The EMBO Journal vol.12 no.6 pp.2483-2494, 1993

The *Escherichia coli* FIS protein is not required for the activation of *tyrT* transcription on entry into exponential growth

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Communicated by A.A.Travers

The *Escherichia coli* DNA bending protein factor for inversion stimulation (FIS), is neither necessary nor responsible for the stimulation of transcription from the wild type promoter for the *tyrT* operon (encoding a species of tyrosine tRNA) that occurs upon resumption of exponential growth. This conclusion is unexpected given that the regulatory element required for optimal transcription of *tyrT* contains three binding sites for FIS protein. In addition, it is in apparent conflict with reports from other laboratories which have described FIS-dependent activation of the stable RNA promoters *rrnB* P1 and *thrU(tufB) in vivo*. However, *tyrT* transcription is stimulated in a FIS-dependent manner both *in vivo* and *in vitro* when promoter function is impaired by mutation of the promoter itself or by the addition of the polymerase effector guanosine 5′-diphosphate 3′-diphosphate. These conditions, which expose a requirement for activation of stable RNA synthesis by FIS, suggest that FIS serves an adaptive role permitting high levels of stable RNA transcription on nutritional shift-up when RNA polymerase levels are depleted. In principle such a mechanism could confer a significant selective advantage thus accounting for the conservation of FIS binding sites in the regulatory regions of stable RNA promoters.

Key words: DNA bending/FIS/stable RNA/transcription/*tyrT*

Introduction

The rRNA and tRNA operons of *Escherichia coli* are expressed at very high levels in exponentially growing cells (Nomura *et al.*, 1984). Their metabolically stable products account for ~98% of the total RNA content of the bacterial cell (Baracchini and Bremer, 1987). In the interests of cellular economy, their rates of synthesis must reflect the nutritional value of the medium and be rapidly adaptable to variations in growth conditions. Two mechanisms, growth rate and stringent control, are known to regulate stable RNA production at the level of transcription initiation (for reviews see Gausing, 1977; Nierlich, 1978; Lamond and Travers, 1985a; Cashel and Rudd, 1987). In general, stable RNA promoters have a suboptimal spacing of the −35 and −10 promoter sequences and their relatively poor matches to the canonical hexamers, especially at −35, are conserved features. Yet, in order to maintain the high levels of expression required for maximum rates of growth, stable RNA operons must possess extremely strong promoters. The high activity of at least some promoters of this class is dependent on a *cis*-acting sequence element upstream of the −35 region. Deletion of these upstream activation sequences (UASs) causes a drastic reduction in promoter activity *in vivo* (Lamond and Travers, 1983; Gourse *et al.*, 1986; van Delft *et al.*, 1987; Bauer *et al.*, 1988) and also, to a lesser extent, *in vitro* (Leirmo and Gourse, 1991; Newlands *et al.*, 1992).

A molecular mechanism for co-ordinating the regulation of stable RNA operons has recently been proposed. This mechanism involves binding of a *trans*-activator protein to the UASs (factor-dependent activation of Newlands *et al.*, 1992). One such protein is the DNA bending protein FIS, the factor for inversion stimulation (Nilsson *et al.*, 1990; Ross *et al.*, 1990) which was originally identified for its role in stimulating the homologous Hin and Gin site-specific DNA recombinases of *Salmonella* and phage Mu (Johnson and Simon, 1985; Kahmann *et al.*, 1985). It has also been implicated in a number of other cellular processes known to be mediated by complex nucleoprotein structures (reviewed by Finkel and Johnson, 1992).

E.coli operons that are known to be co-ordinately regulated contain homologous DNA sequences in their promoter regions in addition to the canonical −35 and −10 hexamers. Thus, one would expect to find common structural or sequence elements in the promoter regions of stable RNA genes. A comparison of the upstream regions of a large number of rRNA, tRNA and ribosomal protein genes revealed no overall sequence homology (Travers, 1984). However, there are some indications that DNA curvature is a characteristic of promoters that exhibit high transcription initiation rates *in vivo* (Plaskon and Wartell, 1987). More recently, putative binding sites for the FIS protein have been recognized within these upstream regions (Josaitis *et al.*, 1990; Verbeek *et al.*, 1990; Condon *et al.*, 1992) based on their homologies to a degenerate binding site consensus sequence (Hübner and Arber, 1989).

To date, functional interactions between FIS and its potential binding sites within stable RNA promoters have only been demonstrated in a few cases [*rrnB* P1, Ross *et al.*, 1990; *thrU(tufB)* and *tyrT*, Nilsson *et al.*, 1990; *metY*, Verbeek *et al.*, 1990]. In addition to *in vitro* binding studies with promoter fragments, the absolute level of transcription from the *thrU(tufB)* operon was shown to be raised ~3-fold by FIS *in vivo* (Bosch *et al.*, 1990; Nilsson *et al.*, 1990). Ross *et al.* (1990) concluded that FIS stimulated the activity of a truncated *rrnB* P1 promoter (retaining only one of three putative FIS binding sites) by ~5-fold. However, this value is a relative measure incorporating differences in the response to FIS-activation between promoters retaining or lacking a single FIS binding site. By contrast the absolute difference in transcriptional activity from the longer construct when measured in FIS-producing (Fis^+) cells relative to *fis* mutants (Fis^-) was only ~13%. To address these apparently contradictory findings we have reinvestigated the function of FIS using another stable RNA promoter, that of the *E.coli* tyrosine tRNA, *tyrT*. This was a likely candidate

promoter for regulation by FIS since DNA fragments derived from *tyrT* had been found to compete for binding of a common factor (FIS) with sequences from other stable RNA promoters (Bosch *et al.*, 1990; Nilsson *et al.*, 1990).

Here we identify three strong binding sites for FIS within the UAS of *tyrT*. Using promoter fusion constructs we established that the highly active *tyrT* promoter is not subject to additional stimulation by FIS on resumption of exponential growth in rich media. FIS-dependent activation could be demonstrated after partially disabling promoter function. Since FIS-dependence could be induced by several inhibitory factors this suggested that they shared a common mode of action. An analogous reduction in promoter activity was found to be responsible for the observed FIS-dependent activation of the *thrU*(*tufB*) operon (described above). Thus at least two wild type stable RNA promoters exhibit FIS-independent activity. This property may extend to all stable RNA operons.

The conservation of FIS binding sites upstream of many stable RNA promoters coupled with the maximal expression of these operons with that for *fis* at the onset of logarithmic growth (Thompson *et al.*, 1987; Ninnemann *et al.*, 1992) supports a legitimate role for FIS in the co-ordinate regulation of stable RNA genes. However, since FIS is dispensable for normal growth (Johnson *et al.*, 1988; Koch *et al.*, 1988) we propose that the physiological function of FIS may be to overcome the effects of RNA polymerase limitation that prevail under certain growth conditions. Thus FIS may confer a selective advantage by enabling rapid adaptation to a changing environment. We propose a mechanism based on protein-induced DNA bending whereby FIS mediates its positive effects on transcription. Similar mechanisms have been implicated for some other cellular functions of FIS, such as site-specific recombination (Hübner *et al.*, 1989) and regulation of DNA replication (Gille *et al.*, 1991).

Results

In vitro binding sites for FIS on the tyrT promoter

The ability of purified FIS protein to bind to a series of *tyrT* promoter fragments to form stable nucleoprotein complexes was analysed by gel retardation and DNase I footprinting techniques. Previous investigations using a crude preparation of FIS demonstrated that a *tyrT* promoter fragment retaining upstream sequences to position −98 (relative to the *in vivo* start site defined as +1) supported the formation of two complexes with FIS, whilst further truncation to −76 removed these binding sites (Nilsson *et al.*, 1990). It was also suggested that an additional site or sites might be present at a more distal location as found for the UASs of the *thrU*(*tufB*) and *rrnB* P1 promoters (Bosch *et al.*, 1990; Ross *et al.*, 1990 and see Discussion).

We observe that the full-length promoter, with up to 248 bp upstream of the start site, supports the formation of three specific complexes with FIS (cI, cII and cIII, Figure 1, lanes 1−3). Removal of sequences between −248 and −150 does not alter the FIS-binding capacity of the *tyrT* fragment (lanes 4−6). Thus the existence of strong FIS binding sites upstream of −150 can be excluded. Further deletion of sequences to −98 removes one binding site as demonstrated by the formation of only two complexes (cI and cII) with this shorter fragment in agreement with Nilsson and co-workers (lanes 7−9). This places the most distal site between

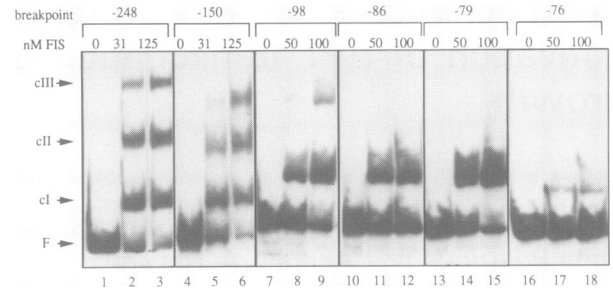

Fig. 1. Gel retardation analysis of sequentially deleted fragments of the *tyrT* promoter upstream region. Restriction fragments were labelled, incubated with purified FIS and analysed on non-denaturing polyacrylamide gels as described in Materials and methods. Lanes 1−3: a *SmaI*−*AvaI* fragment from pTyr2 (breakpoint −248) was titrated with purified FIS protein. Lanes 4−6: titration of a labelled *EcoRI*−*AvaI* restriction fragment from pΔ-150 (breakpoint −150) with FIS. Lanes 7−18: *BsaHI*−*AvaI* fragments were obtained for the shorter *tyrT* constructs (retaining 98 bp or fewer upstream of the start site) for ease of handling and to provide spacer DNA between the *tyrT* sequences and the 5′ fragment terminus. The *BsaHI* site lies ~80 bp upstream of the *EcoRI* site that marks the upstream end of the *tyrT* sequences in Figure 2B. The arrows indicate the different species that can be resolved by this analysis: F = free DNA; cI = complex I; cII = complex II; cIII = complex III.

