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FIS activates sequential steps during transcription initiation at a stable RNA promoter

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FIS (factor for inversion stimulation) is a small dimeric DNA-bending protein which both stimulates DNA inversion and activates transcription at stable RNA promoters in *Escherichia coli*. Both these processes involve the initial formation of a complex nucleoprotein assembly followed by local DNA untwisting at a specific site. We have demonstrated previously that at the *tyrT* promoter three FIS dimers are required to form a nucleoprotein complex with RNA polymerase. We now show that this complex is structurally dynamic and that FIS, uniquely for a prokaryotic transcriptional activator, facilitates sequential steps in the initiation process, enabling efficient polymerase recruitment, untwisting of DNA at the transcription startpoint and finally the escape of polymerase from the promoter. Activation of all these steps requires that the three FIS dimers bind in helical register. We suggest that FIS acts by stabilizing a DNA microloop whose topology is coupled to the local topological transitions generated during the initiation of transcription.

Keywords: DNA microloops/FIS/RNA polymerase/surface plasmon resonance/transcription activation

Introduction

FIS (factor for inversion stimulation) is a small homodimeric DNA-bending protein from *Escherichia coli* which both facilitates DNA inversion (Huber *et al.*, 1985; Johnson and Simon, 1985; Kahmann *et al.*, 1985) and activates transcription from stable RNA promoters (Nilsson *et al.*, 1990; Ross *et al.*, 1990). Both stable RNA transcription and DNA inversion are stimulated strongly by negative supercoiling of DNA (Mertens *et al.*, 1984; Lamond, 1985; Bowater *et al.*, 1994) and involve the initial formation of a complex nucleoprotein assembly followed by DNA untwisting at the transcription startpoint and crossover sites respectively (Ohlsen and Gralla, 1992a; Klippel *et al.*, 1993).

The promoters of stable RNA (tRNA and rRNA) operons of *E.coli* can achieve the highest rates of initiation of all bacterial promoters. Under physiological conditions, these promoters are probably not saturated by RNA polymerase (Zhang and Bremer, 1995), and their regulation, which reflects the importance of their products for essential cellular functions, allows the rate of initiation to be varied over a wide range. The stable RNA promoters contain two important regulatory elements, a GC-rich discriminator between the –10 region and the transcription startpoint (Travers, 1980a) and an upstream activating sequence (UAS) which extends to ~120–150 bp upstream of the startpoint and is required for optimal expression (Lamond and Travers, 1983; Gourse *et al.*, 1986; van Delft *et al.*, 1987). The discriminator is a necessary response element for a stringent control system which abrogates stable RNA synthesis in response to amino acid starvation (Cashel *et al.*, 1996) and is mediated by the nucleotide ppGpp *in vivo* and *in vitro* (Travers, 1980b; Lamond and Travers, 1985; Hernandez and Cashel, 1995; Josaitis *et al.*, 1995; Zhang and Bremer, 1995).

The UAS DNA is anisotropically flexible (Drew and Travers, 1985; Gourse *et al.*, 1986; Plaskon and Wartell, 1987) and contains, in addition, three binding sites for the FIS protein positioned in helical register (Nilsson *et al.*, 1990; Ross *et al.*, 1990; Condon *et al.*, 1992; Lazarus and Travers, 1993), suggesting that bending of the UAS is necessary for transcriptional activation. Consistent with this notion, the UAS can function *in vitro* both with and without FIS (Newlands *et al.*, 1991; Zacharias *et al.*, 1992; Gaal *et al.*, 1994). However, bending of the UAS DNA by FIS, although necessary, is not sufficient for activation *in vivo* since a class of FIS mutants has been isolated which bind and bend DNA but fail to activate transcription (Gosink *et al.*, 1993). Some of these mutants are impaired in cooperative binding to DNA, indicating that transcriptional activation *in vivo* may require the participation of more than one FIS dimer (L.Lazarus, O.Ninnemann, R.Kahmann and A.A.Travers, unpublished results). In agreement with this observation, we have shown recently that, *in vitro*, FIS forms a specific nucleoprotein complex at the UAS which recruits polymerase to the *tyrT* promoter (Muskhelishvili *et al.*, 1995), an effect which requires all three FIS-binding sites positioned in helical register. On this basis, we proposed that the UAS forms a microloop which is stabilized by FIS.

The formation of the transcription initiation complex at bacterial promoters is a sequential process in which the initial formation of a closed polymerase–promoter complex is followed by structural transitions in both the enzyme and DNA, which eventually result in the untwisting of DNA at the transcription startpoint (Buc and McClure, 1985). It is this latter step which is antagonized by ppGpp (Ohlsen and Gralla, 1992a). The polymerase then initiates transcription and escapes from the promoter. Each of these steps is potentially rate-limiting and subject to control by transcriptional activators. There is substantial evidence that at the *rrnB* P1 promoter FIS recruits RNA polymerase into a closed complex and thus increases the

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K_B (Bokal *et al.*, 1995). However, other experiments indicate that FIS may also activate subsequent steps in the initiation pathway. In particular, FIS overrides the inhibitory action of ppGpp on *tyrT* transcription (Lazarus and Travers, 1993) and at *rrnD* P1 FIS facilitates the transition to the elongating complex (Sander *et al.*, 1993). In this study, we show directly that FIS affects sequential steps on the initiation pathway, thereby optimizing the interaction of polymerase with the promoter and facilitating high rates of initiation.

Results

Kinetics of FIS–RNA polymerase complex formation at the tyrT promoter

Surface plasmon resonance (SPR) techniques measure small local changes in refractive index at a surface containing a fixed ligand, and can be used to monitor relative affinities of proteins binding to immobilized DNA fragments (Fisher *et al.*, 1994; Buckle *et al.*, 1996). A unique advantage of this technique is the ability to study the real-time kinetics of very early steps in the initiation process. To examine the effects of FIS on ternary complex formation at the *tyrT* promoter, we immobilized biotin end-labelled promoter fragments containing the FIS sites to streptavidin surfaces in a BIAcore SPR machine (BIAcore AB). Two fragments were used in this study: a 197 bp wild-type sequence containing the three FIS sites in helical register upstream of the *tyrT* promoter and a 203 bp mutant fragment with a 5 bp insertion at position –98 immediately upstream of FIS site II (Figure 1A). This insertion weakens the central FIS-binding site (site II) and disrupts the helical register of sites I and III. Consequently FIS should no longer induce a coherent bend in the UAS. Functionally the mutation prevents the formation of a FIS-dependent polymerase–promoter complex, as observed by gel retardation (Lazarus and Travers, 1993; Muskhelishvili *et al.*, 1995).

Binding of proteins was monitored after their injection into the flowcell containing the surface-immobilized DNA fragments. SPR analysis of the binding of RNA polymerase alone to the wild-type and mutant fragments revealed that the enzyme has a 10-fold higher affinity for the wild-type than for the mutant promoter. This is illustrated by an enhanced overall association rate, leading, however, to final complexes of comparable stability (Table I). We therefore conclude that the rate of polymerase–promoter complex formation at the wild-type promoter is 10 times as rapid as at the mutant promoter and that this difference is due uniquely to the presence of a 5 bp insertion at position –98 in the UAS.

