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Supercoiling response of a bacterial tRNA gene

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The effect of DNA supercoiling on transcription of a bacterial tRNA gene has been analysed *in vitro* and *in vivo*. The *Escherichia coli tyrT* gene is found to be completely dependent on negative supercoiling when transcription is assayed *in vitro* at physiological salt concentrations and this supercoiling dependence is shown to be a property of the primary promoter sequences. The supercoiling sensitivity can however be removed by reducing the salt concentration below 50 mM KCl. The effect of supercoiling *in vivo* was analysed by measuring the activity of the *tyrT* promoter on a *galK* fusion vector in *E. coli* strains that have mutations in either the DNA gyrase or topoisomerase I genes. Surprisingly, in view of the dramatic *in vitro* effects, supercoiling could be either increased or decreased relative to the wild-type *in vivo* level without causing any change in *tyrT* promoter activity.

Key words: DNA supercoiling/transcription/tRNA gene

Introduction

Bacterial cells maintain both chromosomal and episomal DNA *in vivo* in a negatively supercoiled state (Bauer and Vinograd, 1970; Worcel and Burgi, 1972; Pettijohn and Pfenninger, 1980; Sinden *et al.*, 1980). In both *Escherichia coli* and *Salmonella typhimurium* the DNA is thought to be kept negatively supercoiled by a dynamic equilibrium between the opposing enzymatic activities of DNA gyrase, which introduces negative supertwists, and topoisomerase I, which removes them (DiNardo *et al.*, 1982; Richardson *et al.*, 1984). The complete removal of either enzymatic activity, in the absence of any compensatory mutations (Sternglanz *et al.*, 1981), is lethal to the cell, but mutant alleles of both DNA gyrase and topoisomerase I have been isolated that are viable and which yield strains that propagate their DNA with a superhelical density significantly altered from wild-type (DiNardo *et al*; Pruss *et al.*, 1982; Richardson *et al.*, 1984).

Supercoiling is believed to influence many cellular reactions of DNA including replication, recombination, transposition and transcription (for review, see Gellert, 1981). Initial studies reported that RNA synthesis *in vitro* was also stimulated by negative supercoiling (Botchan *et al.*, 1973; Richardson, 1974; Wang, 1974). However, it is now clear that the *in vitro* transcriptional response to supercoiling is extremely heterogeneous and promoter specific. While many genes are modestly stimulated by supercoiling only a few show large effects and a significant fraction are not affected at all (Wei-jue *et al.*, 1982). Recently it has even been shown that the genes encoding the two subunits of DNA gyrase are actually inhibited *in vitro* by negative supercoiling (Menzel and Gellert, 1983). It also remains to be established whether changes in supercoiling levels *in vivo* can be used by the cell to regulate gene expression (reviewed by Smith, 1981).

This paper shows that an *E. coli* tRNA gene, *tyrT*, is extremely sensitive to supercoiling *in vitro*. The *tyrT* gene has one of the strongest recorded promoters *in vivo*, commensurate with the highly efficient rRNA promoters (Lamond and Travers, 1983). By contrast, *in vitro* the *tyrT* promoter has been reported to initiate transcription very poorly and to be highly sensitive to salt concentration (Kupper *et al.*, 1975; Travers *et al.*, 1983). It is shown here that the salt inhibition is removed if transcription is analysed on supercoiled DNA templates. At physiological salt concentration the *tyrT* promoter is only active *in vitro* on supercoiled DNA. It is proposed that the sensitivity to both salt concentration and DNA supercoiling may be a result of *tyrT* having a very GC-rich promoter region. However, analysis *in vivo* shows that supercoiling levels can be either increased or decreased without changing the activity of the *tyrT* promoter. The data suggest that supercoiling is not used by the cell to regulate tRNA synthesis. They also question whether many of the changes in gene expression observed in *E. coli* strains carrying mutations in the DNA topoisomerase enzymes are really caused by direct effects of supercoiling changes on promoter activity.

Results

High salt concentration specifically inhibits the tyrT promoter

Transcription from the *tyrT* promoter was analysed *in vitro* using a run-off assay. Figure 1 shows a 610-bp *Hinc*II-*Ava*I fraction that was isolated from plasmid pΔ-98 (Lamond and Travers, 1983) and which contains both the *tyrT* and *Amp* promoters. These two promoters direct transcription on opposite strands of the helix and produce non-overlapping RNAs of ~56 and 275 nucleotides, respectively. The response of the *tyrT* and *Amp* promoters to increasing KCl concentration is dramatically different [Figure 2(a)]. Transcription from the *tyrT* promoter is strongly inhibited by an increase in KCl concentration from 50 to 75 mM. By contrast, transcription from the *Amp* promoter is slightly stimulated by the same rise in ionic strength.

