Both fis-dependent and factor-independent upstream activation of the *rrnB* P1 promoter are face of the helix dependent

Janet T. Newlands, Cathleen A. Josaitis, Wilma Ross and Richard L. Gourse

Department of Bacteriology, University of Wisconsin, 1550 Linden Drive, Madison, WI 53706, USA

Received December 2, 1991; Revised and Accepted January 10, 1992

ABSTRACT

Transcription from the *Escherichia coli rrnB* P1 promoter is increased by a cis-acting sequence which extends upstream of the –35 hexamer to about –150 with respect to the transcription initiation site, the Upstream Activation Region (UAR). Activation by the UAR involves two components: (1) a trans-acting protein, Fis, which binds to three sites in the UAR between –60 and –150, and (2) the UAR sequences themselves which affect RNA polymerase (RNAP) activity independent of other proteins. We refer to the latter as Factor-Independent Activation (FIA). In addition to its interactions with the –10 and –35 hexamers typical of *E. coli* promoters, RNAP makes contacts to the –53 region of *rrnB* P1, which may be related to the FIA effect. We constructed a series of insertion mutants containing integral and non-integral numbers of helical turns at position –46, between the Fis binding sites and the –35 region, and the resulting promoter activities were measured *in vitro* and *in vivo*. The data suggest that both Fis-dependent and factor-independent activation are face of the helix dependent: the Fis binding site and the sequences responsible for factor-independent activation must be correctly oriented relative to RNA polymerase in order to activate transcription. These results, in conjunction with other evidence, support a model for the involvement of direct Fis-RNAP interactions in upstream activation. We also demonstrate that RNAP interacts with the –53 region of the *rrnB* P1 UAR even when these sequences are displaced upstream of the RNAP binding site, and that these interactions correlate with factor-independent activation.

INTRODUCTION

Each of the seven *Escherichia coli* ribosomal RNA operons has two promoters, P1 and P2. Most rRNA synthesis originates from the P1 promoters at high growth rates (1,2) when rRNA promoters account for more than half of the transcriptional activity of the cell (3). The exceptional strength of the *rrn* P1 promoters derives primarily from their upstream activation regions (UAR)(4). The *rrnB* P1 UAR has been defined functionally as a region of DNA from approximately –154 to –40 with respect to the transcription initiation site (4–9).

Upstream activation is not required for growth rate dependent regulation or stringent control of rRNA transcription (4,7).

Upstream activation of *rrnB* P1 can be separated into two distinct components: Fis-dependent and factor-independent activation. Fis protein binds to three sites in the UAR and increases the transcription rate (7,10). Fis Site I [centered at –71, the site closest to the RNA polymerase (RNAP) binding site] is necessary and sufficient for the majority of the effect of Fis (Ref. 7 and Bokal, Ross, and Gourse, unpublished). Factor-independent activation (FIA) results from an AT-rich region between –40 and –60 which interacts directly with RNAP and increases transcription in the absence of Fis or other factors (8,9,11).

Site-specific DNA binding proteins that increase the rate of transcription have been identified in many prokaryotic and eukaryotic systems. Transcription activation may involve direct contact between activator protein(s) and RNAP (12,13). In some cases, evidence consistent with direct activator protein-RNAP contacts comes from 'phasing experiments' in which the distance between the activator protein and the RNAP binding sites is altered by insertion of integral and non-integral numbers of helical turns. Promoter activity is then evaluated for its dependence on the preservation of the specific rotational orientation of these sites (as would be expected for the maintenance of direct protein-protein contacts; 14–21).

To determine whether a precise distance and orientation of Fis binding site I relative to the RNAP binding site is required for Fis-mediated activation, we inserted integral and non-integral numbers of helical turns of DNA between the two sites and measured the promoter activity *in vivo* and *in vitro*. We show that Fis can activate *rrnB* P1 when its binding site is moved one, two, or three 11 base pair (bp) turns upstream of the RNAP binding site. In addition, we find that FIA also shows 11 bp periodicity. The FIA region can be displaced by one helical turn with no effect on either factor-independent activation or *rrnB* P1-RNAP interactions in the –53 region. The ability of RNAP to recognize and interact with this region may be important for factor-independent activation.

MATERIALS AND METHODS

General

Restriction enzymes were obtained from New England Biolabs. Restriction fragments were 3' end-labelled and purified as described (11). Purified Fis protein was provided by Reid

Johnson. RNA polymerase was provided by Dayle Hager and Richard Burgess. All plasmids used in this study were either purified by CsCl centrifugation (22) or with Qiagen columns (Qiagen, Inc.) followed by two phenol/chloroform extractions and ethanol precipitation. Concentrations of DNAs used for *in vitro* transcription experiments were determined using a Hoefer TKO100 fluorometer to eliminate contributions from possible contaminating RNA.

