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Analysis of the Fis-Dependent and Fis-Independent Transcription Activation Mechanisms of the *Escherichia coli* Ribosomal RNA P1 Promoter

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ABSTRACT: The role of the curved DNA sequence upstream to the *Escherichia coli* ribosomal RNA P1 promoter in transcription activation was studied. This sequence region had been shown to activate transcription from P1 in vivo and in vitro and to harbor binding sites for the trans-activating protein Fis. We have constructed a series of linker scanning mutants spanning the region −104 to −47, relative to the transcription start site. DNA fragments carrying the mutations show altered gel electrophoretic mobilities, consistent with reduced DNA bending angles compared to the wild-type sequence. Using gel retardation assays, qualitative as well as quantitative differences in the binding of the trans-activating protein Fis to the mutant DNA fragments could be observed. The effects of the mutations on *rrnB* P1 promoter activation were studied in vivo in *fis*$^+$ and *fis*$^-$ backgrounds. A reduction in the promoter strength for some of the linker mutants correlates with altered Fis binding to two of the known Fis binding sites. Shifting the Fis binding region by half a helical turn, relative to the promoter core sequence, abolishes Fis-mediated activation almost totally, whereas activation is partly restored by a shift of a complete helical turn. For one mutant, which does not show alterations in Fis binding, a decrease in the promoter strength was observed in a *fis*$^-$ strain. From the results, we conclude that two upstream activating mechanisms, one Fis-dependent and one Fis-independent, influence the *rrnB* P1 promoter strength. Sequence determinants for the Fis-independent mechanism are closer to the promoter core region than the Fis binding sites. In addition, the study demonstrates that both the helical geometry and the absolute distance of the UAS region relative to the promoter are crucial for transcription activation.

In bacteria, transcription initiation is known to start from consensus DNA promoter structures (McClure, 1985). In addition to the conserved −35 and −10 core promoter elements, sequences upstream and downstream from the consensus region determine the efficiency of the initiation process (Deuschle et al., 1986). The strong expression of many stable RNA genes (ribosomal RNA and tRNA genes) is known to be dependent on the presence of AT-rich upstream sequences, located between nucleotides −40 and −150, relative to the transcription start site (UAS regions) (Bossi & Smith, 1984; Lamond & Travers, 1983; Bauer et al., 1988; Plaskon & Wartell, 1987). In the case of the *rrnB* ribosomal RNA promoter P1, a sequence region between nucleotides −50 and −88 was identified which stimulates the transcription about 20-fold (Gourse et al., 1986). Although the upstream promoter sequences of stable RNA genes do not show significant primary sequence homology, they share a common high AT content and a peculiar physical property: DNA fragments containing UAS sequences are generally associated with a stable DNA curvature. These DNA conformational distortions can be identified by an altered electrophoretic mobility of the corresponding DNA fragments. Replacing the natural UAS region of the *rrnA* P1 promoter by a curved DNA fragment from the unrelated organism *Crithidia fasciculata*, as shown recently, restores the original activity to 70% (Nachaliel et al., 1989).

Today there are no experimentally justified mechanistic explanations of how the DNA conformation participates in promoter activation, and there is growing evidence that DNA-binding proteins are involved in the activation mechanism.

The *Escherichia coli* Fis protein, originally identified to stimulate phage Mu *gin* and Salmonella *hin* site-specific DNA inversion (Kahmann et al., 1985; Johnson & Simon, 1985), was shown to bind upstream of *rrnB* P1, *tufB*, and *tyrT* promoters. Addition of Fis can activate transcription from these promoters in vitro (Nilsson et al., 1990; Ross et al., 1990). Gel retardation can resolve at least three different Fis–UAS complexes. In the case of the *rrnB* P1, the corresponding three Fis binding sites were characterized by DNase I footprinting experiments. Although in vitro effects of Fis on the transcription from *rrnB* P1 and *tufB* promoters are dramatic under certain conditions, the promoter strength of the above pro-

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moters in *fis*⁻ strains did not differ strongly from those in *fis*⁺ strains (Nilsson et al., 1990; Ross et al., 1990). It is not clear if the lack of Fis is compensated either by relaxation of the proposed ribosome feedback control or by an additional Fis-independent activation mechanism. Upstream promoter activation by two different mechanisms was also proposed by Hsu et al. (1991). Their studies on the *argT* promoter indicated that activation is mediated by two different upstream elements influencing different steps of the transcription initiation process.

We have undertaken a mutational study of the *rrnB* P1 upstream activating sequence by constructing a nested set of base changes. In addition, we have inserted sequences of variable length between the promoter core region and the known Fis binding sites, and we have thereby shifted the Fis binding sites further upstream. Using these constructs, we have attempted to correlate the different promoter activities with the extent and location of the DNA curvature and binding of Fis. It turned out that mutations affecting Fis binding sites, as well as sequence variants with no effect on Fis binding, can reduce promoter activity in vivo. Apparently, in *fis*⁻ background, compensatory mechanisms seem to operate which may restore the promoter activity of the mutants to that of the wild type for most, but not all, of the mutations.

The results are consistent with the assumption of the existence of two nonadditive activation mechanisms, which under certain conditions are partially compensating. One of the mechanisms is Fis-dependent, while a second one, Fis-independent, is based on sequence elements close to the promoter core region and seems to influence the *rrnB* P1 activity in vivo.

The systematic shift of the Fis binding regions relative to the *rrnB* P1 promoter core region by one helical turn can restore original activity to some degree whereas displacement of half a helical turn or two turns nearly abolishes activation. Therefore, Fis-mediated activation seems to depend on a precise helical orientation of the curved or bent DNA.

