Transcriptional regulation of *fis* operon involves a module of multiple coupled promoters

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The transcription of the *Escherichia coli* *fis* gene is strongly activated during the outgrowth of cells from stationary phase. The high activity of the promoter of the *fis* operon requires the transcription factor IHF. Previously, we identified a divergent promoter, *div*, located upstream of the *fis* promoter. In this study we demonstrate that at least two additional promoters, designated *fis* P2 and *fis* P3, are located in the control region of the *fis* operon. The *fis* P2 and *div* promoters overlap completely, whereas *fis* P3 and *div* P are arranged as face-to-face divergent promoters. We show that the *div* and the tandem *fis* promoters counterbalance each other, such that their activity is kept on a lower than potentially attainable level. Furthermore, we demonstrate an unusual activation mechanism by IHF, involving a coordinated shift in the balance of promoter activities. We infer that these coupled promoters represent a regulatory module and propose a novel ‘dynamic balance’ mechanism involved in the transcriptional control of the *fis* operon.

Keywords: activation/divergent promoter/*fis*/IHF/tandem promoters

Introduction

The transcription initiation regions of bacterial genes and operons often contain multiple promoters (for a recent review see Opel *et al.*, 2001). Although the requirement for this organization is not always obvious, in many cases it is apparent that the coupling of multiple promoters to a single operon increases the flexibility in the adjustment of gene expression to particular growth conditions. Notably, such arrangements are often found in regulatory regions of genes with pleiotropic functions. For example, the rRNA operons contain two tandem promoters, one of which is constitutively expressed, whereas the other is strongly activated on nutritional shift-up to enable rapid cell growth (Sarmientos *et al.*, 1983; Gourse *et al.*, 1986; Liebig and Wagner, 1995). Of the two tandem promoters driving the expression of the *crp* gene coding for the global transcriptional regulator, CRP, only one requires DNA

negative supercoiling and is subject to stringent control (González-Gil *et al.*, 1998; Johansson *et al.*, 2000). Four of the five tandem promoters in the *cydAB* operon involved in oxygen regulation are differentially utilized in response to changes in oxygen availability (Govantes *et al.*, 2000). Finally, the tandem promoters of the *topA* gene involved in the homeostatic control of DNA supercoiling utilize multiple initiation σ factors in adaptation to different growth conditions (Qi *et al.*, 1997).

Furthermore, depending on their arrangement, the multiple promoters themselves can interact with each other in different ways. One such example is topological coupling between divergent promoters, which is explained by the twin-supercoiled domain model of Liu and Wang (1987). In this model, the frictional drag of transcribing polymerase induces a downstream domain of positive supercoiling and an upstream domain of negative supercoiling. The latter facilitates DNA untwisting and thus increases the initiation rate of an upstream promoter (for review see Lilley, 1997). However, if the divergent promoters overlap each other, they can interfere, causing repression (Aiba, 1983; Hanamura and Aiba, 1991). Promoter interference can also occur with tandem promoters when a high initiation frequency of an upstream promoter ‘occludes’ a downstream promoter (Adhya and Gottesman, 1982; Zhang and Bremer, 1996; for review see Goodrich and McClure, 1991). It is conceivable that these various interactions between closely spaced promoters, especially when coupled to selective effects of transcription factors, have a potential for assembling regulatory circuits.

FIS is a pleiotropic regulator involved in the coordination of cellular metabolism with DNA topology during adaptation of cells to rapid growth conditions (Nilsson *et al.*, 1992; González-Gil *et al.*, 1996; Schneider *et al.*, 1999). Recently, we identified a divergent promoter, *div*, in the upstream region of the promoter of the *fis* operon (Nasser *et al.*, 2001). The transcription of *fis* increases sharply on the outgrowth of cells from stationary phase and then decreases steeply in mid-exponential phase (Ball *et al.*, 1992; Ninnemann *et al.*, 1992), whereas the *div* promoter activity declines on activation of *fis* expression. The *div* promoter is not associated with any meaningful ORF and interferes with *fis* transcription both *in vitro* and *in vivo*, but the detailed mechanism of this interference is not understood.

In this study, we demonstrate that the regulatory region of the *fis* operon contains, in addition to previously characterized *fis* and *div* promoters, at least two tandem promoters, P2 and P3, transcribing in the *fis* direction. We show that the tandem *fis* promoters respond coordinately to suboptimal transcription conditions *in vivo*. Furthermore, we demonstrate that the *div* and the tandem *fis* promoters are tightly coupled and their activities can be coordinately

Fig. 1. The upstream region of the *fis* promoter contains additional transcription initiation sites. (A) Primer extension analysis of total RNA from CSH50 cells using the primer ORF13'. Lanes 1, 2, 3 and 4 correspond to the products obtained with RNAs extracted from cells 5, 15, 60 and 150 min after the shift-up. *fisP1* corresponds to the previously described promoter. The novel transcripts initiating at -67 and -114 are indicated. The minor transcript indicated by an asterisk is probably a processing product and has not been studied further. (B) Primer extension analysis of the transcripts produced from pWN1 *in vitro*. The concentration of RNAP in these reactions was 25 nM. The primer extension products corresponding to *fisP1*, *fisP2* and *fisP3* + *fisP4* are indicated. The dideoxy sequencing ladder performed with the same primers on pWN1 is shown. (C) Sequence of the *fis* promoter region. The dotted line indicates the binding region for IHF. The RNAP binding elements (-10 and -35 hexamers) of the *div* promoter are boxed, those of the *fis* promoters are indicated in bold. The start points of transcription are shown by broken arrows. The 'down' mutations introduced in the RNAP recognition elements of *div* (-35 hexamer, -75A/C) and *fisP3* (-10 hexamer, -126T/G) promoters are indicated by C and G above the substituted A and T, respectively.

changed by a single transcription factor. Our findings suggest the involvement of a module of multiple coupled promoters in the transcriptional control of the *fis* operon. From these data we propose a novel 'dynamic balance' mechanism of transcription regulation.

