions and may act through slightly different mechanisms at each promoter. These studies have implications for the control of gene expression of unlinked multigene families.

The synthesis of ribosomes in bacteria is determined by the rate of synthesis of rRNA and, at high growth rates in *Escherichia coli*, rRNA promoters account for more than half of the transcription in the cell (10). The large contribution of rRNA transcription to total cellular transcription, the central role played by ribosomes in cell physiology, and the importance of rRNA regulation as a model for the control of global gene expression justify intensive analysis of rRNA promoters.

rRNA is transcribed from two promoters, P1 and P2, at each of the seven *rrn* operons: *rrnA*, *rrnB*, *rrnC*, *rrnD*, *rrnE*, *rrnG*, and *rrnH*. However, most previous work on the factors that contribute to the unique strength and regulation of the *rrn* P1 promoters has been limited to *rrnB* P1. The *rrnB* P1 core promoter contains near-consensus –35 and –10 hexamers. Interactions between RNA polymerase (RNAP) and the *rrnB* P1 core promoter (defined here as –41 to +1 with respect to the transcription start site) are regulated by the concentration of the initiating nucleotide (19) and by the concentration of guanosine tetraphosphate (ppGpp), a nucleotide that inhibits rRNA synthesis in response to amino acid starvation (5, 12). However, the core promoter accounts for <1% of the activity of the full-length promoter (defined here as containing *rrnB* P1 sequence upstream to –154) (21, 22, 41). The strength of the full-length promoter is attributable to two features: (i) the UP element, an A+T-rich region of DNA located upstream of the –35 hexamer and recognized by the carboxy-terminal domain of the α subunit (α CTD) of RNAP (44); and (ii) FIS, an 11.2-kDa DNA-binding protein that binds as a dimer upstream of the UP element (46, 54), bends each of its binding sites 40 to 90° (18), and interacts with the α CTD of RNAP to activate transcription (8).

UP elements are found at both rRNA (31, 44, 47, 54) and non-rRNA (23) promoters, and the degree of match to the consensus generally correlates with the magnitude of a UP element's effect on transcription (42). Near-consensus UP elements are predicted, based on sequence comparisons, to occur more frequently at stable RNA (rRNA and tRNA) promoters than at other promoters (17). UP elements consist of proximal and/or distal subsites, each of which is capable of interacting with a single α CTD (16, 17). Little is known about the relative effects of UP elements on transcription from the different rRNA promoters.

FIS is the most abundant nucleoid protein during exponential growth (1) and activates transcription not only from *rrnB* P1 (46) and *rrnD* P1 (47) but also from many other promoters [e.g., *thrU*(*tufB*) (37), *proP* P2 (53), *tyrT* (34), and *leuV* (45)]. DNase I, hydroxyl radical, and dimethyl sulfate footprinting studies identified three FIS binding sites upstream of *rrnB* P1, centered at –71, –102, and –143 (9, 46). The promoter-proximal FIS site, site I, accounts for most of the activation by FIS at *rrnB* P1 in vivo; sites II and III increase transcription only marginally (20 to 30%) (9, 46). FIS is assumed to activate all seven rRNA operons (15, 29, 35, 51), but only at *rrnB* P1 and *rrnD* P1 has it been shown that the effects of FIS are direct (46, 47). Furthermore, the precise locations of FIS binding sites have not been determined experimentally at *rrn* P1 promoters other than *rrnB* P1.

In order to determine whether our information about *rrn* P1 promoters extends beyond *rrnB* P1, we have defined the inputs to transcription from the other six P1 promoters. We measured the effects of the UP element and the transcription factor FIS at all seven *rrn* P1 promoters in vivo and in vitro. Our major conclusions are that all seven *rrn* P1 promoters have UP elements and are activated by FIS, but the relative contributions of these *cis*- and *trans*-acting factors to transcription differ significantly at the individual *rrn* P1 promoters.

* Corresponding author. Mailing address: Department of Bacteriology, University of Wisconsin, 1550 Linden Dr., Madison WI 53706. Phone: (608) 262-9813. Fax: (608) 262-9865. E-mail: rgourse@bact.wisc.edu.

MATERIALS AND METHODS

Strains and plasmids. The strains and plasmids used in this study are listed in Table 1. All DNA sequences in the text and figures are written 5' to 3' and refer to the nontemplate strand. *EcoRI-HindIII* fragments containing promoter sequences were constructed by PCR as described previously (42). Promoters with an UP element from one operon and a core promoter from another ("hybrid UP element promoters") were constructed by PCR with an upstream primer containing an *EcoRI* site and an UP element sequence from one operon, and ca. 20 bp of core promoter sequence from a different operon used as the PCR template. "Hybrid FIS site promoters" with FIS sites from one operon (operon 1) and a UP element and core promoter from another (operon 2) were constructed in a two-step PCR process. In the first round of PCR, the upstream primer contained ca. 20 bp of sequence from upstream of -61 from operon 1 and ca. 20 bp of sequence from downstream of -61 from operon 2, which was used as the PCR template. The downstream primer contained a *HindIII* site adjacent to, and containing, the transcription start site (position +1). In a second round of PCR, operon 1 was used as a PCR template, the upstream primer contained an *EcoRI* site and a 20-bp sequence containing the final upstream endpoint, and the product of the first PCR was used as the downstream primer.

EcoRI-HindIII fragments were inserted into pRLG770 (46) for in vitro binding and transcription studies and/or were inserted into one of two phage λ *lacZ* fusion systems for in vivo studies. *lacZ* fusion system I is able to tolerate very strong promoters but exhibits relatively high background activity; system II has a much lower background but cannot tolerate very strong promoters (41).