The results shown in Figure 1 are consistent with previous studies which show that the *tyrT* gene is very salt sensitive *in vitro* (Kupper *et al.*, 1973; Travers *et al.*, 1983). Ribosomal RNA also shows a marked *in vitro* sensitivity to salt (van Ooyen *et al.*, 1975). However most mRNA genes are transcribed efficiently *in vitro* at high ionic strength as seen here with *Amp* (Burgess and Jendrisak, 1975). They typically show a salt optimum *in vitro* of 100-150 mM KCl, closely matching the ionic strength actually measured *in vivo*. It therefore appears paradoxical that the *tyrT* promoter, which is transcribed at very high levels *in vivo*, is essentially inactive *in vitro* at physiological salt concentrations. Clearly some facet of the *in vivo* transcription process that is essential for the activity of the *tyrT* promoter is not represented in the run-off

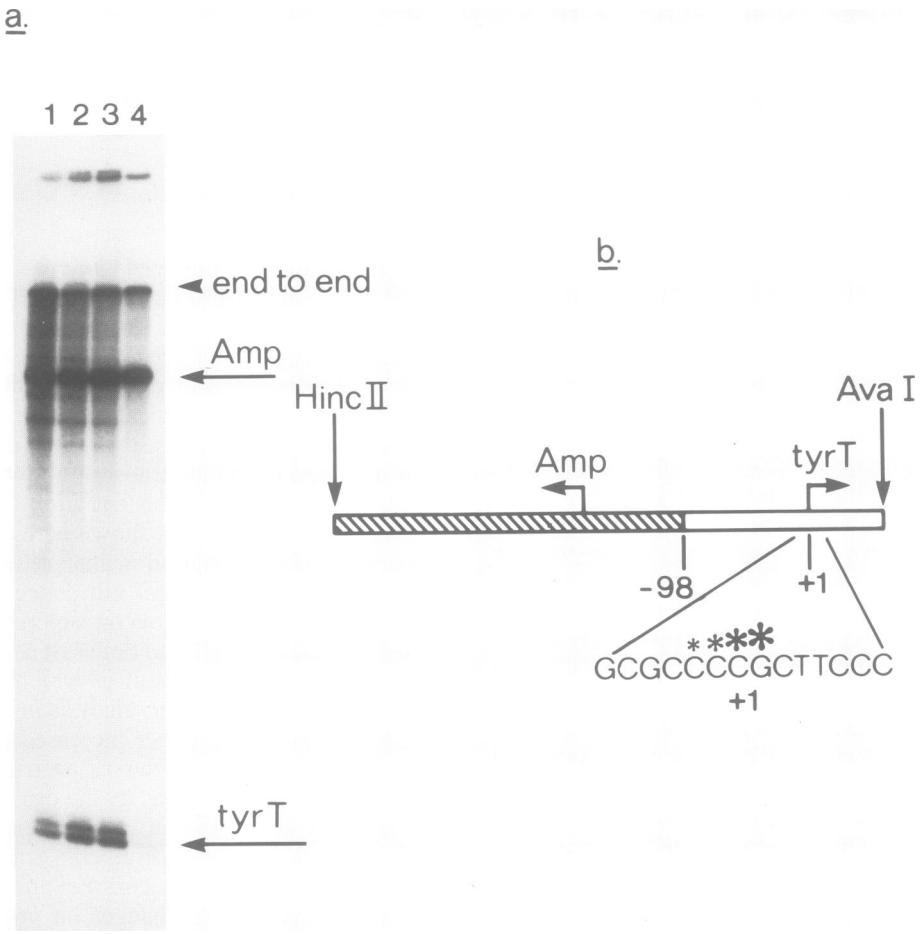

Fig. 1. KCl specifically inhibits the *tyrT* promoter. **(a)** Run-off transcription assays were carried out at 0, 25, 50 and 75 mM KCl concentration **(lanes 1–4)**. Each assay was carried out with 400 ng of RNA polymerase and 50 ng of the purified *Hinc*II-*Ava*I fragment shown in **(b)**. The sequence of the *tyrT* promoter at the transcription start site is shown and the nucleotides used for initiation are marked with asterisks, the size denoting frequency of usage.

assay. One obvious difference between the *in vivo* and *in vitro* conditions is the superhelicity of the DNA template.

Supercoiling stimulates the tyrT promoter in vitro

Plasmid DNA in *E. coli* is maintained *in vivo* in a negatively supercoiled state. As negatively supercoiled plasmid DNA is a covalently closed circular molecule the run-off transcription assay cannot be used. The activity of the *tyrT* promoter on a fully supercoiled plasmid template was therefore analysed by a quantitative S1 mapping method as described in Figure 2. Continuously labelled RNA molecules synthesised *in vitro* were hybridised to unlabelled, single-stranded DNA probes. Probes for both the *tyrT* and *Amp* promoters were made by cloning the relevant fragments from pTyr2 into M13mp9 [Figure 2(c)].