Plasmid construction

Strains containing plasmid constructs are listed in Table 1. A plasmid containing the wild type *rrnB* P1 promoter from -87 (EcoRI) to +50 (HindIII) was digested with DraI (cleaving the promoter region at -46), and 8, 10, or 12 bp BamHI linkers (New England Biolabs) were inserted to make 8, 10, or 12 bp insertion constructs. EcoRI/BamHI and BamHI/HindIII *rrnB* promoter region fragments from the various plasmids were combined to make the 9 and 11 bp insertions. The resulting promoter fragments were subsequently recombined into pRLG770 (7) in front of the *rrnB* T1T2 terminators. To make the 5 bp insertion, the 9 bp insert promoter was digested with BamHI, and the overhanging ends were blunted with Mung Bean Nuclease (Epicentre Technologies) followed by reclosure of the plasmid. To make longer insertions, 12 bp BglII linkers (5'-GGAAGATCTTCC-3') were ligated to each other and digested with BglII, creating DNA fragments with four base overhangs complementary to BamHI. These were inserted into the BamHI site of each of the above plasmids, generating 20–24 and 32–36 bp insertions. To make the 274–278 bp insertions, a 266 bp Sau3A restriction fragment from the *rrnB* 16S rRNA gene (positions 305 to 571) was inserted into the BamHI site of the 8, 9, 10, 11 and 12 bp insert plasmids. Plasmids with the 266 bp insert in the same orientation were recovered for each size. To make the 282 bp insertion, the 278 bp insertion plasmid was cut with AvrII (which recognizes a unique site in the plasmid within the insertion, corresponding to position 562 of 16S rRNA) and the overhanging ends were filled in with dNTPs using Sequenase (US Biochemicals), followed by closure of the plasmid.

Figure 1. Sequences of mutant and wild type *rrnB* P1 promoters. *rrnB* P1 sequence from -87 to +50 is shown. The region protected by Fis in DNaseI footprints (7) and the region necessary for factor-independent activation (9) are indicated. The -35 consensus hexamer for RNA polymerase binding is boxed. EcoRI linker upstream of -87 is overlined. Insertions were constructed at the DraI restriction site (TTTAAA) at -46. Numbers on the left indicate the number of base pairs inserted. Only the promoter proximal sequences of the 274–282 bp inserts are shown (dots indicate that approximately 220 bp of the insertion extend further upstream).

Bacterial strains and β -Galactosidase Measurements

Plasmid-containing strains and λ lysogens are listed in Table I. Plasmids were maintained in *E. coli* strain CAG1574 (*recA56*, *araD139*, Δ (*ara-leu*)7697, Δ *lacX74*, *galU*, *galK*, *hsdR*, *strA*, *srl*, *F*⁻). For measurements of promoter activity *in vivo*, promoter-*trpB*'A *lacZ* fusions were constructed using a bacteriophage λ vector in LL309 (*F'* *lacZ*^R ZAM15 Y⁺, *pro*⁺/ Δ (*pro-lac*), *thi*, *nalA*, *supE*, *bfe*^R) as described previously (4). *Fis*::*kan* derivatives of monolysogenic strains were constructed by P1 transduction from strain CSH50 *fis*::*kan* (=RLG855; Ref. 7).

β -Galactosidase activities were determined as in Miller (23). Cells were assayed after 4–5 generations of growth at 30°C in LB media. Monolysogeny was determined by measurements of six to twelve independently isolated lysogens for each promoter construct. *Fis*⁻ derivatives were grown at 27–28°C due to increased temperature sensitivity of the LL309 *fis*⁻ lysogens.

PCR and DNA Sequencing

The promoter insert in each monolysogen was amplified from the chromosome by PCR and sequenced to confirm the identity of the promoter inserts as follows: several colonies were removed from LB/XGal (40 μ g/ml) plates with a sterile toothpick and resuspended in 50 μ l distilled water in a 1.5ml Eppendorf tube. The mixture was boiled for 10 minutes, spun for 1 minute in a microcentrifuge, and stored at -20°C until used. 75 pmoles each of primer complementary to lambda DNA upstream of the EcoRI cloning site (primer sequence: 5'-TTATATTGGACTC-AAGAATG-3' at lambda coordinates 26170–26152) and to the *trpB*' sequence downstream of the HindIII site (primer sequence: 5'-AGTTTTTCAGCAGGTCGTTG-3') were added to 5 μ l of lysate, then 10 μ l of 10 $\times$ Replitherm (Epicentre Technologies) buffer, 5 μ l of 5mM (each) dNTPs, and 1 μ l Replitherm Thermostable DNA Polymerase (Epicentre Technologies, 5 units)