The first A of the *HindIII* site (AAGCTT) was positioned to serve as the transcription start site for the cloned P1 promoter constructs of *rmA*, *rmB*, *rmC*, *rmE*, *rmG*, and *rmH*. The *rmD* P1 promoter constructs contain the *HindIII* sequence immediately downstream of the +1 transcription start site (G). Therefore, the transcribed sequences were identical for the *rm* P1 promoters starting with ATP; transcripts from *rmD* P1 started with a GTP and were 1 bp longer than those from the other *rm* P1 promoters.

Core promoter constructs (-41 to +1) contained a 2-bp CA insertion between the *EcoRI* site (GAATTC) and the -41 endpoint. The 2-bp CA insert, originally introduced during cloning using synthetic "linkers" (21, 41), reduced promoter activity relative to a construct with the *EcoRI* site immediately adjacent to -41 (data not shown). Presumably, the *EcoRI* site functions as a weak UP element (17), and the CA insertion moves the *EcoRI* site out of phase for interactions with α CTD.

Promoter activity determinations. Promoter activities in vivo were determined from *lacZ* fusions as described previously (see above and references 6 and 32). β -Galactosidase activities were measured from cells grown for at least three generations in exponential growth (to an optical density at 600 nm [OD₆₀₀] of 0.3 to 0.35), when FIS levels are maximal (2, 4). The activities of promoters in system I are reported directly in Miller units with background activity subtracted (determined from RLG4999). In order to derive a factor for converting system II activities to system I activities, we compared four promoters tolerated by both fusion systems. The activities of *rmB* P1 (-41 to +1), *rmB* P1 (-41 to +50), *rmD* P1 (-41 to +1), and *lacUV5* (-46 to +1) differed by 6.1-, 7.7-, 5.3-, and 7.7-fold, respectively, in the two systems, with an average of 6.7-fold, which was used as the conversion factor.

The slightly lower *rmB* P1 UP element effect in vivo reported here relative to the 30-fold effect reported previously (41, 42) appears to be attributable to strain differences (NK5031 in the previous reports, VH1000 here) and in the background subtraction used for calculating the activity of the promoter lacking a UP element. Furthermore, the magnitude of FIS-dependent activation reported here is slightly higher than the four- to fivefold value reported previously (9). This difference is attributable primarily to a difference in the construct used for calculating the activity of a promoter-*lacZ* fusion lacking FIS sites; i.e., the activity of an *rmB* P1 construct with a -61 upstream endpoint is slightly lower than that of a construct with an endpoint of -88 containing the Δ 72 mutation, a 1-bp deletion within the FIS site.

In vitro transcription reactions were carried out in 10- μ l volumes containing 1 nM supercoiled plasmid DNA template, 170 mM NaCl, 10 mM Tris-Cl (pH 8.0), 10 mM MgCl₂, 1 mM dithiothreitol, and 100 μ g of bovine serum albumin/ml, 500 μ M ATP and GTP, 50 μ M CTP, 10 mM UTP, and 1 μ Ci of [α -³²P]UTP (NEN/DuPont). FIS concentrations were varied from 0 to 400 nM or from 0 to 800 nM. Transcription was initiated by addition of 1 nM RNAP (a generous gift of R. Landick). After 15 min, reactions were terminated with an equal volume of 95% formamide, 10 mM EDTA, 0.05% xylene cyanol, and 0.05% bromophenol blue. Samples were electrophoresed on 7 M urea-5% acrylamide gels. Dried gels were visualized and quantified by phosphorimaging (ImageQuant Software; Molecular Dynamics).

FIS purification. FIS was purified from RLG3669 containing pRJ1077 using a procedure obtained from R. Johnson (University of California at Los Angeles). One liter of cells was grown in Luria broth (LB) to an OD₆₀₀ of 0.7, and FIS protein expression was then induced with 1 mM IPTG for 1 h at 37°C. Cells were pelleted, washed in cold 20 mM Tris-Cl (pH 7.5) and 0.15 M NaCl, and resuspended in half their final volume in 50 mM Tris-Cl (pH 8.0) and 10% sucrose. Cells were lysed in a final volume that was five times their mass. Phenylmethylsulfonyl fluoride (0.1 mM), 2 mM dithiothreitol, 15 mM EDTA, and 0.5 M NaCl (final concentrations) were added, and then cells were lysed by sonication. DNA was removed with polyethyleneimine (PEI) and pelleted by centrifugation at 25,000 $\times$ g for 30 min. Residual PEI was removed with 0.2 volumes of phosphocellulose slurry. The lysate was dialyzed against 0.2 M NaCl-HSB buffer (20 mM Tris-Cl [pH 7.5], 0.1 mM EDTA, 10% glycerol), passed through an SP Sepharose (1 ml) column, and eluted with a linear gradient of 0.2 to 1.0 M NaCl. Fractions containing FIS were identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), dialyzed against 0.2 M NaCl-HSB buffer overnight, subjected to fast-protein liquid chromatography by using a Resource-S (1-ml) column, and eluted with a 30 ml of a gradient of 0.2 to 0.7 M NaCl. Fractions containing FIS were pooled and dialyzed overnight into 0.5 M NaCl-20 mM Tris-Cl (pH 7.5)-0.1 mM EDTA-50% glycerol. FIS concentrations were determined with the Bradford protein assay reagent using bovine serum albumin as a standard and was confirmed by SDS-PAGE.

