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Regulation of *crp* transcription by oscillation between distinct nucleoprotein complexes

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FIS belongs to the group of small abundant DNA-binding proteins of *Escherichia coli*. We recently demonstrated that, *in vivo*, FIS regulates the expression of several genes needed for catabolism of sugars and nucleic acids, a majority of which are also transcriptionally regulated by cAMP–cAMP-receptor protein (CRP) complex. Here we provide evidence that FIS represses transcription of the *crp* gene both *in vivo* and *in vitro*. Employing *crp* promoter–*lacZ* fusions, we demonstrate that both FIS and cAMP–CRP are required to keep the *crp* promoter in a repressed state. We have identified in the *crp* promoter other transcription initiation sites which are located 73, 79 and 80 bp downstream from the previously mapped start site. Two CRP- and several FIS-binding sites with different affinities are located in the *crp* promoter region, one of them overlapping the downstream transcription initiation sites. We show that initiation of transcription at the *crp* promoter is affected by the composition of nucleoprotein complexes resulting from the outcome of competition between proteins for overlapping binding sites. Our results suggest that the control of *crp* transcription is achieved by oscillation in the composition of these regulatory nucleoprotein complexes in response to the physiological state of the cell.

Keywords: cAMP–CRP/*crp* promoter/FIS/nucleoprotein complex/transcriptional regulation

Introduction

Escherichia coli is able to utilize a wide range of carbon and nitrogen sources. Many of these compounds are metabolized by inducible pathways. Catabolite repression in bacteria is a phenomenon by which glucose and its analogs decrease the intracellular level of both cAMP and its receptor protein, CRP. This in turn causes repression of a set of catabolite-sensitive operons such as *lac*, *ara*, *mal* and others. As glucose is consumed, cAMP and CRP levels increase and the cAMP–CRP complex activates a set of operons whose products are necessary for the recruitment and metabolism of alternative carbon sources (Ullmann and Danchin, 1983; Ishizuka *et al*., 1993; Kolb *et al*., 1993).

In addition, the cAMP–CRP complex participates in the regulation of a large number of other processes in *E.coli*. It mediates activation of genes whose expression is necessary for the metabolism of some amino acids (*tnaA*, *ilvB*, etc., Friden *et al*., 1982; Ullmann and Danchin, 1983), for the metabolism of nucleic acids (*deo*, *cdd*, etc., Ullmann and Danchin, 1983) and the synthesis of some membrane proteins (*ompA* and *ompF*, Scott and Harwood, 1980). Furthermore, cAMP–CRP is reported to be a repressor of its own transcription (Aiba, 1983; Cossart and Gicquel-Sanzey, 1985; Okamoto and Freundlich, 1986; Hanamura and Aiba, 1991), of genes for adenylate cyclase (Majerfeld *et al.*, 1981; Mori and Aiba, 1985), of genes encoding enzymes participating in glutamine metabolism (Prusiner *et al*., 1972) and of genes for the outer membrane protein III (Mallick and Herrlich, 1979).

CRP is composed of two identical subunits comprising 209 amino acids (Aiba *et al*., 1982; Cossart and Gicquel-Sanzey, 1982). When complexed with its allosteric effector, cAMP, it undergoes a conformational transition and binds to a 22 bp target site within, or close to, promoters. At its site of interaction, cAMP–CRP induces sharp bends in DNA ranging from 90 to 180° (Wu and Crothers, 1984; Liu-Johnson *et al*., 1986). X-ray crystallography and *in vitro* binding experiments have revealed that the C-terminal domain of the protein interacts with DNA, while the large N-terminal domain binds cAMP (Ogden *et al*., 1980; Lee *et al*., 1981; McKay and Steitz, 1981).

FIS is a small, homodimeric DNA-binding protein which, similarly to cAMP–CRP, performs a variety of different roles in *E.coli*. FIS stimulates stable RNA synthesis upon binding to the upstream activating sequences (UAS) of the corresponding promoters (Nilsson *et al*., 1990; Ross *et al*, 1990; Lazarus and Travers, 1993; Emilsson and Nilsson, 1995). FIS influences chromosomal replication by binding to *oriC* (Gille *et al*., 1991; Filutowicz *et al*., 1992; Hiasa and Marians, 1994; Wold *et al*., 1996), and is a repressor of its own transcription (Koch *et al*., 1991; Ninnemann *et al*., 1992). More recently, FIS has been shown to modulate expression of some RpoS-regulated genes (Xu and Johnson, 1995a,b,c), to regulate the promoters of the *hupA* and *hupB* genes encoding HU (Claret and Rouviere-Yaniv, 1996) and of the *hns* gene encoding H-NS (Falconi *et al*., 1996), and to regulate expression of several operons involved in the catabolism of sugars and nucleic acids (González-Gil *et al*., 1996). The intracellular FIS concentration varies during cell growth, being highest after nutritional upshift and leveling off during exponential growth (Thompson *et al*., 1987; Ball *et al*., 1992).

FIS and CRP exhibit related structural features which

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lead to similar modes of action. Both proteins contain a helix–turn–helix DNA-binding domain located at the C-terminus (McKay and Steitz, 1981; Kostrewa *et al*., 1991). Both proteins are homodimers in solution and bend the DNA upon binding (Liu-Johnson *et al*., 1986; Thompson and Landy, 1988). Their regulatory properties strongly depend on helical phasing (Gaston *et al*., 1990; Newlands *et al*., 1992). Additionally, both FIS and CRP bind to highly degenerate sequences (Ebright *et al*., 1984; Hübner and Arber, 1989), suggesting that not only sequence specificity, but other features like DNA topology and the nucleoprotein context in which a binding site is located (Pan *et al*., 1996) determine their interaction with DNA.