Results

By analysing transcription of the *fis* operon in exponentially growing CSH50 cells, we detected two messages in addition to the previously described transcript (Figure 1A; Ball *et al.*, 1992; Ninnemann *et al.*, 1992). These additional transcripts were also growth phase regulated and their 5'-ends were mapped to positions -67 and -114 with respect to the transcription initiation start point of the *fis* operon at +1 (Figure 1C). The transcript starting at -67 was most abundant and already highly expressed 5 min after subculturing overnight cultures in fresh medium (the procedure termed hereafter nutritional shift-up). By contrast, the two other transcripts achieved their maximum levels only after 15 min, but all three messages decreased strongly 60 min after the shift-up. To discriminate whether these additional transcripts represent processing products

Fig. 2. Effects of the altered quality of RNAP and of template DNA topology on the activity of the chromosomal *fis* promoters *in vivo*. (A) Chromosomal *fis* promoter activities in the isogenic strains CF2010 and CF2016. The latter strain carries the 'stringent' polymerase with *rpoB114* mutation (Zhou and Jing, 1998). (B) Chromosomal *fis* promoter activities at different overall DNA superhelical densities in strains with mutant gyrase and/or topoisomerase IV alleles. Addition of norfloxacin leads to relaxation of DNA (rel) in strain LZ54 and its *fis* derivative, and to hypernegative supercoiling (hyp) in strain LZ54 and its *fis* derivative, whereas the superhelical density in strains LZ23 and LZ23 *fis::cm* remains largely unaffected (norm). Norfloxacin was added for 5 min to strains grown in early exponential phase (OD = 0.1). The transcripts originating from *fisP1*, *fisP2* and *fisP3* were detected as in Figure 1A. The superhelicity of DNA was estimated from the overall superhelical densities of plasmids isolated from the respective strains (Schneider *et al.*, 2000).

or independent initiation events, *in vitro* transcription reactions were carried out with the *fisP-lacZ* fusion construct pWN1, containing the extended *fis* promoter region from position -308 to +106 (Nasser *et al.*, 2001). By primer extension of the transcription products, three novel messages were detected, two of which (initiating at positions -67 and -114) coincided with those identified *in vivo* (Figure 1B). We thus infer that at least two tandem promoters initiating at -67 and -114 (denoted P2 and P3, respectively) in addition to the previously identified promoter (hereafter denoted P1) are present in the regulatory region of the *fis* operon. The deduced *fisP2* promoter region overlaps *div* completely, whereas *fisP3* and *div* are arranged as face-to-face divergent promoters with their initiation sites separated by 5 bp (Figure 1C).

To test whether these multiple promoters may have distinct physiological roles, we used two simple approaches that change the transcription conditions of cellular promoters *in vivo*. It has been shown that the *fisP1* promoter is subject to stringent control (Ninnemann *et al.*, 1992). We therefore analysed the tandem *fis* promoter activities in the strain CF2010 and its derivative CF2016, which carries a polymerase altered in its interaction with stringent promoters by mutation (Zhou and Jin, 1998). Primer extension analysis of RNA isolated from exponentially growing cells demonstrated that in the CF2016 strain with 'stringent' polymerase, only *fisP2* was transcribed efficiently, whereas *fisP1* and *fisP3* activities were substantially reduced (Figure 2A). The *fisP1* promoter is also very sensitive to changes in negative superhelical density of DNA (Schneider *et al.*, 2000). We therefore measured next the amount of different *fisP* transcripts in isogenic strains LZ54, LZ41 and LZ23, in which addition of the topoisomerase inhibitor norfloxacin allows the establishment of different overall negative superhelical

densities *in vivo* (Zechiedrich *et al.*, 1997; Schneider *et al.*, 2000). We found that both the relaxation and hyper-negative supercoiling of DNA reduced the *fis* P1 and *fis* P3 transcription to different extents, whereas the activity of *fis* P2 remained high (Figure 2B). Essentially similar dependence of promoter activity on superhelical density was observed in *fis* mutant derivatives of these strains (Figure 2B), ruling out the role of FIS autoregulation in this effect (Ball *et al.*, 1992). We thus infer that the tandem *fis* promoters respond coordinately to the impairment of transcription conditions *in vivo*: *fis* P1 and P3 promoters decrease with altered polymerase and suboptimal template quality, whereas P2 remains largely insensitive to these changes.

To gain more insight into the regulation of tandem *fis* promoters, we performed deletion analyses using the *fisP-lacZ* fusion construct pWN1. A deletion to position -42, removing the *fis* P1 promoter, led to a 5-fold increase in β -galactosidase activity (pWN2 in Figure 3A), consistent with high activity of *fis* P2 observed *in vivo*. However, removal of the *fis* P2 promoter by deletion to positions -90 (pWN5) or -108 (pWN6) led to a further >5-fold increase, such that in total β -galactosidase activity increased 28-fold compared with that of the *fis* extended promoter (pWN1). Notably, this latter deletion also removes the overlapping *div* promoter (see Figure 1C). Further deletions to positions -117 (pWN7), -137 (pWN8) and -150 (pWN9) all decreased β -galactosidase activity by 3- to 4-fold, but it was still substantially higher than that produced from the *fis* extended promoter. Only after deletion to position -163 (pWN10), which removes the putative upstream promoter initiating at -150 (denoted hereafter as P4; see Figure 1B and C), did the β -galactosidase activity decrease strongly, and further deletions did not change these low levels of β -galactosidase produced (data not shown). Thus, the successive deletions to positions -42 and -90/-108, removing first the *fis* P1 and then the overlapping P2/*div* promoter regions, derepress the remaining *fis* promoters.