DNase I footprinting. pRLG770 plasmid derivatives containing promoter sequences from *rmA* P1 (-199 to +1), *rmC* P1 (-201 to +1), *rmD* P1 (-198 to +1), *rmE* P1 (-209 to +1), *rmG* P1 (-199 to +1), and *rmH* P1 (-205 to +1) were digested with *HindIII*, ³²P end labeled with Sequenase (U.S. Biochemicals) and [α -³²P]dATP on the nontemplate strand, and digested with *EcoRI*. The resulting promoter fragments were isolated from 5% acrylamide gels and purified by using Elutip-D columns (Schleicher & Schuell). DNase I footprinting was carried out and analyzed as described previously (43, 46).

RESULTS

Sequences of *rm* P1 promoters. Studies on *rmB* P1 indicated that sequences upstream of -154 contributed little to promoter activity (21). Therefore, we examined sequences no further upstream than about -200 at the other promoters. To limit the potential for variation in reporter gene activity in our promoter-*lacZ* fusions attributable to differential mRNA stability, we chose a downstream endpoint of position +1 for the constructs from the different operons (see Materials and Methods).

Examination of the DNA sequences indicated that, as for *rmB* P1, each of the other *rm* P1 promoters contained an A+T-rich region upstream of the -35 hexamer (Fig. 1A), corresponding in position to the *rmB* P1 UP element. The core promoters and A+T-rich regions of *rmA* P1, *rmB* P1, and *rmC* P1 are identical up to -68; promoter constructs containing sequences identical in these three promoters are referred to as *rmABC* P1. The core and UP element regions of *rmD* P1, *rmE* P1, *rmG* P1, and *rmH* P1 differ from *rmABC* P1 and from each other. Each of the seven operons differs in sequence upstream of the *rm* P1 UP elements (Fig. 1A).

Identification of FIS binding sites upstream of position -61 in all seven *rm* P1 promoters. FIS activates transcription by binding to three sites upstream of position -61 in *rmB* P1. Therefore, we first determined experimentally whether there were FIS sites in the upstream regions of each of the other *rm* P1 promoters. DNase I footprints were performed on DNA fragments containing sequences from about -200 to +1 of the P1 promoters of *rmA*, *rmC*, *rmD*, *rmE*, *rmG*, and *rmH* by using a range of FIS concentrations. FIS binding sites were identified by a characteristic pattern of three regions of protection that span two regions (separated by 11 to 14 bp), where the DNA either is not protected or subject to enhanced cleavage (Fig.