Materials and Methods

Strains and Media. *E. coli* strains CSH50, Δ (*lac pro*) *strA thi* (Miller, 1972) and CSH50*fis*::*kan* (Koch et al., 1988) were used for all transformations and subsequent promoter activity determinations. Cells were grown in standard YT medium (Maniatis et al., 1982) at 37 °C.

Assay for Chloramphenicol Acetyltransferase (CAT). Cells were lysed as described recently (Zacharias & Wagner, 1989) and the CAT activity was determined as the rate of chloramphenicol acetylation according to published procedures (Gorman et al., 1982) using [^{14}C]chloramphenicol. Acetylated reaction products were separated from nonreacted material by thin-layer chromatography. The synthesis rates were determined as nanomoles of acetylated chloramphenicol synthesized per minute and were normalized to a cell density equivalent 1 OD_{600}.

β-Lactamase (BLA) Activity Measurements. The activity of β-lactamase was measured according to a standard procedure (Lupski et al., 1984). 1 BLA unit is defined as the decrease in optical density at 255 nm per minute of a 0.1 mM cephalosporin solution. Data were finally normalized to a cell density equivalent 1 OD_{600}.

Construction of Linker Scanning Mutations. The plasmids pKL1, pKL2, pKL3, pKL4, pKL5, pKL7, and pKL12 are derivatives of the promoter test vector pKK232-8 (Brosius, 1984). The plasmid pKL1 contains the *rrnB* P1 promoter as a *Dde*I insert (2184 bp) ligated to the *Sma*I site of pKK232-8. The other plasmids differ from pKL1 in containing clustered point mutations in the UAS region of the P1 promoter. The different mutations were generated by introducing deletions to the UAS region of the *rrnB* P1 promoter on plasmid pKK3535 (Brosius et al. 1981). Deletions were created by exonuclease BAL31, starting either downstream or upstream from the UAS element. Short pieces of linker DNA containing the recognition sequence for the restriction enzyme *Hin*dIII were subsequently ligated to the endpoints of the shortened DNA fragments. The endpoints were verified by DNA sequencing after the deletion derivatives were cloned into phage M13mp18/19. A cloned upstream and a downstream deletion derivative were digested with *Hin*dIII and a second enzyme cutting upstream (downstream) of the UAS region. The two DNA fragments were ligated at the *Hin*dIII site to form a UAS derivative with alterations at the fusion site due to the linker DNA. Only those fragments were combined, which resulted in a UAS derivative with the same length as that of the original UAS element or very small deletions of one or two base pairs. Finally, the different modified UAS regions were cloned together with the *rrnB* P1 promoter in front of the CAT reporter gene on plasmid pKK232-8 (see above). The DNA sequence of the linker mutants compared to the wild type UAS is shown in Figure 1. Plasmid pKL-DHP1 contains the *rrnB* P1 promoter where the UAS region was deleted upstream of position −47 (*Dra*I/*Hin*dIII fragment from pKL7, cloned in the *Hin*dIII/*Sma*I opened pKK232-8). It contains the same promoter downstream region as pKL1 and the linker derivatives. pKL-DP1 is a pKK232-8 derivative with the UAS region but is missing the *rrnB* P1 promoter downstream from position −70 (114-bp *Hin*dIII deletion from pKL7).

Construction of Clones with Altered Spacing between Promoter Core Region and Upstream Fis Binding Sites. Mutants with altered spacing between promoter core region and the upstream Fis binding site are derivatives of construct pKL12.

A fill-in reaction of the single *Nhe*I cutting site in pKL12 using T4 polymerase and subsequent religation (Maniatis et al., 1982) yielded plasmid pKL12+4 with a 4-bp insertion. Plasmids pKL12+10 and pKL12+22 were constructed by insertion of oligonucleotides of either 10 or 22 bases into the *Nhe*I site of plasmid pKL12.

The two oligonucleotides had the following DNA sequence: CTAGAGATCT (pKL12+10) and CTAGATCTCTAGATCTAGAGAT (pKL12+22).

Plasmid pKL12+22 contained two *Bgl*II restriction sites in a distance of 16 base pairs generated by the oligonucleotide insertion. After digestion of the plasmid with *Bgl*II the 16-bp fragment was separated from the vector fragment using agarose electrophoresis. The vector fragment was religated resulting in plasmid pKL+6 with a 6-bp insertion between the Fis binding region and the promoter core sequence.

DNA Fragments. DNA fragments containing wild-type or linker mutant UAS were prepared from pKK232-8 plasmid derivatives containing the corresponding UAS linker mutations (see above). After digestion of the plasmids with *Bam*HI and *Ssp*I, the UAS-containing fragments were isolated by gel electrophoresis and overhanging 5′ ends were filled in using T4 DNA polymerase, 100 μM each dCTP, dGTP, and dTTP, and 10 μCi of [^{32}P]dATP (Maniatis et al., 1982).

Fis–DNA Binding. Reaction mixtures with a total volume of 10 μL containing 10 mM Tris-HCl, pH 8.0, 50 mM NaCl, 5 mM $MgCl_2$, 20 μg/mL BSA, and 1–4 nM labeled DNA fragment were incubated with the Fis concentrations indicated (0–200 nM). Purified Fis protein was kindly provided by C. Koch and R. Kahmann. After 15 min at room temperature, 3 μL of loading buffer (50% glycerin, 0.2% bromophenol blue)