By analysing the kinetics of FIS binding (Figure 1B), we assumed that the three FIS sites in the UAS are characterized by two distinct affinities (sites I and III being of higher affinity than site II, Lazarus and Travers, 1993). The calculated binding constants are shown in Table II. The results are consistent with FIS saturating sites I and III on both fragments but only poorly binding site II on the mutant fragment.

The formation of a ternary complex between FIS, polymerase and the promoter DNA reached a steady-state equilibrium at the mutant promoter (Figure 1C), but at the wild-type promoter an anomalous profile was obtained

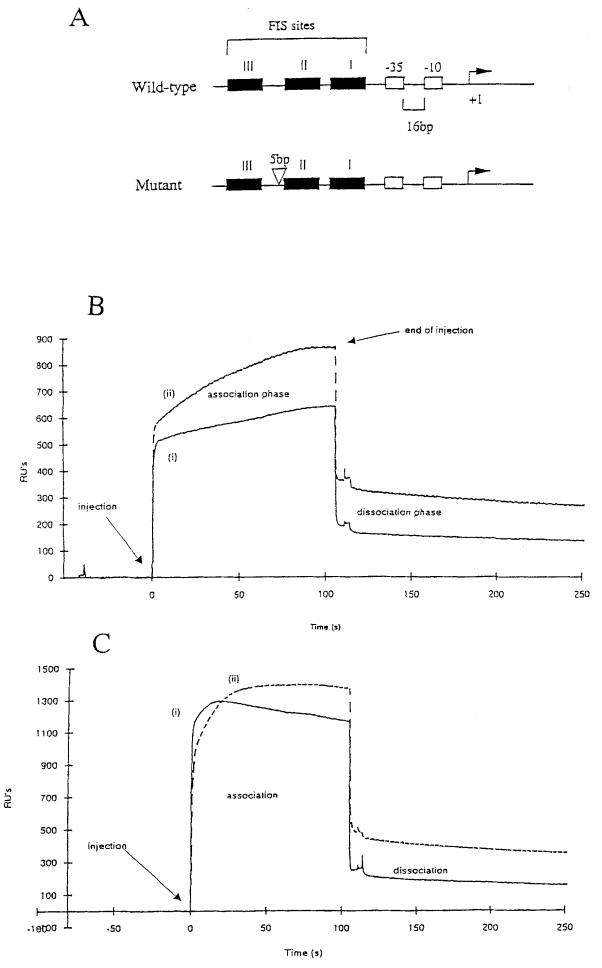

Fig. 1. (**A**) The *tyrT* promoter fragments used in this study. The startpoint of transcription, the –10 and –35 hexamers, the UAS region with three FIS-binding sites and the 5 bp insertion which disrupts the helical phasing of sites I and III are indicated. Note that the spacer length between the –10 and –35 hexamers is 16 bp rather than the consensus 17 bp. (B and C) SPR measurements on RNA polymerase and FIS binding independently and together to immobilized wild-type and mutant fragments. The relative change in refractive index expressed as a change in resonance angle (RU) as protein binding to an immobilized wild-type (i) or mutant (ii) fragment on a sensor surface in the BIAcore (BIAcore AB) apparatus was monitored over time as described in Materials and methods. The initial large increase as protein was injected is due to the large refractive index effect of the extraneous glycerol carrying over from the protein; the ensuing increase leading to steady-state refers to the binding of protein to the DNA and represents the phase used for kinetic analysis. At the end of the injection period, buffer alone flows across the surface and the decrease in RUs reflects the dissociation of protein from the DNA. (**B**) FIS (35 nM monomer) injected at 5 μl/min at 37°C across immobilized wild-type (i) and mutant (ii) DNA fragments in 20 mM Tris–HCl, pH 7.9, 50 mM NaCl, 50 mM KCl, 0.1 mM DTT, 0.005% surfactant P20 (BIAcore AB), at 37°C. (**C**) RNA polymerase (44 nM) and FIS (35 nM monomer) were pre-incubated at 37°C in 20 mM Tris–HCl, pH 7.9, 50 mM NaCl, 50 mM KCl, 0.1 mM DTT, 0.005% surfactant P20 (BIAcore AB) and then injected across immobilized wild-type (i) or mutant (ii) DNA fragments. The different maximum levels attained reflect differing amounts of DNA bound to the surface (see Materials and methods).

in which after reaching a maximum value the signal then decreased during the injection of proteins. Such a profile may be indicative of an evolving interaction in which the rapidly attained steady-state shifts to a final equilibrium state that is different from that originally established. In

Table I. Rate and equilibrium constants derived from sensorgrams of RNA polymerase on wild-type and mutant promoters

Promoter	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_D (M)
Wild-type	6.7 (± 0.13)×10^5	2.9 (± 0.03)×10^{-3}	4.3×10^{-9}
Mutant	4.5 (± 0.15)×10^4	2.3 (± 0.03)×10^{-3}	51×10^{-9}

For the wild-type promoter (197 bp), ~7.39×10^{-16} mol of DNA was immobilized at the surface (equivalent to an effective concentration in the dextran of 7.4 μM) and for the mutant (202 bp) 1.18×10^{-15} mol (11.8 μM). In order to obtain the rates associated with the formation (k_a) and dissociation (k_d) of a given complex, sensorgrams of the type shown in Figure 1A were fitted to the algorithms provided by the BIAcore instrumentation. For the dissociation process, the rate of change of resonance units (R in RUs) as a function of time was fitted to a simple exponential: $R_t = R_0 \exp^{-k_d t}$. The association phase (k_a) was described by the equation: $R_t = R_{eq}\,(1–\exp^{-(k_a C + k_d)(t-t_0)})$. The expected response R_t as a function of the steady-state response level (R_{eq}, which may not necessarily be attained in the sensorgram) is calculated as a function of the concentration (C) of added soluble protein. The errors refer to the fitting procedure for a given sensorgram. For a given concentration of RNA polymerase, k_d values are first estimated from the dissociation part of the sensorgram and used to calculate the k_a values from the association part of the curve. In all the fitting procedures, t is the independent variable; k_d, k_a and R_{eq} are floating parameters and C, R_0 and t_0 are fixed parameters. Best fits to this simple model passed the residuals test and gave χ^2 values <1.