TABLE 1. Strains and plasmids in this study

Strain or plasmid	Genotype	Source or reference
Strains		
VH1000	RLG3499 = MG1655 <i>lacZ lacI pyrE</i> ⁺	19
RJ1617	MC1000 <i>fis::kan-767</i>	27
RLG3669	BL21 (DE3) pLysS	Novagen
λ system I lysogens		
RLG4297	VH1000 λ <i>rrnB</i> P1 (−88 to +1)- <i>lacZ</i>	This work
RLG4787	VH1000 λ <i>rrnE</i> P1 (−89 to +1)- <i>lacZ</i>	This work
RLG4788	VH1000 λ <i>rrnE</i> P1 (−209 to +1)- <i>lacZ</i>	This work
RLG4789	VH1000 λ <i>rrnE</i> P1 (−61 to +1)- <i>lacZ</i>	This work
RLG4999	VH1000 λ M13 polylinker- <i>lacZ</i>	This work
RLG5940	RLG4999 <i>fis::kan-767</i>	This work
RLG5949	VH1000 λ <i>rrnB</i> P1 (−154 to +1)- <i>lacZ</i>	This work
RLG5950	VH1000 λ <i>rrnABC</i> P1 (−61 to +1)- <i>lacZ</i>	This work
RLG6150	VH1000 λ <i>rrnG</i> P1 (−199 to +1)- <i>lacZ</i>	This work
RLG6152	VH1000 λ <i>rrnH</i> P1 (−205 to +1)- <i>lacZ</i>	This work
RLG6153	VH1000 λ <i>rrnH</i> P1 (−62 to +1)- <i>lacZ</i>	This work
RLG6360	VH1000 λ <i>rrnG</i> P1 (−61 to +1)- <i>lacZ</i>	This work
RLG6361	RLG4297 <i>fis::kan-767</i>	This work
RLG6362	RLG5949 <i>fis::kan-767</i>	This work
RLG6363	RLG5950 <i>fis::kan-767</i>	This work
RLG6369	RLG6150 <i>fis::kan-767</i>	This work
RLG6370	RLG6152 <i>fis::kan-767</i>	This work
RLG6371	RLG6153 <i>fis::kan-767</i>	This work
RLG6372	RLG6360 <i>fis::kan-767</i>	This work
RLG6373	RLG6390 <i>fis::kan-767</i>	This work
RLG6374	RLG6392 <i>fis::kan-767</i>	This work
RLG6375	RLG6504 <i>fis::kan-767</i>	This work
RLG6376	RLG6394 <i>fis::kan-767</i>	This work
RLG6377	RLG6505 <i>fis::kan-767</i>	This work
RLG6378	RLG6396 <i>fis::kan-767</i>	This work
RLG6379	RLG6397 <i>fis::kan-767</i>	This work
RLG6380	RLG6398 <i>fis::kan-767</i>	This work
RLG6381	RLG6368 <i>fis::kan-767</i>	This work
RLG6390	VH1000 λ <i>rrnG</i> P1 (−86 to +1)- <i>lacZ</i>	This work
RLG6392	VH1000 λ <i>rrnH</i> P1 (−82 to +1)- <i>lacZ</i>	This work
RLG6394	VH1000 λ <i>rrnA</i> P1 (−199 to +1)- <i>lacZ</i>	This work
RLG6396	VH1000 λ <i>rrnC</i> P1 (−201 to +1)- <i>lacZ</i>	This work
RLG6397	VH1000 λ <i>rrnD</i> P1 (−81 to +1)- <i>lacZ</i>	This work
RLG6398	VH1000 λ <i>rrnD</i> P1 (−198 to +1)- <i>lacZ</i>	This work
RLG6503	VH1000 λ <i>rrnD</i> P1 (−60 to −42)- <i>rrnB</i> P1 (−41 to +1)- <i>lacZ</i>	This work
RLG6504	VH1000 λ <i>rrnA</i> P1 (−81 to +1)- <i>lacZ</i>	This work
RLG6505	VH1000 λ <i>rrnC</i> P1 (−81 to +1)- <i>lacZ</i>	This work
RLG6512	VH1000 λ <i>rrnABC</i> P1 (−61 to −42)- <i>rrnD</i> P1 (−41 to +1)- <i>lacZ</i>	This work
RLG6513	VH1000 λ <i>rrnD</i> P1 (−61 to +1)- <i>lacZ</i>	This work
RLG6516	VH1000 λ <i>rrnB</i> P1 (−154 to −62)- <i>rrnD</i> P1 (−61 to +1)- <i>lacZ</i>	This work
RLG6517	VH1000 λ <i>rrnD</i> P1 (−198 to −62)- <i>rrnABC</i> P1 (−61 to +1)- <i>lacZ</i>	This work
RLG6522	VH1000 λ <i>rrnE</i> P1 (−209 to −62)- <i>rrnABC</i> P1 (−61 to +1)- <i>lacZ</i>	This work
RLG6523	VH1000 λ <i>rrnE</i> P1 (−88 to −62)- <i>rrnABC</i> P1 (−61 to +1)- <i>lacZ</i>	This work
RLG6524	VH1000 λ <i>rrnB</i> P1 (−154 to −62)- <i>rrnE</i> P1 (−61 to +1)- <i>lacZ</i>	This work
RLG6525	VH1000 λ <i>rrnB</i> P1 (−88 to −62)- <i>rrnE</i> P1 (−61 to +1)- <i>lacZ</i>	This work
RLG6527	RLG6513 <i>fis::kan-767</i>	This work
RLG6528	VH1000 λ <i>rrnB</i> P1 (−82 to +1)- <i>lacZ</i>	This work
λ system II lysogens		
RLG6352	VH1000 λ <i>rrnE</i> P1 “CA” (−41/+1)- <i>lacZ</i>	This work
RLG6353	VH1000 λ <i>rrnH</i> P1 “CA” (−41/+1)- <i>lacZ</i>	This work
RLG6354	VH1000 λ <i>rrnG</i> P1 “CA” (−41/+1)- <i>lacZ</i>	This work
RLG6358	VH1000 λ <i>rrnABC</i> P1 “CA” (−41/+1)- <i>lacZ</i>	This work
RLG6502	VH1000 λ <i>rrnD</i> P1 “CA” (−41/+1)- <i>lacZ</i>	This work
Plasmids		
pRJ1077	pET11a with <i>fis</i>	39
pRLG770	Vector (no insert)	46
pRLG5943	<i>rrnB</i> P1 (−154 to +1) in pRLG770	This work
pRLG4782	<i>rrnE</i> P1 (−209 to +1) in pRLG770	This work
pRLG5945	<i>rrnG</i> P1 (−199 to +1) in pRLG770	This work
pRLG5947	<i>rrnH</i> P1 (−205 to +1) in pRLG770	This work
pRLG6385	<i>rrnA</i> P1 (−199 to +1) in pRLG770	This work
pRLG6387	<i>rrnC</i> P1 (−201 to +1) in pRLG770	This work
pRLG6398	<i>rrnD</i> P1 (−198 to +1) in pRLG770	This work

FIG. 1. (A) Sequence alignment of the *rrn* P1 promoters. Sequences were compiled from the *E. coli* K-12 MG1655 complete genome (GenBank accession no. U00096). The −35 and −10 hexamers are in boldface and boxed. FIS binding sites identified by DNase I footprints are in boldface and shaded. (B) Representative FIS footprint with DNase I (*rrnE* P1 FIS site I). The bracket indicates the extent of FIS protection. Positions within the FIS footprint that are either accessible to DNase I or display enhanced cleavage are indicated by capital letters in panel A and arrows in panel B. (C) Schematic alignment of the *rrn* P1 promoters showing the locations of FIS sites relative to the core promoter (−10 and −35 hexamers) and UP element regions.

1B). The sites of enhanced cleavage are indicative of the FIS-induced bend in the DNA (46). We conclude from the footprinting studies that (i) there are three to five FIS sites upstream of position −61 at each *rrn* P1 promoter (Fig. 1A and C), (ii) site I at six of the seven *rrn* P1 promoters is centered at position −71 relative to the transcription start site and at *rrnE* P1 site I is centered at position −72, and (iii) the positions of the FIS binding sites upstream of site I differ at each promoter (Fig. 1C).