Table I. Comparison of potential FIS binding sites in the *tyrT* UAS

5′ end	Potential FIS binding sites	Match to consensus	Relative FIS binding affinity
consensus	G--YR--A--YR--C T T A	7/7	
−219	GCGCGCAGGTCGTGA	6/7	>4 μM
−129*	GATTATTCTTTAATC	6/7	7.5−15 nM
−98*	GGTTACCTTTAATCC	6/7	30−60 nM
−78*	GGATGAAAATTACGC	7/7	15−30 nM
−69	TTACGCAACCAGTTC	6/7	>2 μM
−58	GTTCATTTTTCTCAA	6/7	1−2 μM
−26	GCGCGTCATTTGATA	7/7	4−8 μM

Nucleotides matching the Hübner and Arber (1989) binding site consensus sequence are underlined (column 2) and scored out of 7 for a perfect match (column 3). Asterisks indicate those sites found to be protected from DNase I digestion by FIS *in vitro* (see Figure 2A). Synthetic DNA duplexes (30mers) centred on the 15 bp binding site sequences (with 8 and 7 bp of upstream and downstream flanking sequences, respectively) were titrated with purified FIS. The FIS monomer concentration required for 5% of the DNA to be complexed (as measured by gel retardation assay) is given in the fourth column as a measure of the FIS binding affinity of these putative sites. FIS titrations were suspended in the case of the −219 and −69 sequences before 5% binding was attained.

−98 and −150. The second complex (cII) is unable to form upon truncating the promoter to −86 (lanes 10−12) thus restricting the location of a second FIS site to the sequence between −98 and −86. No specific complexes were obtained when fewer than 55 nucleotides upstream of the start site were present (unpublished data) and 79 bp were necessary for one stable complex to form (lanes 13−15). Therefore the third and final site must lie downstream of −79. Four independent lines of evidence suggest that the potential 7/7 match to the binding site consensus terminating at position −78 corresponds to this promoter-proximal site (see Table I). (i) Weak complex formation was achieved with a fragment deleted to −76 (unpublished data). This residual

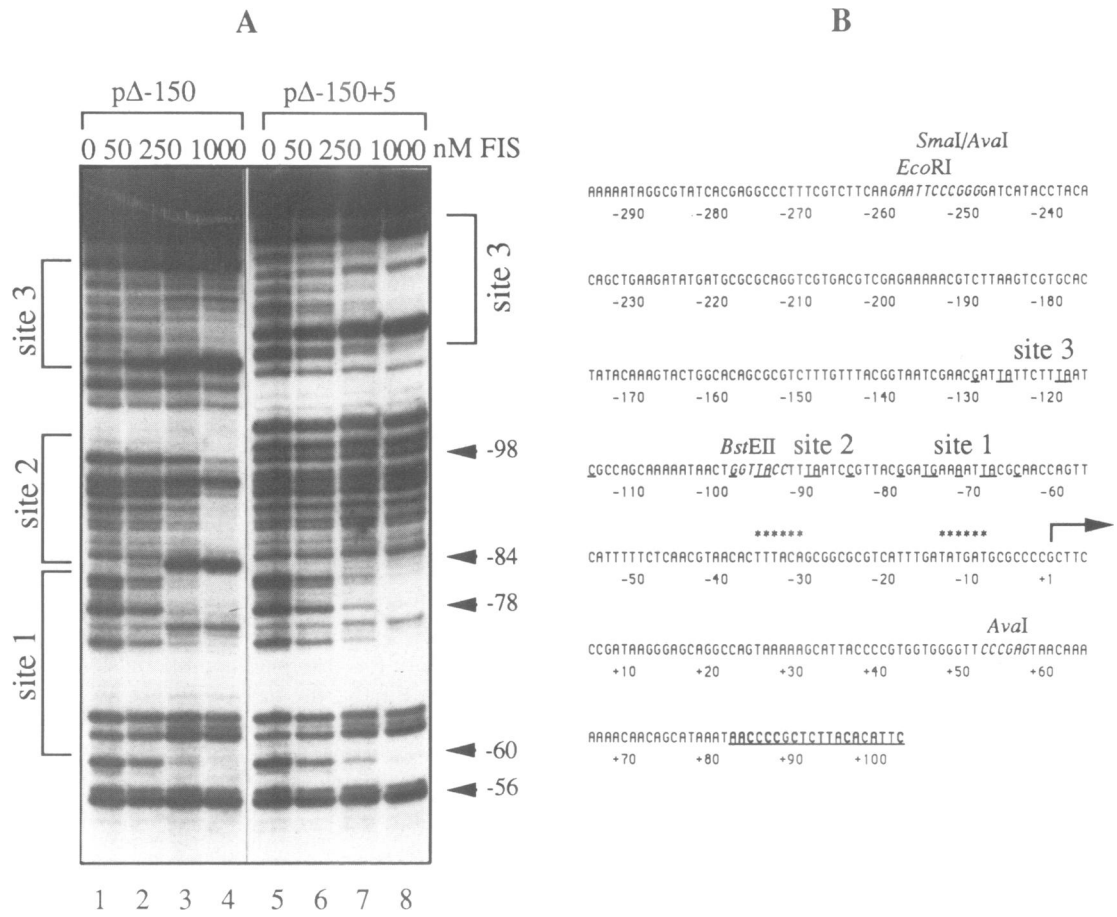

Fig. 2. (**A**) DNase I footprint analysis of FIS bound to the *tyrT* UAS. Lanes 1–4: the 210 bp *Eco*RI–*Ava*I restriction fragment from pΔ-150 labelled at its *Ava*I end (identical to that used in Figure 1, lanes 4–6) was titrated with purified FIS at the concentrations indicated. Lanes 5–8: titration of the equivalent restriction fragment (215 bp) derived from the mutated construct pΔ-150+5. The regions protected from nuclease attack by bound FIS (footprints) are bracketed. Some prominent features of the DNase I digestion pattern are indicated by arrowheads labelled with the positions to which they correspond in the *tyrT* promoter. (**B**) Sequence of the *tyrT* promoter. The *in vivo* transcription start site (+1) is depicted by an arrow above the sequence. The core promoter hexamers are indicated by asterisks. The sequence complementary to the primer used for primer extension analysis is shown in bold-face type and underlined. Conserved elements of the three identified FIS binding sites are underlined. Restriction sites mentioned in the text are italicized.

binding activity was stabilized by the presence of non-*tyrT* sequences upstream of −76 (lanes 16–18) suggesting that the binding site is adjacent to the 5′ terminus of the fragment. (ii) An oligonucleotide corresponding in sequence to the region from −86 to −57 (−78 of Table I) shares a high affinity for FIS with the two other footprinted sites (see below). (iii) A 2 bp deletion within this putative proximal binding site caused a severe reduction in DNA binding affinity of the order of 15- to 20-fold (Lazarus, 1992). (iv) This location for a FIS binding site (centred at −71) appears to be highly conserved amongst stable RNA promoters (Josaitis *et al.*, 1990; Verbeek *et al.*, 1990).

Comparison of the DNase I cleavage patterns for a *tyrT* fragment terminating at −150 in the absence (Figure 2A, lane 1) or presence (lanes 2–4) of FIS protein permitted precise definition of the three FIS binding sites. All indications of DNA–protein interaction (i.e. protected or enhanced cleavages relative to lane 1) are found upstream of the −35 consensus element as expected from the deletion analysis. Protection from nuclease cleavage at position −60 marks the 3′ boundary of the promoter-proximal site (site 1). The footprint extends distally to around −100, but is interrupted by a number of hypersensitive cleavages (most notably at −74 and −84). The gel retardation studies predict that this 40 bp stretch of sequence should harbour two FIS binding sites. Incomplete protection of an upstream region is also observed, with a prominent hypersensitive site at about −116. This represents the effects of FIS bound to the distal site (site 3).

The boundaries of protection conferred by FIS dimers bound to the adjacent sites (sites 1 and 2) were established by analysis of a mutated promoter fragment in which the putative site 2 sequence had been disrupted by filling-in of the natural *Bst*EII restriction site at position −98 (see Figure 2B). The effects of this mutation on binding of FIS are shown in Figure 2A (lanes 5–8). As expected, this mutated DNA fragment only supports the formation of two complexes with FIS (Lazarus, 1992). This correlates with loss of the DNase I hypersensitive site at −84 and protection of the adjacent upstream region which, by subtraction, represent the effects of FIS bound to site 2. Gel retardation and DNase I footprint analysis of the full-length promoter fragment (Figure 1, lanes 1–3; Lazarus, 1992) revealed no additional FIS binding sites upstream of position −150. Therefore all the necessary sequence information for complex formation between the *tyrT* promoter and FIS is found in an equivalent location to that determined for the *thrU*(*tufB*) and *rrnB* P1 promoters (see Discussion).

A sequence homology search of the 250 bp immediately upstream of the start point of *tyrT* transcription identified five perfect (7/7) and eighteen 6/7 matches to the 15 bp degenerate FIS binding site consensus motif (as determined by Hübner and Arber, 1989). The three sequences displaying the closest correspondence in position to the footprinted regions are shown in Table I [i.e. those terminating at −129 (site 3), −98 (site 2) and −78 (site 1)]. The ability of these sequences to bind FIS was tested directly by gel retardation assays using synthetic duplexes 30 nucleotides long (30mers) centred on the putative binding sites. The choice of 30mers was based on DNase I footprinting data for FIS at other chromosomal locations where the homodimeric protein was shown to make contacts with DNA spanning 25 bp or 2.5 turns of the helix (Bruist *et al.*, 1987; Ross *et al.*, 1990; Gille *et al.*, 1991). Indeed truncation of a 30mer to a 20mer reduced FIS binding affinity some 5- to 10-fold (unpublished data) consistent with a requirement for more than just the consensus sequence DNA for stable interaction with FIS.

Additional 6/7 and 7/7 matches to the Hübner and Arber consensus were included in this analysis for comparative purposes. The data in Table I illustrate that a 7/7 match to the consensus does not guarantee stable binding of FIS since the sites terminating at −78 and −26 exhibit a difference in affinity of at least two orders of magnitude. Likewise, not all 6/7 matches exhibit equal affinity for FIS and in fact some bind better than 'perfect' sites. It is clear therefore that homology to this degenerate consensus sequence is not adequate to predict whether the site will bind FIS *in vitro*.