Table II. Calculated rate constants of FIS binding to the wild-type and mutant promoter fragments immobilized on a sensor surface

Promoter	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_D (M)
All sites			
Wild-type	5.1 (± 0.05)×10^5	9.3 (±0.05)×10^{-4}	1.8×10^{-9}
Mutant	4.8 (± 0.06)×10^5	7.1 (±0.05)×10^{-4}	1.5×10^{-9}
Sites I and III			
Wild-type	4.7 (±1.3)×10^5	5.7 (± 0.2)×10^{-4}	1.2×10^{-9}
Mutant	4.8 (±0.1)×10^5	4.1 (±0.3)×10^{-4}	0.8×10^{-9}
Site II			
Wild-type	1.1 (±2.2)×10^5	6.3 (±0.3)×10^{-3}	5.7×10^{-8}
Mutant	1.4 (±0.4)×10^5	1.5 (±0.5)×10^{-3}	1.1×10^{-8}

Data were calculated from sensorgrams of the type shown in Figure 1B. A simple fit of the curves using the single site model as described in the legend to Table I gave the values shown in the table marked 'all sites'. The resulting fit was poor and gave χ^2 values >20 for the mutant promoter. A two-site model (see below) was assessed as having a 100% probability of success with respect to the single site model. FIS dimers were assumed to bind to the three sites on each fragment with equal affinities for sites I and III and a lower affinity for site II. In this case, where parallel association to two sites is assumed, the two association rate constants (k_{a1} and k_{a2}) and associated steady-state response (R_{eq1} and R_{eq2}) for each were calculated by fitting curves of the type shown in Figure 1B to the equation

$$R = R_{eq1}(1–e^{-(k_{a1}C_{n1} + k_{d1})(t-t_0)}) + R_{eq2}(1–e^{-(k_{a2}C_{n2} + k_{d2})(t-t_0)}).$$

This model gave a higher probability of correctness than the single site but was still relatively poor with respect to the χ^2 test (χ^2 >2). Because of this, the calculated value shown for site II on the mutant promoter has a high level of uncertainty, although the other calculated values for sites I and III on both promoters are in reasonable agreement with values obtained by gel retardation experiments (Lazarus, 1992). Parallel dissociation was calculated by fitting the curves to the equation:

$$R = R_{eq1}e^{-(k_{d1}(t-t_0))} + (R_{eq0}–R_{eq1})\,e^{-k_{d2}(t-t_0)}$$

In this model a good fit with χ^2 <2 was obtained.

this particular case, the ternary complex between FIS, polymerase and the wild-type promoter forms more rapidly than at the mutant promoter and then undergoes a transition to a more stable complex. This kinetic profile was observed only on the simultaneous addition of FIS and polymerase. With polymerase alone, a profile consistent with normal steady-state binding was obtained (data not shown). We note that the observed reduction in signal measured by SPR takes place in the continued presence of free FIS and polymerase and is greater than that observed during the dissociation phase at the end of injection. This phenomenon could be due either to an alteration of the conformation of the complex or to an effectively irreversible dissociation of one or more of the components of the complex.

The data obtained by SPR are consistent with our previous findings (Muskhelishvili *et al.*, 1995) that the wild-type and +5 mutant *tyrT* promoters differ in their ability to support FIS-dependent trapping of polymerase. In addition, these data imply sequential and unidirectional effects of FIS at the *tyrT* promoter: an initial facilitation of polymerase binding followed by a structural change in the complex.

Destabilization of polymerase–promoter complexes by FIS

To investigate further the nature of the transition observed after the initial formation of the polymerase–FIS–DNA ternary complex at the wild-type *tyrT* promoter, we carried out DNase I footprinting of FIS–polymerase complexes under experimental conditions close to those used for the SPR measurements. In the time-course experiment, we observed a substantial weakening of the protection by polymerase but not by FIS (Figure 2A). The lessening of protection by polymerase proceeded more rapidly at the wild-type than at the mutant promoter (compare lanes at 30 s for the wild-type with the same for the mutant). This effect was not due to the occlusion of the promoter by FIS because no FIS-specific hypersensitive sites within the promoter region were observed (G.Muskhelishvili, unpublished observations). Displacement of stably bound polymerase molecules did not, however, preclude contacts made by polymerase in the vicinity of the –35 region, as indicated by the retention of the strong DNase I hypersensitivity at position –37 (Figure 2A).

If FIS destabilizes polymerase, this should be reflected in reduced amounts of transcript produced if the transcription were initiated with a delay after addition of FIS. We tested this possibility in a runoff assay by adding all four nucleoside triphosphates to incubation mixtures for a fixed time but at different intervals after mixing FIS and polymerase with the promoter DNA (Figure 2B). This experiment showed that within 20 s, FIS reduced transcription from the wild-type promoter by nearly 60%, a value that was only attained at later times at the mutant promoter. Taken together, these results suggest that the initial recruitment of polymerase by FIS at the *tyrT* promoter is followed by a rapid weakening of polymerase–promoter contacts in a majority of the complexes formed.

Both the SPR measurements and the solution transcription and cleavage protection experiments indicate that complexes at the mutant promoter are more resistant to destabilization by FIS than those at the wild-type promoter. However, the apparent extent of this difference appears

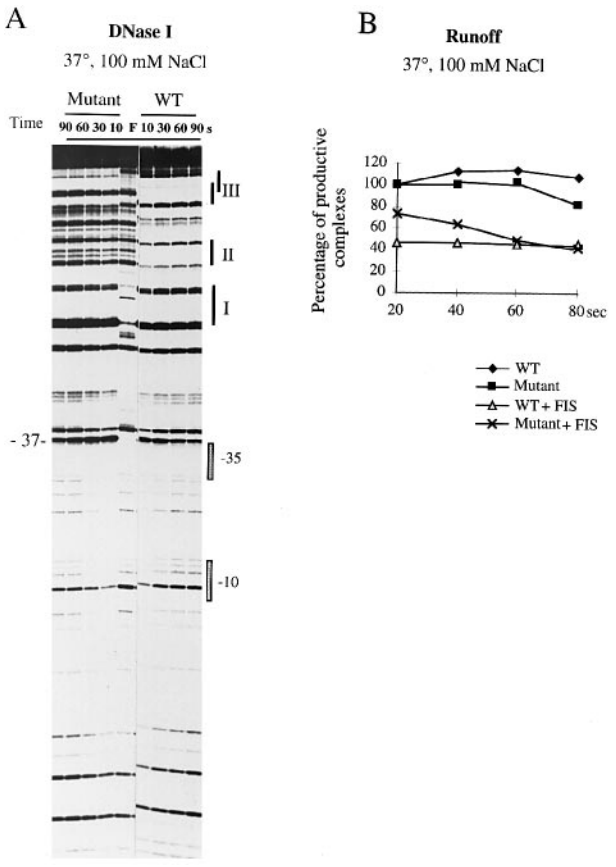

Fig. 2. (**A**) Time-course of destabilization of polymerase by FIS. The reaction conditions were similar to those described in the legend to Figure 1B and C, except that the concentrations of polymerase and FIS were 100 and 40 nM (dimer) respectively. The radiolabelled *tyrT* DNA was mixed with polymerase and FIS and digested for 10 s by DNase I after different time intervals as indicated. The letter F over the middle lane in the autoradiogram indicates free DNA digested for 10 s in the absence of proteins. The FIS sites I to III are shown by vertical lines. The FIS site III is indicated twice and shifted to account for the 5 bp insertion at position –98 in the mutant fragment. The regions of the –10 and –35 hexanucleotides are indicated by grey rectangles. (**B**) Graphical representation of the decay of productive initiation complexes in the presence of FIS. The transcription was initiated by adding NTPs to the incubation mixtures containing the 299 bp wild-type and 304 bp E*co*RI–*Nsi*I *tyr*T DNA fragments (see Materials and methods) and proteins. The concentrations of DNA, polymerase and FIS were 5, 20 and 8 nM (dimer) respectively. The NTPs were added into the incubation mixture at different time intervals after mixing proteins with DNA as indicated. The duration of runoff in each case was 30 s and the amount of the synthesized product was quantified by phosphorimaging as described in Materials and methods. The percentage of productive complexes was deduced from the amount of synthesized transcript and normalized in each case to the value obtained at 20 s for polymerase alone.

to depend on the method used. We note that the local environment of the immobilized DNA in the SPR experiments is significantly different from that of DNA free in solution, and this difference could contribute to observed differences in apparent residence times. Thus, although both conditions show qualitatively that the ternary complex is destabilized, precise quantitative comparisons between SPR and other methods may not, in this case, be justified.