Figure 3 compares the effect of increasing ionic strength on transcription from the *tyrT* promoter, using both negatively supercoiled and linear plasmid DNA as a template. The data show very clearly that the linear plasmid DNA mimics the results obtained with restriction fragments using the run-off assay; transcription from the *tyrT* promoter is essentially abolished above 50 mM KCl. However, *tyrT* transcription from the supercoiled plasmid is actually stimulated by the equivalent transition from low to high salt concentration. The sensitivity of *tyrT* transcription to the superhelical density of the DNA template thus varies significantly at different salt concentrations. Up to 50 mM KCl there is little or no effect at all of supercoiling upon promoter activity. Above 50 mM KCl, at more physiological salt concentrations, only supercoiled *tyrT* templates are active.

The data also show that supercoiling affects the level of correctly initiated *tyrT* transcripts. There is no evidence that RNA polymerase is induced to select different RNA start sites according to the superhelicity of the DNA template. Supercoiling *in vitro*, therefore, modulates the abundance, but not the primary structure, of *tyrT* RNA.

Supercoiling effect is promoter specific

Figure 4 compares the effect of supercoiling upon the *tyrT* and *Amp* promoters on *pTyr2*. Fully relaxed, covalently closed, circular plasmid DNA was prepared by treating supercoiled plasmid DNA with topoisomerase I. Both the relaxed and supercoiled plasmids were used as *in vitro* transcription templates and the resulting *in vitro* synthesised RNA probed separately for *tyrT* and *Amp* transcripts. The relaxed, circular template does not show any *tyrT* promoter activity; the same result that was obtained with linear plasmid DNA. However, the *Amp* promoter, which was not salt sensitive in the run-off assay, shows only a modest inhibitory effect due to decreased supercoiling *in vitro*. Loss of supercoiling causes at most a 2- to 3-fold reduction in *Amp* transcription. This is in general agreement with the data of Wei-jue *et al.* (1982), who report

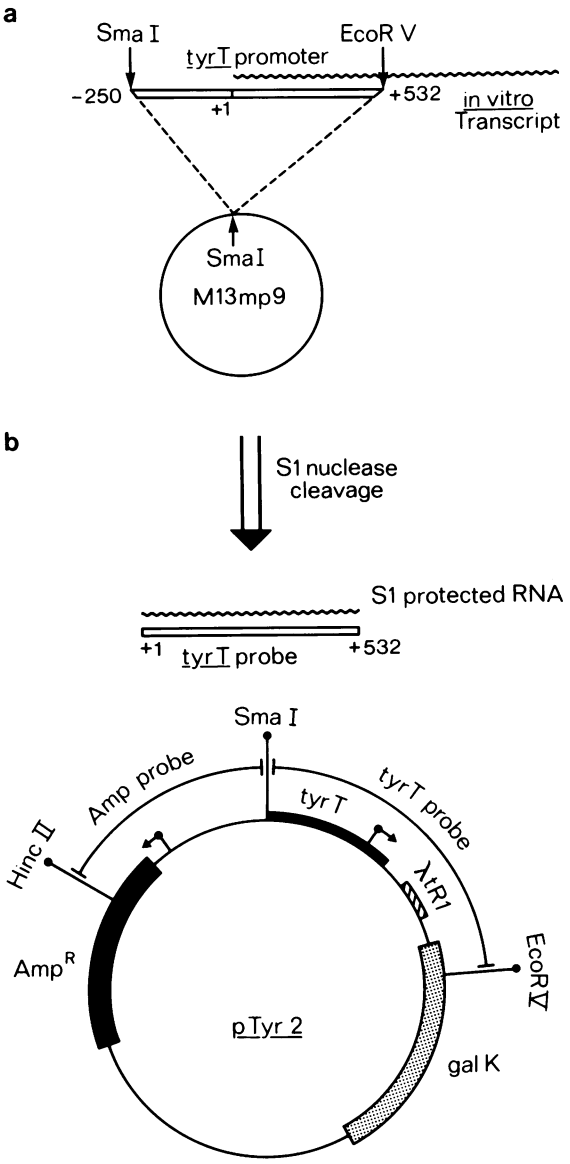

Fig. 2. Analysis of transcripts by nuclease mapping. **(a)** Single-stranded M13 DNA probes were made for both the *tyrT* and *Amp* genes. Uniformly labelled RNA is synthesised *in vitro* with [α-^{32}P]UTP, hybridised to the single-stranded M13 probes and **(b)** the hybrids are digested with S1 nuclease. Probe-protected RNA is then analysed on a denaturing polyacrylamide gel. **(c)** The structure for the *galK* fusion plasmid pTyr2 is shown and the fragments used to make M13 probes for the *tyrT* and *Amp* genes are indicated.

no effect at all of supercoiling on *Amp* promoter function. In contrast, *tyrT* promoter activity is reduced at least 100-fold by the exact same change in supercoiling.