The effects of FIS on stable RNA transcription in vivo

The presence of strong binding sites for FIS within the upstream regions of the stable RNA operons *rrnB* and *thrU*(*tufB*) has been correlated with FIS-dependent transcriptional activation *in vivo* (Nilsson *et al.*, 1990; Ross *et al.*, 1990). However, as described in the Introduction, there was a considerable difference in the absolute effects of FIS on these two promoters *in vivo*. Since the *in vitro* binding results presented above were highly suggestive of a role for FIS in *tyrT* regulation, the response of this stable RNA promoter to the presence of FIS was examined with the aim of resolving the discrepancy between the *rrnB* and *thrU*(*tufB*) reports.

The *in vivo* transcriptional response of the wild type *tyrT* promoter to a simple nutritional upshift was monitored by measuring the levels of galactokinase produced from the *tyrT*−*galK* reporter construct pTyr2 (Lamond and Travers, 1983). The upshift conditions (see Materials and methods for details) were chosen for consistency with previous analyses of the effects of FIS on stable RNA promoter activity (Nilsson *et al.*, 1990; Ross *et al.*, 1990). Since pTyr2 harbours *tyrT* sequences extending to −248 upstream and +53 downstream of the transcription start site it is assumed to harbour all the necessary sequence information to confer wild type regulation. Its activity is compared in DS941 (Fis^+) and DS941 *fis*::Kan (Fis^-) cells in Figure 3A. The initial rise in transcriptional activity in the Fis^+ cells was followed by a steady decline. An almost identical response was detected in a strain lacking functional FIS protein demonstrating that this activity profile is FIS-independent. Indeed, a qualitatively indistinguishable result was obtained when the two strains were transformed with constructs lacking *tyrT* sequences upstream of position −51 (i.e. retaining none of the identified FIS binding sites, Figure 3F). Therefore the peak of *tyrT* promoter activity in the early logarithmic phase of growth although coinciding with maximal expression of *fis* (Ninnemann *et al.*, 1992), must reflect inherent properties of the minimal promoter, whilst the level of transcription is probably determined by the *cis*-acting effects of the UAS (Lamond and Travers, 1983). One potentially significant difference between the two strains was a temporal one. The activity peak was delayed by ~15 min in the Fis^- background relative to Fis^+ and probably stems from the slower growth of this mutant strain.

The absence of any increase in transcription in a Fis^+ strain relative to Fis^- is similar to that observed for the *rrnB* P1 promoter (Ross *et al.*, 1990), but apparently contradicts the results with the *thrU*(*tufB*) operon (Bosch *et al.*, 1990; Nilsson *et al.*, 1990). To control against the possibility that differences in experimental procedure were responsible for the failure to detect FIS-dependent activation of *tyrT*, the *tyrT* and *thrU*(*tufB*) promoters were compared directly. The effect of upshift on the full-length *thrU*(*tufB*) reporter construct ptufBM (identical to +UAS of Nilsson *et al.*, 1990) is compared in Fis^+ and Fis^- cells in Figure 3D. Two major differences are detected between the response of this promoter and the full-length *tyrT* construct (pTyr2). Firstly, the *thrU*(*tufB*) promoter is stimulated 2- to 3-fold in a FIS-dependent manner in good agreement with previous experiments (Nilsson *et al.*, 1990). However, peak activity is attained more rapidly following upshift (after only 45 min instead of the 60−80 min interval observed by Nilsson *et al.*). Since this earlier onset of activity is also observed with the *tyrT* constructs (Figure 3A and F) it must be due to strain and/or procedural differences. Secondly, the fully activated *thrU*(*tufB*) promoter has only one-third of the activity of pTyr2. This conflicts with expectations from the relative abundance of the two tyrosine inserting tRNA species in *E.coli* (Gross and Raab, 1972). The more abundant tyrosine tRNA is transcribed from the *thrU*(*tufB*) promoter (*tyrU* being one of four tRNA genes co-transcribed with *tufB*) whilst the minor component is produced from the tandemly duplicated genes of the *tyrT* locus.

Sequencing the *thrU*(*tufB*)-derived sequences of ptufBM identified a substitution mutation at position −9. This converts the wild type −10 hexanucleotide sequence from TAGAAT (An and Friesen, 1980) to TAGAgT, thus increasing the divergence of this critical promoter element from the established consensus (TATAAT, Harley and Reynolds, 1987). In order to test the significance of this mutation (which is expected from the 'consensus is strength' rule to reduce promoter activity) the altered base pair was restored to wild type. The activity of this wild type construct (ptufB, Lazarus, 1992) is 10-fold higher than that of its mutated counterpart (compared in Figure 3B) and 3-fold greater than pTyr2 (compare scales of Figure 3A and B). The increased expression of the wild type promoter (ptufB) was accompanied by a loss of dependence on *fis* function (Figure 3B). Thus the mutation present in the *thrU*(*tufB*) constructs employed by Nilsson *et al.* accounts not only for the 3-fold reduction in activity relative to *tyrT*, but apparently determines the FIS-dependency of the *thrU*(*tufB*) promoter.

As further proof of the involvement of the −10 region in conferring FIS-dependence, the analogous mutation was introduced into the *tyrT* promoter (pTyr2M, Lazarus, 1992). This single nucleotide substitution alters the wild type −10 sequence from TATGAT to TATGgT. The effect on promoter activity was substantial; transcription was reduced

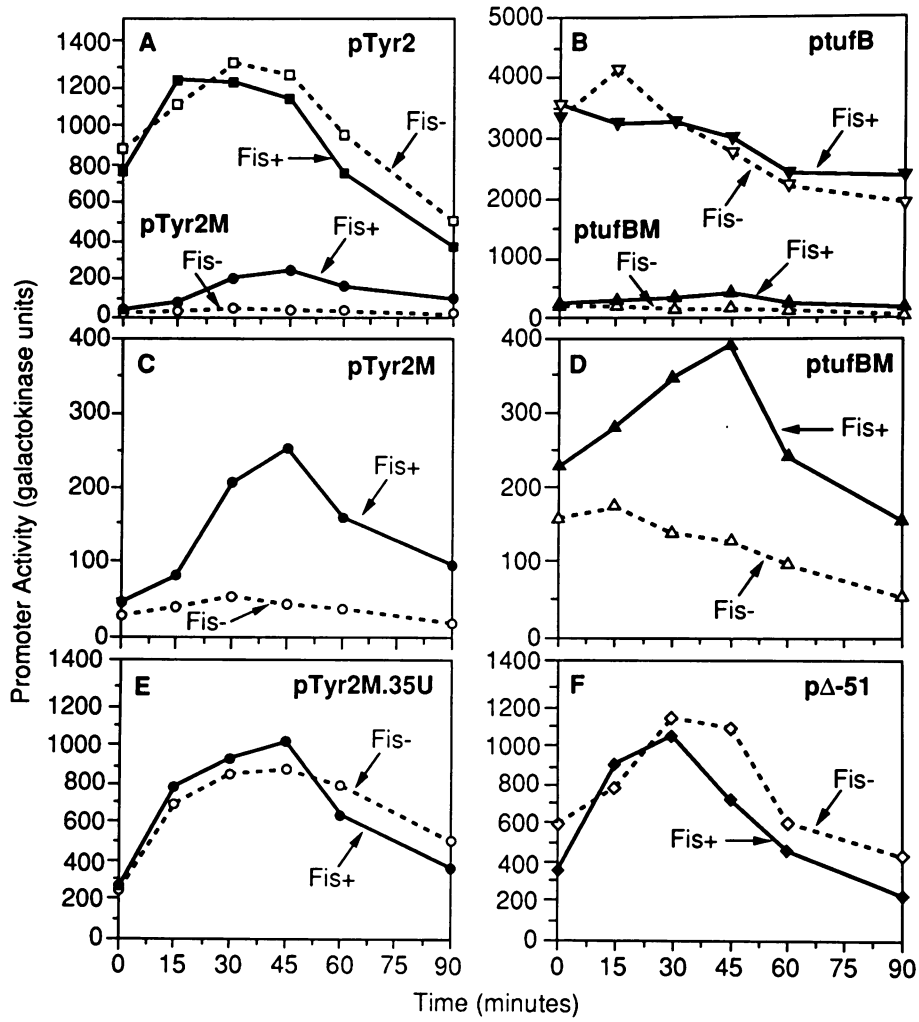

Fig. 3. *In vivo* activities of wild type, truncated and mutated stable RNA promoters compared in Fis$^+$ and Fis$^-$ strains following nutritional upshift. Overnight cultures of freshly transformed DS941 (Fis$^+$) and DS941 *fis*::Kan (Fis$^-$) cells were diluted 50-fold into fresh medium and grown at 37°C for 90 min. Samples were removed for optical density and GalK activity measurements as described in Materials and methods. Initial OD_{650} were typically between 0.1 and 0.15 units. The alterations in promoter activity with time are shown for representative experiments, all of which were performed at least three times. Differences in copy number were found to be <2-fold as previously determined (Lamond and Travers, 1983). Plain lines with filled symbols are for Fis$^+$ transformants and dashed lines with open symbols are for Fis$^-$. (**A**) Full-length wild type (pTyr2) and −10-mutated (pTyr2M) *tyrT* promoter constructs compared. (**B**) Full-length wild type (ptufB) and −10-mutated [ptufBM is identical to +UAS of Nilsson *et al.* (1990)] *thrU*(*tufB*) promoter constructs compared. (**C** and **D**) pTyr2M and ptufBM on the same (expanded) vertical scale. (**E**) Compensatory effect of a secondary 'up' mutation in the core promoter region of *tyrT* illustrated by the double mutant pTyr2M.35U. (**F**) Activity of the truncated *tyrT* promoter construct pΔ-51 lacking FIS binding sites.

5- to 6-fold in the Fis$^+$ strain and ~20-fold in the Fis$^-$ strain (Figure 3A). Re-stated, this demonstrates that mutation of the −10 hexamer can convert the FIS-independent *tyrT* promoter into one which exhibits a 5-fold enhancement of activity in the presence of the *fis* gene (shown on an enlarged scale in Figure 3C).