FIS activates sequential steps in the initiation process

The experiments described above indicate that the formation of a FIS–polymerase–DNA ternary complex at the wild-type promoter is followed by changes in the structure of the complex and provide direct evidence that FIS can affect sequential steps in the dynamic transitions undergone by the complex. To assess the relevance of these changes to the initiation process, we chose different conditions that allowed us to distinguish the effects of FIS on the initial binding of polymerase, on promoter opening and finally on polymerase escape.

We first analysed polymerase–promoter complex formation at 30°C and elevated salt concentrations (140 mM), conditions known to impair the transition from the closed to open complex at the *rrnB* P1 promoter (Ohlsen and Gralla, 1992b). Using DNase I as a probe for complex formation, we observed that under these conditions the interaction of polymerase with both the wild-type and mutant promoter fragments was characterized solely by an enhanced DNase I cleavage at position –37, with little or no protection apparent within the remainder of the polymerase-binding site (Figure 3A and B, arrowheads). However, upon addition of FIS, protection was apparent at the wild-type but not the mutant promoter (Figure 3B), although in the latter case the enhancement of cleavage at –37 was increased. The downstream limit of the observed protection varied in different experiments between positions +8 and +17 as mapped by using DNA fragments of different lengths. The former value is consistent with the limit of the initial or closed complex formed at the *rrnB* P1 promoter but the latter does not extend to the +25 limit of the open complex on the same promoter (Ohlsen and Gralla, 1992a). This result confirms our previous conclusion that under restrictive conditions FIS site I alone is insufficient to stabilize polymerase binding at the *tyrT* promoter (Muskhelishvili *et al.*, 1995).

To test whether FIS affected subsequent steps in the initiation process, we then monitored the effect of FIS on promoter opening. On addition of the two nucleoside triphosphates necessary for the synthesis of the first dinucleotide bond, RNA polymerase forms comparatively stable complexes at both the *rrnB* P1 (Gourse, 1988; Ohlsen and Gralla, 1992a) and *tyrT* (Küpper *et al.*, 1975; Debenham, 1979) promoters. These complexes, termed initiation complexes, are characterized by a high reactivity of the promoter DNA in the –10 hexamer region to permanganate, a reagent that is specific for untwisted DNA (Gralla *et al.*, 1993), and by a DNase I footprint extending to near position +25 (Gourse, 1988; Ohlsen and Gralla, 1992a; G.Muskhelishvili, unpublished observations).

By using a high molar ratio of RNA polymerase to DNA in the presence of initiating nucleoside triphosphates so that initial complex formation at both promoters was independent of FIS, we asked whether FIS influenced the reactivity to potassium permanganate of thymine residues around the –10 region and transcription startpoint. At 140 mM salt concentration, addition of FIS substantially increased permanganate reactivity of thymines within the –10 hexamer region (Figure 4A, positions –12 and –9) at the wild-type but only to a slight extent at the +5 mutant promoter (Figure 4B). The observation that FIS increases the accessibility of this region to permanganate suggests an increase in the extent of untwisting of DNA within the –10 region necessary for promoter opening. Again,

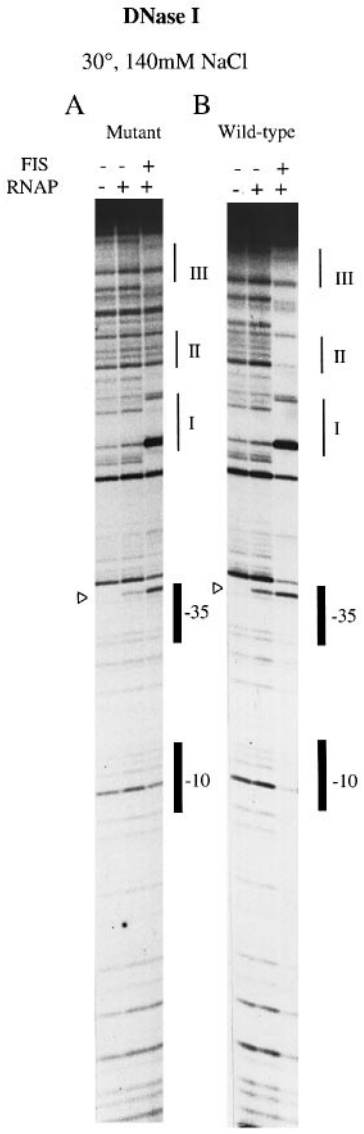

Fig. 3. Differential effect of FIS on polymerase–promoter complex formation at the (**A**) 197 bp wild-type and (**B**) 203 bp +5 mutant *tyrT* promoter fragments. The radiolabelled DNA (10 nM) was mixed with RNA polymerase (200 nM) or with polymerase and FIS (20 nM) at 30°C in the presence of 140 mM NaCl and digested for 10 s by DNase I immediately after mixing proteins with DNA. Note the appearance of a region protected by polymerase in the presence of FIS at the wild-type promoter fragment.

this effect requires all three FIS sites to be positioned in helical register.

The regulatory nucleotide ppGpp inhibits promoter opening at the *rrnB* P1 promoter (Ohlsen and Gralla, 1992a) but FIS is known to override the negative effect of ppGpp on transcription initiation at the *tyrT* promoter (Lazarus and Travers, 1993). We therefore asked whether FIS could overcome the effect of ppGpp on promoter opening at the *tyrT* promoter. We observed that the addition of ppGpp prevented the enhancement of the permanganate reactivity in the –10 region by polymerase alone and that FIS partially overcame the negative effect of ppGpp (Figure 4C). This effect of FIS was apparent at both the wild-type and +5 mutant promoters.

We next asked whether FIS could affect any reaction steps subsequent to the formation of an initiation complex. Since the addition of heparin destabilizes the binding of FIS to site II (G.Muskhelishvili, unpublished observations), we could not use this compound to remove unstable pre-initiation complexes. We therefore pre-formed initiation complexes by the addition of the two nucleoside triphosphates, GTP and CTP, necessary for the synthesis of the first dinucleotide bond. To the pre-formed initiation complexes we added UTP to allow more extensive RNA synthesis, up to a nonanucleotide (Figure 5A). Addition of this nucleotide alone further increased the permanganate reactivity of the bases within the –10 hexamer region at the wild-type promoter and increased the permanganate reactivity of the base at position +1 (Figure 5B and C), indicating a conformational alteration of the complex. Quantitation of the extent of permanganate reactivity within the –10 hexamer region (Figure 5C) showed that on addition of UTP the signal obtained after 10 s for the bases at –9 and –12 with polymerase alone (2.4 ± 0.6) significantly increased in the presence of FIS (3.7 ± 0.7) at the wild-type but not at the mutant *tyrT* promoter. These results suggest that in the presence of UTP, binding of FIS to helically phased sites in the UAS facilitates a conformational transition of initiation complexes.