Transcription of *crp* is both activated and repressed by its own product. Activation occurs through binding of cAMP–CRP to the CRP II site located upstream of the *crp* promoter (Hanamura and Aiba, 1992), while repression depends on the CRP I site located downstream of the *crp* promoter (Aiba, 1983). It has been proposed that repression is accomplished through synthesis of a divergent RNA molecule whose transcription starts 2 bp upstream from the *crp* mRNA, runs in the opposite direction and is strongly dependent on cAMP–CRP (Okamoto and Freundlich, 1986; Okamoto *et al*., 1988). This scenario has been questioned by Hanamura and Aiba (1991) who reported that the divergent RNA does not repress *crp* transcription. They demonstrated that in the presence of cAMP–CRP the RNA polymerase preferentially binds to the divergent RNA promoter, while in its absence it binds to the *crp* promoter.

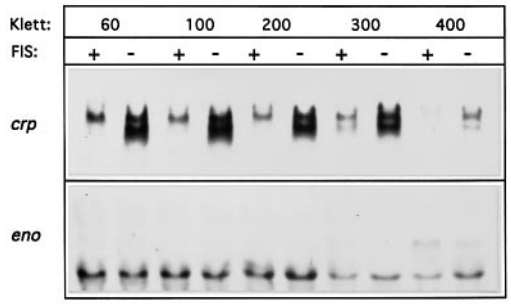
In a recent publication, we showed that FIS regulates expression of several operons involved in catabolism of sugars and nucleic acids (González-Gil *et al*., 1996). In most cases, the observed FIS regulation of the structural genes was indirect and was mediated through effects of FIS on the transcription of the respective repressor genes. A common feature of these FIS-regulated operons was that their transcription is activated by cAMP–CRP. This led us to investigate whether FIS also affects *crp* expression. In this study, we demonstrate that *crp* transcription is regulated by FIS and that both FIS and cAMP–CRP are required to keep the *crp* promoter in a repressed state. Furthermore, we present evidence that the transcriptional regulation of *crp* during cellular growth is achieved by changes in the composition of nucleoprotein complexes formed at the *crp* promoter.

Results

crp transcription is regulated by FIS

In order to assess whether FIS influences *crp* transcription, we prepared RNA from the isogenic strains CSH50 and CSH50 Δ*fis* at different time points during growth. Interestingly, Northern analysis revealed that *crp* was transcribed more actively in the absence than in the presence of FIS, and this held true for all time points analyzed, spanning early logarithmic to stationary phase. During logarithmic phase, up to 5-fold more *crp* RNA was detected in CSH50 Δ*fis* than in CSH50 (Figure 1).

The *crp* hybridization signal corresponding to RNA isolated from CSH50 Δ*fis* always consisted of two bands, the faster migrating band being more abundant than the slower migrating band. In RNA prepared from CSH50, the higher molecular weight *crp* signal was present at all time points, while the faster migrating RNA species was only detectable in late logarithmic and stationary phase cells (Figure 1). This result suggested that in the absence of FIS (CSH50 Δ*fis*) or at low FIS concentration (CSH50 at late logarithmic or stationary phases), two transcripts of different length originate from the *crp* promoter.



*crp**	0.27	0.21	0.19	0.30	0.56

*RNA ratio of *fis*$^+$ versus *fis* normalized to the *eno* control.

Fig. 1. Transcription of *crp* in *fis*$^+$ and *fis* cells by Northern analysis. Total RNA was prepared from CSH50 and CSH50 Δ*fis* at different time points during growth. The 60 Klett units point corresponds to the start of the logarithmic phase, 100 and 200 Klett to logarithmic phase, 300 Klett to late logarithmic phase and 400 Klett to stationary phase. Analysis of *eno* served as a control for constitutive expression (Weng *et al*., 1986). FIS+: CSH50 RNA. FIS–: CSH50 Δ*fis* RNA.

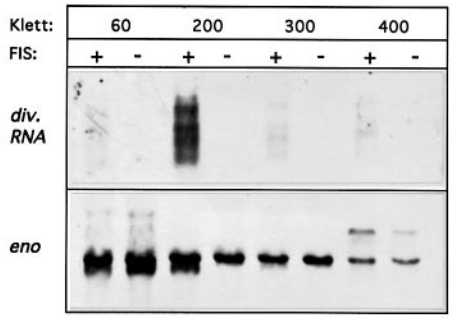


Fig. 2. Transcription of divergent RNA in *fis*$^+$ and *fis* cells by Northern analysis. RNA was isolated from CSH50 and CSH50 Δ*fis* at various time points during growth (see Figure 1). *eno* was analyzed as a control for constitutive expression. FIS+: CSH50 RNA. FIS–: CSH50 Δ*fis* RNA.

Since transcription of *crp* has been postulated to be autoregulated by activation of divergent RNA synthesis, one might expect that transcription from the divergent promoter would also be affected by FIS. Northern analysis revealed that in CSH50 the divergent RNA was most abundant at mid-log phase (200 Klett units in Figure 2). Since, in this strain, the amount of *crp* transcript was fairly constant during growth (Figure 1), divergent RNA synthesis apparently does not reduce *crp* transcription. Northern analysis of RNA isolated from CSH50 Δ*fis* demonstrated that the divergent RNA could not be detected at any time during growth (Figure 2). These results suggest that FIS is required for activation of the divergent promoter. Thus, FIS and cAMP–CRP regulate transcription from both the *crp* and divergent RNA promoters.