Determinants of *rrn* P1 promoter strength in vivo. The FIS sites were located upstream of the UP element-like regions in all seven promoters. To determine the contributions of the

predicted UP elements and FIS sites to promoter activity, we constructed promoter-*lacZ* fusions containing (i) only the core promoter (upstream endpoint, −41); (ii) the core promoter and the predicted UP element region (−61); (iii) the core promoter, the predicted UP element, and the FIS site I (−81); and (iv) the full-length promoters (−200) (see Table 1 for individual promoter endpoints). Upstream endpoints from previously characterized promoter-*lacZ* fusions were used for *rrnB* P1 (46). Although inputs to promoter strength had been determined previously for *rrnB* P1, it was included in the following analyses for comparison.

The *rm* P1 promoter-*lacZ* fusions were integrated into the chromosome in single copy at the λ att site. Since the activities of all of the promoters were assayed at the same location in the chromosome, the potential for positional effects resulting from differences in gene dose or local chromosome structure was eliminated.

All *rm* P1 core promoter activities were low, similar to that of *rmB* P1, at which the core promoter activity accounts for less than 1% of the full-length promoter activity (21, 41). The *rmABC* P1 core promoter was ~1.5 to 2-fold stronger than the *rmD* P1, *rmE* P1, *rmG* P1, and *rmH* P1 promoters (Fig. 2A).

The activities of six of the *rm* P1 promoters were increased to similar extents by the UP element regions, i.e., 17- to 29-fold, while *rmD* P1 was activated more by its UP element, i.e., 54-fold (Fig. 2B). We emphasize the relative UP element effects for the different operons rather than the absolute values of the activation ratios (see Materials and Methods). Transcription from each of the promoters in vitro with or without the UP element region confirmed that the effects of the UP element were mediated through RNAP, since they were observed in a purified system in the absence of other protein factors (42, 44; data not shown).

UP element effects depend upon the identity of both the UP element and the core promoter sequences. To determine whether the large effect of the *rmD* P1 UP element is a function of its sequence (a closer match to the UP element consensus than the other *rm* P1 UP elements) or of the ability of its core promoter to be activated, hybrid promoters were constructed in which the UP element of *rmD* P1 was fused to the *rmABC* P1 core promoter or in which the *rmABC* P1 UP element was fused to the *rmD* P1 core promoter. These constructs allowed us to compare (i) two different UP elements in the context of the same core promoter and (ii) the same UP element on two different core promoters. The *rmABC* P1 core promoter was activated 29-fold by the *rmD* P1 UP element compared to 17-fold activation by its own UP element; the *rmD* P1 core promoter was activated 11-fold by the *rmABC* P1 UP element and 54-fold by its own UP element (Fig. 3). These results indicate that both the identity of the core promoter and the identity of the UP element contribute to the extent of activation.

Activation of *rm* P1 promoters by sequences upstream of ~-61 in vivo is FIS dependent. All *rm* P1 promoters except *rmD* P1 were activated ~6- to 8-fold by sequences located between positions ~-61 and ~-200 in vivo (Fig. 2C, Fig. 4; see also Materials and Methods). *rmD* P1, whose activity was higher than the other promoters in the absence of any FIS sites, was activated to a much smaller degree (<3-fold). Thus, although the presence of the region between ~-200 and ~-61 increased the activity of the *rm* P1 promoters to different extents, the activities of all seven full-length *rm* P1 promoters (containing FIS sites) were very similar (6,800 to 8,400 Miller units; Fig. 2C).

The effect of the sequences upstream of ~-61 was drastically reduced in a strain lacking FIS, confirming that the activation in vivo from sequences upstream of ~-61 is primarily dependent on FIS (Fig. 4). Upstream sequences increased transcription 1.9-fold or less in the absence of FIS, whereas FIS increased transcription up to 8-fold. As previously reported for *rmB* P1 (46), the activation ratio (full-length promoter/pro-

FIG. 2. β -Galactosidase activities from *rm* P1 promoter-*lacZ* fusions. (A) Promoters (-41 to +1) containing only the core promoter were fused to *lacZ* by using system II; the activities have been converted to system I units as described in Materials and Methods. (B) Promoter-*lacZ* fusions (~-61 to +1) containing the core promoter plus UP element. Numbers above bars refer to fold activation by the UP element (ratio of activities in panels B and A for each promoter). (C) Promoter-*lacZ* fusions (~-200 to +1) containing all FIS sites, UP element, and core promoter. Numbers above bars refer to the fold activation by sequences upstream of ~-61 (ratio of activities in panels C and B for each promoter). Means and standard deviations are derived from at least three independent assays.

motor from ~-61 to +1) decreased in a *fis::kan* strain not because of a decrease in the activities of the promoter constructs containing FIS sites but rather because of an increase in the activities of the *rm* P1 promoters lacking FIS sites (C. A. Hirvonen, W. Ross, V. H. Newburn, and R. L. Gourse, unpub-

FIG. 3. Both the UP element and the core promoter sequence determine the extent of activation by UP elements. The sources of the UP element and core promoter regions are indicated below each bar. The fold activation is the ratio of the β -galactosidase activity of the UP element-containing promoter (-61 or -60 to $+1$) to that same promoter lacking a UP element (-41 to $+1$). Means and standard deviations are derived from at least three independent assays.

lished data [see also reference 15]). This increase in activity results from a feedback mechanism that compensates for the loss of activation of the seven *rmn* operons when the *fis* gene is inactivated (22, 46).

All seven *rmn* P1 promoters are activated directly by FIS in vitro. In vitro transcription was used to verify that FIS was directly responsible for the effect of the sequences upstream of ~ -61 in each *rmn* promoter. Transcription from each of the seven full-length *rmn* P1 promoters was increased by purified FIS in vitro (Fig. 5A), while transcription from *rmn* P1 promoter variants extending upstream only to ~ -61 was not (data not shown).