To confirm that this effect of FIS was related to the efficiency of transcription initiation, we carried out a runoff transcription assay under similar conditions. First, initiation complex formation was allowed in the presence of GTP and CTP and then [α-^{32}P]UTP and ATP were added. FIS markedly increased the amount of the synthesized product at the wild-type, but not at the mutant promoter (Figure 5D). This result is consistent with FIS stimulating a rapid transition of the complexes to the elongation mode. Again, this effect requires the wild-type configuration of three FIS-binding sites in UAS.

Discussion

We have demonstrated that the FIS–polymerase nucleoprotein complex formed at the *tyrT* promoter is a dynamic structure which undergoes conformational transitions driven by FIS dimers bound to the UAS. It thus appears that, in contrast to other prokaryotic transcriptional activators, FIS activates transcription initiation by enabling efficient polymerase recruitment and also by facilitating promoter opening and subsequent post-initiation events.

Sequential effects of FIS on transcription initiation

We have shown previously that FIS forms a nucleoprotein complex with RNA polymerase at the *tyrT* promoter, a process which requires the participation of three FIS dimers (Muskhelishvili *et al.*, 1995). We have now shown that under restrictive conditions (30°C, 140 mM KCl) FIS promotes the establishment of a polymerase–promoter complex at the wild-type, but not the +5 mutant promoter (Figure 3). Similarly, SPR measurements show that the overall rate of formation of a FIS–polymerase complex is higher at the wild-type than at the mutant promoter. These results confirm our previous findings and show that under these conditions FIS recruits RNA polymerase to the *tyrT* promoter. This observation is similar to that of Bokal *et al.* (1995) who showed that FIS facilitated the initial binding of polymerase to the *rrnB* P1 promoter. However,

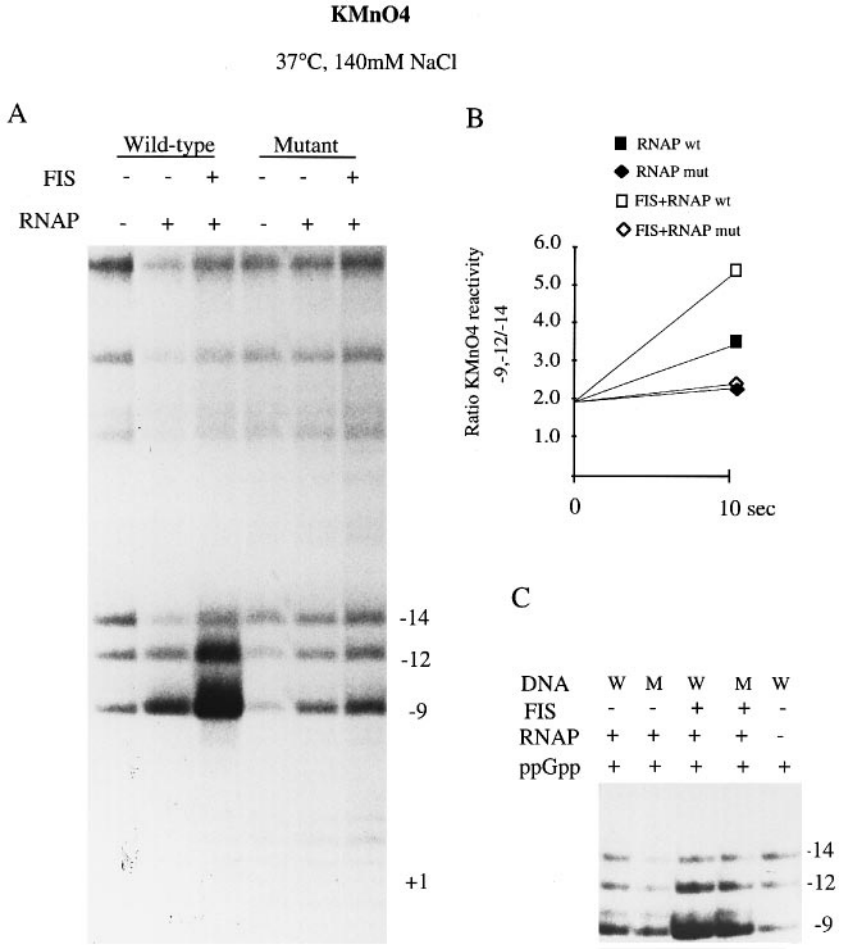

Fig. 4. (**A**) Stimulation of promoter opening by FIS. The incubation was at 37°C in the presence of 2 nM DNA, 200 nM RNA polymerase, 20 nM FIS, 140 mM NaCl and 1 mM each of GTP and CTP. Permanganate was added for 10 s immediately after mixing proteins with DNA. The reactive bases within the –10 region are those at positions –9 and –12. (**B**) Graphical representation of a PhosphorImager quantification of $KMnO_4$ reactivity. The reactivity of bases in different lanes (A) was normalized by using the ratios of the obtained signals rather than absolute values (see Materials and methods for details). The abscissa indicates the duration of probing with $KMnO_4$. The intercept on the ordinate indicates the $KMnO_4$ reactivity of the same bases on the naked DNA. (**C**) FIS overrides the inhibitory effect of ppGpp on promoter opening. The reaction conditions were as in (A), except that $KMnO_4$ was added for 1 min. The letters W and M indicate the wild-type and mutant promoter fragments respectively.

whereas recruitment at the *rrnB* P1 promoter required only the proximal FIS-binding site, this site, especially under restrictive conditions, is not sufficient at the *tyrT* promoter. Although the properties of the two promoters clearly differ in this respect, it is unclear whether the observed difference is biologically relevant or is simply a consequence of differences in assay conditions.

FIS also facilitates a second step in the initiation process, the untwisting of DNA in the –10 region. Again this effect is strong at the wild-type but barely apparent at the +5 mutant promoter. Since the extent of untwisting is similar to that observed in other polymerase initiation complexes, we infer that FIS is promoting initiation complex formation. This view is also consistent with the antagonistic effects of FIS and ppGpp, a nucleotide which is known to block the transition to the initiation complex at the *rrnB* P1 promoter (Ohlsen and Gralla, 1992a). FIS partially counteracts the negative effect of ppGpp on untwisting but, interestingly, this effect is observed with both wild-type and mutant promoters, suggesting that the intact UAS may not be required in the presence of the inhibitory nucleotide. Further genetic studies are under way to clarify this point.

At a higher temperature (37°C) FIS weakens the interaction of polymerase with the promoter DNA, an effect again requiring the participation of all three FIS-binding sites in the UAS. In the absence of nucleoside triphosphates, this results in the dissociation of bound polymerase. However, under conditions which allow RNA chain elongation, FIS facilitates both post-initiation structural changes in the –10 region and also transcription itself. These effects are quantitatively similar to the FIS-induced enhancement of transition of open to transcribing complexes observed at the *rrnD* P1 promoter (Sander *et al.*, 1993).