Mutation of the −10 region is not the sole determinant of FIS-dependence

To investigate how the −10 region confers FIS-dependency, a secondary mutation was introduced within the core promoter region. This single nucleotide substitution (at position −33) creates a canonical −35 hexamer (TTgACA) from the slightly imperfect wild type sequence (TTTACA) and should therefore constitute a promoter 'up' mutation. The activity of this doubly mutated *tyrT* promoter (pTyr2M.35U, Lazarus, 1992) was analysed in Fis$^+$ and Fis$^-$ cells as before (Figure 3E). Upon comparison with the single point mutant construct pTyr2M (Figure 3C), two obvious effects of the mutation within the −35 hexamer are detected. It not only restores promoter strength to a level intermediate between pTyr2 and pTyr2M, but also abolishes FIS-dependence. Another more drastic mutation in the −10 region which converts the 89% conserved T at −8 to an A (*tyrTp27*, Berman and Landy, 1979) and retains only 1−2% of wild type activity fails to be activated in a Fis$^+$ background (unpublished data). Thus 'down' mutations within the −10 region *per se* are not sufficient to determine FIS-dependency and suggest that the inherent 'transcribability' of the promoter governs the response to *fis*.

Effects of FIS on tyrT transcription in vitro

Thus far, FIS-dependent effects on *tyrT* transcription have only been observed under rather artificial conditions (i.e. following alterations to the primary sequence information). In order both to test the transcribability theory and to determine whether promoter mutagenesis has a physiological counterpart, we analysed the response of the *tyrT* promoter

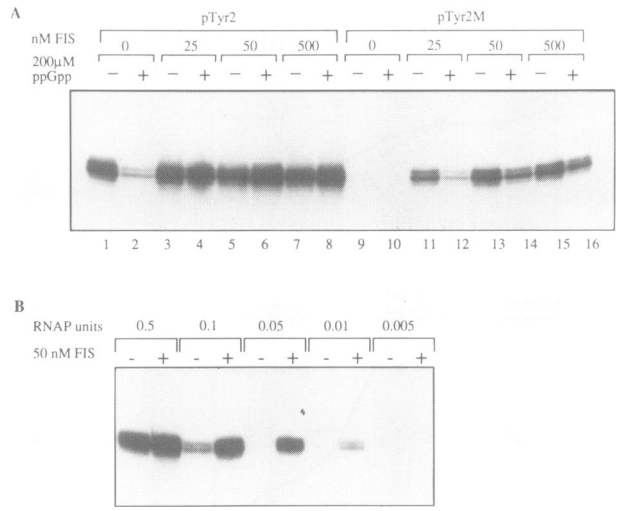

Fig. 4. (**A**) Analysis of the combined effects of FIS and ppGpp on *in vitro* transcriptional activity from the wild type (lanes 1–8) and −10-mutated (lanes 9–16) *tyrT* promoter constructs. Standard *in vitro* transcription reaction conditions were employed with pTyr2 (see Materials and methods) but the ionic strength was reduced to 250 mM KCl for pTyr2M. Levels of *tyrT*-specific transcript were measured by primer extension as described in Materials and methods. (**B**) Effects of FIS on wild type *tyrT* transcriptional activity measured over a 100-fold range of RNA polymerase (RNAP) concentrations at 300 mM KCl.

to the putative activator FIS under a variety of *in vitro* conditions. Transcription from stable RNA promoters is known to be sensitive to a number of factors that fluctuate according to the growth phase such as DNA supercoiling (McClellan *et al.*, 1990), ionic strength (Schultz and Solomon, 1961) and guanosine tetraphosphate (Cashel and Rudd, 1987).

The first stage of these investigations was to establish the basal level of *tyrT* promoter activity with respect to general factors that influence transcription initiation such as ionic strength and RNA polymerase concentration. Thus for a duplex DNA template at a superhelical density roughly double that found *in vivo* (Drlica, 1992) and in the presence of a 10- to 20-fold molar excess of RNA polymerase, two major transcripts are initiated from the wild type *tyrT* promoter (see Figure 4A for example). The shorter and slightly more abundant transcript of 102 nucleotides maps to the correct *in vivo* start site at +1 and the longer product maps to position −1 (Lazarus, 1992). Transcription initiation was previously inferred to originate from these two positions on linear templates by the comparative influence of different ribonucleoside triphosphates on initiation (Küpper *et al.*, 1975) and from run-off transcription experiments (unpublished data). Therefore alterations in template topology do not affect start site selection by RNA polymerase. In fact, of all the factors considered in this study only the sequence context of the start sites was found to have a significant effect on the pattern of transcripts generated from the *tyrT* promoter *in vitro* (see below).

Transcriptional activity of the wild type *tyrT* promoter is remarkably salt tolerant and can be detected at all concentrations of KCl within the range 0–350 mM, with a broad optimum at 200 mM ± 100 mM (Lazarus, 1992). This conflicts with the inhibition of *tyrT* promoter activity observed with linearized templates at concentrations above 50 mM KCl (Küpper *et al.*, 1975), but is consistent with the reported increase in salt-tolerance of this promoter when present on a supercoiled rather than a relaxed or linear template (Lamond, 1985). It should be noted that the standard *in vitro* transcription conditions employed here (for details see Materials and methods) probably involve higher concentrations and different preparations of RNA polymerase compared with other researchers which could account for the apparent increase in salt-tolerance of this promoter in our hands.

All subsequent *in vitro* experiments involving the wild type *tyrT* promoter were performed in the presence of 300 mM KCl. This is in reasonable agreement with physiological determinations of potassium ion concentrations which peak at ~250 mM in the early logarithmic phase of growth (Schultz and Solomon, 1961). In spite of these somewhat unfavourable ionic conditions (i.e. at the upper limits of the optimum range), the wild type promoter is refractory to stimulation by FIS (Figure 4A, compare lanes 1, 3, 5 and 7). In contrast, the disabled pTyr2M promoter is not only susceptible to inhibition by high salt [having barely detectable transcript levels at 250 mM KCl (lane 9)], but is also activated by FIS some 20- to 30-fold. This activation is clearly saturable since no further increase in transcriptional activity is detected when FIS concentrations are raised above 50 nM (compare lanes 9, 11, 13 and 15). The responses of these two *tyrT* promoter constructs pTyr2 and pTyr2M to FIS *in vitro* are therefore qualitatively similar to those *in vivo*.

A similar effect of suboptimal ionic strength on FIS-dependent activation has been reported for another stable RNA promoter, *rrnB* P1. In that instance, the salt-sensitivity of transcription [reflecting the instability of initiation complexes formed between the promoter and RNA polymerase at salt concentrations above 30 mM (Gourse, 1988)] was found to be alleviated by FIS [i.e. the stimulatory effect of FIS on transcription was enhanced with increasing ionic strength (Ross *et al.*, 1990)]. An alternative and not necessarily exclusive role for FIS in *trans*-activation has been suggested based on the observed dependence of *thrU*(*tufB*) promoter activity on RNA polymerase concentration. Nilsson *et al.* (1990) proposed that binding of FIS to the UAS facilitated binding of RNA polymerase to the promoter, thereby stimulating transcription initiation. Analogous modes of transcriptional activation for the wild type *tyrT* promoter might be masked by the exceptionally high salt tolerance of this promoter and/or the inclusion of RNA polymerase at a 10- to 20-fold molar excess relative to DNA template in standard reactions. To address this latter possibility the effects of FIS were examined over a 100-fold range of RNA polymerase:template ratios. The concentration of FIS chosen for this experiment (50 nM) was sufficient to saturate the available binding sites on pTyr2M (Figure 4A, compare lanes 13 and 15). RNA polymerase is evidently in functional excess at the highest concentration tested (in Figure 4B 0.5 units corresponds to a 15- to 30-fold molar excess) since FIS does not stimulate basal activity. However, in the presence of ≤0.1 units of RNA polymerase (3- to 6-fold molar excess) basal activity is increased substantially by FIS implying that enzyme:template ratios below this level are rate-limiting.

FIS overrides inhibition of transcription by guanosine tetraphosphate in vitro

Stable RNA promoters are preferentially inhibited upon amino acid starvation by the stringent response which

operates at the level of transcription initiation to abolish new ribosome synthesis whilst maintaining housekeeping functions. The cellular levels of guanosine 5′-diphosphate 3′-diphosphate (ppGpp) have been shown to be inversely related to amino acid availability (Ryals *et al.*, 1982) rising rapidly to ~1 mM concentration upon induction of the stringent response (Cashel and Gallant, 1969). This has led to the proposal of a stringent control mechanism whereby ppGpp interacts directly with RNA polymerase to induce a conformational change that prevents efficient recognition of stringent promoters. Amino acid starvation therefore represents a physiological and reversible mechanism for inducing transcriptional down-regulation of stable RNA promoters.

Stringent regulation of stable RNA transcription has previously been demonstrated for the *tyrT* promoter *in vivo* by induction of amino acid starvation (Lamond and Travers, 1985b). Preferential inhibition of this promoter present on a linear DNA fragment has also been reproduced *in vitro* by inclusion of the stringency effector molecule, ppGpp (Travers, 1980a). These studies used the synthetic promoter SSU1, which retains only 51 bp of *tyrT* sequences upstream of the transcription start site. Substitution of four AT base pairs within the GC-rich region lying between the −10 hexamer and the start site (the so-called discriminator motif) created construct SSU2 (Ryan *et al.*, 1979). In contrast to SSU1, SSU2 was not subject to stringent control *in vivo* (Lamond and Travers, 1985b) nor was it inhibited by ppGpp *in vitro* (Travers, 1980a). The discriminator region which is conserved amongst stringently controlled and hence stable RNA promoters (Travers, 1980b, 1984) was thus identified as a sequence determinant of stringent regulation. The differential sensitivity of SSU1 and SSU2 to ppGpp *in vitro* is maintained when transcription is initiated from supercoiled templates (Lazarus, 1992). However, SSU1 and SSU2 have much lower promoter activity than the full-length *tyrT* constructs presumably due to loss of *cis*-activation by upstream sequences. Therefore these constructs were not well-suited for quantitative analysis of changes in transcriptional activity. To overcome this difficulty a discriminator-mutated derivative of the full-length *tyrT* promoter was employed (pTyr2D, Lazarus, 1992) having the same alteration of nucleotides −1 to −4 as found in SSU2 (for sequence see Figure 6B), but with *tyrT*-derived sequences extending to position −248.