Since *fis* expression is known to be modulated by three DNA architectural proteins, IHF, CRP and FIS itself (Ball *et al.*, 1992; Ninnemann *et al.*, 1992; Pratt *et al.*, 1997; Nasser *et al.*, 2001), the differences in relative β -galactosidase activities of deletion mutants could be explained by binding of these regulatory proteins. We therefore analysed the deletion mutants in the *fis*, *ihf* and *crp* mutant cells. We observed that although the inactivation of these regulators clearly modulated the relative β -galactosidase activities of deletion mutants, the overall pattern remained essentially unchanged (Figure 3C). These data suggest that the pattern obtained reflects intrinsic differences in the activities of deletion mutants and is not determined uniquely by these regulatory proteins.

To evaluate the relative strengths of tandem *fis* promoters, we created minimal promoter-*lacZ* fusion constructs by cloning the sequences from +106 to -42 (pminiP1), from -42 to -108 (pminiP2; we note that this construct also contains the overlapping *div* promoter), from -90 to -156 (pminiP3) and also from -137 to -204 (pminiP4), upstream of the *lacZ* gene. Consistent with deletion analyses, with the exception of *fis* P1 all these minimal promoter constructs demonstrated higher β -galactosidase activities than the extended *fis* promoter

Fig. 3. Deletion analysis of the *fis-lacZ* promoter fusion. (A) The extended promoter fusion construct pWN1(-308 to +106) and deletion mutants are represented by gray horizontal bars. The β -galactosidase activity of pWN1 is normalized to 1. The broken arrows indicate transcription initiation sites. The downstream deletion boundaries are indicated on the right side of the horizontal bars and correspond to the *fis-lacZ* fusion junctions. The relative β -galactosidase activities (ratios) of overnight cultures containing corresponding *lacZ* fusion constructs are indicated. The ratios obtained in three separate experiments varied by <15%. (B) The relative β -galactosidase activities of the minimal promoter constructs and constructs containing two contiguous promoters. Indications and normalization are as in (A). (C) Relative β -galactosidase activities of deletion mutants in different genetic backgrounds. The β -galactosidase activity of the extended *fis* promoter fusion construct pWN1(-308 to +106) is normalized to 1 separately for each mutant strain, allowing comparison of relative activities of deletion mutants within, but not between, the different genetic backgrounds.

(Figure 3B). Notably, both the P2 and P3 promoters were more active on minimal constructs than on the construct pWN11 comprising the regions of overlapping *fis* P2/*div* and *fis* P3 promoters. Likewise, the P2 promoter was more active on the minimal construct than on the construct

Fig. 4. Deletion analysis of the *div-lacZ* promoter fusion pWN3. The β -galactosidase activity of pWN3 is normalized to 1. The denotations are as in Figure 3A. The ratios obtained in three experiments varied by <15%.

pWN15 comprising the regions of overlapping *fis* P2/*div* and *fis* P1 promoters. This indicates the possibility of competitive interactions between these promoters.

To understand the role of the *div* promoter in these interactions, we carried out deletion analyses using the construct pWN3, which is identical to pWN1 but contains the extended *fis* promoter (+106 to -308) region in opposite orientation with respect to the *lacZ* reporter gene, thus allowing β -galactosidase activity produced from the *div* promoter to be measured. Deletion of the region between +106 and -42 containing the *fis* P1 promoter had only a small, if any, effect on β -galactosidase activity produced from the *div* promoter (Figure 4, compare pWN3 and pWN12). Nevertheless, the same deletion substantially increased *div* P activity in the absence of the *fis* P3 and P4 promoters (compare pWN13 with pminiDiv), suggesting that the effect of the +106 to -42 region is context dependent. Deletion of the *div* downstream region comprising the *fis* P4 promoter led to a >2-fold increase in *div* P activity (compare pWN12 and pWN14), whereas deletion of the *fis* P3 promoter region led to an additional 30-fold increase (compare pWN14 and pminiDiv), indicating that the P3 promoter exerts a strong negative effect on *div* P activity. Again, in the presence of the region between +106 and -42, comprising the *fis* P1 promoter and downstream sequences, this increase was less pronounced (compare pWN3 and pWN13). Thus, the negative effects exerted by both the downstream and upstream regions on β -galactosidase activity produced from the *div* promoter are context dependent. We infer that in the context of the extended *fis* promoter (+106 to -308), the major negative effect is exerted by the P3 promoter since it represses, though to different extents, the *div* promoter both in the presence and absence of the region comprising *fis* P1 (compare pWN3 with pWN13, and pWN14 with pminiDiv).

Taken together, our deletion analyses presented in Figures 3 and 4 indicate that the face-to-face *fis* P3 and *div* promoters may counteract each other. This predicts that inactivation of one of these promoters (e.g. by mutation) should increase the activity of the other. To test this hypothesis, we used a construct carrying a 'down' mutation in the -35 element of the *div* promoter (-75A/C), which abolishes *div* transcription *in vitro* (Nasser *et al.*,

2001). We observed that this mutation increased the permanganate reactivity of the *fis* P3 promoter DNA, indicative of RNA polymerase (RNAP) open complex formation and also *fis* P3 transcription *in vitro* (Figure 5A and B). In turn, we next introduced a 'down' mutation in the putative -10 region of the P3 promoter (a T to G transversion at position -126, see Figure 1C) to impair its activity. As expected, this mutation decreased the opening and transcription of the P3 promoter *in vitro*, whereas both the opening and the transcriptional activity of the *div* promoter were increased (Figure 5C and D). Similar reciprocal effects of these promoter mutations were observed *in vivo* (Figure 5E). We thus infer that the *div* and *fis* P3 promoters counterbalance each other such that their activity is kept on a lower than potentially attainable level.

