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The supercoiling sensitivity of a bacterial tRNA promoter parallels its responsiveness to stringent control

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In *Salmonella typhimurium*, expression of the *hisR* locus, a tRNA operon, decreases upon inhibiting DNA gyrase. Here, the *hisR* promoter dependence on negative DNA supercoiling was examined *in vivo* and *in vitro*. Mutant analysis showed the sequence determinants of this dependence to lie in the region between the –10 box and the transcription start site. As with most promoters subject to stringent control, this portion of the *hisR* promoter is C–G-rich. Replacing a C/G bp with T/A at position –7 partially relieves the supercoiling response while changing the sequence between –5 and +1 (-CCCCCG-) for -GTTAA- abolishes the response *in vitro* and *in vivo*. The relief of the supercoiling dependence closely correlates with increased promoter susceptibility to melting *in vivo* and a lesser requirement for initiating nucleotides in the formation of stable initiation complexes *in vitro*. Studies in isoleucine-starved cells showed that such sequence changes mitigate and abolish the *hisR* promoter response to stringent control, respectively. The data presented suggest that the *hisR* promoter's sensitivity to stringent regulation arises from the same physical property that confers supercoiling sensitivity, i.e. resistance to melting. We propose that the stringent control mechanism acts by hampering the ability of RNA polymerase to melt the DNA helix.

Keywords: discriminator/*hisR*/melting/stringent control/supercoiling

Introduction

In bacteria, expression of genes encoding components of the translational machinery—notably ribosomal RNA and transfer RNA (collectively called 'stable' RNA)—is coordinately regulated in response to nutrient availability. An extreme form of control is known as the 'stringent response', the abrupt inhibition of stable RNA gene transcription when bacteria experience amino acid starvation (recently reviewed by Cashel *et al.*, 1996; see also Condon *et al.*, 1995; Gourse *et al.*, 1996). Although known for many years, the stringent response remains incompletely understood. A large body of evidence assigns the key effector role to guanosine tetraphosphate (ppGpp), a signal nucleotide that rapidly accumulates at the onset of the response in wild-type strains but not in 'relaxed' (*relA*$^-$) mutants (Cashel and Gallant, 1969). The mechanism of ppGpp-specific inhibition of stable RNA gene transcription remains uncertain. The promoters of stringently controlled genes share some structural features including an imperfect match to the canonical promoter sequence and the presence of a C–G-rich sequence between the –10 box and the transcription initiation site (Travers, 1984). The latter element—named the 'discriminator'—was shown to be necessary for the response to stringency of the *tyrT* tRNA gene (Travers, 1980; Lamond and Travers, 1985), the *rrnB* operon (Josaitis *et al.*, 1995) and the *thrT-tufB* operon (Mizushima-Sugano and Kaziro, 1985). Thus, a widely held view is that ppGpp acts at the level of transcription initiation, presumably by binding to RNA polymerase and somehow changing its promoter selectivity (Travers, 1976; Kajitani and Ishihama, 1984; Hernandez and Cashel, 1995). The apparent lower affinity of RNA polymerase for stringently controlled promoters led some authors to explain selectivity in terms of ppGpp-mediated modulation of RNA polymerase availability as proposed by 'partitioning' or 'sequestering' models (Travers *et al.*, 1980; Ryals *et al.*, 1982; Baracchini and Bremer, 1988; Jensen and Pedersen, 1990; Vogel *et al.*, 1992; Vogel and Jensen, 1994). An alternative scenario emerges from the work of Ohlsen and Gralla (1992a,b,c) who showed that ppGpp can directly affect RNA polymerase function at the *rRNA* P1 promoter *in vitro* by specifically inhibiting the formation of open complexes. Intriguingly, the ppGpp effects demanded DNA melting-limiting conditions (linear DNA templates and high salt) to be observed (Ohlsen and Gralla, 1992b). These studies, as well as the previous work of Gourse (1988), have revealed that the *rrnB* P1 promoter is atypical with respect to its initiation properties. Unlike most *Escherichia coli* promoters that readily isomerize into an open complex upon binding to RNA polymerase in the absence of ribonucleotides, constitution of a stable open complex at the *rrnB* P1 promoter requires formation of the first phosphodiester bond and it is therefore dependent on the presence of ribonucleotide substrates (Gourse, 1988; Ohlsen and Gralla, 1992a,c). A similar dependence was recently demonstrated *in vivo* and the concentration of ribonucleotides proposed to play a role in the regulation of ribosomal RNA transcription (Gaal *et al.*, 1997).