Figure 2. Promoter activities of wild-type and insertion constructs *in vivo*. β -galactosidase activities of promoter-*lacZ* fusions were measured in a *fis*⁺ strain. The percentage of wild-type (*rrnB* P1 sequences from -87 to +50, insert size = 0) promoter activity is plotted versus the size of the insertion in base pairs. For each experiment, β -galactosidase activities of the wild-type were averaged and designated as 100%, and β -galactosidase activities of insert constructs were determined as a percentage of the wild-type level. Results of at least 3 separate assays for each promoter are shown with standard errors. The activity of an *rrnB* P1 promoter with no *rrnB* sequences upstream of a BamHI linker at position -46 (B46) is 18–20% of wild type *rrnB* P1 containing *Fis* Site I in a *fis*⁺ strain. The activity of B46 was nearly twice that of another *rrnB* P1 promoter lacking sequences upstream of -46 (EcoRI linker at -46 = R46; Ref. 11) which may indicate partial activation from sequences in the BamHI linker.

in a final volume of 100 μ l. 50–100 μ l of mineral oil was added to cover the reaction. A Coy Tempcycler was used for the PCR reactions. PCR products were phenol extracted twice, ethanol precipitated with 2 μ g glycogen (Boehringer-Mannheim) as carrier, and purified away from the unincorporated nucleotides using a Centricon 30 column (Amicon). Sequencing of PCR products and supercoiled plasmid templates was performed with a Pharmacia T7 (dideoxy) Sequencing Kit and a 32 P 5'-end-labelled primer. Plasmids were sequenced using the New England Biolabs primer 1204; PCR products were sequenced using one of the primers used for amplification. In order to eliminate premature terminations due to structural abnormalities when sequencing the 12, 24 and 36 bp inserts, 10 units Terminal dNTP Transferase (US Biochemicals) and 1 mM each dNTP in Sequenase buffer (US Biochemicals) were added following the sequencing reactions and incubated at 37°C for 30 minutes (24). Sequences were electrophoresed on 7M Urea, 8% or 9.6% acrylamide gels.

In vitro Transcription assays

In vitro transcription reactions for testing Fis-dependent activation were performed in a high salt buffer (150 mM NaCl; Ref. 7). For measuring promoter activities in the absence of Fis, reactions were carried out in low-salt buffer (30 mM KCl; 8,25). Reactions (10 μ l) contained 0.2 nM RNAP and 0.2 nM supercoiled plasmid and were incubated for 10 minutes at room temperature (approx. 25°C) with 5 μ Ci each of 32 P UTP and CTP, 500 μ M ATP, 50

μ M GTP, and 10 μ M each of CTP and UTP. Transcription was stopped with the addition of 5 μ l 3 $\times$ transcription stop buffer (30% glycerol, 3 $\times$ Tris-Borate-EDTA buffer, 3% SDS, 30 mM NaEDTA (pH 8.0), 0.05% Xylene cyanol FF, 0.025% Bromophenol blue, 7M urea), and the entire sample was electrophoresed on a 6% acrylamide (30:1) 7M urea denaturing gel and dried on a Savant gel drier. Radioactive bands were quantitated with a radioanalytic imaging system (Ambis Systems, San Diego, CA) and/or by autoradiography and densitometry using a Hoefer GS300 densitometer and GS365 software. Exposures were analyzed with bands in the linear film response range.

Table I.

Promoter Construct ^a	Plasmid ^b Containing Strain	Fis ⁺ ^c Lambda Lysogen	Fis ⁻ ^d Lambda Lysogen
0 (wild type promoter)	RLG 1616	RLG 581	RLG 1643
5	RLG 1950	RLG 975	RLG 1631
8	RLG 1951	RLG 707	RLG 1633
9	RLG 1952	RLG 747	—
10	RLG 1953	RLG 969	—
11	RLG 1954	RLG 970	RLG 1635
12	RLG 1955	RLG 971	—
20	RLG 1956	RLG 976	—
21	RLG 1957	RLG 977	RLG 1637
22	RLG 1958	RLG 978	RLG 991
23	RLG 1959	RLG 979	—
24	RLG 1960	RLG 980	RLG 993
32	RLG 1961	RLG 981	—
33	RLG 1962	RLG 982	RLG 995
34	RLG 1963	RLG 983	—
35	RLG 1964	RLG 984	—
36	RLG 1965	RLG 985	RLG 997
274	RLG 1966	RLG 986	—
275	RLG 1967	RLG 987	—
276	RLG 1968	RLG 988	—
277	RLG 1969	RLG 989	—
278	RLG 1970	RLG 990	RLG 1645
282	RLG 1971	—	—
<i>rrnB</i> P1 -46 to +50 (B46)	RLG 1972	RLG 974	RLG 1641

(a) All constructs contain the *rrnB* P1 promoter (-87, +50 with respect to the transcription initiation site) with insertions of the indicated number of base pairs at position -46, except -46 to +50 construct (B46; Ref. 11)

(b) Plasmid vector= pRLG770 (7), host= CAG1574 (see Methods).