The amount of FIS required for half-maximal transcription from each full-length *rmn* P1 promoter was determined by in vitro transcription at a range of FIS concentrations (Fig. 5B). *rmnE* P1 required significantly higher concentrations of FIS (145 nM) for half-maximal transcription than any of the other six promoters (15 to 48 nM) (Fig. 5B). The maximal extent of activation by FIS in vitro varied from <3 -fold (*rmnD* P1) to 12-fold (*rmnE* P1) (Fig. 5C). Thus, although *rmnE* P1 required higher FIS concentrations than the other promoters for full activation, it was activated more at the highest FIS concentrations than the other promoters. However, since FIS did not increase the activity of *rmnE* P1 more than the other promoters in vivo (Fig. 4), this suggests that the concentration of FIS must not be high enough in cells (at least under the conditions tested) to fully utilize the *rmnE* FIS sites.

Intrinsic features of the *rmnD* P1 promoter limit the degree of activation by FIS. We tested whether the relatively small effect of FIS at *rmnD* P1 in vivo and in vitro was the result of features of its FIS sites or features of its promoter. Hybrid promoters were constructed in which the *rmnD* P1 FIS sites were fused to the *rmnB* P1 promoter at -61 or the *rmnB* P1 FIS sites were fused to *rmnD* P1 at -61 . The *rmnD* P1 FIS sites activated the *rmnB* P1 promoter to about the same extent (~ 6 -fold) as the *rmnB* P1 FIS sites activated their own promoter in vivo (Fig. 6). Thus, the small effect of FIS at *rmnD* P1 is not due to intrinsic features of its FIS sites. Likewise, the *rmnB* P1 FIS sites activated the *rmnD* P1 promoter to the same extent as its

own FIS sites, <3 -fold (Fig. 6). Therefore, the limited effect of FIS at *rmnD* P1 appears to result from intrinsic features of its core promoter and/or its UP element rather than from its FIS sites.

At five of the seven *rmn* P1 promoters, FIS sites upstream of site I play a larger role in transcription than at *rmnB* P1. At *rmnB* P1, ca. 80% of the effect of FIS in vivo is attributable to the promoter proximal FIS site, site I, centered at position -71 (Table 2). To determine whether this is also true at other *rmn* P1 promoters, we compared the extent of activation by FIS site I with the extent of activation by all FIS sites. The effect of FIS site I varied from 1.6-fold at *rmnA* P1 to 7.5-fold at *rmnG* P1 (Table 2). Purified FIS also directly increased transcription in vitro at each of the seven *rmn* P1 promoters containing only site I (data not shown).

The small effect (1.6-fold) of FIS site I at *rmnA* P1 was significantly different from the four- to fivefold effect of site I at *rmnB* P1 and *rmnC* P1 in vivo, even though the core promoter and UP element sequences are the same in each of these operons and FIS site I is centered at the same position (i.e., -71). Therefore, differences in activation by FIS site I at these three promoters must reflect subtle differences in how FIS is positioned on the DNA (39) and/or differences in the concentration of FIS required for binding.

Comparing the activities of each of the promoters with only FIS site I to the same promoter with all FIS sites allowed calculation of the contributions of sites upstream of site I (the "distal FIS sites"; Table 2, column 9). At *rmnG* P1, the distal FIS sites accounted for even less of the total promoter activity (8%) than at *rmnB* P1 (22%). However, the distal sites played a larger role at the other operons (32 to 73%), with 67% of the total effect of FIS resulting from the distal sites at *rmnE* P1 and 73% of the total effect of FIS resulting from the distal sites at *rmnA* P1. The large impact of the distal FIS sites is not unique to *rmn* P1 promoters; at the *thrU*(*tufB*) promoter, FIS sites

FIG. 4. FIS is required for the effects of sequences upstream of position ~ -61 in vivo. *rmn* P1 promoter-*lacZ* fusions from the *rmnA*, *rmnB*, *rmnC*, *rmnD*, *rmnE*, *rmnG*, and *rmnH* operons were measured in wild-type (black bars) or *fis:kan* (gray bars) backgrounds. The effect of sequences upstream of ~ -61 was determined as the ratio of activity from a full-length construct containing all FIS sites (~ -200 to $+1$) to the activity of that same promoter lacking FIS sites (~ -61 to $+1$). A promoter whose activity is not increased by a sequence upstream of ~ -61 would have a fold activation of 1.0 (represented by the dotted line). Means and standard deviations are derived from at least three independent assays.

FIG. 5. FIS concentration requirements and extent of activation of *rm* P1 promoters by FIS in vitro. (A) In vitro transcription reactions were performed as described in Materials and Methods by using plasmid templates carrying the full-length *rm* P1 promoters (~ -200 to $+1$) in the absence or presence of increasing concentrations of FIS. Transcripts from *rm* P1 and vector-encoded RNA 1 promoter are indicated. The RNA 1 transcript is vector encoded and serves as an internal control. (B) The FIS concentration required for half-maximal activation of each full-length *rm* P1 promoter in vitro was determined by quantitation of transcripts from at least two separate assays such as that shown in panel A. Values were normalized in each case to the plateau level, defined as 1.00, for each promoter. *rmA* P1 (●), *rmB* P1 (○), *rmC* P1 (▲), *rmD* P1 (△), *rmE* P1 (■), *rmG* P1 (□), and *rmH* P1 (◆). (C) The fold activation by FIS for each *rm* P1 promoter was determined by comparison of transcripts in the presence or absence of 400 nM FIS.

upstream of site I were also reported to play a relatively large role in activation (52).