We note, however, that the β -galactosidase activities measured from pWN11 and pWN11-75A/C (Figure 5E) actually reflect the contribution of both the *fis* P3 and P2 promoters. To address the question of the relationships between the overlapping *div* and *fis* P2 promoter activities, we first measured their relative activities using the same minimal DNA fragment comprising the overlapping *fis* P2 and *div* promoters (-42 to -108) inserted in two opposite orientations upstream of the *lacZ* gene. The β -galactosidase measurements showed that, depending on the growth stage, the activity of the *div* promoter exceeded that of *fis* P2 by 9- to 29-fold (Figure 6A). A comparison of *div* P and *fis* P2 transcriptional activities *in vitro* showed that the *div* promoter is also a stronger promoter *in vitro* (Figure 6B). Furthermore, we found that on the extended promoter template pWN1, the 'down' mutation of *div* P increases P2 activity *in vitro* (Figure 6C and D) and *in vivo* (Figure 6E), consistent with competitive interactions between these overlapping promoters.

Since *fis* P2 is the strongest among the tandem *fis* promoters *in vivo*, we asked how this high activity could be maintained in the presence of limitations imposed in the context of extended *fis* promoter region. Previous studies implicated IHF in the maintenance of high levels of *fis* expression (Pratt *et al.*, 1997). We therefore investigated the activating effect of IHF both *in vivo* and *in vitro*. As expected, in mutant strains lacking IHF, the β -galactosidase activity produced by the extended *fis* promoter construct pWN1 decreased. However, the β -galactosidase activity produced by the *div* promoter construct pWN3 increased by >5-fold, suggesting that IHF represses *div* (Figure 7A). Furthermore, we found that IHF repressed *div* P on pWN12 and pWN14 but *not* on the pminiDiv construct, implicating the sequences between -108 and -156 in mediating repression (Figure 7B). The *in vitro* transcription and permanganate reactivity assays carried out using pWN1 demonstrated that addition of IHF not only activates the *fis* P1, as expected, but also the *fis* P2 promoter, whereas both P3 and *div* promoters were strongly repressed (Figure 7C). Consistent with this finding, elevated levels of *fis* P3 and lowered amounts of *fis* P2 and *fis* P1 transcripts were detected in *ihf* mutant cells in comparison to wild type *in vivo* (Figure 7D). From these data, we infer that IHF is involved in the maintenance of the high activity of the *fis* P2 promoter *in vivo*.

Discussion

The major finding of this study is the identification of a module of multiple coupled promoters in the regulatory region of the *fis* operon, implying that the control of *fis* expression is much more complex than anticipated. Although we have not explicitly addressed the particular physiological roles of the tandem *fis* promoters described here, they respond coordinately to deviations of transcription conditions from optimum *in vivo*. Furthermore, the balance of their activities is coordinately switched by the transcriptional activator of *fis* expression, IHF. We propose that this organization of multiple promoters in a coupled module potentially provides for a factor-independent mechanism of transcription regulation by RNAP itself. We denote the latter as a 'dynamic balance' regulation.

The *fis* transcription unit contains at least three tandem promoters

Of the three *fis* promoters identified besides P1 (P2, P3 and P4), the relevance of P4 *in vivo* remains unclear and therefore we focused primarily on the P2 and P3 promoters. The putative -35 and -10 elements of the P3 promoter, GTGTAA and TAATAT, are separated by a suboptimal 18 bp spacer and deviate from the consensus -35 (5'-TTGACA-3') and -10 (5'-TATAAT-3') elements in five out of 12 positions (Figure 1C; Lisser and Margalit, 1993). Notably, the -10 element of P3 is identical to that of *fis* P1. Furthermore, both these promoters use a less favourable pyrimidine nucleotide for initiation (Liu and Turnbough, 1994; Walker *et al.*, 1999). It is thus possible that these similarities account for the apparent coherence in the response of P1 and P3 promoters to the alterations of DNA superhelical density and RNAP quality observed *in vivo*. We note, however, that one of the major determinants of both supercoiling sensitivity and stringent control, the GC-rich discriminator sequence (Travers,

1980; Ninnemann *et al.*, 1992; Figueroa-Bossi *et al.*, 1998; Schneider *et al.*, 2000), is present in *fis* P1 but absent in *fis* P3. The putative -35 and -10 hexamers of the *fis* P2 promoter, TTATCG and TGCAAT, are also separated by an 18 bp spacer and deviate from the consensus in five out of 12 positions. However, the *fis* P2 promoter is largely insensitive to negative effects of suboptimal superhelical density and altered polymerase quality. This promoter also shows an extensive DNA untwisting in the AT-rich discriminator region on binding RNAP (T-68, T-69, T-72 and T-73; Figure 6C) and, in contrast to P1 and P3, uses a favourable purine nucleotide for initiation. Another peculiarity is that the -10 element of P2 almost coincides (in five out of six positions) with the -35 element of overlapping *div* promoter and, vice versa, the -10 element of *div* P coincides (in five out of six positions) with the -35 element of P2 (Figure 1C). Given the separation between initiation sites of P2 and P3 of ~4.5 helical turns, this organization places *div* P face to face with the *fis* P3