In vitro transcription from pTyr2D was measured over a range of ppGpp concentrations and compared with pTyr2 (Figure 5A). Whilst basal activity from pTyr2 was repressed by 50−70% by micromolar levels of ppGpp (50−200 μM, Figures 3A and 5A), levels of pTyr2D transcription were marginally stimulated within this physiological range of ppGpp concentrations. In addition to exhibiting an intrinsic increase in transcriptional activity of ~30%, initiation from pTyr2D occurred at a triplet of start sites (Figure 5A). The recruitment of position −2 as an extra start site (the wild type starts at −1 and +1 are preserved) was also observed with SSU2 (Lazarus, 1992) and would therefore appear to be a feature of the discriminator-mutated *tyrT* promoter. This finding differs from a previous report in which there was no observed difference in start site usage between SSU1 and SSU2 from a linear template (Travers, 1980a). The major product now initiates at −1, rather than at +1, and the flanking nucleotides (+1 and −2) are used roughly equally, but to a lesser extent than −1 (Figure 5B). The preference

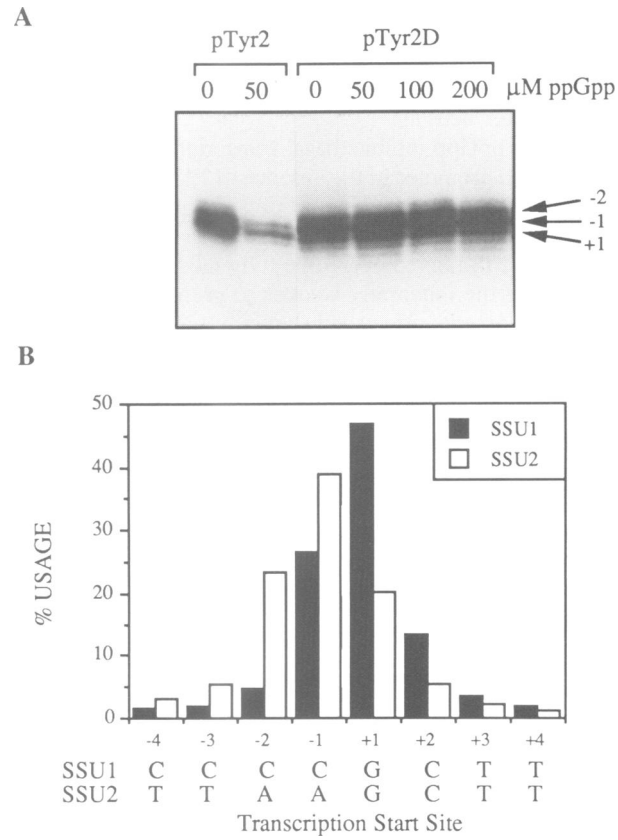

Fig. 5. (**A**) Effects of ppGpp on transcriptional activity of wild type and discriminator-mutated *tyrT* promoters. *In vitro* transcription reactions were performed using pTyr2 and pTyr2D as templates under standard reaction conditions in the presence or absence of ppGpp at the indicated concentrations. The labelled arrows indicate the triplet of start sites from which transcripts initiate from pTyr2D. (**B**) Comparison of *in vitro* start site selection at wild type (SSU1) and discriminator-mutated (SSU2) *tyrT* promoters. Transcription from SSU1 and SSU2 was compared in an equivalent experiment to that of part A and the proportion of transcripts initiated at each of the nucleotides between +4 and −4 was calculated following densitometric scanning of the products formed in the absence of ppGpp.

for −1 is not unexpected since the purine adenosine has been substituted for the pyrimidine cytosine at this position and purines are more commonly used as the initiating nucleotide (Harley and Reynolds, 1987).

Transcription from the wild type *tyrT* promoter was only partially inhibited by ppGpp and was strictly dependent on ionic strength. Thus below 300 mM KCl the nucleotide was ineffective, and at concentrations exceeding this level basal transcriptional activity was diminished (Lazarus, 1992). This salt-dependence probably reflects the naturally high affinity of RNA polymerase for the *tyrT* promoter. There is some evidence to suggest that ppGpp interacts directly with the enzyme thereby inducing a conformational change that prevents efficient recognition of stringent promoters (Travers, 1976; Owens *et al.*, 1987). Consistent with such a model, conditions that weaken polymerase−template interactions, for example template linearization and/or high salt, have now been shown to enhance the inhibitory effects of ppGpp on transcription from two stable RNA promoters, namely *tyrT* (Travers, 1980a; Lazarus, 1992) and *rrnB* P1 (Ohlsen and Gralla, 1992). Conversely, the negative effects of ppGpp on stable RNA transcription should be reversed by factors that stabilize the binding of RNA polymerase to

stringently controlled promoters. The possibility that FIS might serve as such a stabilizing influence was tested directly by examining the combined effects of FIS and ppGpp on *tyrT* promoter activity.

Although ppGpp inhibits basal transcriptional activity of the wild type promoter in the absence of FIS, the nucleotide is completely ineffective in the presence of even modest concentrations of FIS (compare lanes 2 and 4 of Figure 4A). In contrast, FIS fails to overcome fully the ppGpp-mediated inhibition of the inherently weaker pTyr2M promoter, but does antagonize its effects substantially (compare lanes 15 and 16). Reversal of ppGpp-mediated inhibition of the wild type *tyrT* promoter was saturable at low concentrations of FIS consistent with a dependence on binding of FIS to its sites within the UAS. The requirement for at least one FIS binding site was confirmed by the failure of SSU1 (which lacks all the identified FIS binding sites of the *tyrT* UAS) to be stimulated by FIS in the presence or absence of ppGpp (Lazarus, 1992). This binding site requirement is entirely consistent with the other known functions of FIS (see Introduction).

Discussion

FIS is not required for stimulation of tyrT transcription upon resumption of exponential growth

The principal conclusion from the experiments reported here is that FIS is neither required nor responsible for the initial burst of transcription from the wild type *tyrT* promoter under our experimental conditions. Although transcription from the *fis* gene is maximal within 15 min of upshift (Ninnemann *et al.*, 1992) the same transcriptional response of the *tyrT* promoter is observed in cells lacking functional FIS protein. This observation extends to the wild type *thrU(tufB)* promoter. We note that this lack of stimulation is not an intrinsic property of the *tyrT* promoter itself nor of its genetic context since exogenously supplied FIS will, under the appropriate conditions, enhance *tyrT* expression from the same plasmid *in vivo* (L.R.Lazarus, O.Ninnemann, C.Koch, R.Kahmann and A.A.Travers, unpublished observations). Similarly our results do not preclude the existence of another transcription factor that is both essential and specific for UAS function.

This response of the *tyrT* promoter on upshift is similar to that of transcription of the *fis* gene (Ninnemann *et al.*, 1992) but is in apparent conflict with conclusions from other laboratories that FIS activates the wild type *rrnB* P1 and *thrU(tufB)* promoters *in vivo* (Nilsson *et al.*, 1990; Ross *et al.*, 1990). In the case of the *rrnB* P1 promoter constructs retaining a single FIS binding site, the absolute difference in transcriptional activity between FIS-producing (Fis^+) cells and a *fis* null mutant (Fis^-) was only ~13%. In these experiments activity was examined at a single time point after five cell generations had elapsed whereas under the conditions where we observe FIS-dependent activation *in vivo* (see Results), it was found to be short-lived and restricted to the early logarithmic phase of growth, coincident with *fis* expression (Thompson *et al.*, 1987; Ball *et al.*, 1992; Ninnemann *et al.*, 1992). Therefore we would not expect any substantial absolute stimulation to occur after five cell doublings. Any differences after such prolonged growth are likely to reflect secondary adaptations rather than primary effects of *fis*. The reported FIS-dependent activation of the

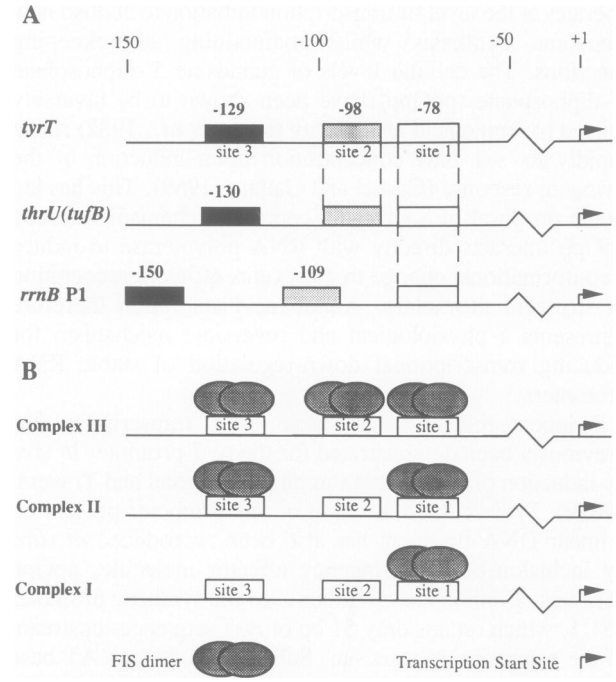

Fig. 6. (**A**) Schematic representation of the FIS binding site distribution in the *tyrT*, *thrU(tufB)* and *rrnB* P1 UASs. The proximal (site 1), central (site 2) and distal (site 3) FIS sites are represented by boxes. The 5′ ends of the 15 bp binding site sequences are indicated above the boxes. (**B**) Predicted order of complex formation between FIS dimers and stable RNA promoter UASs harbouring three FIS binding sites in the conserved arrangement shown in part A.

thrU(tufB) promoter (Nilsson *et al.*, 1990) is accounted for by an undetected down mutation in the −10 region. We conclude that FIS does not significantly stimulate stable RNA synthesis from wild type promoters under the upshift protocols so far tested. However, we argue below that FIS could confer an adaptive advantage under particular growth conditions.