To determine whether the large effect of the distal FIS sites in *rmE* P1 was a property of these sites or of features of the promoter, we constructed hybrid promoters in which either FIS site I or all of the FIS sites from *rmE* P1 and *rmB* P1 were exchanged. *rmE* P1 FIS site I activated the *rmB* P1 and *rmE* P1 promoters similarly (2.8-fold versus 2.5-fold; Table 2), but *rmB* P1 FIS site I activated the *rmE* P1 promoter less than *rmB* P1 (2.9-fold versus 5.1-fold). When all of the *rmB* P1 and *rmE* P1 FIS sites were exchanged, their abilities to activate transcription were somewhat reduced, i.e., both hybrid promoters had slightly lower activities than the natural constructs (Table 2). Thus, the magnitude of activation mediated by the FIS sites was affected by both the identity of the FIS sites and of the RNAP binding regions. We calculated the relative contribution of the upstream FIS sites in the different hybrid promoters. Distal *rmE* P1 FIS sites made a smaller relative contribution to total activity when positioned upstream of the *rmB* P1 promoter than when in their native context (43% versus 67%; Table 2). However, the distal FIS sites of *rmB* P1 had a slightly larger effect on total activity when positioned upstream of *rmE* P1 than in their native context (37% versus 22%). Therefore, it appears that the relative contribution of the FIS sites upstream of site I to total transcription is dependent on multiple aspects of promoter architecture, e.g., on the identity of the

FIS sites and the RNAP binding region (from positions ~ -61 to $+1$).

DISCUSSION

***rm* P1 promoter activities derive from different relative contributions of the same components.** Our studies have shown that UP elements and the transcription factor FIS contribute to the strength of each *rm* P1 promoter (Fig. 1). If we assume that the seven *rm* operons arose through gene duplication, it is not surprising that FIS sites and UP elements (albeit degenerate) have been retained in the course of *E. coli* evolution as the mechanisms responsible for high activity at all seven *rm* P1 promoters. Selective pressure has apparently acted at each operon to maintain sensitivity to the same inputs while maintaining the same overall activity for each full-length *rm* P1 promoter.

However, several different solutions have been found to account for high activity in the different *rm* operons. (i) The effect of FIS at *rmD* P1 is small relative to that at the other *rm* P1 promoters; *rmD* P1 derives its strength from a larger contribution of its UP element to total expression (relative to the effects of the other *rm* P1 UP elements). (ii) *rmB* P1 and *rmG* P1 are activated more than the other *rm* P1 promoters by the promoter proximal FIS site, site I. (iii) *rmA* P1 and *rmE* P1 are activated more than the other *rm* P1 promoters by FIS sites

FIG. 6. Intrinsic features of the *rrnD* P1 promoter (−61 to +1) limit its activation by FIS in vivo. *rrnB* P1 or *rrnD* P1 promoters were either wild type (containing native FIS sites) or hybrid (containing FIS sites from the other promoter). The fold activation by FIS was determined as the ratio of the β -galactosidase activity of a promoter-*lacZ* fusion containing the indicated FIS sites to that of the same promoter lacking FIS sites (−61 to +1). Means and standard deviations are derived from at least three independent assays.

upstream of site I. In the case of *rrnE* P1, high concentrations of FIS could potentially make an even greater contribution to transcription, but the sites have evolved with relatively low affinity for FIS, limiting their ability to activate transcription by the FIS concentrations actually present in vivo (at least under the conditions tested). It has been noted previously in other systems that different regulatory assemblies can result in a similar transcriptional outcome (11).

Why do all seven full-length *rrn* P1 promoters have similar activities? The *rrn* P1 promoters are extremely active (perhaps the most active of the cell's promoters) at high growth rates. Although we have examined expression of the seven *rrn* P1 promoters under conditions where transcription activity is high, our reporter system is not saturated for β -galactosidase (e.g., double lysogens containing these promoter-*lacZ* fusions have twice the enzyme activity of monolysogens [data not shown]). Although we cannot provide a conclusive answer to why the seven full-length *rrn* P1 promoters have evolved to have similar activities, we suggest that these activities are not

set by an approach of each promoter to some theoretical limit. For example, transcriptional output from *rrn* operons can increase further, even in rich medium, to compensate for reductions in gene dose (3), and transcription initiation at rRNA promoters increases when the gene for the elongation factor NusB is inactivated (50). Furthermore, the activity of a full-length *rrnD* P1 promoter can increase ~50% in a *fis::kan* mutant (data not shown).

Changing FIS concentrations may influence the relative contribution of different *rrn* operons to total rRNA synthesis. FIS is undetectable in stationary-phase cells, and levels increase dramatically as cells enter the exponential phase. After only two generations of growth, there are as many as 50,000 FIS molecules per cell when cultured in rich medium (2, 4). The occupancy of FIS sites at *rrnB* P1 in vivo correlates with these changes in cellular FIS levels (2). Since the extent of activation by FIS at different promoters varies with the FIS concentration in vitro (especially at *rrnE* P1 versus the other *rrn* P1 promoters) and since the unactivated level of transcription at *rrnD* P1 is higher than at the other promoters in vivo and in vitro, changes in the amount of FIS could potentially result in differences in the relative contributions of different operons to total rRNA synthesis in vivo. We note that the products of the different *rrn* operons (both tRNAs and rRNAs) are not identical (26). Whether the differences in the expression of different operons have physiological consequences remains to be determined.