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                    -110          -90          -70          -50          -35
pKL1 (wild type)..... TTCGAAACGCTCGAAAAACTGGCAGTTTTAGGCTGATTTGGTTGAATGTTGCGCGGTCAGAAAATTATTTTAAATTTCCTCTTGTCA
                          II                        I
pKL2..... TTCGAAACGCTCGCAAGCTTGGCAGTTTTAGGCTGATTTGGTTGAATGTTGCGCGGTCAGAAAATTATTTTAAATTTCCTCTTGTCA
pKL3..... TTCGAAACGCTCGAAAAACTCAAGCTTGGGCTGATTTGGTTGAATGTTGCGCGGTCAGAAAATTATTTTAAATTTCCTCTTGTCA
pKL4..... TTCGAAACGCTCGAAAAACTGGCAGTTTTACAAGCTTGGGTTGAATGTTGCGCGGTCAGAAAATTATTTTAAATTTCCTCTTGTCA
pKL5..... TTCGAAACGCTCGAAAAACTGGCAGTTTTAGGCCAAGCTTGGAATGTTGCGCGGTCAGAAAATTATTTTAAATTTCCTCTTGTCA
pKL7..... TTCGAAACGCTCGAAAAACTGGCAGTTTTAGGCTGATTTGGTTCAAGCTTGGCGGTCAGAAAATTATTTTAAATTTCCTCTTGTCA
pKL12..... TTCGAAACGCTCGAAAAACTGGCAGTTTTAGGCTGATTTGGTTGAATGTTGCGCGGTCAAGCTAGCTTGGGAAATTTCCTCTTGTCA
pKL12+4.....TTCGAAACGCTCGAAAAACTGGCAGTTTTAGGCTGATTTGGTTGAATGTTGCGCGGTCAAGCTAGCTAGCTTGGGAAATTTCCTCTTGTCA
pKL12+6.....TTCGAAACGCTCGAAAAACTGGCAGTTTTAGGCTGATTTGGTTGAATGTTGCGCGGTCAAGCTAGATCTAGCTTGGGAAATTTCCTCTTGTCA
pKL12+10.....TTCGAAACGCTCGAAAAACTGGCAGTTTTAGGCTGATTTGGTTGAATGTTGCGCGGTCAAGCTAGAGATCTCTAGCTTGGGAAATTTCCTCTTGTCA
pKL12+22 AACGCTCGAAAAACTGGCAGTTTTAGGCTGATTTGGTTGAATGTTGCGCGGTCAAGCTAGATCTCTAGATCTAGAGATCTAGCTTGGGAAATTTCCTCTTGTCA
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FIGURE 1: DNA sequence of the linker and oligonucleotide insertion constructs. In the top line, the wild-type sequence (positions −117 to −31, relative to the transcription start) is given. The names of the linker and insertion constructs are shown on the left side. The −35 region is indicated by a line above and in bold type. Underlined are the Fis binding regions I and II, according to Ross et al. (1990). Restriction sites introduced by the linkers and inserted sequences are boxed.

was added and the samples were loaded on a 5% polyacrylamide gel (29:1 acrylamide:bisacrylamide). The gel electrophoresis was run in 50 mM Tris–borate, pH 7.5, 1 mM EDTA (Maniatis et al., 1982) for 1.5 h at 10 V/cm, dried, and autoradiographed. Band intensities were quantified by densitometric scanning of unsaturated films.

Results

We took advantage of the linker scanning technique (Haltiner et al., 1985) to construct a nested set of base changes in the upstream region of the ribosomal RNA P1 promoter. The base changes caused by *Hin*dIII linkers are located between nucleotide positions −47 and −104, relative to the transcription start site, resulting in six linker scanning mutations. In some of the constructs, one or two nucleotide deletions were introduced in addition to the base changes. The constructs were fused to the chloramphenicol acetyltransferase (CAT) reporter gene and termed pKL2, pKL3, pKL4, pKL5, pKL7, and pKL12, respectively. Plasmid pKL12 was used to construct pKL12+4, pKL12+6, pKL12+10, pKL12+22, with additional 4, 6, 10, or 22 base pairs inserted between the Fis binding site I and the promoter core region (see Materials and Methods for details). The exact sequence changes were verified in each case by dideoxy sequencing and are shown in Figure 1.

Electrophoretic Mobility of DNA Fragments Containing the UAS Derivatives. DNA sequences upstream of several *E. coli* stable RNA promoters contain patterns known to have a curved helical structure (Lamond & Travers, 1983; Bossi & Smith, 1984; van Delft et al., 1987; Bauer et al., 1988). For example, DNA fragments with the UAS regions of the *tyrT*, *leuV*, *hisR*, and *rrnB* P1 promoters exhibit a reduced electrophoretic mobility compared to uncurved DNA of similar length.

Using the different UAS mutations, the influence of the base changes on the abnormal electrophoretic behavior of DNA fragments of 250–252 bp length, depending on the presence of the small deletions of some of the linker mutations, was investigated. The fragments contain the corresponding UAS region and the *E. coli rrnB* P1 promoter in an approximately central position. Figure 2 shows the result of a gel electrophoretic separation on 8% polyacrylamide for the wild-type fragment (pKL1) and the different linker mutations (pKL2, pKL3, pKL4, pKL5, pKL7, and pKL12). While the fragments containing the wild-type UAS sequence and the linker mutation pKL12 show about the same degree of retardation, a gradual increase in mobility of fragments where the linker

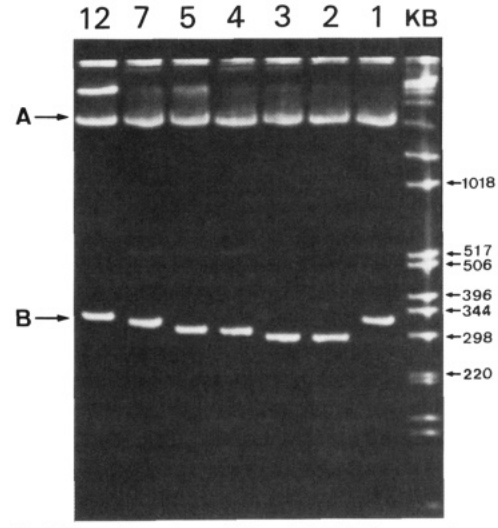

FIGURE 2: Electrophoretic mobility of DNA fragments containing linker mutations. *Bam*HI/*Ssp*I digests of the different linker construct containing plasmids were separated on an 8% polyacrylamide gel and stained with ethidium bromide. The clone number is indicated at the top. In KB, a DNA marker digest is separated. The arrow A shows the remaining vector fragments, and B points to the fragments containing the linker mutations.