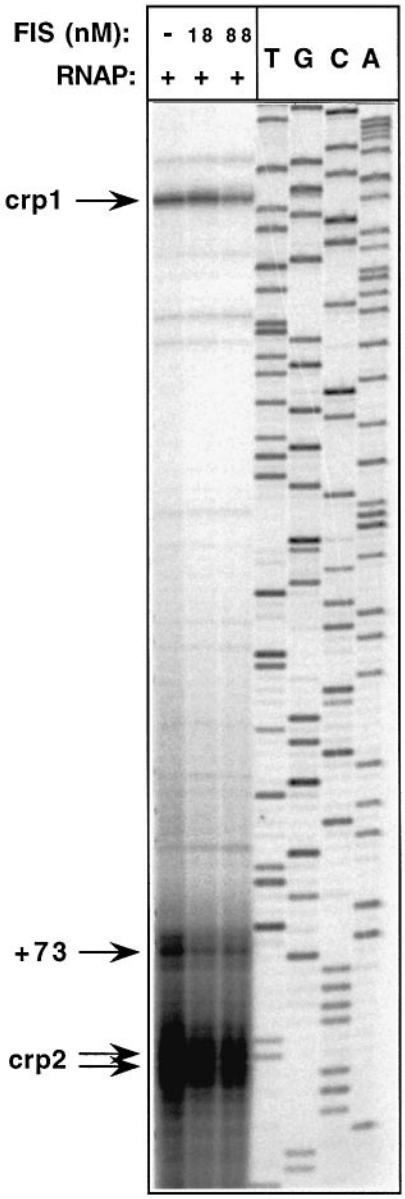


Fig. 3. Analysis of transcription initiation at the *crp* promoter and its sensitivity to FIS. A standard *in vitro* transcription reaction was performed with supercoiled pGC1 and 200 nM RNA polymerase (RNAP). Levels of specific transcripts were measured by primer extension. Dideoxy sequencing of pGC1 with the same primer was carried out for mapping transcription initiation sites. The four transcription initiation sites are indicated by arrows.

FIS prevents crp transcription initiation

To discriminate whether the two *crp* mRNA species detected in CSH50 and CSH50 Δ*fis* result from a processing of the major transcript or from transcription initiating at different sites, we analyzed the products of *crp* transcription *in vitro* using supercoiled templates. The initiation sites were detected by primer extension. Four transcripts were observed, the longest one (*crp1*) mapping to the start site at +1 (Figure 3). This band corresponded to the transcription start site previously mapped for the *crp* gene (Aiba, 1983; Okamoto and Freundlich, 1986). The shortest and most abundant message (*crp2*) comprised two transcripts differing by 1 bp in length and mapping to positions +79 and +80 respectively. A fourth transcript initiating at position +73 was also detected. The initiation frequency at position +73 was similar to that at *crp1* and noticeably lower than at *crp2*. Initiation at *crp2* and +73 sites was reduced in the presence of low FIS concentrations, while initiation at the *crp1* site was inhibited only at high FIS concentrations (Figure 3). Since all *crp1*, *crp2* and *crp* +73 messages are readily detectable in a purified *in vitro* system, we conclude that the *crp* promoter contains three transcription initiation sites.

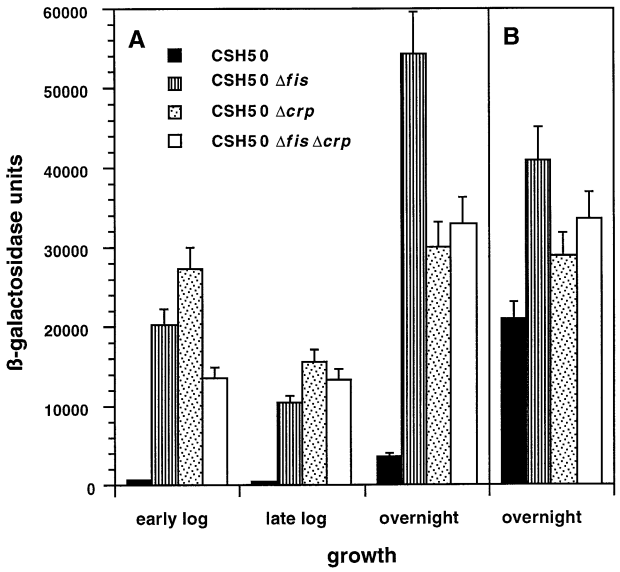


Fig. 4. Activity of the *crp* promoter in the four isogenic strains CSH50, CSH50 Δ*fis*, CSH50 Δ*crp* and CSH50 Δ*crp* Δ*fis*. Promoter activity was determined at different growth times with the help of the *crp* promoter–*lacZ* fusion plasmid pGClac. Early logarithmic phase corresponded to 60 Klett units for CSH50 and CSH50 Δ*fis*, and to 30 Klett units for CSH50 Δ*crp* and CSH50 Δ*crp* Δ*fis*. Late logarithmic phase for CSH50 and CSH50 Δ*fis* was reached at 250 Klett units, for CSH50 Δ*crp* at 120 Klett units and for CSH50 Δ*crp* Δ*fis* at 80 Klett units. β-Galactosidase units are averages from two experiments and were determined as described in Materials and methods. (**A**) cAMP (1 mM) was added to the culture; (**B**) culture grew without added cAMP.

Analysis of CRP expression in vivo

To elucidate further how repression of *crp* transcription takes place, we cloned the *crp* promoter (–161 to +200) upstream of a *lacZ* gene in the low copy number expression vector pRS415. The resulting plasmid (pGClac) harboring the *crp* promoter–*lacZ* fusion was introduced in the isogenic strains CSH50, CSH50 Δ*fis*, CSH50 Δ*crp* and CSH50 Δ*crp* Δ*fis*. β-Galactosidase activity of cell extracts prepared from overnight cultures of the respective strains revealed that the *crp* promoter was 2-fold more active in the absence than in the presence of FIS (Figure 4B). As expected, deletion of *crp* also led to an increased activity of the *crp* promoter (Figure 4B).

It has been described that CRP lowers the intracellular level of cAMP (Botsford and Drexler, 1978; Ishizuka *et al*., 1993). In order to determine whether the increased activity of the *crp* promoter in *fis* cells is the result of a reduced cAMP level such that no active cAMP–CRP complexes are formed, we grew the cultures in the presence of 1 mM cAMP. β-Galactosidase activity was determined at early and late logarithmic phase and from overnight cultures. As expected, addition of cAMP caused repression

of the *crp* promoter in CSH50 and remained without effect in CSH50 Δ*crp* and CSH50 Δ*crp* Δ*fis* (Figure 4A). In CSH50 Δ*fis*, cAMP addition did not repress the *crp* promoter, indicating that FIS is essential for repression of *crp* gene transcription. In the presence of 1 mM cAMP, β-galactosidase levels were up to 10-fold higher in CSH50 Δ*crp* than in CSH50 and up to 30-fold higher in the absence than in the presence of FIS. In addition, there was no difference in β-galactosidase activity between CSH50 Δ*crp* and CSH50 Δ*crp* Δ*fis*, except for early logarithmic growth. These results are consistent with the Northern analyses and indicate that both FIS and cAMP–CRP are required throughout the growth phase to keep the *crp* promoter in a repressed state.