(c) host strain= LL309 (see Methods, Ref. 4).

(d) host strain= LL309 *fis::kan* (see Methods, Ref. 7).

Figure 3. Activation by Fis *in vitro*. a) Multiple round transcription reactions containing 0, 30, or 60 nM Fis were performed on supercoiled plasmid DNAs containing the *rrnB* P1 promoter with the indicated insertions at position -46 (see Methods and Ref. 7). The *rrnB* P1 transcript is approximately 220 bases long and terminates at an *rrnB* T1 terminator. The transcript labeled RNA I originates from the plasmid vector (26). b) Fold-activation by Fis is the ratio of transcription with Fis to that obtained without Fis and represents the mean of at least two experiments. For each experiment, the fold-activation obtained for the wild-type promoter was designated 100%, and no activation=0%. The fold-activation for each of the insertion constructs was plotted as a percentage of the wild type. Negative activation results from a slight inhibitory effect of Fis in the transcription assay on some constructs.

Footprinting

Hydroxyl radical and DNaseI footprinting with RNAP was performed as described in Newlands *et al.*, (11). Autoradiographs were quantitated using a Hoefer GS300 scanner and software. DNaseI footprinting with purified Fis protein was performed as in Ross *et al.*, (7) except that low salt buffer (8,25) was used.

RESULTS

I. Upstream activation *in vivo*

In order to elucidate the mechanism by which Fis activates transcription from *rrnB* P1, we tested the effects of insertions between the binding sites for RNAP and Fis. A series of BamHI and BglII/BamHI linkers were inserted at a naturally-occurring DraI restriction site at position -46 (Figure 1). This separated the RNAP binding site from Fis Site I, the site responsible for the majority of Fis-dependent activation of this promoter (7), and also interrupted the region responsible for factor-independent activation (FIA; 8,9). We first assayed the mutant promoter activities as *rrnB-lacZ* fusions *in vivo*.

Five or eight base pair insertions at -46 decreased promoter activity five-fold *in vivo* (Figure 2), to an activity comparable with that of a control promoter lacking *rrnB* sequences upstream of -46 (B46; Table I and Ref. 11). As the spacing between the RNAP and Fis sites was increased further (to 9–11 bp), promoter activity increased until it reached the wild-type level at 11 bp.

Figure 4. DNaseI footprints of Fis protein bound to wild-type, 5 and 11 base pair insert promoter fragments. Fis protein at the indicated concentration (nM) was preincubated with DNA fragments (end-labelled at the EcoRI site at -87 on the bottom strand) in a low salt transcription buffer (see Methods) for 10 minutes at 37°C and cleaved with DNaseI for 30 seconds. Products were run on a 12% acrylamide (30:1) 8M urea gel. '0' = wild-type promoter (insert size = 0); '5' = 5 bp insert construct; '11' = 11 bp insert construct. G+A = sequencing ladder prepared by the method of Maxam and Gilbert (27). Arrows at -78 and -66 indicate characteristic enhanced DNaseI cleavages within the Fis binding site (wild-type coordinates given).

With a 12 bp insertion, the promoter activity decreased again. This periodic behavior was also seen when two or three helical turns (20–24 bp, 32–36 bp) were introduced between Fis Site I and the RNAP binding site except that wild type activity was not fully restored even with optimal spacing. Maximum activity for each series was obtained when a multiple of 11 bp was inserted (11, 21–22, or 33 bp). Inserting 24–25 helical turns (274–278 bp) resulted in little or no activation.

Both Fis-dependent and Fis-independent components contribute to upstream activation *in vivo* (7). We therefore proceeded to determine the effects of UAR placement on each of these systems independently.

II. Fis mediated activation *in vitro*

The dependence of promoter activity *in vivo* on the size of the insertion at -46 suggested that the ability of Fis protein to activate transcription was being affected. We therefore assayed the extent of Fis-mediated activation *in vitro* with a subset of the insertion mutants. As found for activation *in vivo*, Fis-mediated activation *in vitro* was dependent on the size of the insert (Figures 3A, 3B). Insertions of 11 or 22 bp allowed activation by Fis comparable to that found with the wild type promoter. Some activation was also observed with the 21 and 33 bp insertion constructs. However, little or no activation by Fis was observed with insertions of non-integral numbers of turns (5, 8, 24, or 36 bp) or with large insertions (276 or 282 bp).