Previous *in vivo* studies indicated that the *tyrT* promoter is subject to positive activation (Lamond and Travers, 1983). The very high transcriptional activity of the wild-type promoter *in vivo* requires the presence of an upstream promoter element. This element is located between 40 and 98 bp upstream of the transcription start point. Deletion of the upstream element reduces *tyrT* expression by 10- to 12-fold. However, analysis of deletion mutants *in vitro* shows that the observed sensitivity to supercoiling is a property of the primary *tyrT* promoter region and is not dependent on the integrity of the upstream element. Figure 5 shows the supercoiling sensitivity of a *tyrT* promoter mutant that has had deleted all the wild-type sequence upstream of −40. This mutant displays the same dependence on negative supercoiling for promoter activity at high salt concentrations as does wild-type (cf. Figures 3 and 4). The extreme supercoiling sensitivity of the *tyrT* gene must therefore result from the particular structural configuration present within the canonical promoter region.

In vivo studies

The *in vitro* dependence on negative supercoiling shown by the *tyrT* promoter suggested that changes in the level of supercoiling *in vivo* might influence *tyrT* expression and could be used to control stable RNA synthesis. To examine the *in vivo* effects of supercoiling on the *tyrT* promoter the *tyrT-galK* fusion plasmids were analysed in *E. coli* strains that carry mutant alleles of the genes encoding either DNA gyrase or topoisomerase I. The steady-state supercoiling level of bacterial DNA is thought to reflect a dynamic balance between the action of gyrase, which introduces negative supercoils, and topoisomerase I which removes them. Viable mutations have been isolated in both enzymes, yielding strains that maintain their DNA at a superhelical density either higher or lower than wild-type. Thus by measuring GalK activity from plasmid vectors in isogenic pairs of *E. coli* strains, that are either mutant or wild-type for one of the topoisomerase enzymes, it is possible to ask whether the alteration in DNA supercoiling causes a corresponding change in promoter activity.

Table I(a) shows a comparison of *galK* expression in a strain (*SD104-20*) with a DNA gyrase mutation, *gyrB225*, and an isogenic wild-type sibling (SD104-14). The *gyrB225* allele decreases the supercoiling levels of both chromosomal and plasmid DNA *in vivo* (Pruss *et al.*, 1982). The wild-type *tyrT* promoter in *pTyr2* gives ~25% less GalK activity in the *gyrB* mutant strain than it does in the wild-type. This difference could be due to an effect of supercoiling on either *tyrT* promoter activity, termination efficiency of λtR1 [see Figure 2(c)], plasmid copy number or some combination of these parameters. To try and distinguish the cause of the reduced GalK activity in the gyrase mutant the *lacUV5* promoter was analysed in the same pair of *E. coli* strains, using *galK* vectors both with and without the λtR1 terminator. The *lacUV5* promoter has been shown not to be sensitive to supercoiling *in vitro* (Wei-jue *et al.*, 1982) and thus constitutes a negative control for *in vivo* studies on the *tyrT* promoter, which *in vitro* is highly sensitive to supercoiling.

As shown in Table I the *lacUV5* promoter exhibits a similar difference in *galK* expression between the wild-type and *gyrB* strains as did *tyrT*. Furthermore, this difference is observed with *lacUV5* whether or not the vector also contains a terminator between the promoter and the *galK* gene. It is therefore most likely that the observed difference in *galK* expression results from the *gyrB225* allele causing a general reduction in plasmid copy number and not because of a direct effect on promoter activity. This view is supported by direct measurements of plasmid copy number (data not shown) and by the *in vitro* experiments of Kaguni and Kornberg (1984), who demonstrate a requirement for DNA gyrase to promote efficient replication of plasmid templates.

A similar analysis was carried out using a separate isogenic pair of strains *JTT1* and *RS2*, one of which (*RS2*) carried a mutation in the topoisomerase I gene. This mutation, *topI0*, has been shown to increase the supercoiling level of both chromosomal and plasmid DNA relative to wild-type (Pruss