FIS and CRP compete for binding to overlapping binding sites at the crp promoter

To investigate the mechanism by which FIS represses *crp* transcription, we mapped by DNase I footprinting the FIS- and CRP-binding sites on a *crp* promoter fragment encompassing sequences from –126 to +119. *In vitro*, FIS bound the *crp* promoter at sequences both upstream and downstream of the transcriptional start point, *crp1* (Figure 5A and B). The downstream protected region encompasses at least three adjacent FIS-binding sites which differ slightly in affinity. The site centered at +68 is occupied first, and the sites centered at +48 and +30 are protected only at higher FIS concentrations (Figure 5B, compare lanes 2 and 3; sites matching the consensus FIS-binding site are underlined in Figure 5A). Consistent with this observation, the site centered at +68 has the best match to the consensus FIS-binding site (Figure 5C). The far upstream site centered at –92 was occupied simultaneously with the site centered at +68 and showed a good fit to the consensus sequence (Figure 5B, lane 2, and Figure 5C). The FIS site centered at +30 overlapped the –35 element of the divergent RNA promoter. Binding of FIS at this site caused strong DNase I hypersensitivity at position +33, suggesting a substantial DNA distortion in this region. The high affinity FIS-binding site centered at +68 overlapped the transcription initiation site at +73 and almost overlapped the *crp2* site, suggesting that binding of FIS may prevent transcription initiation from these sites by steric hindrance.

Binding of cAMP–CRP alone protected the region between positions +29 and +49 and between positions –50 and –70, corresponding to CRP sites I and II respectively (Figure 5B, lanes 5–8). These CRP-binding sites were mapped previously by Aiba (1983) and Hanamura and Aiba (1992). Binding of cAMP–CRP at site I increased DNase I cleavage at positions +35 and +45 (Figure 5B, lanes 8 and 13). Likewise, binding of cAMP–CRP at site II increased DNase I cleavage at two positions within the –50 to –70 region (Figure 5B, lanes 8 and 13).

The FIS-binding sites centered at +48 and +30 overlap CRP-binding site I (Figure 5B, compare lanes 4 and 8). Addition of FIS (10 nM) together with cAMP–CRP (250 nM) resulted in the simultaneous occupation of CRP site I and the non-overlapping FIS site centered at +68, producing an extensive protected region downstream of the *crp* promoter. Similarly, CRP site II and the FIS site centered at –92 were occupied simultaneously (Figure 5B, lane 14). Increasing FIS concentrations relative to cAMP–CRP prevented binding of cAMP–CRP at site I, but had no effect on cAMP–CRP binding at site II, as evidenced from both the protection pattern by cAMP–CRP and the DNase I-hypersensitive sites (Figure 5B, lane 15). At a FIS/cAMP–CRP ratio of 100:250 nM, CRP-binding site II, as well as the –10 and –35 regions of the *crp* promoter, became occupied by FIS (Figure 5B, lane 16). In accordance with our primer extension analysis, this result suggests that occupation of secondary binding sites in the upstream region of the *crp* promoter at high FIS concentrations may both displace cAMP–CRP from binding to site II and prevent transcription initiation from the *crp1* site.

Analysis of crp transcription on linear templates in vitro

In order to determine the effect of the distinct nucleoprotein complexes formed at the *crp* promoter on transcriptional activity, we performed multiple round runoff assays using linear DNA templates encompassing the *crp* promoter sequences from –126 to +119. In contrast to the result obtained using supercoiled DNA templates, in the absence of other proteins, RNA polymerase (RNAP) initiated transcription from *crp1*, but not from *crp2* or +73 sites (Figure 6, lane 1). In line with previous reports (Hanamura and Aiba, 1992), addition of cAMP–CRP activated transcription from the divergent promoter and markedly reduced initiation at *crp1* (Figure 6, lanes 2–5). At high cAMP–CRP concentrations, the repression of the *crp1* promoter was relieved (Figure 6, lane 5). In contrast, FIS repressed *crp1* transcription in a concentration-dependent manner (Figure 6, lanes 6–9) without activating the divergent RNA promoter. Furthermore, FIS prevented activation of the divergent promoter even in the presence of cAMP–CRP (Figure 6, lanes 10–13). Addition of cAMP–CRP in combination with FIS slightly alleviated the repression of the *crp1* promoter, but not of the divergent promoter, indicating that the divergent promoter is repressed by FIS even more efficiently than is the *crp1* promoter. These results are consistent with the DNase I protection experiments which show that FIS displaces cAMP–CRP more efficiently from binding site I than from site II (see Figure 5B). Taken together, these results indicate that the nucleoprotein complexes formed at the *crp* promoter differentially affect promoter activity.

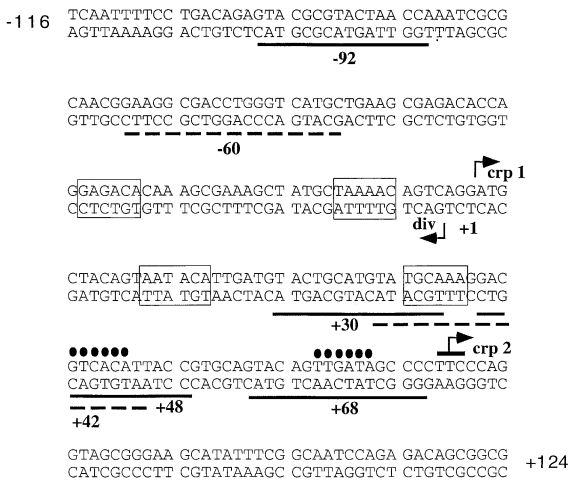
Discussion

Regulation of crp transcription

Here we have shown that in addition to cAMP–CRP, another *trans*-acting factor, FIS, is indispensable for repression of *crp*. In CSH50 Δ*fis* cells, the endogenous *crp* transcription was strongly enhanced during all phases of growth. Moreover, the *crp* promoter on a low copy number plasmid was derepressed in CSH50 Δ*fis* after overnight culture (measured by β-galactosidase activity), although these cells overproduced CRP and cAMP was added to the culture. These *in vivo* results clearly indicate that both proteins, FIS and cAMP–CRP, are required for *crp* repression, since the lack of either transcriptional regulator leads to a derepressed *crp* promoter.