To determine whether the inability of Fis to activate the 5 bp insert construct was due to altered or reduced Fis binding to Fis Site I, we assayed Fis binding with DNaseI footprints of the wild-type, 5, and 11 bp insert promoters. No differences in Fis binding to Site I were observed (Figure 4). All constructs showed characteristic DNaseI enhancements within Fis Site I (positions -78 and -66 of the wild-type promoter). Therefore, the inability of Fis to activate the 5 bp insert promoter is unlikely to be the result of reduced or altered Fis binding to Fis Site I.

We note the presence of additional Fis-dependent enhancements in the -50 region of all three promoters at higher concentrations of Fis. However, we think it is unlikely that binding of Fis in the -50 region of the 5 bp insert construct is responsible for the inability of Fis to activate this promoter, since the wild-type

Figure 5. Promoter activities of wild-type and insert constructs in a *fis*⁻ strain. The percentage of wild-type (-87 to +50) promoter-*lacZ* activity is plotted versus size of insert in base pairs. β -galactosidase activities were calculated as for Figure 2.

and 11 bp insert promoters are still activated at these higher Fis concentrations, and the 5 bp insertion construct is not activated even at lower concentrations of Fis where binding occurs only to Fis Site I and not to the -50 region (data not shown). At these same low Fis concentrations, Fis-dependent activation does occur with the wild type promoter (data not shown).

III. Factor-independent activation *in vitro* and *in vivo*

We have found that the -40 to -60 region of *rrnB* P1 increases the rate of transcription in the absence of auxiliary proteins *in vitro* and in the absence of the *fis* gene *in vivo* (7–9). (For simplicity, we refer to upstream activation of *rrnB* P1 in *fis*[−] strains as factor-independent activation, FIA, although we cannot exclude the possibility of other unknown factors in the cell contributing to the upstream activation phenomenon *in vivo*). We evaluated the effect of the insertions at position -46 on FIA by measuring the transcription activities of a subset of the insertion mutants in a strain lacking a functional *fis* gene and by comparing transcription activities of these constructs *in vitro* in the absence

of Fis. We found that an insertion of 11 bp at -46 did not impair transcription *in vivo* or *in vitro* in the absence of Fis (Figures 5 and 6). Insertions of 2 or 3 turns, however, gave promoter activities in *fis*[−] strains similar to those obtained for a construct lacking *rrnB* sequences upstream of -46 (Figure 5), and the stimulatory effect of the -46 to -87 component of the UAR on transcription of these constructs *in vitro* without Fis was greatly reduced (Figures 6A, 6B).

The insertions displace only the portion of the FIA region upstream of -46 . Even those promoters with insertions of non-integral numbers of base pairs, as well as B46, have higher activities than *rrnB* P1 promoters which contain sequences only to -40 (9). Therefore, our data show that the FIA region can be interrupted and the distal section displaced upstream, but only by one helical turn, without disturbing its ability to stimulate transcription.

IV. RNA polymerase contacts to the UAR can be displaced upstream

RNAP binding results in altered contacts with the -53 region of *rrnB* P1 when compared to promoters with non-*rrnB* sequences

Figure 6. Promoter activities in the absence of Fis *in vitro*. a) Multiple round *in vitro* transcription reactions were carried out without Fis, using supercoiled templates containing the indicated insertions at position -46 . Duplicate samples are shown for each promoter. See also legend to Figure 3 and Methods. b) Promoter activities from *in vitro* transcription reactions such as pictured in Figure 6A are presented as a percentage of the wild type promoter activity. A promoter lacking sequences upstream of -46 (B46) had about 25% activity.

Figure 7. Hydroxyl radical footprints of RNAP on wild-type, 5, 11, 22 and 33 base pair insert promoter constructs. a) Footprinting was performed as described in Newlands *et al.* (11). Bottom strand (labelled at EcoRI site at -87) is shown. Size of insert at position -46 (0 = wild type, 5, 11, 22, or 33 bp) is indicated. Arrows point to position -53 in wild-type *rrnB* P1, displaced to -58 , -64 , -75 , and -86 in the respective insert constructs. Solid line indicates position of BamHI linker insert DNA. G + A = sequencing ladder prepared by the method of Maxam and Gilbert (27). Uncut = untreated DNA fragment, shown to depict background. b) Scans of autoradiograms in Fig 7a. Scans were prepared as described in Newlands *et al.*, (11). Heavier line = hydroxyl radical cleavage in the presence of bound RNAP; lighter line = hydroxyl radical cleavage in the absence of bound RNAP. Arrows point to base at -53 in wild-type *rrnB* P1, displaced to -58 , -64 , -75 , and -86 in the respective insert constructs. Solid line indicates inserted DNA.