Fig. 5. Analysis of the mutual effects of 'down' mutations in the RNAP recognition elements of the *fis* P3 and *div* promoters *in vitro* and *in vivo*. (A) *In vitro* effect of the 'down' mutation in the -35 hexamer of the *div* promoter on the *fis* P3 promoter opening and transcription. Lanes 1 and 2, transcription of *fis* P3 from pWN1; lanes 3 and 4, transcription of *fis* P3 from pWN1-75A/C; lanes 5 and 6, permanganate reactivity of the *fis* P3 and *div* promoters on pWN1; lanes 7 and 8, permanganate reactivity of the *fis* P3 and *div* promoters on pWN1-75A/C; lanes 9, 10 and 11, 12, transcription of the *bla* promoter from pWN1 and pWN1-75A/C respectively (an internal control). The concentration of RNAP is indicated. The dideoxy sequencing ladder performed with the same primer on pWN1 is shown on the left. (B) Quantitative analysis of *fis* P3 activity on pWN1 and pWN1-75A/C. The data were normalized to the value obtained for pWN1 at 50 nM RNAP, which was set at 100%. (C) *In vitro* effect of the 'down' mutation in the -10 element of the *fis* P3 promoter on *div* promoter opening and transcriptional activity on pWN14 and pWN14-126T/G. The concentration of RNAP is indicated. The dideoxy sequencing ladder performed with the same primer on pWN14 is shown. (D) Quantitative analysis of *div* activity on pWN14 and pWN14-126T/G. The data were normalized to the value obtained for pWN14 at 30 nM RNAP, which was set at 100%. (E) *In vivo* effects of 'down' mutations in the RNAP recognition elements of the *fis* P3 and *div* promoters. Representation and indications are as in Figure 3A. Only those promoters that contribute to the measured β -galactosidase activities are indicated. The point mutations are indicated by crosses on the top or bottom strand of DNA. The ratios (WT/Mutant) obtained in three separate experiments varied by <15%.

Fig. 6. Comparative analysis of the *fis* P2 and *div* promoters. (A) The β-galactosidase activities of pminiDiv and pminiP2 were measured in overnight cultures (ON) and 5, 30 and 150 min after the shift-up. The ratios (*div* P/*fis* P2) are shown below. (B) *In vitro* transcription of the *fis* P2 and *div* promoters from pWN11 and pWN14 was monitored by primer extension using the same radiolabelled primer in order to directly compare the respective promoter activities. The concentrations of RNAP used in the reactions are indicated. Note the additional transcripts (asterisks) increasing with RNAP concentration and presumably due to aberrant initiation within the presumably flexible DNA stretch of ~40 bp in length containing the overlapping P2 and *div* promoters. (C) The effect of 'down' mutation in *div* P on the transcription of the *fis* P2 promoter. The constructs used to monitor transcription *in vitro* and the RNAP concentrations are indicated. (D) Quantitative analysis of *fis* P2 activity on pWN1 and pWN1-75A/C. The data were normalized to the value obtained for pWN1 at 50 nM RNAP, which was set at 100%. (E) The β-galactosidase activities of pminiP2 and pminiP2-75A/C carrying a 'down' mutation in *div* P. The abbreviations are as in (A).

promoter, with their initiation sites separated only by half a helical turn. This unique arrangement of three promoters over a DNA stretch of ~85 bp prompted us to investigate the interactions between *fis* P2, *fis* P3 and *div* P in more detail.

The *div* and the tandem *fis* promoters are coupled

We studied the interactions between the *div* P and tandem *fis* promoters *in vivo* using transcriptional fusions with the *lacZ* gene on multicopy plasmids. Since the plasmid copy number may vary in different mutant strains, we separately compared the relative promoter activities of deletion constructs within each genetic background. To avoid possible variation due to different mRNA stability dependent on the particular *fis* (or *div*) promoter-*lacZ* junction sequence in the transcriptional fusions (Liang *et al.*, 1998), we verified the critical genetic data either by measuring the transcriptional activity *in vitro* or from chromosomal *fis* promoters *in vivo*. We note that the chromosomal transcripts were detected using a primer complementary to a region in ORF1 upstream of the *fis* gene and, therefore, their apparent abundances may not reflect their contribution to translatable *fis* message.

Our genetic analyses revealed that a deletion of the region from +106 to -42 encompassing the *fis* P1, and a further deletion to -90/-108 encompassing the overlapping P2 and *div* promoters, both unmask a high activity of the upstream *fis* promoters. A qualitatively similar pattern was observed in wild type and three mutant strains

lacking the known transcriptional regulators of the *fis* promoter activity, IHF, CRP and FIS itself. This argues that the repression cannot be uniquely ascribed to any of these factors, but rather is modulated by them.

Previous studies indicated that a deletion of the region between +7 and +107 derepresses the minimal *fis* P1 promoter by ~2-fold (Pratt *et al.*, 1997). Although this suggests an existence of repressing factor(s) targeting the region between +7 and +107, we cannot exclude the possibility that, in addition, the *fis* P1 promoter itself could limit the transcription from the upstream P2 promoter to a certain extent. This notion is consistent with the 6-fold lower β-galactosidase activity of the construct pWN15 containing both the P1 and P2 promoters in comparison to the minimal P2 promoter construct pminiP2 (Figure 3B). Furthermore, we have shown here that in the absence of *fis* P3, the region between +106 and -42 comprising the *fis* P1 promoter reduces β-galactosidase activity produced from the *div* promoter 10-fold (Figure 4). These observations are consistent with limitations imposed by the downstream region comprising the *fis* P1 promoter on both the P2 and *div* promoters.