Table I: Electrophoretic Anomaly of Mutant Activator Regions

mutant	pKL1	pKL2	pKL3	pKL4	pKL5	pKL7	pKL12
fragment length (bp)	252	252	250	251	250	251	252
k^a	1.42	1.18	1.17	1.24	1.23	1.33	1.40

mutant	pKL12+4	pKL12+6	pKL12+10	pKL12+22
fragment length (bp)	256	258	262	274
k^a	1.55	1.53	1.41	1.40

[a] *k*-factor: apparent length (in base pairs) relative to fragment length (in base pairs). *k*-factors were determined according to the gel electrophoretic mobility in 8% polyacrylamide.

insertion lies further upstream (mutants pKL2 to pKL7) could be observed. For a better comparison, the mobility differences have been expressed in terms of *k*-values (apparent size to actual size of the DNA fragments) and are summarized in Table I. It can be seen that the reduction in gel electrophoretic mobility which is known for the DNA fragment with the wild-type UAS sequence is diminished in all the linker mutants (there is a general decrease in *k*-values). This indicates a reduced bending angle of the mutated fragments. The degree of this reduction varies with the position of the linker mutation, and it seems to be more pronounced for

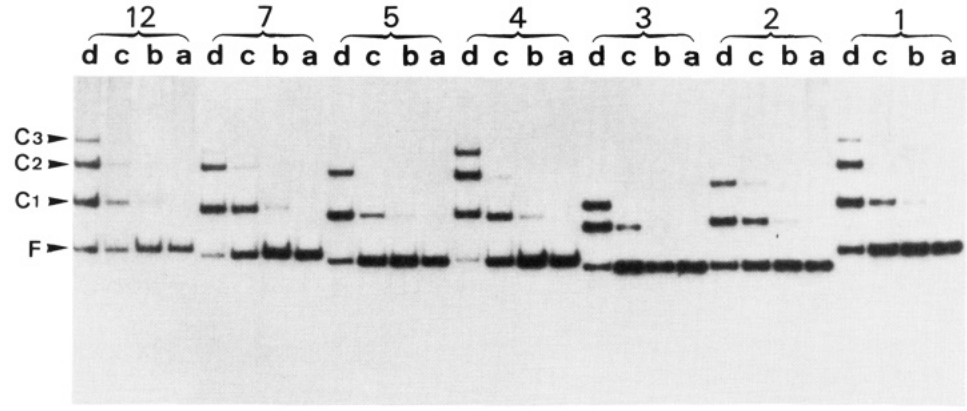

FIGURE 3: Fis binding to DNA fragments containing the mutant UAS regions of *rrnB* P1. *Bam*HI/*Ssp*I UAS fragments (4 nM) of the wild-type pKL1 (1) and linker mutants pKL2, pKL3, pKL4, pKL5, pKL7, and pKL12 (2, 3, 4, 5, 7, and 12, respectively) were filled in and labeled with ^{32}P. They were incubated with various amounts of purified Fis protein: (a) 0 nM; (b) 0.2 nM; (c) 2 nM; (d) 20 nM and separated by gel electrophoresis. Electrophoresis was performed in 5% polyacrylamide (29:1) for 1.5 h at 10 V/cm. An autoradiogram of a dried gel is shown. Three different Fis–DNA complexes (C1, C2, C3) are indicated. The position of the free DNA fragment is indicated by F.

mutations further upstream. One has to conclude, therefore, that mutations more distant to the transcription start site result in a weaker degree of overall bending compared to the fragments where the base changes are located closer to the promoter core region. Although the DNA sequence altered in linker mutant pKL12 contains tracts of A and T (which are common in curved DNA), no significant difference in the electrophoretic mobility compared to the wild-type fragment can be observed. Together, these findings indicate that the center of bending is very likely located in the region around −105 to −80.

Surprisingly, the linker insertion constructs pKL12+4 and pKL12+6 show a further decrease in the electrophoretic mobility compared to that of the wild-type fragment. However, the mobilities of insertion mutants pKL12+10 and pKL12+22 do not differ strongly from that of the wild type. It should be noted that the inserted sequences by themselves do not seem to affect the B-form DNA by any criteria we applied (none of the current models for the calculation of DNA bending showed a bend in the inserted sequences). However, the region downstream from the insertion, including the promoter core region itself, has a slightly curved structure (k = 1.15 in 8% polyacrylamide). The increase in curvature of the insertion mutants pKL12+4 and pKL12+6 might be explained by the assumption that the overall curvature consisting of the bend centered around position −90 and the bend located in the promoter core region, both of which are partly compensated in the wild-type situation, become additive in the mutants by a rotational rearrangement. The insertion of 4 or 6 bases (half a helical turn) between the two regions changes the dihedral orientation in a way that both fragment ends are now pointing in the same direction, which in turn increases the overall curvature of the DNA. Insertion of 10 bases (one turn) or 22 bases (two turns) between the Fis binding site and the promoter core region restores the wild-type situation and results in the same electrophoretic anomaly. Therefore, there is good reason to assume that the increase in electrophoretic mobility of the linker mutants pKL2, pKL3, pKL4, pKL5, and pKL7, relative to that of the wild-type fragment, is the result of alterations in DNA sequences responsible for the curved DNA structure, whereas the decreased mobilities caused by the oligonucleotide insertions are mainly due to the altered arrangement of the relative orientation of two curved regions within the DNA fragment.