substituted for the UAR (11). We performed footprints on the promoter insertion mutants to test whether the upstream contacts would be maintained in promoters displaying FIA, and if so, whether the contacts would be displaced upstream or be located within the linker sequences. The hydroxyl radical cleavage patterns of the wild type, 5, 11, 22, and 33 bp insert promoters with and without RNAP are shown in Figure 7A. Densitometric scans of these footprints are shown in Figure 7B. In all cases, RNA polymerase protections between -40 and $+20$ are essentially identical. Surprisingly, the interactions between RNAP and the -53 region (arrows in Figure 7A, 7B) are observed not only in the wild type, but also are maintained in the 11 bp insertion mutant, where the -53 sequence is now at position -64 with respect to the transcription initiation site, and to a lesser extent with the 22 bp insertion construct, where protection is observed at -75 . RNAP is unable to protect the displaced -53 region in the 5 bp insertion construct, nor is protection of this sequence observed at position -86 in the 33 bp insert construct. Thus, there is a correlation between factor-independent activation of the wild type and 11 bp insertion constructs and protection of the -53 region, whether it is at its normal position or displaced by one helical turn. In addition, in both the 22 and 33 bp constructs, there is partial protection by RNAP within the BamHI linker DNA, and in the 5 bp construct, there is partial protection just upstream of the linker.

We also footprinted the *rrnB* P1-RNAP complexes with DNaseI (Figure 8). The -40 to $+20$ region is essentially the same in all the footprints, except for position -37 . Cleavage by DNaseI at this position is enhanced in the wild type, the 11 and 22 bp insert promoter constructs (asterisks in Figure 8), and

in a construct lacking *rrnB* sequences upstream of -46 (B46; Ref. 11), but not in the 5 and 33 bp insertion constructs. DNaseI hypersensitivity occurs also at many positions in the linker DNA in the 11 and 22 bp constructs, generally on each side of positions protected from hydroxyl radical or DNaseI cleavage. The 5 and 33 bp insert promoters showed only one slightly enhanced cleavage in the intervening linker DNA, at approximately -48 . RNAP partially protects the 33 bp construct from DNaseI cleavage within the linker DNA at -53 , -62 and -65 from the transcription initiation site.

Complete protection by bound RNAP is observed at -50 in the wild type and is maintained in the 11 bp insert construct, where the T-A base pair at -50 has been displaced to position -61 (arrows in Figure 8). Less complete protection of this position (displaced to -55) is observed in the 5 bp insert construct. There is a correlation between factor-independent activation of the wild type and 11 bp insertion constructs and full protection of the -50 region. Thus, strong protection of the -53 region in hydroxyl radical footprints and of position -50 in DNaseI footprints coincides with FIA.

DISCUSSION

Fis-mediated upstream activation

A number of reports suggest that protein-RNA polymerase interactions play a role in prokaryotic transcriptional activation (28–37). Phasing experiments similar to those reported here indicate that the relative positions of the binding sites for RNAP and the activator protein are crucial for activation and are consistent with the presence of protein-protein interactions (14–21). Our results indicate that activation by Fis also depends on the relative orientation of its binding site and that for RNAP. Thus, we suggest that Fis-dependent activation of the *rrnB* P1 promoter is mediated through direct contacts with RNAP. At the least, the restoration of upstream activation with multiples of 11 bp insertions implies that Fis-mediated activation can occur if the rotational orientation of Fis and RNAP relative to each other is maintained. This conclusion is in agreement with that drawn from the reduced activity of an *rrnB* P1 promoter containing a 3 bp deletion at positions -38 to -40 reported previously (5,6). As observed with some other activators, the degree of activation of *rrnB* P1 by Fis decreases with each successive turn (16,18–20). The *rrnB* P1 system may differ from some other systems in that the insertion of one turn results in *full* restoration of wild-type activation.

Several additional lines of evidence are consistent with a role for Fis-RNAP interactions in the upstream activation mechanism. First, footprinting studies from our laboratory indicate that Fis binds to the same face of the helix as RNAP, assuming a 10–11 bp helical repeat (Ross and Gourse, unpublished data). Second, mutant Fis proteins have been identified which are able to bind and bend the *rrnB* UAR, but are unable to activate *rrnB* P1 transcription (38). These mutants are functionally analogous to the positive control mutants described in other systems (28,30,36). Third, transcription of restriction fragments containing *rrnB* P1 but ending at position -87 with respect to the transcription start site (i.e. containing little or no DNA upstream of Fis Site I) is fully activated by Fis *in vitro* (38). This result indicates that the role of Fis is not merely to bring DNA from further upstream into the proximity of the promoter. Fourth, the presence of RNAP in footprinting reactions influences Fis binding to the UAR (Ross and Gourse, unpublished).