The ability of FIS to stimulate sequential steps in the initiation process at the *tyrT* promoter *in vitro* is consistent with the otherwise disparate observations that it promotes initial complex formation at the *rrnB* P1 promoter (Bokal *et al.*, 1995) but increases the rate of both promoter opening and polymerase escape at the *rrnD* P1 promoter (Sander *et al.*, 1993). More compellingly, this property provides an explanation for the observation that *in vivo* FIS stimulates expression from both down and up polymerase-binding site mutants but not from the wild-type *tyrT* promoter (Lazarus, 1992; Lazarus and Travers, 1993;

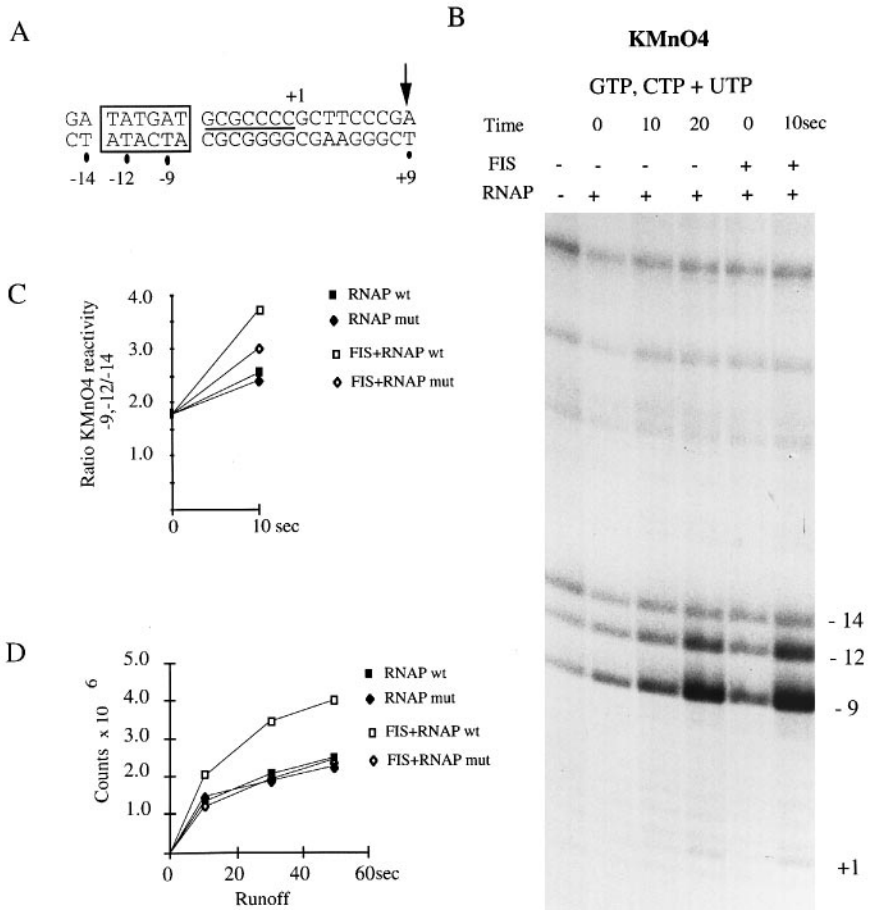

Fig. 5. FIS drives conformational transition of initiation complexes. (**A**) The sequence of the *tyrT* promoter from position –15 to +9. The –10 hexamer element is boxed, the GC-rich discriminator region marked by a horizontal line, and the startpoint of transcription at +1 is indicated. The arrow indicates the first thymine base in the sequence at which polymerase would stall in the presence of GTP, CTP and UTP but in the absence of ATP in the incubation mixture. The black ellipses indicate the permanganate-reactive thymines in the naked DNA. Only thymines within the –10 region show increased permanganate reactivity with polymerase. (**B**) Time-course of conformational transition of initiation complexes. Mixtures of 197 bp wild-type promoter DNA with polymerase and GTP/CTP were pre-incubated for 45 min at 37°C in a buffer containing 140 mM NaCl before addition of 1 mM UTP with or without FIS. Permanganate was added for 10 s at the indicated time intervals after the addition of UTP or UTP + FIS to the reaction mixtures. Zero indicates that permanganate was added immediately after UTP. Note that FIS enhances permanganate reactivity within the –10 region and at position +1 already after 10 s, whereas in the absence of FIS this effect shows up later. (**C**) Graphical representation of the effect of FIS on initiation complexes. The abscissa indicates the duration of probing with $KMnO_4$. Values obtained from five independent experiments similar to that shown in (B) were averaged after quantification of corresponding signals by phosphorimaging as described in the legend to Figure 4. The value obtained for the naked DNA is indicated by the intercept on the ordinate. FIS significantly increases the permanganate reactivity only at the wild-type promoter (3.7 ± 0.7 with FIS versus 2.4 ± 0.6 without FIS). (**D**) FIS stimulates transcription by initiation complexes pre-formed at the wild-type *tyrT* promoter. The reaction conditions were as described for (B) (above) except that the 299 bp wild-type and 304 bp +5 mutant *Eco*RI–*Nsi*I fragments (see Materials and methods) were used as templates and [α-^{32}P]UTP and ATP were added with and without FIS as indicated. The graph shows the amount of the product (ordinate) synthesized during the runoff experiment and quantified by phosphorimaging.

H.Auner and G.Muskhelishvili, unpublished observations). We surmise that in the absence of FIS, initiation at the wild-type promoter is finely tuned so that under optimum conditions the different steps in the initiation process are kinetically coordinated, i.e. no one step is strongly rate-limiting. The role of FIS in such a situation would be to act as a facultative activator overcoming any kinetic bottlenecks caused by substrate or polymerase limitation. Similarly both up and down promoter mutations could also create kinetic blocks (Ellinger *et al.*, 1994a) which again could be relieved by FIS.

Certain prokaryotic activators have the potential to activate different steps dependent on their placement with respect to the polymerase-binding sites. For example, the cAMP receptor protein (CRP) accelerates polymerase recruitment at the *lac* promoter (Malan *et al.*, 1984), isomerization to the open complex at the *gal* promoter (Herbert *et al.*, 1986) and polymerase escape at the *mal*T promoter (Menendez *et al.*, 1987). However, to our knowledge, FIS is the first example of a prokaryotic transcriptional activator that is involved throughout the initiation process.

Active role of DNA microloops

As measured by SPR in the absence of FIS, the wild-type *tyrT* promoter has an ~10-fold higher affinity for RNA polymerase than the +5 mutant promoter. This result is comparable with the 14-fold enhancement of association rate conferred by an intact UAS at the *rrnB* P1 promoter (Newlands *et al.*, 1991) and implies that at the *tyrT* promoter, sequences upstream of position –98 are necessary for full factor-independent UAS function *in vitro*. One interpretation of this extended sequence requirement is that the *tyrT* UAS forms a microloop making an

additional contact with RNA polymerase upstream of the 5 bp insertion point (Muskhelishvili *et al.*, 1995). The existence of such loops has been inferred from the enhancement of promoter activity by upstream curved DNA (Bracco *et al.*, 1989; Gartenberg and Crothers, 1991; Ellinger *et al.*, 1994b) and from the activation of the λ pL and *malT* promoters by the DNA-bending protein IHF (Giladi *et al.*, 1990; Déthiollaz *et al.*, 1996). More direct evidence for an upstream polymerase contact at the *lac* UV5 promoter has also been presented (Buckle *et al.*, 1992). We suggest that the 5 bp insertion mutation alters the phasing of the anisotropically flexible *tyrT* UAS region (Drew and Travers, 1985) and so reduces, but does not necessarily eliminate, the probability of loop formation.