It is important to note that the bending analysis of the different DNA fragments by the computer program AUGUR (Tan et al., 1988) using the ApA wedge model (Trifonov & Sussman, 1980) exactly predicts the above illustrated scenario (data not shown).

Binding of Fis Protein to the Linker Mutants. The trans-activating protein Fis is known to bind upstream of many stable RNA promoters (Nilsson et al., 1990; Ross et al., 1990). There are potential binding sites in all rRNA operons (Verbeek et al., 1990), and specific interactions with at least three different sites upstream to the *rrnB* P1 promoter core have been shown (Ross et al., 1990; Nilsson et al., 1990). The sites almost perfectly match the degenerated consensus sequence -T/G--YR--T/A--YR--A/C- proposed previously (Hübner & Arber, 1989) to be important for Fis binding. These complexes (C1, C2, and C3), consisting of one, two, or three Fis dimers, respectively, can be resolved by gel electrophoresis. Gel mobility shift analysis was therefore used to study binding of Fis protein to the UAS linker mutations (see Figure 3). Gel retardation resolves these complexes in the case of the wild-type UAS region at 20 nM Fis. Similar retardation patterns at the same Fis concentration are observed for linker mutants pKL4 and pKL12, in line with the fact that both mutations did not change the consensus Fis binding sequences in the UAS region (see Figure 1). The same was true for derivatives with oligonucleotide insertions (mutants pKL12+4, pKL12+6, pKL12+10, and pKL12+22; data not shown). Base changes in constructs pKL2 and pKL3 alter the Fis binding sequence II, whereas in pKL5 and pKL7 Fis binding site I is affected (Figure 1). Consequently, the ability to form the third complex (C3) in the gel retardation study is reduced (Figure 3). Note that the C1 complex in Figure 3 is a mixture of one Fis dimer bound to either one of the three binding sites. A loss of one of these sites should reduce the ability to form the complex C1. This is indeed observed for pKL2 and pKL3, as well as for pKL5 and pKL7, where the band intensities for C1 relative to those of free DNA are lowered compared to those of the wild type (data not shown).

Interestingly, the linker mutation pKL2 does not change the Fis binding sequence further away from consensus but does affect binding properties. Only one position already nonconsensus in the wild type was altered. Nevertheless, construct pKL2 affects the curvature of the Fis binding region which may influence Fis affinity. Obviously, DNA conformation and primary sequence determine Fis binding specificity.

Mutants pKL2 and pKL7 were still able to form the third complex, however, with a 3–5 times reduced affinity compared to that of the wild type (the C3 complexes became visible at Fis concentrations above 20 nM). For the linker mutants pKL3 and pKL5, the third complex occurred only at much

higher Fis concentrations (100–200 nM, data not shown).

With the exception of linker mutant pKL3, the distance between the bands corresponding to free DNA and the different complexes is similar to that of the wild type. The difference in the electrophoretic mobility between complexes C2 and C1 for the mutant pKL3 is significantly smaller than in all other constructs, including the wild-type UAS (Figure 3). Linker mutation pKL3 not only affects the Fis binding site II but also reduces the distance between site I and III by two base pairs, and it thereby alters the rotational arrangement of Fis molecules bound to site I and III (a 2-bp deletion is equivalent to a rotation of about 70° around the helical axis). It is known that Fis induces a bend upon binding to DNA (Osuna et al., 1991). A reduction of the distance between the two bent regions affects the overall curvature in a way that the electrophoretic mobility of the DNA–protein complex is increased relative to the other C2 complexes.

Determination of the in Vivo Promoter Activity of UAS Mutants in fis⁺ and fis⁻ Strains. Activation of ribosomal RNA transcription from promoter P1 in vivo may be subject to different kinds of mechanisms. While intrinsic DNA curvature by itself is known to stimulate transcription in some cases (Hsu et al., 1991; Nachaliel et al., 1989), for most in vivo studies exploring the effects of UAS regions the contribution of the trans-activator protein Fis is not known. To distinguish between effects based on DNA conformational changes only or Fis-mediated effects, we determined the promoter strength of UAS mutants in vivo in *fis*⁺ and *fis*⁻ backgrounds.

E. coli CSH50 (*fis*⁺) or CSH50*fis*::*kan* (*fis*⁻) cells transformed with the different plasmid constructs were grown in YT medium at 37 °C. Stationary bacterial cultures were diluted 1:100 into fresh and prewarmed medium. Chloramphenicol acetyltransferase (CAT) and β-lactamase (BLA) activities of the transformants were determined at an optical density of 0.35 at 600 nm. The determination of the plasmid-encoded BLA activity served as an internal control to correct for differences in the plasmid copy numbers. Since all plasmids with the *rrnB* P1 promoter produce the same CAT mRNA, the CAT:BLA ratio directly reflects the relative promoter strength of each construct and should not be influenced by different mRNA stabilities. Figure 4 shows the CAT:BLA ratios of the different clones relative to the activity of the construct pKL1. A plasmid derivative without the *rrnB* P1 promoter but with the natural UAS region exhibited a more than 500 times lower CAT:BLA ratio than pKL1 (data not shown), and in accordance it was chloramphenicol sensitive (10 μg/mL chloramphenicol). This demonstrates that transcription is dependent on the presence of the rRNA P1 promoter and that no additional transcription start points were present in the construct.