Figure 8. DNaseI footprints of RNAP on wild-type, 5, 11, 22 and 33 base pair insertion constructs. Footprints were performed as described in Newlands *et al.*, (11). Bottom strand (labelled at EcoRI site at -87) is pictured. Duplicate samples are shown. Size of insert at position -46 (0 = wild type, 5, 11, 22, or 33 bp) is indicated above each panel. Arrows point to position at -53 in wild-type *rrnB* P1, displaced to -58 , -64 , -75 , and -86 in the respective insert constructs. Solid line indicates position of BamHI linker insert DNA. G + A = sequencing ladder prepared by the method of Maxam and Gilbert (27). Asterisks mark position -37 [mislabeled $-39/40$ in ref. 25], a hypersensitive site in the presence of RNAP in the wild type, 11, and 22 bp constructs.

Factor-independent activation

The -40 to -60 sequences of *rrnB* P1 constitute an 'extended promoter region,' responsible for at least a 20-fold increase in promoter strength (4,7-9). Although we cannot exclude the possibility of other unknown factors in the cell contributing to the upstream activation phenomenon *in vivo*, we refer to upstream activation of *rrnB* P1 in *fis*⁻ strains as factor-independent activation, since activation by the -40 to -60 region is also observed in *in vitro* transcription reactions containing only RNAP (9). Both the -41 to -50 and the -51 to -60 regions are responsible for part of this promoter activation (9). Our insertions displaced the distal section of the extended promoter region relative to the -10 and -35 hexamers.

We saw a position dependence of FIA with an 11 bp periodicity. Factor-independent activation was fully restored with the 11 bp insertion at -46, indicating that a discontinuity in the extended promoter region does not eliminate FIA. However, the insertion of two or three helical turns resulted in reduced promoter activity relative to the wild type or 11 bp insertion promoters, implying that activation was impaired when the distal FIA section was moved too far from the RNAP binding site.

We have shown previously that RNAP protects DNA backbone positions well upstream of the -35 recognition hexamer. Displacing this upstream region results in maintenance of the interaction with RNAP, as shown by protection of the -64 region of the 11 bp insert construct in hydroxyl radical footprints. However, RNAP bound to the 5 bp insert construct is unable to contact this region effectively, presumably because it is on the opposite face of the helix. It is possible that the *rrnB* P1 UAR DNA backbone contains a particular structure, determined by the base sequence, with which RNAP interacts. Alternatively, RNAP could make base-specific contacts in this region.

The FIA region increases the equilibrium constant, K_B , for formation of the closed complex, RP_C (8). We have suggested that this activation may result, at least in part, from interaction of RNAP with the FIA sequences, conceivably with the -53 region, assuming that the -53 interaction visualized in the footprint of the final complex, (RP_{init}), is present in RP_C (11). This hypothesis is supported by the correlation observed here between factor-independent activation and protection from hydroxyl radical attack of the -64 region of the 11 bp insertion construct.

We emphasize that the activity of the wild-type and mutant *rrnB* P1 promoters *in vivo* (Figure 2) is determined by the combination of the effects of the Fis and factor-independent activation systems, and the two systems may not respond in the same fashion to a particular insertion. For example, the 22 bp insert construct is fully activated by Fis *in vitro*, but is two fold less active than the wild type promoter in a Fis⁺ strain. This implies that the decreased promoter activity of the 22 bp insert construct in a Fis⁺ strain is the result of decreased FIA. On the other hand, the 24 bp insert promoter is deficient in both FIA and Fis-dependent activation. These data indicate that full upstream activation requires effective factor-independent activation plus the correct alignment of Fis relative to RNAP.

The two components of the upstream activation system (Fis and the extended promoter region) could function independently, or alternatively activation by Fis could require the contacts afforded by the -40 to -60 region. In fact, it is conceivable that Fis could facilitate transcription at *rrnB* P1 by stabilizing RNAP interactions with the extended promoter region. Recent experiments (9) show that Fis activates transcription with reduced

efficiency when FIA is abolished, implying that the extended promoter region is important for both factor-independent and Fis-dependent activation.

Some of the conclusions reported here have been reached independently by M. Zacharias, H.U. Goring, and R. Wagner (personal communication). It has also been shown recently that upstream activation of the *E. coli thrU(tufB)* operon is face of the helix dependent (39).

ACKNOWLEDGEMENTS

The authors wish to thank Reid Johnson for purified Fis, and Dayle Hager and Richard Burgess for purified RNA polymerase. We also thank Joan Meccas, Sigrid Leirno, Tamas Gaal, and other members of our lab for helpful discussions, and Rolf Wagner for communicating unpublished results. We thank Hossain Alavi, Lonny Palubicki and Jeremy Kohler for help in the initial stages of this work. This work was supported by NIH grants R01 GM37048 and RCDA AI90035 to R.L.G. J.T.N. thanks the University of Georgia for support during the initial stages of this work.