In CSH50, a fairly constant level of *crp* mRNA was transcribed during growth (see Figure 1, upper band). The

A



C

G NN YR NNNNN YR NN C	consensus
G TA CG CGTAC TA AC C	-92
G TA C**T** GCATG TA TG C	+30
G GA CG TCACA T**T** AC C	+48
G TA CA GTTGA TA GC C	+68

B

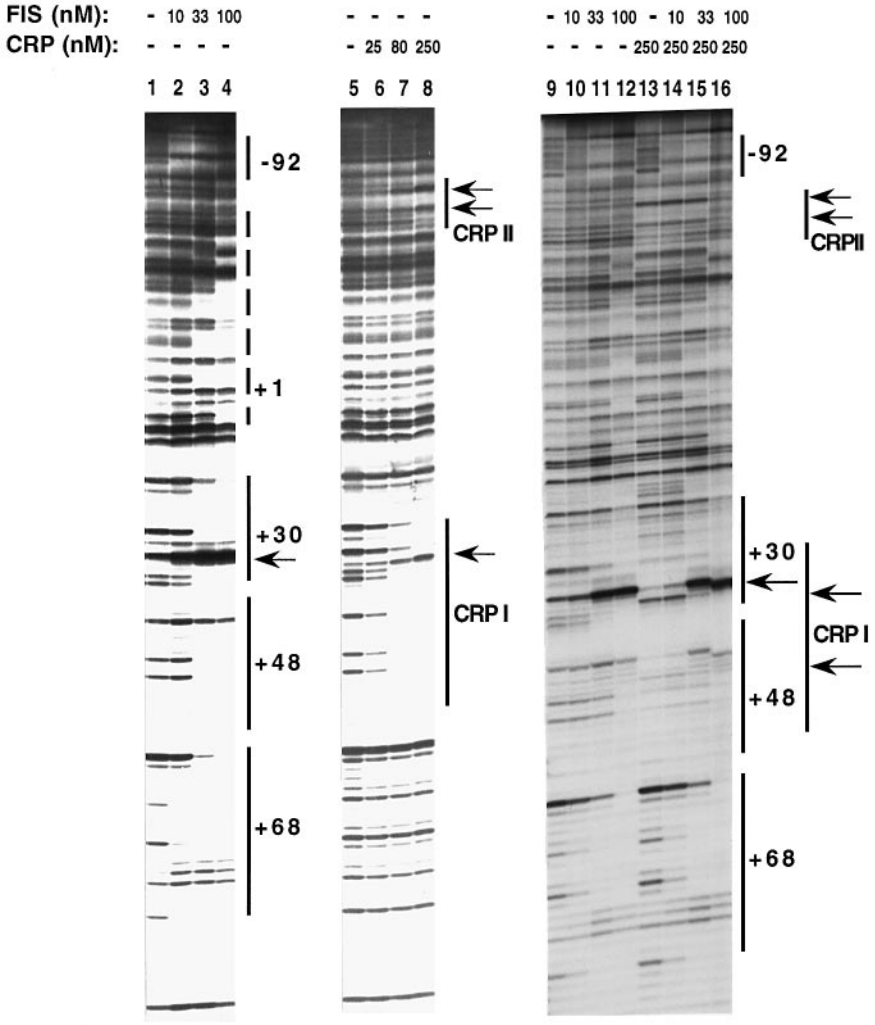


Fig. 5. DNase I footprinting of FIS- and CRP-binding sites in the *crp* promoter. (**A**) Sequence of the *crp* promoter. The footprinting results are indicated: the FIS-binding sites are underlined by solid lines and the CRP-binding sites by dashed lines. The –10 and –35 hexamers of the *crp1* and divergent promoters are boxed. The putative –10 and –35 hexamers corresponding to the *crp2* promoter are indicated by ellipsoids above the sequence. Numbering is with respect to the *crp1* initiation site positioned at +1. (**B**) DNase I footprints obtained on a 245 bp *crp* fragment uniquely 5′ end-labeled on the bottom strand. Lanes 1, 5 and 9 represent free DNA. In lanes 2–4, the sites protected by increasing FIS concentrations centered at positions –92, +30, +48 and +68 (numbered with respect to the start point of *crp1* promoter at +1) are indicated. The protected region overlapping the *crp1* initiation site is indicated by a dashed line. In lanes 6–8, increasing concentrations of cAMP–CRP were added. The CRP-binding sites I (centered at +42) and II (centered at –60) are indicated. In lanes 13–16, CRP binding was assayed with increasing concentrations of FIS. Note that FIS at a concentration of 33 nM displaces CRP from the CRP site I and at 100 nM also from the CRP site II. Arrows point to DNase I-hypersensitive sites. (**C**) Comparison of the mapped FIS-binding sites with the degenerate consensus FIS-binding site (Hübner and Arber, 1989).