How does FIS mediate its effects on the transcription initiation process? The coherent DNA bending induced by FIS in the UAS could increase both the probability of forming a microloop and its subsequent stability. Such an effect would be consistent with the inability of the +5 mutant to support the formation of a FIS–polymerase complex (Muskhelishvili *et al.*, 1995) or to promote FIS-dependent DNA untwisting in the –10 region. Similarly, the FIS dependence of post-initiation events at the wild-type promoter implies that the integrity of the loop is maintained during the initial stages of transcription elongation. Mechanistically, the role of FIS in facilitating the initiation process could be explained most easily by assuming that FIS stabilizes a left-handed writhe. In this model, the writhed microloop captures the polymerase in the initial complex, and then a rotation of RNA polymerase writhes the loop in a right-handed sense, thereby generating torsion in the microloop (Figure 6). FIS subsequently drives a reversion to left-handed writhe. This motion both transmits untwisting to the separate topological domain formed by the initiation bubble and accommodates the negative superhelicity generated upstream by the movement of the elongation bubble. In this model, torsional transmission could be mediated by either direct FIS–polymerase contacts (Muskhelishvili *et al.*, 1995) or polymerase contacts with UAS DNA or, alternatively, by both types of contacts.

We and others (Gosink *et al.*, 1993; Muskhelishvili *et al.*, 1995) have observed previously that high concentrations of FIS can compete with RNA polymerase for its binding site at the *rrnB* P1 and *tyrT* promoters. We have now shown here that FIS can destabilize pre-formed complexes, as indicated by a reduction in the SPR signal (Figure 1), by the loss of an extensive polymerase footprint and by loss of transcriptionally productive complexes (Figure 2). However, under these conditions, the enhanced DNase I cleavage immediately upstream of the –35 region suggests that polymerase can still interact with and distort the DNA at this position. Unlike protection, a protein-induced enhanced DNase I cleavage signal may only require a transient distortion to be detectable and is not necessarily indicative of high occupancy by the protein. It seems unlikely that the FIS-induced destabilization of polymerase binding we have reported here is a consequence of competition between FIS and polymerase since we observe no FIS-related footprint within the polymerase-binding region under our assay conditions. At higher FIS concentrations, invasion of this region by FIS is readily apparent (G.Muskhelishvili, unpublished observations). An alternative possibility is that the structural transitions in the nucleoprotein complex that occur between the initial and initiation complexes may directly drive the observed FIS-dependent destabilization of polymerase binding.

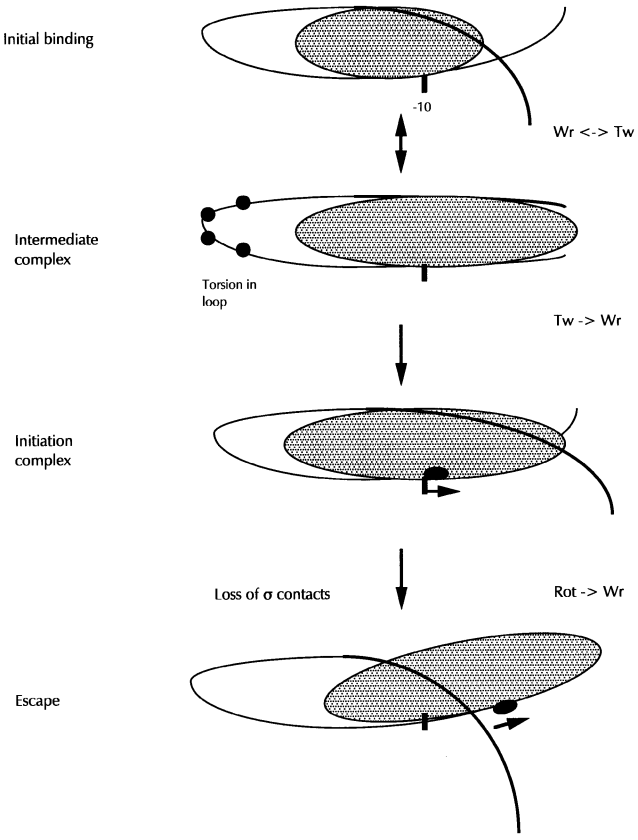

Fig. 6. The torsional transmission model for transcription activation by FIS. The types of polymerase complexes, as well as the topological alterations in twist (Tw) and writhe (Wr) accompanying the transitions between the complexes and rotation of polymerase (Rot) are indicated. The arrowheads indicate the direction in which the alterations proceed. The polymerase is drawn as an ellipse, DNA is represented by a thin line. The filled circles in the DNA loop indicate the accommodated torsion, the small dark grey ellipse represents the initiation and elongation bubbles. FIS is omitted from the drawing for clarity. For further details see text.

Biological implications

The rapid synthesis of stable RNA species is a prerequisite for the efficient growth of *E.coli*. Such optimized synthesis requires a concomitant optimization of the initiation process, from the initial capture of polymerase by the promoter to its subsequent escape as an actively transcribing enzyme. The ability of FIS to overcome the barriers to differing rate-limiting steps in initiation is consistent with the notion that the primary biological role of FIS is to optimize the rate of transcription initiation at stable RNA promoters under otherwise non-ideal conditions (Lazarus and Travers, 1993; Muskhelishvili *et al.*, 1995). However, if *in vivo* conditions were sufficiently unfavourable, for example if concentrations of the initiating triphosphates were low, FIS potentially could abort initiation by forcing the dissociation of bound polymerase. Taken together, these results suggest that FIS functions as a molecular machine which optimizes the turnover of polymerase holoenzyme at the *tyrT* promoter. The ability of FIS to stimulate both the

assembly of the transcription complex and the subsequent promoter opening parallels its function in promoting Gin-mediated recombination. The binding of FIS to the recombinational enhancer is thought to facilitate both the assembly of the synaptic complex (Merker *et al.*, 1993) and the subsequent DNA untwisting at the sites of strand exchange (Klippel *et al.*, 1993). We note that the mechanism of torsional transmission inferred for promoting transcription initiation would provide a means for channelling the free energy of negative supercoiling, thereby localizing untwisting at biologically relevant sites.

Materials and methods

Biotinylated DNA substrates

The uniquely end-biotinylated wild-type and the +5 mutant *tyrT* extended promoter fragments (positions –150 to +47 and –155 to +47 respectively) were obtained by PCR (Saiki, 1989) using the 5′-biotinylated primer R-bio (5′-CACCACGGGGTAATGCTTT-3′), the primer UAS-L (5′-CTTTGTTTACGGTAATCGAACG-3′) and the *tyrT* promoter constructs ptyrTΔ150 and ptyrTΔ150+5 (Lazarus, 1992; Lazarus and Travers, 1993) as templates for amplification respectively. In these fragments, the biotinylated terminus was downstream of the transcription startsite. The +5 mutant refers to the promoter construct bearing a 5 bp insertion at position –98 which impairs the FIS site II and changes the helical phasing between FIS sites I and III (Lazarus and Travers, 1993; Muskhelishvili *et al.*, 1995).