The repressing effect exerted by the region between -42 and -90/-108 on the *fis* P3 promoter is clearly due to the activity of the *div* promoter, whereas the latter is itself repressed by *fis* P3. This notion is supported by both our *in vivo* and *in vitro* analyses. First, the disruption of the face-to-face arrangement of *div* and *fis* P3 promoters substantially increases the β-galactosidase activity of both

Fig. 7. Effect of IHF on the *fis* and *div* promoter activities. (A) The β -galactosidase activities of pWN1 and pWN3 measured in the cultures of CSH50 and CSH50 Δ *ihf* cells. The β -galactosidase activities of overnight (ON) cultures and 15 and 180 min after the shift-up are shown. (B) The β -galactosidase activities of pminiDiv, pWN14 and pWN12 measured in the cultures of CSH50 and CSH50 Δ *ihf* cells. The activity ratios (*ihf*/WT) and the corresponding constructs are indicated. (C) The effect of IHF addition on promoter opening and transcription *in vitro*. The concentration of RNAP was 20 nM; the concentration of IHF was 2 nM in lanes 2 and 6, 5 nM in lanes 3 and 7, and 50 nM in lanes 4 and 8. The transcription of the *fis* P1, P2, P3 and *bla* (used as an internal control) promoters was evaluated from pWN1; the *div* promoter transcription was measured from pWN14. (D) Primer extension of mRNA isolated from CSH50 and CSH50 Δ *ihf* cultures 5, 15 and 30 min after the shift-up. The reactions were carried out as in Figure 1A. The amount of the transcripts was normalized to the maximal values obtained in wild-type cells for *fis* P1 (15 min), *fis* P2 (5 min) and *fis* P3 (15 min), which were each set at 100%.

promoters (Figures 3A and 4). Secondly, the introduction of ‘down’ mutations in the RNAP recognition elements of the *div* and *fis* P3 promoters reciprocally increases the RNAP open complex formation and transcription *in vitro*, suggesting that these two promoters counterbalance each other. Notably, the –75A/C ‘down’ mutation in the –35 region of *div* P also impairs the match of the *fis* P2 promoter –10 element to the consensus, yet it increases the activity of P2. One possible explanation is that since *div* P appears to be much stronger than *fis* P2, the net effect of the mutation that abolishes *div* transcription *in vitro* (Nasser *et al.*, 2001) results in increased P2 activity. Furthermore, in the context of the extended promoter, this mutation also increases the activity of *fis* P1 (Nasser *et al.*, 2001). Taken together, these data argue for a tight coupling of the *div* and the tandem *fis* promoters.

We cannot exclude the possibility that topological coupling is involved in the complex interactions between these multiple promoters. However, while this mechanism

easily applies to two gene promoters organized as divergent transcription units (for review see Opel *et al.*, 2001), the organization of divergent promoters in the *fis* control region is much more complex. Three types of arrangement can be discerned here, including the closely spaced (*div* and *fis* P1), overlapping (*div* and *fis* P2) and face-to-face (*div* and *fis* P3) divergent promoters. Furthermore, in the case of topological coupling, inactivation of one of the promoters is predicted to impair the activity of the coupled promoter (Opel *et al.*, 2001), whereas inactivation of *fis* P3 activates *div* P and, vice versa, inactivation of *div* P activates all of the three *fis* promoters (this study; Nasser *et al.*, 2001). We think it unlikely that the ‘down’ mutation in *div* P could have a direct activating effect on all three *fis* promoters (Goodrich and McClure, 1991), or that activation of one of the tandem *fis* promoters subsequently activates the others. The observed higher β -galactosidase activities of minimal *fis* promoters in comparison to constructs containing two

Fig. 8. Dynamic balance model of regulation of *fis* operon transcription. (A–D) The sequential joining of promoter regions to the basal element (A) results both in the repression and shifts in the balance of transcription, leading to the establishment of a new dynamic balance in the context of the extended promoter (D). This balance can be perturbed on binding of a transcriptional factor (E), by changes in general transcription conditions or by promoter mutation. The RNAP molecules interacting with *fis* P1, P2, P3 and *div* promoters are indicated. The coupling (competitive interactions) between the promoters is indicated by connecting double arrows. Horizontal blue and red arrows indicate the relative strengths of transcription in *fis* and *div* directions, respectively. With the exception of (A), the sizes of the arrows within each pair are roughly approximated to values obtained by β -galactosidase assays. For details see the text. (E) Coordinated switch of the *fis* promoter activities by IHF. In this model, the sharp bend (U-turn) introduced by IHF (Rice *et al.*, 1996) on binding to the site centred at –114 precludes binding of RNAP at *fis* P3 and *div* promoters with resultant ‘channeling’ of transcription towards *fis* P2 and P1. For details see the text.

contiguous *fis* promoters (Figure 3B) are rather indicative of competitive interactions. For example, the α -CTD of RNAP bound at P2 could use the AT-rich sequence around the start point of P3 as a proximal UP element (Ross *et al.*, 1993) and thus occlude binding of RNAP at P3.