The average growth rate μ of the bacterial cultures in YT medium was 2.0 h^{-1} for *fis*⁺ and 1.95 h^{-1} for *fis*⁻ transformants and did not differ between the various transformants by more than ±0.1 h. It has been shown before that the degree of growth rate dependence of the *rrnB* P1 promoter is independent from the UAS region (Gourse et al., 1986). Therefore, one may use the known growth rate dependence of this promoter to estimate the error due to differences in the growth rates of the various transformants. The CAT:BLA ratio of the *rrnB* P1 promoter varies by 8% when the growth rate is increased from 1.9 to 2.0 h^{-1} (Zacharias et al., 1989, 1990). The maximal error introduced by the different growth rates of the various constructs is therefore by a factor of 2 to 3 lower than the differences in the activities given in Figure 4. Hence,

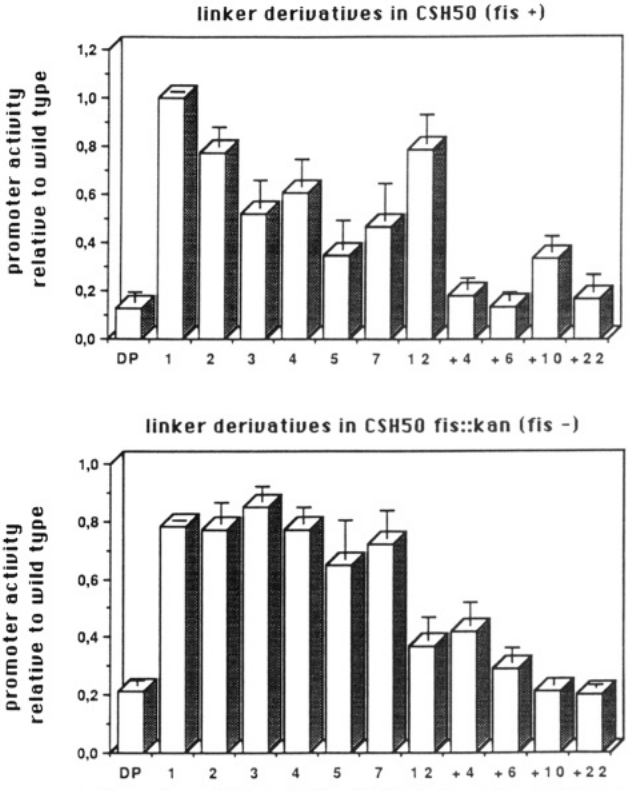

FIGURE 4: Promoter activity in *fis*⁺ and *fis*⁻ strains. The CAT:BLA ratio of the different constructs was normalized to the wild-type activity (pKL1 in CSH50) grown under identical conditions which was set to 1. The error bars indicate the standard deviations of a minimum of three independent measurements performed with different extracts. DP represents the clone pKL-DHP1 which lacks the UAS region (see Materials and Methods). Numbers 1–12 represent the linker mutants pKL1–pKL12, and +4, +6, +10, and +22 stands for the insertion mutants pKL12+4, pKL12+6, pKL12+10, and pKL12+22, respectively.

the observed changes in activity are not simply due to difference in growth rate regulation of the P1 promoter.

Correlation of Fis Binding Properties with in Vivo Promoter Strength of the Linker Mutants. Determination of promoter activity in a *fis*⁺ and *fis*⁻ background offers the possibility to distinguish between Fis-mediated activation effects and possible Fis-independent mechanisms. Consequently, if activation would only be achieved by a Fis-dependent mechanism, one should not expect different promoter activities of UAS mutants in a fis⁻ strain.

As can be seen in Figure 4, in the presence of Fis (strain CSH50) there is a 10 times higher activity of *rrnB* promoter P1 containing the intact UAS region (pKL 1), compared to the clone pKL-DHP1 which lacks the UAS region. In the absence of Fis (CSH50*fis*::*kan*) the promoter activity of pKL1 drops by approximately 20% whereas construct pKL-DHP1 showed an increase of promoter activity by a factor of 2. Promoter activation due to the UAS region is therefore larger in the presence of Fis (a factor of 10) than in its absence (a factor of 4). Whereas the various linker mutations result in rather uniform promoter activities in the absence of Fis, they reduce the activation effect of the wild-type UAS to different degrees in CSH50 strains. In the *fis*⁺ strain, linker mutants pKL5 and pKL7, with base changes in the Fis binding site I, give rise to the largest reduction in promoter strength (60 or 40%, respectively). The linker mutants pKL2 and pKL3 have base changes in the Fis binding site II. However, compared with the wild type, the changes introduced into pKL2 do not affect the consensus sequence compiled by Hübner and Arber (1989) which is believed to be important for Fis binding. As

already apparent from the data presented in Figure 3, Fis binding to these mutants is reduced. In accordance, promoter activity is reduced in these constructs, although to a different degree: 50% reduction by pKL3, 20% reduction by pKL2.

Linker mutations pKL4 and pKL12, on the other hand, do not interfere with Fis binding (in fact, binding might be slightly enhanced; see Figure 3); nevertheless, both exhibit a reduced activation of transcription down to about 80% of the wild-type level in the case of pKL12 and 60% for pKL4.

An insertion of 4 or 6 bases between Fis binding sites and promoter core region completely abolishes activation, whereas a shift by 10 bases (one helical turn) can restore activation to about 30%. A further shift by 22 base pairs (approximately two helical turns) in pKL12+22 does not activate transcription of the P1 promoter noticeably. The data suggest that the Fis-dependent activation mechanism is linked to the helical arrangement of the curved UAS structure relative to the promoter core region. Activation is only observed if the curved UAS region and the promoter core are in a precise angular orientation, presumably on one face of the helix. Due to the absence of Fis residual differences in the promoter activity of the linker derivatives of CSH50*fis*::*kan* cells reflect disturbances of a Fis-independent activation mechanism.

In the *fis*⁻ strain, promoter activities of all linker mutants, with the exception of pKL12, do not differ significantly from wild type. We take this as an indication that DNA sequences altered in linker mutants pKL2, pKL3, pKL4, pKL5, and pKL7 mainly interfere with a Fis-mediated mechanism but do not disturb possible Fis-independent activation.