Proteins

FIS and RNA polymerase were isolated as described previously (Koch and Kahmann, 1985; Metzger *et al.*, 1993).

Surface plasmon resonance (SPR)

SPR measurements were conducted using a BIAcore instrument from BIAcore AB. The units of measurement are expressed in resonance units (RUs) where a change of 10^{-4} degrees is equivalent to a change of 1 RU and the machine has an effective dynamic range from 3–4 RUs to 30 000 RUs. The actual response in RU as a function of the change in surface molecule depends to an extent upon the differential refractive index of the solute, but for many globular proteins 1 kRU is equivalent to a change in surface concentration of ~1 ng/mm^2.

Immobilization of DNA fragments. The uniquely end-biotinylated 197 bp wild-type and the 203 bp +5 mutant *tyrT* extended promoter fragments (0.125 μg/ml) in 75 μl were injected independently across streptavidin-pre-treated dextran sensor surfaces *in situ* in the BIAcore apparatus at 5 μl/min in 20 mM Tris–HCl, pH 7.9, 50 mM NaCl, 50 mM KCl, 0.1 mM dithiothreitol (DTT), 0.005% surfactant P20 (BIAcore AB), at 37°C. In the experiments shown, ~7.39×10^{-16} mol of wild-type promoter DNA was immobilized at the surface (equivalent to an effective concentration in the dextran of 7.4 μM) and 1.18×10^{-15} mol (11.8 μM) of the mutant DNA.

Protein binding. FIS or RNA polymerase singly or in combination were applied at various concentrations to the different immobilized surfaces in 20 mM Tris–HCl, pH 7.9, 50 mM NaCl, 50 mM KCl, 0.1 mM DTT, 0.005% surfactant P20 (Biosensor Pharmacia), at 37°C. The surface was regenerated by washing with a 10 ml pulse of 1 M NaCl for 2 min, which removed all bound protein.

Interpretation of sensorgrams. In order to obtain the rates associated with the formation (k_a) and dissociation (k_d) of a given complex, sensorgrams were fitted to the algorithms provided by the BIAcore instrumentation. For the dissociation process (k_d), the rate of change of resonance units (R in RUs) as a function of time was fitted to a simple exponential ($R_t = R_0\exp^{-k_d t} + R_{drift}$). The association phase (k_a) was described by the equation:

$$R_t = \frac{k_a C R_{max}}{k_a C + k_d}\,(1-e^{-(k_a C + k_d)}) + R_{bulk} + R_{drift}$$

The expected response R_t as a function of maximal analyte binding capacity (R_{max}) is calculated as a function of the concentration (C) of added soluble protein. The bulk contribution is made by the sample refractive index (R_{bulk}). Careful temperature control minimized the baseline drift (R_{drift}).

DNase I footprinting

DNase I footprinting was performed with *tyrT* promoter fragments uniquely radiolabelled at the bottom strand as previously described (Muskhelishvili *et al.*, 1995). The 197 bp wild-type and 203 bp mutant DNA fragments were uniquely end-labelled by PCR amplification using radioactively 5′ end-labelled primer R3 (5′-CACCACGGGGTAATGC-3′) and primer UAS-L (see above). The primers R3 and S90 were radiolabelled using [γ-^{32}P]ATP (NEN; 3000 Ci/mmol) and T4 polynucleotide kinase. The ptyrΔ50 and ptyrΔ50+5 constructs (see above) were used as templates in these PCR reactions. The fragments obtained were purified by PAGE using a neutral 0.5× TBE gel. Unless otherwise indicated, the incubation mixtures contained 10 mM Tris–HCl, pH 7.9, 0.1 mM DTT, 0.005% Triton X-100, NaCl (as indicated) and various concentrations of polymerase and FIS in a 20 μl total volume. The reaction was initiated by adding polymerase, or FIS and polymerase, to a mixture containing DNA and other ingredients. Before mixing, all the components were pre-equilibrated for 5 min at the required temperature. After incubation for different time intervals, a freshly prepared mixture of DNase I and $MgCl_2$ (adjusted to the required temperature) was added to 5 μg/ml and 10 mM final concentrations respectively. The reaction was stopped after 10 s by adding 80 μl of the solution containing 0.5% SDS and 50 mM EDTA. After digestion by proteinase K for 45 min at 42°C, the samples were deproteinized by phenol extraction and the aqueous phase precipitated with ethanol. The pellets were washed with 70% ethanol, dried, dissolved in the loading dye and analysed on 6% sequencing gels.

Potassium permanganate reactivity assay

The reactions for potassium permanganate reactivity assays were assembled and processed similarly to those used for DNase I footprinting unless otherwise indicated. GTP and CTP were added to 1 mM each and, where used, UTP to 50 μM and ppGpp to 100 μM. The reaction was initiated by adding only polymerase, or FIS and polymerase, to a mixture containing radiolabelled DNA. Before mixing, all the components were pre-equilibrated at the required temperature. After the incubation, 2 μl of 100 mM permanganate solution was added to 20 μl reaction mixtures containing DNA and proteins for either 10 s or 1 min as indicated in the figure legends. The reactions were stopped by addition of 2 μl of 14 M β-mercaptoethanol, 8 μg of sonicated salmon sperm DNA and sodium acetate to 0.3 M, precipitated with 3 volumes of ice-cold ethanol and washed with 70% ethanol. The pellets were resuspended in 100 μl of 10% piperidine and incubated at 90°C for 20 min. Then LiCl was added to 0.5 M, the DNA precipitated with 3 volumes of ice-cold ethanol and washed at least twice with 100% ethanol. The pellets were dried, dissolved in the loading dye and analysed on 6% sequencing gels. The signals due to permanganate reactivity of bases were quantified by using the PhosphorImager (Storm 840, Molecular Dynamics). The absolute values of the signals obtained by this procedure may vary and need to be normalized for comparative analysis. We normalized the reactivity of bases in different lanes by using the ratios of the sum of signals obtained for bases at –9 and –12 divided by the value obtained for the base at –14 (which is the first thymine outside of the –10 region) in each lane. The ratios obtained were averaged and subjected to statistical analysis. The ratio obtained for the naked DNA at both promoters was similar and varied within a narrow range (1.79 ± 0.22).

In vitro transcription assay

The 299 bp wild-type and 304 bp mutant *tyrT* DNA templates used in the runoff assay were obtained by *Eco*RI–*Nsi*I digestion of the ptyrTΔ150 DNA and ptyrTΔ150+5 DNA followed by agarose gel purification of the respective fragments. The runoff transcription assays were performed at 37°C in a buffer containing 10 mM Tris–HCl, pH 7.9, 0.1 mM DTT, various concentrations of NaCl, 10 mM $MgCl_2$, 5 nM of the *Eco*RI–*Nsi*I *tyrT* DNA fragment, various concentrations of polymerase and FIS, 1 mM each of GTP and CTP, 0.05 mM [α-^{32}P]UTP and 0.4 mM ATP. The reactions were stopped after different time intervals by directly adding equal amounts of the formamide loading dye to aliquots of incubation mixtures. The reaction products (145 bp) were analysed on 6% sequencing gels and quantified by using the PhosphorImager (Storm 840, Molecular Dynamics).

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