Since *E. coli* containing only five *rrn* operons grows at near wild-type rates on rich medium (13) and the deletion of additional *rrn* operons is not lethal (3), it has been proposed that the presence of all seven *rrn* operons is necessary for swift adaptation to environmental changes (14, 30). The different ways of achieving the same final output might help to allow for such swift adaptations.

Structural considerations for activation by distal FIS sites. The discovery that distal FIS sites play a more significant role in transcription than predicted from studies on *rrnB* P1 suggests that the structure of the P1 activation complex varies in the different *rrn* operons. Physical contacts (if they exist) between FIS bound at distal sites and RNAP has not been ex-

TABLE 2. Relative contribution of upstream FIS sites to total *rrn* P1 promoter activity

Promoter	Origin of −61 to +1	Origin of FIS sites ^a	Activity ^b		Fold effect (site I) ^c	Activity (all sites) ^b	Fold effect (all sites) ^c	% From upstream sites ^d
			No sites	Site I				
Wild type	<i>rrnA</i> P1	A	1,260	1,959	1.6	7,350	5.8	73
	<i>rrnB</i> P1	B	1,260	6,468	5.1	8,332	6.6	22
	<i>rrnC</i> P1	C	1,260	5,090	4.0	8,407	6.7	39
	<i>rrnD</i> P1	D	2,795	3,841	1.4	7,361	2.6	48
	<i>rrnE</i> P1	E	889	2,255	2.5	6,756	7.6	67
	<i>rrnG</i> P1	G	862	6,498	7.5	7,039	8.2	8
	<i>rrnH</i> P1	H	1,146	5,635	4.9	8,248	7.2	32
Hybrid	<i>rrnE</i> P1	B	889	2,505	2.9	3,981	4.5	37
	<i>rrnB</i> P1	E	1,260	3,455	2.8	6,027	4.9	43

^a The sources of FIS sites found upstream of −61 at each promoter are indicated.

^b β -Galactosidase activities (in Miller units) from promoters lacking FIS sites (−61 to +1) or containing only FIS site I or all FIS sites are the mean from at least three separate assays. The error was generally 10% or less.

^c The fold effect is the ratio of β -galactosidase activities of FIS site-containing promoter-*lacZ* fusions and −61 to +1 *rrn* P1 promoter-*lacZ* fusions. Fold effect (site I) is the ratio of the activities of constructs containing site I and no sites; fold effect (all sites) is the ratio of the activities of constructs containing all sites and no sites.

^d The contribution of the upstream FIS sites is expressed as a percentage of total activity: $100 \times (\text{activity of a construct containing all sites} - \text{activity of a construct containing site I}) / \text{activity of construct containing all sites}$.

pored in detail at *rm* P1 promoters. Present information strongly suggests that there are no cooperative interactions between bound FIS dimers (9, 28) and that FIS bound at site I contacts the α CTD of RNAP (8).

Since the α CTD and α NTD are connected by a flexible linker of only ca. 13 amino acids (~ 46 Å if fully extended) (25), α CTD should not be able to reach further upstream than positions ~ -80 to -90 in the absence of DNA distortion (36, 38). Assuming FIS bound at the distal sites exerts its effects on transcription by interacting directly with RNAP, we suggest that FIS bound at the proximal site(s), possibly in conjunction with intrinsic DNA curvature (20), distorts the DNA to facilitate these contacts. Differences in the positions of the upstream FIS sites at different operons, differences in the angles of the bends induced by FIS bound to its various binding sites, and differences in intrinsic DNA curvature in different operons could all contribute to differences in the extents of activation by the different *rm* P1 upstream regions. Consistent with this model, mutations at *rmE* P1 that prevent binding of FIS to site I eliminate activation by distal FIS sites (C. A. Hirvonen and R. L. Gourse, unpublished data). We cannot eliminate the possibility that other protein factors, as yet unknown, might be required for the activation attributed to the sequences upstream of FIS site I. However, the effects of these sequences would depend on *fis* (Fig. 4) and FIS bound at site I.

Further studies will be needed to determine where the two α CTDs are located in complexes containing multiple FIS sites, as well as other details about promoter architecture in the different operons. However, initiation complexes in which there are multiple activator dimers and intrinsic DNA bends facilitating upstream binding by α CTD would not be unique to *rm* P1 promoters. For example, when multiple CAP-cyclic AMP dimers are bound upstream of a promoter, the α CTD can reach to at least position -100 (7, 33), and at the *Pu* promoter in *Pseudomonas putida*, integration host factor (IHF) bends the DNA so that α CTD can interact with specific DNA sequences located upstream of the IHF site (40).

Prediction of FIS binding sites. We compared the FIS binding sites defined in our footprinting assays (Fig. 1) with FIS sites predicted by the most recent computational means (24, 48, 49). Our conclusions are as follows: (i) sites predicted as having high probability of being FIS sites were detectable by DNase I footprinting; (ii) sites with a lower probability of being FIS sites were usually not detected even as partially occupied by FIS in footprints; and (iii) sites detected with relatively weak affinity for FIS were often not predicted by the computational analysis. Since the footprints presented here have increased the number of confirmed FIS sites substantially, this study should allow further refinement of FIS site prediction.

Concluding remarks. In summary, we have shown that the same inputs contribute to the strength of all seven *rm* P1 promoters, although the relative contributions of these inputs vary. Understanding the contributors to transcription of the different *rm* P1 promoters allows us to begin to integrate information about how the multiple inputs contributing to rRNA expression act together to regulate rRNA promoter output.

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