Distribution of FIS binding sites in the UASs of stable RNA operons

Three FIS binding sites are located between positions −64 and −150 in the three stable RNA promoters for which *trans*-activation by FIS has been studied. The UASs of these promoters are compared schematically in Figure 6A. Several conserved features are immediately apparent. (i) The conservation of FIS site locations between the *tyrT* and *thrU(tufB)* promoters is almost perfect, but the ribosomal RNA promoter *rrnB* P1 exhibits greater divergence. The position of the promoter-proximal site (terminating at −78) is, however, fully conserved between these three stable RNA promoters and has also been identified by sequence homology in the UASs of many additional stable RNA operons (Josaitis *et al.*, 1990; Verbeek *et al.*, 1990). Although the ability to bind FIS *in vitro* by no means guarantees a functional role for these sequences, their widespread occurrence is wholly consistent with a selective pressure maintaining an involvement of FIS in co-ordinating stable RNA synthesis. (ii) In all three of these promoters the FIS binding sites are organized such that their mid-points are in the same double-helical phase (Figure 6A). The direction of protein-induced bending would be essentially the same as that preferred by the *tyrT* DNA in the absence

Table II. Redefining the FIS binding site consensus

FIS site (5′ end)	YR alignment	Match to consensus	G(13 bp)C
tyrT site 2 (−98)	GGTTACCTTTAATCC	6/7 → 7/7	GGTTACCTTTAATCC
thrU(*tufB*) site 2 (−98)	GGCTGTTTCTTCATC	6/7 → 7/7	GGCTGTTTCTTCATC
thrU(*tufB*) site 3 (−130)	GTTGAAAAAGTGTGC	6/7 → 7/7	GTTGAAAAAGTGTGC
rrnB P1 site 2 (−109)	GCTCGAAAAACTGGC	6/7 → 7/7	GCTCGAAAAACTGGC
consensus	T/G YR A/T YR C/A	7/7	$Gn_{(1-3)}$ $YRn_{(4-6)}$ $YRn_{(1-3)}C$

The match to the consensus according to Hübner and Arber (aligned on the YR base pairs, column 2) is compared with the match to a revised consensus (based on alignment of the terminal residues and conservation of a 13 bp separation, where the sum of n is 9, column 4) for selected 'imperfect' FIS binding sites located upstream of stable RNA genes. Note the prevalence of A or T residues in the central region.

of the protein (Drew and Travers, 1985) while at full occupancy of the FIS binding sites the magnitude of the bend would be maximally ~270° (Thompson and Landy, 1988). The average separation between the FIS binding sites in these three UASs is a multiple of 10.2−10.3 bp. This value is significantly less than the intrinsic helical repeat of supercoiled DNA in the cell (Lee and Schleif, 1989), a relationship which argues that FIS organizes the UAS DNA in the form of a shallow left-handed toroidal loop. Conservation of a particular spatial geometry has also been shown to be important for the stimulation by FIS of site-specific recombination (Hübner *et al.*, 1989) and for FIS-dependent activation of the *rrnB* P1 and *thrU*(*tufB*) operons (Josaitis *et al.*, 1990; Verbeek *et al.*, 1991; Newlands *et al.*, 1992). However, since *cis*-activation of *rrnB* P1 shares the need for a precise orientation of the UAS relative to the RNA polymerase binding site (Newlands *et al.*, 1992), the phasing may reflect a requirement for appropriately directed DNA curvature (see below). (iii) The two most proximal sites of *tyrT* and *thrU*(*tufB*) have only 6 bp between the 5′ end of site 1 (−78) and the 3′ end of site 2 (−85). This spacing must represent the minimal possible separation that conserves the periodic arrangement of binding sites. (iv) Not only are the number, location and spacing of the FIS sites conserved, but their order of occupancy (reflecting their different affinities for FIS) is also conserved. The studies with synthetic binding sites described here for the *tyrT* promoter and elsewhere for *thrU*(*tufB*) (Lazarus, 1992) indicate that the proximal and distal sites (sites 1 and 3 in Figure 6A) have a greater affinity for FIS than the central site 2. This same binding preference is also observed for the *rrnB* P1 promoter (Ross *et al.*, 1990). The probable composition of the specific FIS−UAS complexes is illustrated in Figure 6B.

Re-defining the FIS binding site consensus sequence

The dependence of the selectivity of FIS binding on both DNA sequence and structure recognition (Travers, 1991) has led to a variety of proposals for the preferred consensus binding sequence (Bruist *et al.*, 1987; Hübner and Arber, 1989; Verbeek *et al.*, 1990; Yanagi *et al.*, 1991; Finkel and Johnson, 1992; Kostrewa *et al.*, 1992). We have addressed this question with extensive *in vitro* studies with synthetic FIS binding sequences (Lazarus, 1992 and unpublished data) from which we deduce a functional consensus shown in Table II. There are two key differences from the earlier consensus sequences; (i) the boundary nucleotides themselves are quite strictly conserved (guanosine at the 5′ end and cytosine at the 3′ end) as is their 13 bp separation; (ii) since the binding sequences were aligned on the terminal residues of the 15 bp binding sequence rather than the YR base steps the positions of these latter conserved elements are no longer rigidly defined with respect to the fixed ends. This results in a more flexible consensus retaining overall symmetry. As a consequence, the imperfect central sites (sites 2) of the *tyrT*, *thrU*(*tufB*) and *rrnB* P1 promoters can be re-defined (see Table II). The much lower affinity for FIS of the other potential sites upstream of *tyrT* which only have a 6/7 match to the Hübner and Arber consensus (Table I) can now be accounted for by the fact that they do not exhibit a superior fit to this revised consensus.

Although this revised consensus is still degenerate with respect to precise sequence requirements, the observed conservation of the YR base steps (which possess an unusual degree of conformational flexibility) suggests that specificity of FIS binding is determined in part by structural features of the DNA. The ability of the DNA to bend or kink at these positions is reflected in the diagnostic pairs of DNase I hypersensitive cleavages associated with FIS footprints (see Figure 2A for example), a characteristic which is also observed in footprints of the catabolite activator protein (CAP). In the co-crystal of this latter protein with DNA the induced bending is principally a consequence of two ~40° kinks between TG/CA base pairs in each half of the symmetric CAP binding site (Schultz *et al.*, 1991).

Partially disabling promoter function allows FIS-dependent activation of stable RNA operons

We have demonstrated FIS-dependent activation of *tyrT* transcription in three experimental situations: (i) a down mutation of the −10 hexamer which confers FIS-dependence both *in vivo* and *in vitro*, (ii) *in vitro* transcription under conditions of high ionic strength/limiting RNA polymerase and (iii) addition of ppGpp *in vitro* to mimic the stringent response. It is clear from the experiments of the second type that FIS *trans*-activates the *tyrT* promoter both by alleviating salt-sensitivity (readily detected with the disabled pTyr2M promoter, Figure 4A) and by facilitating transcription at low RNA polymerase concentrations (Figure 4B). How does this fit with the known effects of salt and polymerase concentrations on the kinetics of transcription initiation? Following promoter location, RNA polymerase binds specifically to the DNA to form the 'closed' complex. Formation of this complex is governed by the equilibrium constant K_B which is largely determined by the concentrations of the reactants (i.e. RNA polymerase and DNA template). It therefore presents an obvious target for polymerase concentration-dependent effects. Inhibition by high salt may also affect this step by increasing the rate of dissociation of the closed complex. Subsequent stabilization of polymerase−promoter interactions to form the 'open' complex is essentially

irreversible and is described by the isomerization constant, k_2. Low salt concentrations, negative supercoiling (Borowiec and Gralla, 1985, 1987) and AT-richness (Lazarus, 1992) all facilitate this thermodynamically unfavourable transition whereby the DNA is locally unwound and melted thus exposing the transcription start point.

It is evident from recent experiments on the *rrnB* P1 promoter that transcriptional inhibition by ppGpp occurs in the absence of post-initiation steps thus ruling out a significant role for altered rates of RNA chain elongation in mediating stringent control. Indeed, the influence of template topology and salt concentration on the effectiveness of ppGpp are consistent with the nucleotide inhibiting isomerization of the closed to the open complex (Ohlsen and Gralla, 1992). The negative effects of ppGpp on the *tyrT* promoter exhibit analogous salt and template dependence. Although the initial *in vitro* experiments described here implicated binding of polymerase to the promoter as the target for FIS action, the ability of FIS to fully counteract the effects of ppGpp-mediated inhibition suggests that these positive and negative regulators target the same rate-limiting step. However, the failure of saturating quantities of FIS (whose binding to the *tyrT* UAS is unaffected by high concentrations of ppGpp, unpublished data) to antagonize completely the inhibitory effects of ppGpp on pTyr2M supports a case for partial rather than total overlap of targets. Since the promoter mutation at −9 could affect either or both K_B (through loss of specific contacts with the sigma subunit of RNA polymerase holoenzyme) and k_2 (through increasing the GC-richness of the nucleating region for DNA strand separation), it is not yet possible to draw definitive conclusions as to the target(s) of FIS action.

How does FIS activate stable RNA promoters?

The UAS regions of stable RNA promoters act by facilitating initiation complex formation (Leirmo and Gourse, 1991). One intact FIS binding site is both necessary and sufficient for activation of the *tyrT* and *rrnB* P1 promoters by FIS *in vitro* under suboptimal transcription conditions (Ross *et al.*, 1990; Lazarus, 1992). However, sequences upstream of −88 are required for FIS-dependent activation of the disabled *thrU*(*tufB*) promoter *in vivo* (Verbeek *et al.*, 1992). The *in vitro* experiments described in this report indicate that FIS may facilitate binding of polymerase to the promoter. There are two distinct ways, not mutually exclusive, in which FIS may enhance RNA polymerase binding; either by assisting the formation of a preferred DNA topology for polymerase binding or by direct protein interactions between the activator and polymerase. Since both the required upstream regions and the FIS binding sites extend to at least 130 bp upstream of the transcription start point, sufficient DNA is available for the formation of a relatively tight loop. In this context we have noted that the disposition of the FIS binding sites would allow the protein to induce the formation of a shallow left-handed toroidal loop in negatively supercoiled DNA, a configuration which occurs naturally at the apices of the interwound (plectonemic) form of such molecules (Boles *et al.*, 1990). These apices are tightly bent (Laundon and Griffith, 1988) and are also known to be preferred binding sites for RNA polymerase (ten Heggeler-Bordier *et al.*, 1992). We therefore propose that FIS localizes stable RNA promoters to an apex of negatively supercoiled DNA where they are easily distinguishable from other promoter sequences in the interwound region of the template.

What is the legitimate role of FIS in stable RNA regulation in vivo?