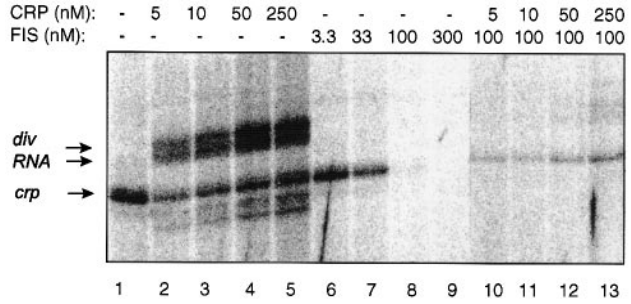


Fig. 6. Effect of CRP and FIS on transcription of the *crp* promoter *in vitro*. A 245 bp *crp* promoter fragment was used as template for *in vitro* transcription as outlined in Materials and methods. Products were analyzed on a 6% denaturing polyacrylamide gel. Transcription reactions contained: RNAP alone (lane 1); RNAP with increasing concentrations of CRP (lanes 2–5); RNAP with increasing concentrations of FIS (lanes 6–9); and RNAP, FIS and increasing concentrations of CRP (lanes 10–13). The 119 bp *crp1* and 124 bp divergent promoter products are indicated. On switching on the divergent promoter, several additional minor transcripts appear, probably due to aberrant initiation by RNAP. Transcription from the downstream *crp* initiation sites is inefficient under these conditions, precluding its experimental analysis.

transcription start site of this mRNA coincides with the one already mapped by two different groups (Aiba, 1983; Okamoto and Freundlich, 1986). From late logarithmic to stationary phase in CSH50, as well as in CSH50 Δ*fis*, a second mRNA species of smaller size was also detected. Transcription of this smaller mRNA is initiated 73, 79 and 80 bp downstream from the first transcriptional start site. These RNA species were probably not detected by the aforementioned groups because they used RNA prepared from *fis*$^+$ cells at early logarithmic growth. Our results indicate that binding of FIS to high affinity sites in the downstream region of the *crp* promoter prevents transcription initiation from +73 and *crp2* sites. Interestingly, the downstream sites were used very inefficiently when transcription was analyzed on linear DNA templates *in vitro*. It is possible that specific template topology, e.g. a high level of negative supercoiling of DNA, is required to activate these sites for transcription. We note that the *crp2* site is used very efficiently in *fis* cells, which are characterized by high levels of negative supercoiling (Schneider *et al*., 1997). In line with this notion, transcription initiation at *crp2* is very efficient on negatively supercoiled templates *in vitro* (Figure 3). The putative RNAP-binding sequence identified within this region contains features characteristic of supercoiling-dependent promoters such as suboptimal –35 and –10 hexamers, a 16 bp spacer and a GC-rich discriminator sequence between the –10 hexamer and the initiation site (Figure 5A; Borowiec and Gralla, 1987; Giladi *et al*., 1992; Jordi *et al*., 1995). However, additional experiments are required to define unequivocally the RNAP-binding elements of the *crp2* promoter as well as those giving rise to transcription initiation at position +73.

The divergent RNA could only be detected in *fis*$^+$ cells *in vivo*. However, it is unlikely that FIS directly activates the divergent RNA promoter for the following reasons. First, we did not see any activation of the divergent RNA promoter by FIS *in vitro*. Furthermore, as shown in our footprint analyses, binding of FIS at a site contained in the divergent RNA promoter caused a strong DNase I hypersensitivity at position +33, suggesting a substantial distortion of DNA in the –35 region of this promoter. Additionally, maximal expression of the divergent RNA was only detected *in vivo* at late log phase, when the level of FIS decreases (Ninnemann *et al*., 1992). Importantly, the *crp2* initiation site is the stronger site in the absence of FIS *in vivo* and on supercoiled templates *in vitro*, as demonstrated by our Northern and primer extension analyses (Figures 1 and 3). We suggest that the absence of the divergent RNA message in *fis* cells is due to the efficient utilization of the downstream *crp* initiation site, which would preclude transcription initiation at the divergent promoter. At late log phase in *fis*$^+$ cells, the FIS concentration might be too low to repress the divergent RNA promoter, but still high enough to prevent initiation at the downstream *crp* sites. Optimal cAMP–CRP levels might then activate divergent RNA synthesis. Indeed, at low FIS concentrations, binding of cAMP–CRP to site I occurs simultaneously with binding of FIS to its high affinity site centered at +68 (Figure 5) which overlaps the –10 region of the putative *crp2* promoter and thus may occlude binding of RNAP at this site.

Our results suggest the following model for regulation of *crp* transcription during the growth phase (Figure 7). After dilution of an overnight culture into fresh medium (nutritional upshift), a large amount of FIS protein is synthesized immediately (Thompson *et al*., 1987; Ball *et al*., 1992; Ninnemann *et al*., 1992). Under these circumstances, it is very likely that the specific FIS-binding sites downstream of the *crp* promoter are occupied by FIS and transcription can only initiate at the *crp1* site. Complete repression of the upstream initiation site by binding of FIS to secondary sites would be prevented by binding of cAMP–CRP at site II, which relieves repression by FIS and activates *crp1* transcription (Hanamura and Aiba, 1992; this study). However, binding of FIS to its downstream sites might also limit transcription initiated at *crp1*. As growth progresses, the intracellular FIS concentration decreases, whereas the cAMP level increases. cAMP–CRP complexes are formed which, at lowered FIS levels, activate divergent RNA synthesis. At this stage, initiation at the *crp1* site would not become limited by FIS, but by the concurrent transcription from the divergent promoter, whereas *crp* transcription from the downstream initiation sites would be repressed completely. From late logarithmic growth onwards, the intracellular FIS concentration is too low to allow saturation of binding sites. Transcription of the divergent RNA would be reduced to its basal level due to transcription of *crp* RNA from the downstream promoter, which becomes activated in the absence of FIS. Under these conditions, *crp* transcription would initiate at *crp1* and downstream sites.