The *rrnB* P1 and *tyrT* promoters share the additional property of being strongly dependent on negative supercoiling *in vitro*, a dependence that can be partially relieved by lowering the salt concentration (Glaser *et al.*, 1983; Lamond, 1985; Leirmo and Gourse, 1991; Ohlsen and Gralla, 1992a,b). In the case of the *tyrT* promoter, a supercoiling response was also demonstrated *in vivo*

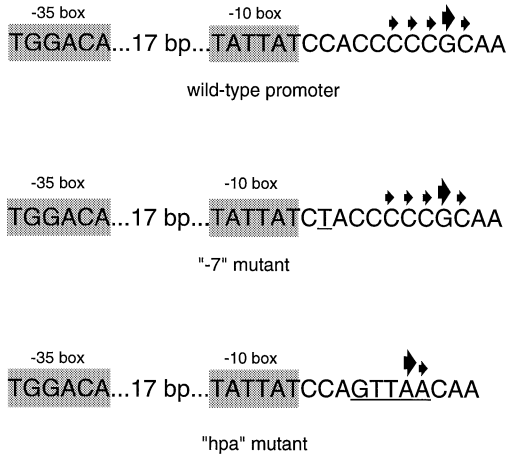

Fig. 1. DNA sequences near the transcription start sites of the *hisR* promoter and mutant variants (non-template strand). Initiation patterns were determined by sequencing the RNA with reverse transcriptase (Figueroa *et al.*, 1991; unpublished results). The size of the arrows reflect the relative initiation frequency *in vivo* at that position. Base pair changes in the promoter variants are underlined.

(Bowater *et al.*, 1994; Free and Dorman, 1994). The C–G-rich discriminator sequence and the abnormal spacing between the –35 and –10 regions of both promoters (16 bp as opposed to 17 bp) were considered as the possible bases for these effects (Lamond, 1985; Ohlsen and Gralla, 1992a).

In *Salmonella typhimurium*, early genetic studies on the mechanism of histidine operon regulation yielded mutants defective in the biosynthesis of $tRNA^{His}$, later found to be a key factor in the regulation mechanism (Johnston *et al.*, 1980). Some of these mutants were impaired in the transcription of the locus that encodes $tRNA^{His}$, an operon specifying four different tRNAs (Bossi and Smith, 1984). [For coherence with early work, we call this operon the *hisR* operon. In *E.coli*, the corresponding locus is named *argT* (from the first gene in the operon) according to the nomenclature of Fournier and Ozeki (1985).] Two classes of mutants resulted from changes in gyrase subunit genes (*gyrA* and *gyrB*), suggesting that the *hisR* operon promoter required negative DNA supercoiling for optimal activity (Rudd and Menzel, 1987). Consistent with this hypothesis, the *his* regulatory defect of a *gyrB* mutant could be suppressed by a mutation within the *hisR* promoter (Figueroa *et al.*, 1991). The change, a C/G→T/A transition within the *hisR* discriminator region (–7), appeared to render the promoter insensitive to the gyrase defect.

In the present study, the supercoiling response of the *hisR* promoter was further analysed both *in vivo* and *in vitro*. Our results suggest that the role of DNA supercoiling stems from the fact that the *hisR* promoter is particularly refractory to melting, apparently due to the high C–G content of the discriminator sequence. The torsional energy of negative supercoiled DNA helps in opening the promoter. Consistent with this view, the sequence changes that lower or remove the barrier to melting lessen or relieve the supercoiling requirement for initiation. Interestingly, the same changes relieve the transcriptional response to the stringent control suggesting that 'resistance to melting' is the basis for the susceptibility to stringent regulation.