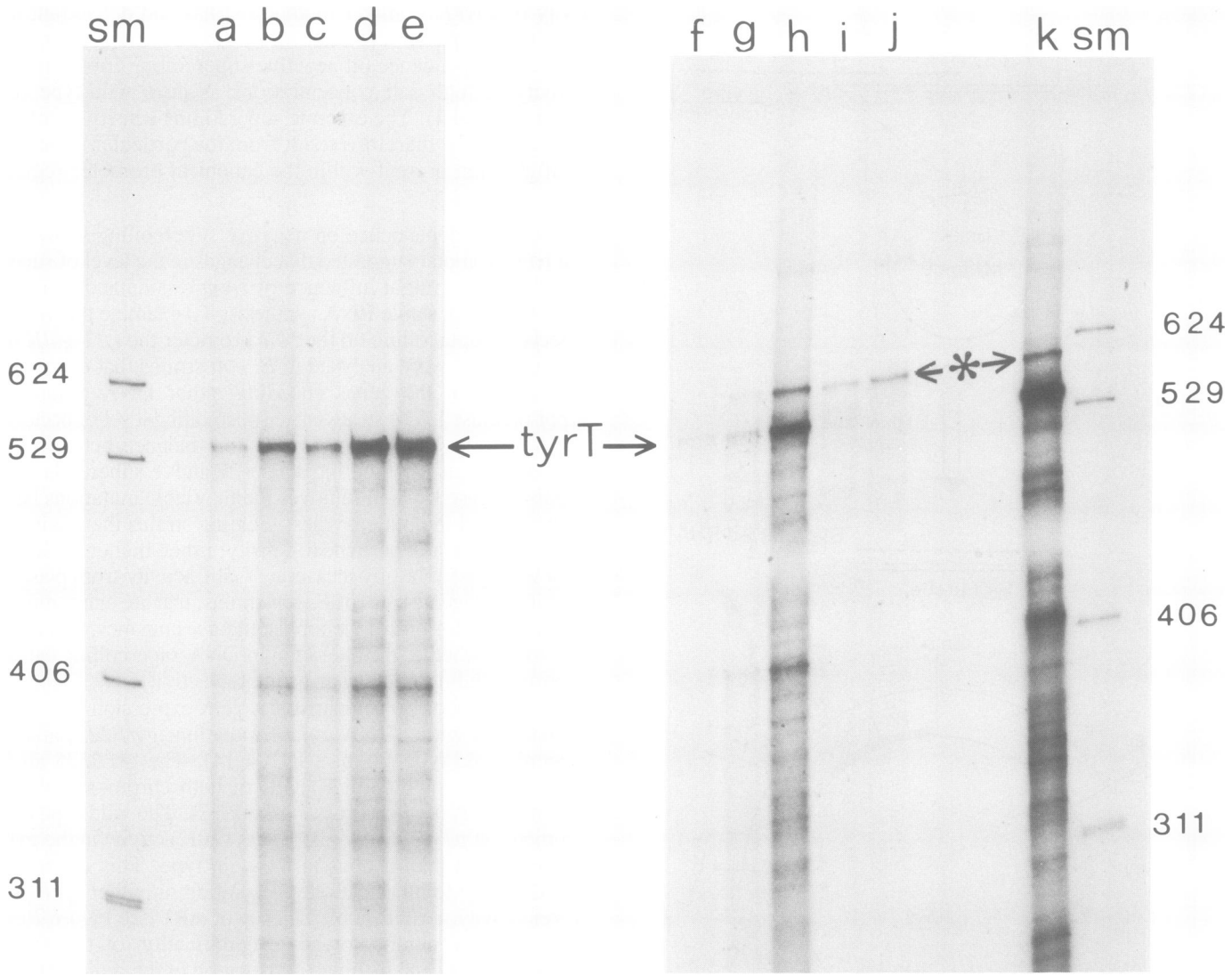

Fig. 3. Supercoiling stimulates the *tyrT* promoter *in vitro*. *In vitro* transcription from plasmid templates was assayed at 0, 25, 50, 100 and 150 mM KCl (**a**–**e** and **f**–**j**) using the method described in Figure 2. The transcripts were visualised by autoradiography at −70°C using pre-flashed Fuji RX X-ray film and an intensifying screen. **Lanes a**–**e** show *tyrT* transcripts made from fully supercoiled pTyr2 plasmid DNA and **lanes f**–**j** *tyrT* transcripts made from pTyr2 that had been linearised by *Pst*I digestion. **Lane k** shows transcripts made from an equimolar mixture of supercoiled and linear plasmid DNA, assayed at 100 mM KCl. 400 ng of RNA polymerase and 150 ng of plasmid DNA were used in each reaction. The large band seen only with the linear template assays (indicated by an asterisk) arises from transcripts initiated at the end of the linear plasmid DNA. The size markers (sm) are DNA fragments, made by *Msp*I digestion of pBR322, and end-labelled with [^{32}P]dCTP.

et al., 1982). As shown in Table I(b) there is essentially no difference in GalK activity for either the wild-type *tyrT* promoter, the −40 *tyrT* deletion mutant or the *lacUV5* promoter when expression is compared between the wild-type and *top10* strains. The results of these experiments indicate that supercoiling *in vivo* can be either decreased or increased relative to the wild-type level without changing the activity of a promoter, *tyrT*, that is highly sensitive *in vitro* to negative supercoiling.

Discussion

The results presented in this paper make two main points. Firstly, it is shown that transcription of the *tyrT* gene *in vitro* is strongly dependent on DNA supercoiling at physiological salt concentrations. This explains why the *tyrT* gene was previously reported to be transcribed poorly on linear templates (Kupper *et al.*, 1975). Secondly, despite the dramatic effects of supercoiling *in vitro* the level of supercoiling *in vivo* can be either increased or decreased relative to wild-type without a concomitant change in promoter activity.