The ‘dynamic balance’ model

Our data can be explained by a ‘dynamic balance’ mechanism, which implies the assembly of the module of coupled promoters by sequential joining of competing promoter regions. We assume that the basal element of the module is the sequence comprising the overlapping *fis* P2 and *div* promoters. The transcription of both these promoters is high, but from our data the *div* promoter activity exceeds that of P2 by at least by an order of magnitude (Figure 8A). The addition of the *fis* P1 promoter

region to this basal element decreases the activity of both *div* and P2 promoters by competition and also shifts their balance, yet the *div* promoter is still transcribed more strongly (Figure 8B). By contrast, addition of the P3 promoter region to the basal element not only decreases the *div* and P2 promoter activities, but also, by being a strong competitor of *div* P, shifts the balance in favour of *fis* transcription (Figure 8C). In the context of the extended promoter, the transcription from the overlapping *fis* P2 and *div* promoters is lowest, being counterbalanced by negative effects now exerted by both the P3 and P1 promoter regions (Figure 8D). The model predicts that activation of P1 and P3 should correlate with reduction of the *fis* P2 activity and, vice versa, high levels of *fis* gene expression could be maintained from P2 under conditions repressing the *fis* P1 and P3 promoters. This is exactly what we observe under respectively optimum (Figure 1) and suboptimum (Figure 2) transcription conditions *in vivo*. This regulation is essentially homeostatic, and also allows a rapid and precise adjustment of *fis* promoter activities by shifting the dynamic balance.

Regulation of *fis* transcription by IHF

The mechanism of activation of *fis* transcription by IHF appears to be such an example of shifting the balance of relative activities of the *fis* P3, P2, P1 and *div* promoters. In line with previous observations, we have found that IHF increases the β -galactosidase activity produced from the *fis* promoter (Pratt *et al.*, 1997), but, as shown here, concomitantly and strongly represses the *div* promoter. This latter effect is wholly consistent with the previously observed inverse correlation between the *div* P and *fis* P activities during the first 15 min after the nutritional shift-up (Nasser *et al.*, 2001). Furthermore, the effect of IHF on tandem *fis* promoters is selective, since IHF increases the transcription of *fis* P2 and P1 but reduces that of *fis* P3 *in vivo*. This notion is further reinforced by our *in vitro* data clearly showing that IHF specifically increases both the opening and transcription of P2 and P1 promoters, but strongly represses those of *div* and P3. The repression by IHF requires sequences between –108 and –156, consistent with a role for the IHF binding site centred at –114 shown to be involved in activation of *fis* transcription (Pratt *et al.*, 1997). This IHF binding site overlaps the initiation start points and –10 regions of both the *fis* P3 and *div* promoters (Figure 1C). Repression of RNAP binding by interaction of IHF with a site overlapping the promoter has also been observed with bacteriophage λ pcin promoter (Griffo *et al.*, 1989). We propose that bending of DNA by binding of IHF at this site may preclude the interaction of polymerase with both the P3 and *div* promoters, supporting early activation of P2 (Figure 8E). The activation could then be a result of both the relief of limitations imposed by *div* P and *fis* P3 on P2, and stabilization of favourable local geometry for binding of RNAP at the P2 and, perhaps, P1 promoters. Thus, IHF appears to ‘channel’ transcription by shifting the balance, as predicted by the dynamic balance mechanism. We emphasize, however, that the regulation of *fis* expression is a complex phenomenon subject to growth phase-dependent and stringent control, and involving in addition to IHF the effects of other factors such as FIS itself, CRP, the superhelical density of DNA and, perhaps, NTP pools (this study; Ball *et al.*, 1992;

Ninemann et al., 1992; Pratt et al., 1997; Walker et al., 1999; Schneider et al., 2000; Nasser et al., 2001).

There are many examples of the organization of gene regulatory regions as coupled overlapping and face-to-face divergent promoters, which in concert with transcription factors, allow balanced gene expression (Aiba, 1983; Jagura-Burdzy and Thomas, 1997; González-Gil et al., 1998; Rhee et al., 1999; for review see Opel et al., 2001). However, the *div* promoter located in the *fis* control region is not associated with any meaningful ORF, a situation reminiscent of the role of the CRP-dependent divergent promoter involved in the complex control of *crp* expression and suggesting an entirely regulatory function (Aiba, 1983; Hanamura and Aiba, 1991). Notably, the tandem *crp* P1 and P2 promoters differ in their sensitivity to superhelical density and stringent control (González-Gil et al., 1998; Johansson et al., 2000). It is conceivable that such modules of multiple coupled promoters represent a transcription factor-independent regulation mechanism mediated by interactions between RNAP molecules. Such mechanisms could have co-evolved with factor-dependent regulation. Alternatively, the control by transcription factors could have been superimposed on primordial RNAP-dependent homeostatic regulation mechanisms in the course of evolution.

Materials and methods

Chemicals and enzymes

Chemicals and enzymes used in this work were obtained from commercial sources.

Bacterial strains, growth conditions and plasmids

Bacterial strains used in this study were *Escherichia coli* K12 derivatives. The genotype of CSH50 is *ara Δ(lac pro) thi rpsL* (Miller, 1972). The construction of CSH50 Δ *fis* is described elsewhere (Koch et al., 1988). The strain CSH26 Δ *ihf* was kindly provided by C.Koch. Strains CF2010 and CF2016 are MG1655 derivatives, and were kindly provided by M.Cashel. Strains LZ41 (*parC^{K84kan}*) and LZ54 (*gyrA^{L834} zei-3143::Tn10kan*) are RS22 [*pyrF287 nirA trpR72 iclR7 gal25 rpsL195 topA10 λ(P80xis1 cl857)*] derivatives. Strain LZ23 (*gyrA^{L834} zei-723::Tn10*) is a C600 (*F- thr-1 leu-6 thi-1 lacY1 supE44 tonA21A*) derivative. The strains with mutant gyrase and/or topoisomerase IV alleles were kindly provided by L.Zechiedrich and were grown at 30°C. The *fis*⁻ derivatives of LZ41, LZ54 and LZ23 were obtained by phage P1 transduction using the strain CSH50*fis::cm* described in González-Gil et al. (1998). All strains were grown in 2× YT medium (16 g of tryptone, 10 g of yeast extract, 5 g of NaCl per litre, pH 7.4). Single colonies were isolated on YT plates (8 g of tryptone, 5 g of yeast extract, 5 g of NaCl, 15 g of agar per litre).