A complex network of control mechanisms exists to ensure tight coupling of ribosome synthesis (i.e. stable RNA regulation) to the nutritional environment. In addition to the well-characterized stringent and growth rate controls, products of stringently controlled promoters such as FIS are themselves implicated in the process (Ninnemann *et al.*, 1992). The conclusion that FIS is not necessary for the activation of so vital a set of genes as the stable RNA operons is not surprising given that FIS is a non-essential gene product (Johnson *et al.*, 1988; Koch *et al.*, 1988). Indeed it provides further evidence of redundancy in the regulatory network previously inferred from the continued operation of growth rate control in the absence of both ppGpp-synthesizing activities (Gaal and Gourse, 1990). Nonetheless, the conservation of FIS binding sites within the UASs of stable RNA promoters is compelling evidence for FIS having a legitimate role in co-ordinating their regulation. Nilsson *et al.* (1992) suggested that FIS is required to allow very fast growth, whilst the growth rate and stringent control of the *fis* operon led Ninnemann *et al.* (1992) to propose that FIS serves both to amplify and to fine-tune these regulatory mechanisms. An alternative hypothesis is that FIS may boost stable RNA synthesis on nutritional upshift following prolonged adaptation to a nutrient-poor environment. Such adaptation could result in a relative depletion of the capacity for initiation at stable RNA promoters. This is largely consistent both with our *in vitro* findings, notably the antagonism of ppGpp-induced inhibition of a stable RNA promoter by FIS, and also with the high level of expression of *fis* immediately prior to the resumption of exponential growth (Ninnemann *et al.*, 1992). Such a boost would in principle confer a significant selective advantage and would explain the conservation of FIS binding sites in the UAS.

Materials and methods

Strains and media

Bacterial strains used in this study were *E.coli* K12 derivatives. DS941 (*thr*-1, *ara*-14, *leu*B6, *lac*Y1, *tsx*-33, *sup*E44, *gal*K2, λ⁻, *rac*⁻, *his*G4 (0c), *rfb*D1, *mgl*-51, *rps*L31, *kdg*K51, *xyl*-5, *mtl*-1, *arg*E3, *thi*-1, Δ(*gpt-pro*A)62, *rec*F, *lac*I^q, *lac*ZΔM15; Stirling *et al.*, 1989) and DS941 *fis*::Kan (a *fis*⁻ derivative of DS941) were kindly provided by D.Sherratt. All strains were grown in 2×TY medium (16 g tryptone; 10 g yeast extract; 5 g NaCl per litre, pH 7.4). Bacterial strains were maintained and single colonies isolated on TYE plates (15 g agar; 8 g NaCl; 10 g Bactopeptone; 5 g yeast extract per litre). For both liquid and solid growth media ampicillin selection was carried out at 100 μg/ml final concentration.

Plasmids and constructs

All plasmid DNA manipulations were performed essentially according to Sambrook *et al.* (1989). The construction of pTyr2, the parental plasmid from which the majority of the constructs discussed in the text are derived, is described elsewhere (Lamond and Travers, 1983; Lamond, 1984). Essentially it consists of *tyrT* promoter sequences (from −248 to +53 relative to the transcription start site, flanked at the 5′ end by an *Eco*RI site and terminating at the 3′ end with a naturally occurring *Ava*I site) cloned upstream of a *gal*K gene derived from the pKO-1 expression vector of McKenney *et al.* (1981). A rho-dependent transcription terminator was inserted between the cloning site and the *gal*K gene (K.McKenney, unpublished) to permit cloning of the wild type *tyrT* promoter whose high level of transcriptional activity was found to interfere with plasmid replication (Lamond, 1984). Derivatives of pTyr2 truncated from the 5′ deletion breakpoint (−248) were

generated by inverse PCR methods (Clackson *et al.*, 1991) and named according to the new breakpoint (x) as pΔ-x. A 5 bp insertion was made at position −97 of construct pΔ-150 by restriction at the *Bst*EII site (see Figure 2B) followed by end-filling and religating to create pΔ-150+5. The point mutants pTyr2M (−10 hexamer TATGgT), pTyr2M.35U (−10 hexamer TATGgT; −35 hexamer TTgACA) and pTyr2D (discriminator GCGttaaG) were created by PCR mutagenesis (for details see Lazarus, 1992). The wild type *thrU*(*tufB*)−*galK* fusion construct ptufB (−10 hexamer wild type) was created from +UAS of Nilsson *et al.* (1990) by similar techniques.

Gel retardation analysis

The gel retardation/mobility shift assay (Fried and Crothers, 1981; Garner and Revzin, 1981) was used to measure the interactions of purified FIS protein (kind gift of C.Koch) with labelled DNA fragments. Binding to synthesized 30mers was performed as follows; the complementary pairs were annealed as for DNA sequencing reactions and 5′-end-labelled using [γ-^{32}P]-ATP and T4 polynucleotide kinase (Sambrook *et al.*, 1989). Binding reactions were carried out in a total of 20 μl of 0.15 M HEG (25 mM HEPES pH 7.6; 0.1 mM EDTA; 1 mM DTT; 150 mM KCl; 10% glycerol) supplemented with 1 mM spermidine, 0.1 μg/μl poly[d(I-C)] DNA, 2 μg/μl bovine serum albumin and 0.1% NP40. After 15 min pre-incubation on ice with purified FIS protein at the indicated (monomer) concentration the labelled double-stranded oligonucleotides were added along with any specific (unlabelled) competitor DNA. The mix was incubated at 30°C for 20 min prior to addition of loading dyes (final concentration of 4 mM Tris−HCl pH 7.5; 1.5 mM NaCl; 0.01% bromophenol blue; 0.01% xylene cyanol and 4% glycerol). The samples were analysed on 0.7% agarose, 0.5×TBE (45 mM Tris−borate; 1 mM EDTA pH 8.0) gels run at 2−4 V/cm. Dried gels were exposed to film and complexes were visualized by autoradiography. Quantitative analysis was performed by densitometric scanning of appropriately exposed autoradiographs using a Molecular Dynamics 300A Computing Densitometer and the ImageQuant software package.

Complexes formed between FIS and longer DNAs (restriction fragments derived from pTyr2 and the various pΔ-x derivatives as described in the legend to Figure 1) were analysed in identical fashion except that (i) the DNA was labelled at the *Ava*I restriction site by filling in with Klenow fragment of *E.coli* DNA polymerase I and [α-^{32}P]-dCTP and gel purified (Sambrook *et al.*, 1989) and (ii) separation of the nucleoprotein complexes was performed on 0.25×TBE, 4%, 20:1 acrylamide:bisacrylamide gels run at 7 V/cm.

Dilutions of FIS protein were calculated for wild type FIS based on an approximate (10 000) rather than an exact (11 200) value for the molecular weight of the FIS monomer resulting in ~12% over-estimation of the number of FIS molecules present at a given concentration.

DNase I footprints with FIS

Restriction fragments were uniquely labelled at their 3′ (*Ava*I cut) ends and complexed with FIS as described for gel retardation analysis but in double the reaction volume and in the absence of detergent (NP40). These complexes were subsequently treated with DNase I at 4 μg/ml in the presence of 6 mM $MgCl_2$ and 6 mM $CaCl_2$ for 60 s at room temperature. Digestion was stopped by addition of 80 μl of stop solution (50 mM EDTA; 0.2% SDS; 10 μg/ml tRNA; 100 μg/ml proteinase K) and the mixture incubated at 42°C for 45 min. The DNA was then phenol extracted, ethanol precipitated and boiled for 2 min before loading on a 1×TBE, 6% denaturing polyacrylamide gel. G-tracks of the labelled DNAs were prepared by the method of Maxam and Gilbert (1980) and used in conjunction with standard size markers to assign the positions of DNase I cleavages.

In vivo transcription assays

Fresh transformants of each construct were assayed in parallel in the two host strains DS941 (Fis$^+$) and DS941 *fis*::Kan (Fis$^-$). Overnight cultures grown in 2×TY at 37°C were diluted 50-fold into fresh medium. This procedure is termed upshift throughout the paper. Samples were removed at intervals, two per time point. Cell extract was prepared from one whilst the optical density at 650 nm (OD_{650}) was measured for the other. Galactokinase (GalK) activity was determined (in duplicate) from 1 ml of cell extract as previously described (McKenney *et al.*, 1981) and expressed as nmol galactose phosphorylated per min per ml cells at OD_{650} of 1.0. Plasmid copy numbers were determined by a modification of the method of Stueber and Bujard (1982).

In vitro transcription assays

In vitro transcription reactions were performed by a modification of standard methods and contained only the following highly purified components; 0.5 μg (~3.5 nM) supercoiled plasmid DNA template [prepared by centrifugation through CsCl gradient or precipitation with PEG, Sambrook *et al.* (1989)], 5 mM each of rATP, UTP, rCTP and rGTP, 300 mM KCl unless otherwise stated, 10 mM Tris−HCl pH 8.0, 10 mM $MgCl_2$, 1 mM DTT and 100 μg/ml BSA and ppGpp (Calbiochem) and FIS where appropriate in a 50 μl reaction volume. Reactions were pre-incubated for 10 min at 30°C. Transcription reactions were initiated by addition of 0.3 units (equivalent to 30−60 nM) *E.coli* RNA polymerase (Boehringer Mannheim) and incubated for 30 min at 30°C. Reactions were terminated by addition of 250 μl of 1 mM EDTA, 50 mM NaOAc, 0.2% SDS, 10 mM Tris−HCl pH 7.4 and 10 μg/ml proteinase K and incubated at 37°C for 20 min. Phenol extraction was followed by two rounds of precipitation from ethanol firstly with 50 μl 5 M NH_4OAc in the presence of 10 μg carrier tRNA and subsequently by addition of 1/10 vol 2.5 M NaOAc. Half the *in vitro* reaction products (~5−10 μg) were annealed with ~1 ng of a 5′ end-labelled synthetic oligonucleotide primer (complementary in sequence to the bold and underlined region of Figure 2B) for 30 min at 37°C in a 10 μl vol containing 40 units RNAsin (Promega) and 2 μl 10×RT buffer (100 mM Tris−HCl pH 8.3; 250 mM NaOAc; 40 mM $MgCl_2$). The primer was extended for 1 h at 37°C by addition of 10 μl dNTP mix (0.5 mM each dATP, dCTP, dGTP, dTTP), 0.5 μl actinomycin D (1 mg/ml) and 10 units super reverse transcriptase (HT Biotechnology Ltd). Reactions were terminated by dilution into TE pH 8.0 (10 mM Tris−HCl; 1 mM EDTA) and the recovered cDNAs were analysed on 6% denaturing polyacrylamide gels. Quantitative analysis was performed as described above.