The marked *in vitro* effect of supercoiling on the *tyrT* tRNA gene is shown here to act on the primary promoter region and to specifically influence the production of correctly initiated transcripts. The *in vitro* transcription of rRNA genes is also very sensitive to supercoiling (Glaser *et al.*, 1983; Yang *et al.*, 1979). Bacterial tRNA and rRNA genes share many common properties; they are transcribed very actively *in vivo* and are coordinately regulated in response to both amino acid starvation and cellular growth rate (reviewed by Nomura *et al.*, 1984). It has been pointed out by Travers (1980, 1984) that both tRNA and rRNA promoters contain a highly GC-rich region between the Pribnow box and the transcription initiation site, the so-called 'discriminator' region. The sequence of the *tyrT* promoter is shown in Figure 7. Almost the entire sequence of the *tyrT* promoter region that is thought to be melted by RNA polymerase prior to transcription

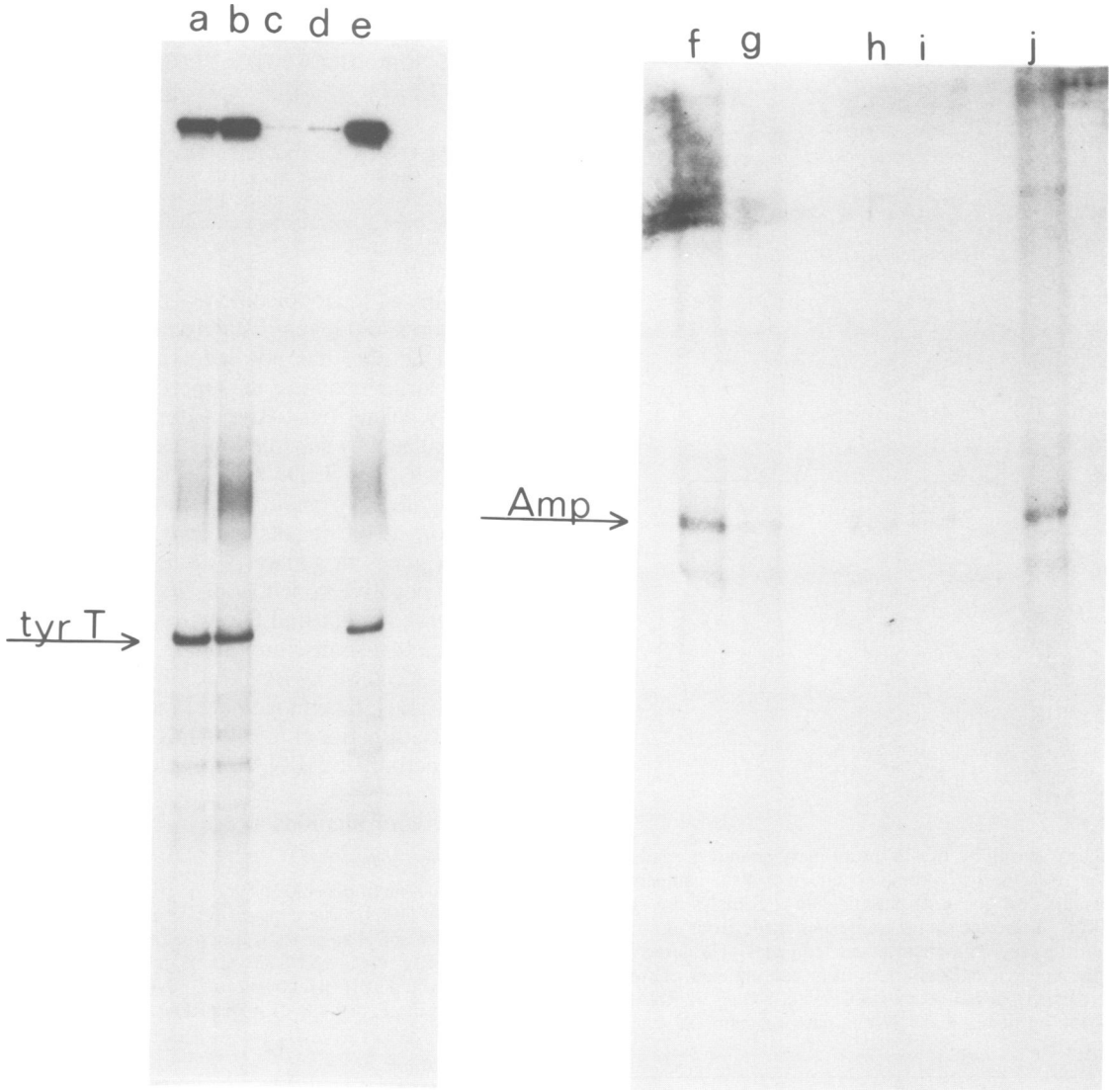

Fig. 4. Supercoiling stimulation is promoter specific. *In vitro* transcription from the *tyrT* **(a – e)** and *Amp* **(f – j)** promoters is compared on both fully supercoiled **(a,b,f,g)** and topoisomerase I relaxed **(c,d,h,i)** pTyr2 DNA. Each reaction was carried out at 100 mM KCl with 400 ng of RNA polymerase and 150 ng of plasmid DNA. The samples are shown assayed in duplicate. **Lanes e** and **j** show transcripts made from an equimolar mixture of supercoiled and relaxed plasmid DNA.

initiation consists of GC base pairs (Siebenlist *et al.*, 1980). A high GC content in the region of the promoter melted by polymerase will increase the energy required to form a stable open complex. Thus factors that facilitate strand separation, such as negative supercoiling and low salt concentration, would be expected to stimulate promoter activity *in vitro*. Since this is exactly what is observed it suggests that *in vitro* transcription of non-supercoiled *tyrT* templates may be limited by the high energy of strand separation imposed by a very GC-rich discriminator region. A similar explanation may apply to rRNA promoters.