REFERENCES

1. Lund, E. and Dahlberg, J.E. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 5480-5484.
2. deBoer, H.A. and Nomura, M. (1979) *J. Biol. Chem.* **254**, 5609-5612.
3. Bremer, H. and Dennis, P.P. (1987) in Neidhardt, F.C. (ed), *Escherichia coli and Salmonella typhimurium, Cellular and Molecular Biology*. American Society for Microbiology, Washington, D.C. pp. 1527-1542.
4. Gourse, R.L., deBoer, H.A. and Nomura, M. (1986) *Cell* **44**, 197-205.
5. Gaal, T., Barkei, J., Dickson, R.R., deBoer, H.A., deHaseth, P.L., Alavi, H. and Gourse, R.L. (1989) *J. Bacteriol.* **171**, 4852-4861.
6. Josaitis, C.A., Gaal, T., Ross, W. and Gourse, R.L. (1990) *Biochim. Biophys. Acta* **1050**, 307-311.
7. Ross, W., Thompson, J.F., Newlands, J.T. and Gourse, R.L. (1990) *EMBO J.* **9**, 3733-3742.
8. Leirno, S. and Gourse, R.L. (1991) *J. Mol. Biol.* **220**, 555-568.
9. Rao, L., Ross, W., Leirno, S. and Gourse, R.L., in preparation.
10. Nilsson, L., Vanet, A., Vijgenboom, E. and Bosch, L. (1990) *EMBO J.* **9**, 727-734.
11. Newlands, J.T., Ross, W., Gosink, K.K. and Gourse, R.L. (1991) *J. Mol. Biol.* **220**, 569-583.
12. Ptashne, M. (1988) *Nature* **335**, 683-689.
13. Gralla, J.D. (1989) *Cell* **57**, 193-195.
14. Dunn, T.M., Hahn, S., Ogden, S. and Schleif, R.F. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 5017-5020.
15. Mandecki, W. and Caruthers, M.H. (1984) *Gene* **31**, 263-267.
16. Maeda, S., Ozawa, Y., Mizuno, T. and Mizushima, S. (1988) *J. Mol. Biol.* **202**, 433-441.
17. Lee, D.H. and Schleif, R.F. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 476-480.
18. Minchin, S.D., Austin, S. and Dixon, R.A. (1989) *EMBO J.* **8**, 3491-3499.
19. Gaston, K., Bell, A., Kolb, A., Buc, H. and Busby, S. (1990) *Cell* **62**, 733-743.
20. Ushida, C. and Aiba, H. (1990) *Nucl. Acids Res.* **18**, 6325-6330.
21. Serrano, M., Barthelemy, I. and Salas, M. (1991) *J. Mol. Biol.* **219**, 403-414.
22. Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
23. Miller, J.H. (1972) *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
24. Fawcett, T.W. and Bartlett, S.G. (1991) *United States Biochemical Editorial Comments* **17**, 19-20.
25. Gourse, R.L. (1988) *Nucl. Acids Res.* **16**, 9789-9809.
26. Morita, M. and Oka, A. (1979) *Eur. J. Biochem.* **97**, 435-443.
27. Maxam, A.M. and Gilbert, W. (1980) *Methods Enzymol.* **65**, 499-560.
28. Hochschild, A., Irwin, N. and Ptashne, M. (1983) *Cell* **32**, 319-325.
29. Hawley, D.K. and McClure, W.R. (1983) *Cell* **32**, 327-333.
30. Irwin, N. and Ptashne, M. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 8315-8319.
31. Ren, Y.L., Garg, S., Adhya, S. and Krakow, J.S. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 4138-4142.

32. Li, X.M. and Krakow, J.S. (1988) *J. Biol. Chem.* **263**, 3448–3453.
33. Wedel, A., Weiss, D.S., Popham, D., Droge, P. and Kustu, S. (1990) *Science* **248**, 486–490.
34. Su, W., Porter, S., Kustu, S. and Echols, H. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 5504–5508.
35. Bell, A., Gaston, K., Williams, R., Chapman, K., Kolb, A., Buc, H., Minchin, S., Williams, J. and Busby, S. (1990) *Nucl. Acids Res.* **18**, 7243–7250.
36. Eschenlauer, A.C. and Reznikoff, W.S. (1991) *J. Bacteriol.* **173**, 5024–5029.
37. Pinkney, M. and Hoggett, J.G. (1988) *Biochem. J.* **250**, 897–902.
38. Gosink, K.K., Leirno, S., Ross, W., Finkel, S., Osuna, R., Johnson, R.C., and Gourse, R.L. in preparation.
39. Verbeek, H., Nilsson, L., and Bosch, L. (1991) *Biochimie* **73**, 713–718.