The constructs pWN1, pWN1-75A/C and pWN3 are described by Nasser et al. (2001). pWN1 (*fis* promoter–*lacZ* fusion) contains a *fis* promoter fragment encompassing positions –308 to +106 (relative to the *fis* operon transcriptional start at +1). pWN1-75A/C contains the same *fis* promoter fragment as pWN1 in which an A-to-C base substitution was introduced at position –75 (see Figure 1C) by a two-step PCR method. pWN3 (*div* promoter–*lacZ* fusion) contains the same DNA fragment as pWN1 inserted in opposite orientation with respect to the *lacZ* reporter gene (+106 to –308). The fragments comprising different deletions of the *fis* and *div* promoter regions, and their respective constructs are represented in Figures 3 and 4, respectively. All these fragments were PCR amplified and cloned in *EcoRI*–*NruI* sites of pTyrTlac (Schneider et al., 1999). Thereby, the *tyrT* promoter of pTyrTlac was replaced by these *fis* or *div* promoter DNA sequences. To construct pWN14-126T/G, the primers pdiv-126T/G comprising positions –156 to –117 and containing a T-to-G base substitution at –126 on the top strand (5'-GCCTATCGCGACTTTGAGTGTAATTTTAGTCACTATTTTCG-AATATGATG-3') (*NruI* site underlined) and p_{fis}-42*EcoRI* (5'-CGGAATTCCTTTGACCAATCCTCTGAG-3') (*EcoRI* site underlined)

were used for the PCR reactions on the pWN1 template. The resulting PCR product was cloned in *EcoRI*–*NruI* sites of pTyrTlac.

Proteins

RNAP was from Boehringer Mannheim. Purified IHF was kindly provided by Dr F.Boccard.

RNA isolation and primer extension analysis

Total *E. coli* RNA was isolated using the Qiagen RNeasy kit. The concentration of RNA in different samples was detected spectrophotometrically, normalized for each sample and verified on a reference gel, using rRNA as loading control. The primers used for specific detection of mRNA were 5' end-labelled ORF13' (5'-AGGCTCTGTCTG-TAATGCCAG-3'), p_{fis}+60 (5'-CTGAGCTGATATTGTCCG-3') and p_{fis}+9 (5'-CTGCAAGGCGGCGTATATTAC-3'), which anneal to mRNA molecules at positions –106 to –87, +60 to +43 and +9 to –12 relative to the *fis* operon transcriptional start, respectively. The radiolabelled primers (0.4 pmol) were annealed with 5–10 µg of RNA and reverse transcription was performed with Superscript II (Gibco-BRL) according to the manufacturer's recommendations. The extension products were resolved on a 6% sequencing gel and visualized by phosphorimaging. The length of the transcripts was identified using the corresponding dideoxy sequencing reactions as a reference.

In vitro transcription

Supercoiled plasmids were used for *in vitro* transcription and primer extension reactions according to Lazarus and Travers (1993). The mRNA obtained after *in vitro* transcription was divided into equal parts and used for primer extension by Superscript II (Gibco-BRL) with radioactively end-labelled primers for detection of different *fis* P, the *div* P and *bla* transcripts. The transcription of the *fis* promoters from pWN1 was evaluated by primer extension using the primers ORF13' for *fis* P1 and *fis* P2, and p_{fis}+9 for *fis* P3 and P4; the primer Δ 61+90 (5'-AAGTGCCACCTGACGTCTAAG-3') for *div* P; and the primer bla3B4 (5'-CAGGAAGGCAAAATGCCGC-3') for *bla*. The transcripts generated from *div* and *fis* P3 on pWN14 were detected by primer extension using the primers lacZ+473' (5'-GCAGGGTAGCCAAATGC-3') and Δ 61+90, respectively. The latter primer was used to directly compare the amount of mRNA transcribed from *div* and P2 promoters on pWN11 and pWN14, respectively. After primer extension, the samples were loaded onto 6% denaturing polyacrylamide gels. The transcripts were identified and visualized as described above and quantified using ImageQuant software. The data derived from at least three independent experiments were averaged before normalization.

Potassium permanganate reactivity assay

The reactions for potassium permanganate reactivity assays were performed with supercoiled templates. The reactions were performed similarly to those used for *in vitro* transcription, with minor modifications: plasmid DNA (1 µg) and proteins as indicated were incubated in 100 µl of a buffer containing 10 mM Tris-HCl pH 8.0, 200 mM NaCl, 0.2 mM dithiothreitol and 100 µM each GTP and CTP. After incubation at 37°C for 30 min, 0.1 vol. of 100 mM potassium permanganate solution was added for 15 s to the reaction mixtures containing DNA and proteins. The reactions were stopped by addition of 0.1 vol. of 14 M β -mercaptoethanol, 8 µg of sonicated salmon sperm DNA and sodium acetate to 0.3 M, precipitated with 3 vols of ice-cold ethanol and washed twice with 70% ethanol. The reaction products were solubilized in water and divided into two halves. Each half was used as a template for five cycles of amplification by *Taq* polymerase with 5'-radiolabelled primers to reveal the modified bases. The amplification products were analysed on 6% sequencing gels. The signals due to permanganate reactivity of bases were visualized, identified and quantified as described above.

β -galactosidase assay

Overnight cultures were diluted 1:50 in fresh 2× YT medium. Triplicate samples taken at the times indicated were assayed for β -galactosidase activity following the protocol of Sadler and Novick (1965). β -galactosidase units were multiplied by 1000 to make them equivalent to those of Miller (1972).

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