Biological implications

The cAMP–CRP complex is often found to be involved in the formation of multiprotein regulatory assemblies. For example, to bind to and achieve repression of the *deoP2*, *cdd* and *udp* promoters, the CytR repressor has to interact with cAMP–CRP (Holst *et al*., 1992; Sogaard-Andersen and Valentin-Hansen, 1993; Brikun *et al*., 1996). The proximity of CRP- and FIS-binding sites in the *crp* promoter suggests that specific nucleoprotein complexes containing FIS and CRP are formed and act as transcrip-

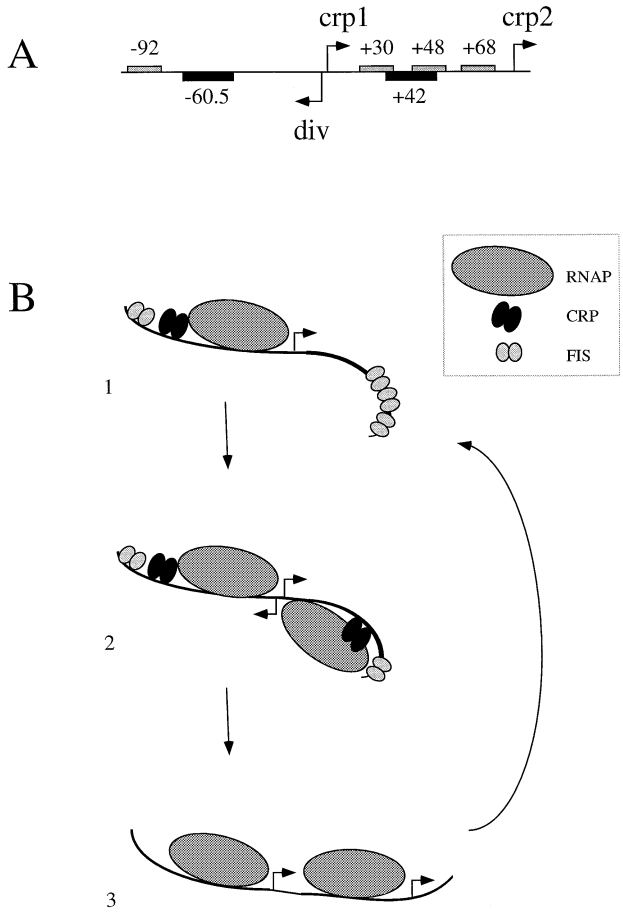


Fig. 7. Oscillation model of *crp* regulation. (**A**) Schematic representation of the *crp* promoter region. The FIS- and CRP-binding sites are indicated by black and gray rectangles, respectively. The binding sites are numbered by positions at which they are centered with respect to the *crp1* initiation site. The upstream and downstream initiation sites of the *crp* promoter, as well as of the divergent promoter are indicated. The transcription initiation sites at positions +73, +79 and +80 are assumed to be used by the same RNAP molecule bound at the *crp2* promoter (see Figure 5A). (**B**) 1. Dilution of an overnight culture into fresh medium induces a high level of FIS which binds to the *crp* promoter, blocking transcription from the downstream initiation sites by steric hindrance and reducing transcription from the *crp1* site. 2. During logarithmic growth phase, the concentration of FIS decreases, whereas increasing cAMP–CRP levels activate divergent RNA synthesis, limiting the frequency of initiation at the *crp1* site. Although the intracellular FIS concentration diminishes as growth progresses, it is sufficient to block *crp* transcription initiation from the downstream sites. 3. During late logarithmic to stationary growth phase, depletion of intracellular FIS levels allows *crp* transcription initiation at the downstream sites. The role of FIS binding at its site centered at –92 is not clear. The DNA is shown as a thin line. The direction of DNA bending by bound proteins is arbitrary.

tional barriers for RNAP. The levels of FIS in the cell vary with growth and nutritional supply, and it has been proposed that FIS may serve as an indicator for environmental conditions (Ninnemann *et al*., 1992; González-Gil *et al*., 1996). We show that the competition between FIS and CRP for occupation of binding sites in the *crp* promoter may be the key mechanism for this sensing to occur. The composition of nucleoprotein complexes formed at this promoter could oscillate in response to the nutritional conditions of the cell. Moreover, sequential loss of FIS from binding sites having different affinities during the growth phase could coordinate such transitions with the phase of the growth cycle. This situation closely resembles oscillations in the composition of regulatory nucleoprotein complexes formed at the origin of chromosomal replication in *E.coli* where FIS is also involved (Cassler *et al*., 1995), and may reflect a general strategy used by the bacterial cell for rapid adaptation to changing growth conditions.

The results presented unravel one more function of the small DNA-binding protein FIS and further substantiate the importance of this protein in transcriptional control in *E.coli*.

Materials and methods

Strains and plasmids

Bacterial strains used in this study were *E.coli* K12 derivatives. CSH50 is *ara* Δ(*lac pro*) *thi* (Miller, 1972). CA8445-1 (Δ*crp*-45; Sabourin and Beckwith, 1975) was kindly provided by B.Bachmann. Construction of CSH50 Δ*fis* was as described elsewhere (González-Gil *et al*., 1996). CSH50 Δ*crp* was constructed by phage P1 transduction of the Δ*crp*-45 mutation from CA8445-1 into CSH50. Δ*crp* mutants were screened by co-transduction of *rpsL* located at 73 min on the *E.coli* map (*crp* is at min 74). CSH50 Δ*crp* Δ*fis* was created by phage P1 transduction of the Δ*fis* mutation from CSH50 Δ*fis* into CSH50 Δ*crp* and selection for chloramphenicol resistance.

A PCR-derived fragment comprising the *crp* promoter from –161 to +200 flanked by *Eco*RI and *Sph*I restriction sites was digested with the same enzymes and cloned in pSP72 (Promega), giving rise to pGC1. Plasmid pGC2 was obtained by restriction of pGC1 with *Hin*dIII (the *crp* sequence from +148 to +200 is eliminated) and religation. pRS415 is a pBR322 derivative designed to measure promoter strength as an operon fusion with *lacZ* (Simons *et al*., 1987). To construct pGClac, the *Eco*RI–*Pvu*II *crp* fragment (–161 to +200) from pGC1 was cloned into pRS415 previously digested with *Bam*HI, the overhang filled with Klenow polymerase and redigested with *Eco*RI.

Proteins

FIS and RNAP were purified as described previously (Koch and Kahmann, 1985; Metzger *et al*., 1993). Purified CRP was kindly provided by A.Kolb.