Alterations in the wild-type supercoiling levels found *in vivo*, caused either by mutations in the topoisomerase enzymes or by antibiotic treatment, have been observed to produce highly pleiotropic phenotypes in bacterial cells, e.g., failure to support growth of bacteriophage Mu, failure to lysogenise bacteriophage lambda, induction of the cryptic *bgl* operon and altered expression of the *lacI* gene and both the lactose and maltose operons (DiNardo *et al.*, 1982; Sanzey, 1979; for review, see Smith, 1981). Since negative supercoiling can be shown to thermodynamically favour the binding of RNA polymerase to a promoter site (Bauer and Vinograd, 1970; Wang, 1980), and since supercoiling has been found to stimulate RNA synthesis in purified *in vitro* systems, it is tempting to assume that the *in vivo* phenotypes associated with altered supercoiling levels arise *via* similar altered polymerase/promoter interactions. The data reported here introduce a note of caution to this simple interpretation, for it is demonstrated that a gene which is extremely sensitive to supercoiling *in vitro* can be propagated in *E. coli* strains that display various supercoiling-associated phenotypes without showing any effect.

The difference between the *in vitro* and *in vivo* effects of supercoiling on *tyrT* expression could arise for many reasons. It cannot be excluded that transcription factors, which are absent from the *in vitro* system, are present *in vivo* and effectively circumvent the supercoiling requirement. It is also possible that supercoiling affects plasmid-encoded genes differently from genes in the bacterial chromosome. It should be noted however that the changes in supercoiling levels analysed *in vivo* are smaller than those compared *in vitro*. This could mean that there exists a threshold effect of supercoiling density on promoter activity rather than a gradient response. If so, then

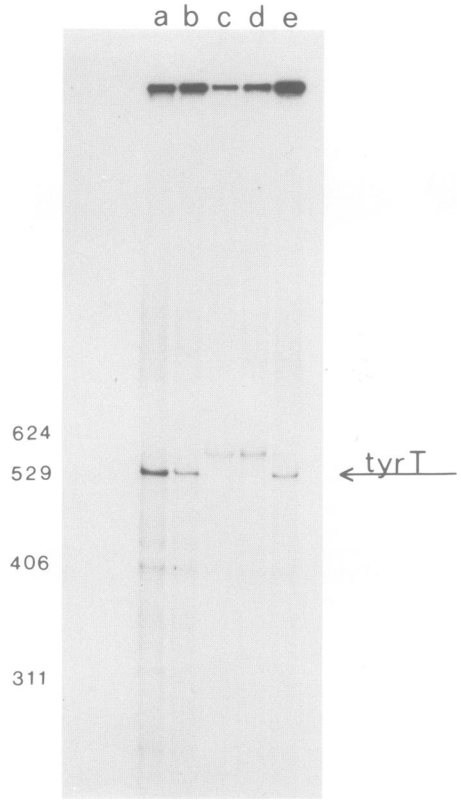

Fig. 5. Supercoiling sensitivity lies in the primary promoter region. *In vitro* transcription from the *tyrT* promoter mutant Δ-40 is compared on supercoiled **(a,b)** and *Pst*I cut **(c,d)** plasmid DNA. Samples are shown assayed in duplicate. **Lane (e)** shows transcripts made from an equimolar mixture of supercoiled and linear templates. The large band seen only with the linear templates arises from end-initiated transcripts. All assays were carried out at 100 mM KCl with 400 ng of RNA polymerase and 150 ng of plasmid DNA in each reaction.

Table I. *GalK* expression in topoisomerase mutant strains

(a) Effect of a DNA gyrase mutation on *galK* expression

Promoter	Terminator (λtR1)	Wild-type (SD10-14)	*gyrB225* (SD104-20)	% Wild-type
tyrT (wild-type)	+	570 ∓ 8%	430 ± 8%	75%
lacUV5	+	94 ± 9%	68 ± 6%	72%
lacUV5	−	220 ± 7%	167 ± 5%	76%

(b) Effect of a topoisomerase I mutation on *galK* expression

Promoter	Terminator (λtR1)	Wild-type (JTT1)	*topI0* (RS2)	% Wild-type
tyrT (wild-type)	+	289 ± 4%	287 ± 10%	100%
*tyr*Δ-40	−	135 ± 4%	138 ± 5%	102%
lacUV5	−	112 ± 19%	120 ± 7%	107%

The expression of GalK activity from both the *tyrT* and *lacUV5* promoters was analysed in *E. coli* strains that display either decreased **(a)**, or increased **(b)**, levels of DNA supercoiling relative to wild-type. GalK activities are expressed as nmol galactose phosphorylated/min/ml cells at 1 OD_{650}. The GalK values shown were each derived from eight separate assays performed on two independent transformants. The final column in each table shows the GalK activity in the mutant strains expressed as a percentage of the activity measured in the corresponding wild-type sibling.