The increase in activity of most of the linker mutants and the P1 promoter without UAS in a *fis*⁻ background can be interpreted as a result of compensation by the growth rate control system for the loss of Fis-mediated activation (Ross et al., 1990). It is unclear whether this compensation is mediated by the ribosome feedback control mechanism (Jinks-Robertson et al., 1983) which has been challenged recently (Baracchini & Bremer, 1991).

However, neither linker mutant pKL12 nor any one of the oligonucleotide insertion derivatives reached wild-type promoter activity in *fis*⁻ cells. No activity difference to the construct lacking the UAS (pKL-DHP1) was observed for the insertion derivatives pKL12+10 or pKL12+22 in CSH50*fis*::*kan*. Promoter activity of pKL12+4 and pKL12+6 was slightly higher compared to that of pKL-DHP1, approaching approximately the level of linker construct pKL12. Since in *fis*⁻ cells any Fis-mediated activation mechanism should be eliminated, the reduced activity must reflect the partial (pKL12, pKL12+4, pKL12+6) or complete (pKL12+10, pKL12+22) loss of a Fis-independent way of promoter activation.

Correlation of Promoter Activation and Bending of the UAS Region. DNA sequences upstream of many stable RNA promoters are associated with DNA curvature. It has been shown in several cases that this curved DNA structure plays a role in the mechanism of promoter activation by upstream sequence elements (Nachaliel et al., 1989; Lamond & Travers, 1983; Bossi & Smith, 1984; Bauer et al., 1988).

In a previous study using exponentially growing *E. coli* CP78 cells, we found a correlation between activation of *rrnB* P1 promoter and the degree of curvature of the linker-modified UAS (Zacharias et al., 1991). We still find a weak correlation for the promoter activity of the linker mutants in CSH50 cells, although not as pronounced as in strain CP78 (compare Figure 5, first two diagrams). The fact that CSH50 cells grow much faster in YT medium (μ = 2.0 h^{-1}) than do CP78 (μ = 1.5 to 1.6 h^{-1}) could influence the degree of this correlation. It is important to note that in the absence of Fis a correlation can no longer be observed at all (Figure 5, last diagram). This indicates that curvature of the UAS region can influence the Fis-mediated activation (most probably by enhanced binding) but is not correlated with promoter activation in the absence of Fis.

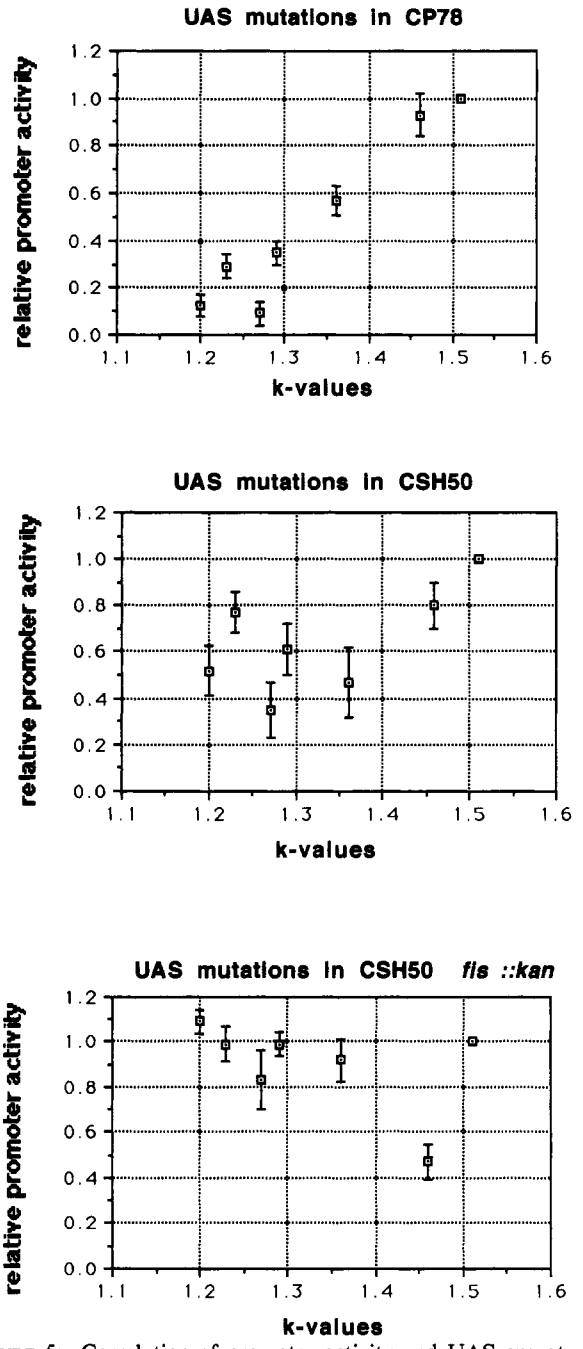

FIGURE 5: Correlation of promoter activity and UAS curvature. Diagrams represent the relative promoter strengths of the various linker scanning constructs as a function of the electrophoretic anomaly of UAS-containing fragments isolated from the corresponding plasmid. The activity was normalized to that of the wild type in each case. The relative promoter activities in strains CSH50 and CSH50*fis*::*kan* were taken from data presented in Figure 4. The *k*-factors, representing electrophoretic anomalies, were obtained in the same way as described in the legend to Table I except that the data was taken from 10% polyacrylamide gels. Promoter activities of the different constructs in CP78 cells (first diagram) were adopted from Zacharias et al. (1991).

The findings underline the complex situation where promoter activity is not simply determined by the degree of

curvature of an upstream promoter element.