Acknowledgements

We thank Christian Koch and Regine Kahmann for generously giving FIS protein and David Sherratt for providing the *galK*$^-$ strains. L.R.L. thanks the Medical Research Council for a studentship.

References

An,G. and Friesen,J.D. (1980) *Gene*, **12**, 33−39.
Ball,C.A., Osuna,R., Ferguson,K.C. and Johnson,R.C. (1992) *J. Bacteriol.*, **174**, 8043−8056.
Baracchini,E. and Bremer,H. (1987) *Anal. Biochem.*, **167**, 245−260.
Bauer,B.F., Kar,E.G., Elford.R.M. and Holmes,W.M. (1988) *Gene*, **63**, 123−134.
Berman,M.L. and Landy,A. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 4303−4307.
Boles,T.C., White,J.H. and Cozzarelli,N.R. (1990) *J. Mol. Biol.*, **213**, 931−951.
Borowiec,J.A. and Gralla,J.D. (1985) *J. Mol. Biol.*, **184**, 587−598.
Borowiec,J.A. and Gralla,J.D. (1987) *J. Mol. Biol.*, **195**, 89−97.
Bosch,L., Nilsson,L., Vijgenboom,E. and Verbeek,H. (1990) *Biochim. Biophys. Acta*, **1050**, 293−301.
Bruist,M.F., Glasgow,A.C., Johnson,R.C. and Simon,M.I. (1987) *Genes Dev.*, **1**, 762−772.
Cashel,M. and Gallant,J. (1969) *Nature*, **221**, 838−841.
Cashel,M. and Rudd,K.E. (1987) In Neidhardt,F.C., Ingraham,J.L., Brooks,L., Magasanik,B., Schaechter,M. and Umbarger,H.E. (eds), *Escherichia coli and Salmonella typhimurium*. American Society of Microbiology, Washington, DC, pp. 1410−1438.
Clackson,T., Güssow,D. and Jones,P.T. (1991) In McPherson,M.J., Quirke,P. and Taylor,G.R. (eds), *PCR—A Practical Approach*. IRL Press, Oxford, pp. 187−214.
Condon,C., Philips,J., Fu,Z-Y., Squires,C. and Squires,C.L. (1992) *EMBO J.*, **11**, 4175−4185.
Drew,H.R. and Travers,A.A. (1985) *J. Mol. Biol.*, **186**, 773−790.
Drlica,K. (1992) *Mol. Microbiol.*, **6**, 425−433.
Finkel,S.E. and Johnson,R.C. (1992) *Mol. Microbiol.*, **6**, 3257−3265.
Fried,M. and Crothers,D.M. (1981) *Nucleic Acids Res.*, **9**, 6505−6525.
Gaal,T. and Gourse,R.L. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 5533−5537.
Garner,M.M. and Revzin,A. (1981) *Nucleic Acids Res.*, **9**, 3047−3060.
Gausing,K. (1977) *J. Mol. Biol.*, **115**, 335−354.
Gille,H., Egan,J.B., Roth,A. and Messer,W. (1991) *Nucleic Acids Res.*, **19**, 4167−4172.
Gourse,R.L. (1988) *Nucleic Acids Res.*, **16**, 9789−9809.
Gourse,R.L., de Boer,H.A. and Nomura,M. (1986) *Cell*, **44**, 197−205.
Gross,H.J. and Raab,C. (1972) *Biochem. Biophys. Res. Comm.*, **46**, 2006−2011.
Harley,C.B. and Reynolds,R.P. (1987) *Nucleic Acids Res.*, **15**, 2343−2361.
Hübner,P. and Arber,W. (1989) *EMBO J.*, **8**, 577−585.

Hübner,P., Haffter,P., Iida,S. and Arber,W. (1989) *J. Mol. Biol.*, **205**, 493–500.
Johnson,R.C. and Simon,M.I. (1985) *Cell*, **41**, 781–791.
Johnson,R.C., Ball,C.A., Pfeffer,D. and Simon,M.I. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 3484–3488.
Josaitis,C.A., Gaal,T., Ross,W. and Gourse,R.L. (1990) *Biochim. Biophys. Acta*, **1050**, 307–311.
Kahmann,R., Rudt,F., Koch,C. and Mertens,G. (1985) *Cell*, **41**, 771–780.
Koch,C., Vandekerckhove,J. and Kahmann,R. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 4237–4241.
Kostrewa,D., Granzin,J., Stock,D., Choe,H-W., Labahn,J. and Saenger,W. (1992) *J. Mol. Biol.*, **226**, 209–226.
Küpper,H., Contreras,R., Landy,A. and Khorana,H.G. (1975) *Proc. Natl. Acad. Sci. USA*, **72**, 4754–4758.
Lamond,A.I. (1984) Ph.D. thesis, University of Cambridge, UK.
Lamond,A.I. (1985) *EMBO J.*, **4**, 501–507.
Lamond,A.I. and Travers,A.A. (1983) *Nature*, **305**, 248–250.
Lamond,A.I. and Travers,A.A. (1985a) *Cell*, **40**, 319–326.
Lamond,A.I. and Travers,A.A. (1985b) *Cell*, **41**, 6–8.
Laundon,C.H. and Griffith,J.D. (1988) *Cell*, **52**, 545–549.
Lazarus,L.R. (1992) Ph.D. thesis, University of Cambridge, UK.
Lee,D.-H and Schleif,R.L. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 476–480.
Leirmo,S. and Gourse,R.L. (1991) *J. Mol. Biol.*, **220**, 555–568.
McClellan,J.A., Boublikova,P., Palecek,E. and Lilley,D.M. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 8373–8377.
McKenney,K., Shimatake,H., Court,D., Schmeissner,U., Brady,C. and Rosenberg,M. (1981). In Chirikjian,J.G. and Papas,T.J. (eds), *Gene Amplification and Analysis 2: Analysis of Nucleic Acids by Enzymatic Methods*. Elsevier, Amsterdam, pp. 383–415.
Maxam,H. and Gilbert,W. (1980) *Methods Enzymol.*, **65**, 499–560.
Newlands,J.T., Josaitis,C.A., Ross,W. and Gourse,R.L. (1992) *Nucleic Acids Res.*, **20**, 719–726.
Nierlich,D.P. (1978) *Annu. Rev. Microbiol.*, **32**, 393–432.
Nilsson,L., Vanet,A., Vijgenboom,E. and Bosch,L. (1990) *EMBO J.*, **9**, 727–734.
Nilsson,L., Verbeek,H., Vijgenboom,E., van Drunen,C., Vanet,A. and Bosch,L. (1992) *J. Bacteriol.*, **174**, 921–929.
Ninnemann,O., Koch,C. and Kahmann,R. (1992) *EMBO J.*, **11**, 1075–1083.
Nomura,M., Gourse,R.L. and Baughman,G. (1984) *Annu. Rev. Biochem.*, **53**, 75–117.
Ohlsen,K.L. and Gralla,J.D. (1992) *Mol. Microbiol.*, **6**, 2243–2251.
Owens,J.R., Woody,A.-Y.M. and Haley,B.E. (1987) *Biochem. Biophys. Res. Commun.*, **142**, 964–971.
Plaskon,R.R. and Wartell,R.M. (1987) *Nucleic Acids Res.*, **15**, 785–796.
Ross,W., Thompson,J.F., Newlands,J.T. and Gourse,R.L. (1990) *EMBO J.*, **9**, 3733–3742.
Ryals,J., Little,R. and Bremer,H. (1982) *J. Bacteriol.*, **151**, 1261–1268.
Ryan,M.J., Ramamoorthy,B., Brown,E.L., Fritz,H.J. and Khorana,H.G. (1979) *J. Biol. Chem.*, **254**, 10803–10810.
Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. Second edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Schultz,S.C., Shields,G.C. and Steitz,T.A. (1991) *Science*, **253**, 1001–1007.
Schultz,S.G. and Solomon,A.K. (1961) *J. Gen. Physiol.*, **45**, 355–369.
Stirling,C.J., Colloms,S.D., Collins,J.F., Szatmari,G. and Sherratt,D.J. (1989) *EMBO J.*, **8**, 1623–1627.
Stueber,D. and Bujard,H. (1982) *EMBO J.*, **1**, 1399–1404.
ten Heggeler-Bordier,B., Wahli,W., Adrian,M., Stasiak,A. and Dubochet,J. (1992) *EMBO J.*, **11**, 667–672.
Thompson,J.F. and Landy,A. (1988) *Nucleic Acids Res.*, **16**, 9687–9705.
Thompson,J.F., Moitoso de Vargas,L., Koch,C., Kahmann,R. and Landy,A. (1987) *Cell*, **50**, 901–908.
Travers,A.A. (1976) *Mol. Gen. Genet.*, **147**, 225–232.
Travers,A.A. (1980a) *J. Mol. Biol.*, **141**, 91–97.
Travers,A.A. (1980b) *J. Bacteriol.*, **141**, 973–976.
Travers,A.A. (1984) *Nucleic Acids Res.*, **12**, 2605–2618.
Travers,A.A. (1991) *Curr. Biol.*, **1**, 171–173.
van Delft,J.H.M., Mariñon,B., Schmidt,D.S. and Bosch,L. (1987) *Nucleic Acids Res.*, **15**, 9515–9530.
Verbeek,H., Nilsson,L., Baliko,G. and Bosch,L. (1990) *Biochim. Biophys. Acta*, **1050**, 302–306.
Verbeek,H., Nilsson,L. and Bosch,L. (1991) *Biochimie*, **73**, 713–718.
Verbeek,H., Nilsson,L. and Bosch,L. (1992) *Nucleic Acids Res.*, **20**, 4077–4081.
Yanagi,K., Privé,G.G. and Dickerson,R.E. (1991) *J. Mol. Biol.*, **217**, 201–214.

Received on December 23, 1992; revised on March 3, 1993