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                                        **********
ACACTTTACAGCGGCGCGTCATTTGATATGATGCGCCCCGCTTCC
  -35                          -10        +1
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Fig. 6. Sequence of the *tyrT* promoter. The DNA sequence of the *tyrT* promoter is shown. The conserved −10 and −35 boxes are underlined and the nucleotides in the region that is thought to be melted by polymerase during open complex formation (Siebenlist *et al.*, 1980) are marked with asterisks.

the viability of the topoisomerase mutant strains may indicate that the threshold level of supercoiling necessary to influence promoter activity has not been reached.

Since large changes in supercoiling levels *in vivo* are extremely deleterious to the bacterial cell it is likely that any genes regulated by supercoiling must be responsive to relatively small changes in DNA superhelicity. This consideration argues against a role for supercoiling in regulating tRNA expression, if the results with *tyrT* are at all representative of tRNA genes in general. Additional experiments point to a similar negative conclusion. No change in linking number was observed in plasmid DNA isolated from cells undergoing the stringent response and ppGpp, the stringent control factor, did not inhibit DNA gyrase *in vitro* (unpublished observations). Thus, taken together, I feel that the *in vivo* evidence is against a functional role for DNA supercoiling in controlling the activity of tRNA promoters.

Materials and methods

Plasmid construction

The construction of plasmids pTyr2 and pTyrΔ-40 is described in Lamond and Travers (1983). The construction of single-stranded M13 probes for both the *tyrT* and *Amp* genes is described in Lamond and Travers (1985). Plasmids pKOlac and pKMlac were made by inserting the 205-bp *Eco*RI fragment from pOP1, (Backman *et al.*, 1976), that contains the *lacUV5* promoter, into the *Eco*RI site of both pKO-1 and pKM-1 (McKenney *et al.*, 1981).

Bacterial strains

SD104-14 DM4100, ΔtrpE63, cysB$^+$, pyrF287 Dinardo *et al.* (1982)
SD104-20 DM4100, ΔtrpE63, cysB$^+$, pyrF287, gyrB225 DiNardo *et al.* (1982)
JTT1 PLK831, trp$^+$ Sternglanz *et al.* (1981)
RS2 PLK831, trp$^+$, topI0 Sternglanz *et al.* (1981)

The supercoiling phenotypes of these strains are also documented in Pruss *et al.* (1982). For this study the phenotypes of all the strains were confirmed by (i) testing the auxotrophic markers by assaying for growth on supplemented minimal media and (ii) checking the topoisomerase I and gyrase mutations by comparing plasmid superhelicity on agarose gels and by testing the supercoiling associated phenotypes, e.g., Mu plating efficiency.

DNA isolation

Plasmid DNA was prepared from 1 litre bacterial cultures grown in 2 x TY, isolated as described by Clewell and Helinski (1969) and banded to equilibrium in an isopycnic caesium chloride/ethidium bromide gradient. After incubation with restriction enzymes or topoisomerase I the DNA was further purified by phenol extraction and preparative chromatography on low gelling temperature agarose gels.

GalK assays

GalK assays were carried out essentially as described by McKenney *et al.* (1981). All the GalK values shown were derived as the average of at least eight separate assays, carried out on two separate transformants.

In vitro transcription

RNA polymerase holoenzyme was prepared as described by Travers *et al.* (1983). The holoenzyme, with 1 mol σ per mol core enzyme, was used for all the *in vitro* transcription experiments. Transcription reactions were carried out in a 50 μl volume at 30°C for 15 min. The assay buffer was 80 mM Tris-HCl pH 7.9, 10 mM $MgCl_2$, 0.2 mM EDTA, 12 mM EtSH, 0.25 mM ATP, CTP and GTP, 0.01 mM UTP and 0 − 150 mM KCl as indicated.

Each sample also contained 10 μCi of α-^{32}P-labelled UTP and RNA polymerase and DNA as shown. Reactions were stopped by extraction with H_2O saturated phenol and then ethanol precipitated.

Analysis of transcripts

RNA synthesised from restriction fragment templates was analysed directly on denaturing polyacrylamide gels (Biggin *et al.*, 1982). RNA synthesised from plasmid templates was first hybridised to single-stranded DNA probes and then digested with S1 nuclease using the conditions described by Miller (1984). The nuclease-resistant RNA was then ethanol precipitated and analysed on denaturing polyacrylamide gels.

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