Discussion

The analysis of linker scanning and oligonucleotide insertion mutations of the *rrnB* P1 upstream activating sequences supports the view that at least two activating mechanisms influence the P1 promoter strength. All linker mutations affecting the binding of Fis protein to either binding site I (centered at position −71) or binding site II (around position −102) also reduce the *rrnB* P1 promoter activity in a *fis*$^+$ strain. No such difference compared to wild type was observed in a *fis*$^-$ strain. We can conclude that in the absence of Fis protein sequence changes introduced by linker mutations between position −65 and −104 relative to the transcription start site do not affect promoter activity significantly, regardless of whether the intrinsic DNA curvature is reduced by the changes. In contrast, promoter activity of linker mutations in the region −47 to −58 (pKL12), with sequence changes downstream of the Fis binding sites, is only slightly lower than wild type in *fis*$^+$ cells but significantly reduced in a *fis*$^-$ strain. This result can be interpreted by a defect in a second promoter activation mechanism, not depending on *E. coli* Fis protein, which under normal conditions is covered or possibly compensated by Fis. The higher activity of pKL12 in CSH50 may therefore be explained by compensation of the defect in the Fis-independent mechanism by Fis.

We note that in pKL12 an AT-rich cluster between position −47 and −57 is disrupted which reminds at the "USR" regions proposed to be responsible, in part, for the strong "early" T5 or T7 promoters (Deuschle et al., 1986).

From the in vivo experiments reported here, we cannot distinguish whether the changes in activity observed are due to variations in RNA polymerase promoter binding (K_b) or to isomerization rates for open complex formation (k_f) (Chamberlin, 1974; McClure, 1980). However, in a different in vitro study, measuring the abortive initiation reaction (McClure et al., 1978) in the presence and absence of Fis, employing a DNA fragment containing promoter P1 and the UAS region, we have shown that the major effect caused by Fis is an increase in RNA polymerase promoter binding (K_b) (Zacharias et al., 1991).

These results are in line with a kinetic analysis of the role of the UAS region on *rrnB* P1 transcription by Leirmo and Gourse. The authors have shown that for Fis-independent activation the UAS increases the rate of the RNA polymerase concentration-dependent step in association (closed complex formation) without affecting the isomerization to open complexes (Leirmo & Gourse, 1991).

Recently, Hsu et al. (1991) suggested the existence of two activating elements in the upstream sequence of the *E. coli* *argT* promoter. The distal element located between −130 and −60 activates the *argT* promoter by stimulating the rate of open complex formation whereas a proximal sequence element between −60 and −38 enhances the binding of RNA polymerase. Our results support a similar picture for the UAS of the *rrnB* P1 promoter. DNA sequences upstream of position −65 (distal element) contribute to Fis-mediated activation of P1 and, according to our results, have no significant effect on a second, Fis-independent mechanism. DNA sequence determinants of this Fis-independent activation are located closer to the promoter core region, downstream of position −65 (proximal element), and were only affected by linker mutant pKL12.

Insertion of 10 or 22 base pairs into the *Nhe*I site of pKL12 completely abolished promoter activation by the Fis-independent mechanism. Therefore, in *fis*$^-$ cells these constructs did not differ in their activity from the construct pKL-DHP1 which lacks the UAS. However, insertion of 4 or 6 base pairs still allows Fis-independent activation to a some degree.

In *fis*$^+$ background, the shift of the Fis binding region by one helical turn (construct pKL12+10) restores *rrnB* P1 promoter activation to about 33% of the wild-type level, whereas shifts of half a helical turn (pKL12+4, pKL12+6) or two helical turns (pKL12+22) almost completely eliminate promoter activation in the presence of Fis. These results indicate that Fis-mediated activation is a function of both the distance of the Fis binding region and the helical arrangement relative to the promoter core region. One has to note that, in addition to the shift of the Fis binding site in pKL12+10, sequence elements important for a Fis-independent activation mechanism are also affected. Therefore, it is possible that the "helical restoration" effect would be much more pronounced if it would not interfere with the Fis-independent activation.

Both Fis Binding Sites I and II Play a Role in Upstream Activation of rrnB P1 Promoter. The analysis of potential Fis binding sites upstream of *E. coli* stable RNA promoters (Verbeek et al., 1990) revealed position −71 (site I), relative to the transcription start, as a strongly conserved binding sequence. This is in accordance with our finding that the correct arrangement of the Fis binding site and promoter core region is critical for activation.

The successive deletion of upstream sequences of the *rrnB* P1 promoter has led to the finding that the major determinants of upstream activation are located between position −88 and −50 (Gourse et al., 1986). This would imply that Fis binding sites II and III (see Figure 1) do play a minor role in upstream activation. Linker mutants pKL5 and pKL7 confirm that sequence changes in Fis binding site I cause the strongest reduction of promoter activity. Nevertheless, we found that DNA sequence alterations interfering with Fis binding in site II can reduce promoter activity significantly (approximately 60% of wild-type level) in the case of mutant pKL3. In linker mutant pKL2, Fis binding in site II is also reduced, but the promoter activity is still 80% of that of wild type. This effect is most likely explained if one considers that the altered helical arrangement in pKL3 due to a 2-bp deletion may additionally interfere with activation.

In summary, by comparing the degree of curvature of UAS mutations and promoter activation, we could present evidence that a reduction of the DNA bending angle, centered around position −90, mainly affects the Fis-mediated activation mechanism. Participation of this region in *Fis*-independent activation should be reflected in a variation of the promoter strength in *fis*$^-$ cells for those mutants which reduce curvature. This was not observed. In contrast, only the linker mutant pKL12, with about the same intrinsic curvature as that of the wild type, showed a reduction in *rrnB* P1 promoter activity in a *fis*$^-$ strain.

Therefore, a possible role of the curved DNA structure found upstream of many stable RNA promoters could be either a determinant facilitating Fis binding in addition to the degenerate primary recognition sequence or a primary bend which is further increased by Fis interaction.

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