Growth of strains

Overnight cultures were diluted 1:200 in dYT medium (Miller, 1972) and grown with vigorous shaking at 37°C until the indicated cell densities. For CSH50 and CSH50 Δ*fis*, 60 Klett units correspond to early logarithmic growth, 100–200 Klett units represent logarithmic growth, 300 Klett units are reached at late logarithmic growth and 400 Klett units are stationary growth cells. Although the time taken by CSH50, CSH50 Δ*fis*, CSH50 Δ*crp* and CSH50 Δ*crp* Δ*fis* to reach stationary phase was the same, the last two strains showed reduced growth in dYT medium, reaching the stationary growth phase at ~170 and 100 Klett units respectively.

RNA isolation and Northern analysis

Samples of ~10^{10} cells were chilled on ice and collected by centrifugation. The pellet was resuspended in 0.6 ml of LETS buffer (100 mM LiCl, 10 mM EDTA, 10 mM Tris–HCl pH 7.5, 0.2% SDS). An equal volume of phenol/chloroform was added and vortexed for 1 min. After centrifugation, the RNA was precipitated by adding LiCl to a final concentration of 0.5 M and 2 vols of ethanol. The RNA was resuspended in 100 µl of H_2O. For Northern analysis, 20 µg of RNA per lane was separated on a 1% agarose gel containing formaldehyde (Sambrook *et al*., 1989) and transferred to GeneScreen Plus filters (DuPont). Filters were hybridized overnight with digoxigenin-labeled oligonucleotides (Boehringer) which were complementary to the RNA. Luminescent detection of the hybrid bands was performed according to instructions given by the supplier.

The following oligonucleotides were used: *eno*, 5′-TGACGGAGCA-GCTGCCATACCGACGA-3′; *crp*, 5′-CTGATTCAGATAGGAGAGG-ATCAT-3′; and divergent RNA, 5′-GAGTACGCGTACTAACCA-AATCGC-3′.

DNase I footprinting

DNase I footprinting was performed with a 245 bp *crp* promoter fragment end-labeled on the bottom strand. The conditions of footprinting were essentially as described earlier (Muskhelishvili *et al*., 1997). The *crp* promoter region (–126 to +119) was PCR amplified using the primers CAP3 (5′-CTGTCTCTGGATTGCCGAAATATG-3′) and CAP5 (5′-CTCCACTGCGTCAATTTTCCTG-3′) and the pGC1 DNA as template. The primer CAP3 was uniquely end-labeled by using [γ-^{32}P]ATP and T4 polynucleotide kinase. The obtained fragment was purified by PAGE using a neutral 0.5× TBE gel. The incubation mixture contained 10 mM Tris–HCl, pH 7.9, 75 mM NaCl, 1 mM dithiothreitol (DTT), 0.2 mM cAMP, and CRP and FIS as indicated in a 20 µl volume. After incubation for 60 min at 37°C, DNase I and $MgCl_2$ were added to 2 µg/ml and 10 mM final concentrations respectively. The reaction was terminated after 10 s by adding 80 µl of a solution containing 0.5% SDS and 50 mM EDTA. After digestion by proteinase K for 45 min at 45°C and phenol extraction, the aqueous phase was precipitated with ethanol. The pellets were washed with 70% ethanol, dried, dissolved in loading dye and analyzed on a 6% denaturing polyacrylamide gel. Protected and hypersensitive bands were identified by using the Maxam–Gilbert G-ladder (Maxam and Gilbert, 1977) of the same DNA fragment as reference.

In vitro transcription

Supercoiled templates. *In vitro* transcription was carried out using 2 µg of supercoiled pGC1 DNA in a buffer containing 10 mM Tris–HCl pH 8, 2 mM DTT, 100 mM NaCl, 10 mM $MgCl_2$, 2.5 mM each ATP, GTP, CTP and UTP, 200 nM RNAP, and FIS as indicated for 30 min at 30°C in a 50 µl reaction volume. The reaction was stopped by adding 250 µl of 1 mM EDTA, 50 nM NaOAc, 0.2% SDS, 10 mM Tris–HCl pH 7.4 and 10 µg/ml proteinase K. After incubation for 30 min at 42°C, the reactions were extracted with phenol followed by two rounds of precipitation with ethanol in the presence of 0.3 M NaOAc and 10 µg of carrier tRNA. Half of the *in vitro* reaction products were used for primer extension.

Linear templates. Multiple round runoff transcription reactions were performed with a 245 bp *crp* promoter fragment (–126 to +119) (10 nM) in a buffer containing 10 mM Tris–HCl pH 7.9, 75 mM NaCl, 1 mM DTT, 0.2 mM cAMP, CRP and FIS as indicated, 66 nM RNAP, 1 mM each GTP, CTP and ATP, and 0.1 mM [α-^{32}P]UTP, in a 20 µl volume at 37°C. The reaction was terminated in 15 min by addition of an equal volume of formaldehyde loading dye and heating to 94°C. The samples were loaded on 6% denaturing polyacrylamide gels and analyzed by phosphorimaging (PhosphorImager Storm 840, Molecular Dynamics).

Primer extension

In vitro transcription products were annealed with ~1 ng of 5′-end-labeled synthetic primer CAP3 (see above) after heating to 70°C for 5 min, quick chilling on ice and subsequent incubation at 42°C for 5 min in a 10 µl volume containing 40 U of RNase inhibitor (Boehringer Mannheim). Primer extension was carried out using 200 U of Super-Script™II reverse transcriptase (Gibco-BRL) in a buffer supplied by the manufacturer. After incubation for 1 h at 37°C, the reverse transcriptase was inactivated by heating at 70°C for 15 min. After addition of an equal volume of formaldehyde loading dye, the reaction products were analyzed on 6% denaturing polyacrylamide gels as described above.

β-Galactosidase determinations

Overnight cultures were diluted 1:200 in fresh dYT medium which was supplemented with 1 mM cAMP where indicated. Samples taken at the indicated times 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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