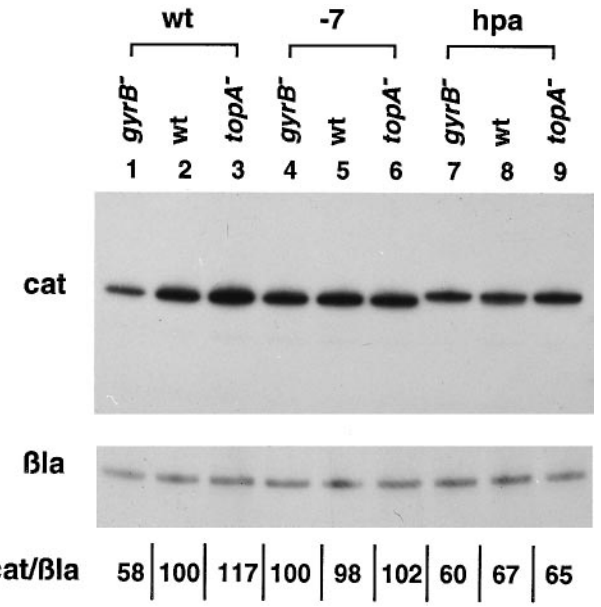

Fig. 2. Effect of DNA topoisomerase mutations (*gyrB*, *topA*) on activity of *hisR* promoter and variants. Plasmids pKP1-13 ($hisR^{wt}$), pKP6-1 ($hisR^{-7}$) and pHP14 ($hisR^{hpa}$) were each introduced into *E.coli* strains JTT1 (wild-type), RS2 (*topA10*) and KD112 (*gyrB226*). Bulk RNA was extracted and *cat* and *bla* transcripts detected by primer extension as described in Materials and methods. Extension products were quantified by phosphorimaging.

Results

Changes in the hisR promoter 'discriminator' sequence affect the transcriptional response to negative supercoiling in vivo and in vitro

The promoter variants used in this work are shown in Figure 1. The '–7' derivative was originally selected *in vivo* as a chromosomal mutation (*hisR10107*) suppressing the defect in *his* operon regulation consequent to a mutation in *gyrB* (Figueroa *et al.*, 1991). It consists of a C/G→T/A substitution 7 bp upstream from the main transcription start site of the *hisR* promoter (Figueroa *et al.*, 1991). The 'hpa' derivative was constructed *in vitro* in the course of this study (see Materials and methods). In this mutant, the C–G stretch of the *hisR* discriminator sequence is replaced by the recognition sequence of *Hpa*I restriction endonuclease (GTTAAC). The three promoter derivatives were analysed on plasmid constructs where they drive transcription of the chloramphenicol acetyl transferase gene (*cat*; see Materials and methods). The transcription initiation sites were determined by sequence analyses of the 5′-end portions of *cat* messenger RNA (data not shown).

As an initial test for probing the response of the three promoters to changes in negative DNA supercoiling, plasmids pKP1-13 ($hisR^{wt}$), pKP6-1 ($hisR^{-7}$) and pKP14 ($hisR^{hpa}$) were introduced in topoisomerase mutants of *E.coli* and *cat* mRNA production measured by primer extension (none of the promoter changes affects *cat* RNA turnover; data not shown). Results in Figure 2 show that the activity of the $hisR^{wt}$ promoter is significantly reduced in a gyrase mutant as compared with the wild-type strain (lanes 1 and 2, respectively). Normalization to the plasmid-encoded β-lactamase transcript (*bla*) shows this reduction to be ~40%. An opposite response is observed in a strain

where average negative superhelical density is higher than normal due to a mutation in the topoisomerase I gene *topA* (Hsieh *et al.*, 1991). In this case, P*hisR*^{wt} is stimulated slightly (~15%; lane 3). By contrast, both promoter variants, P*hisR*$^{-7}$ (lanes 4–6) and P*hisR*^{hpa} (lanes 7–9) appear to be unaffected by either *gyrB* or *topA* alterations. Thus, the wild-type version of the promoter is sensitive to supercoiling variations whereas the sequence changes in P*hisR*$^{-7}$ and P*hisR*^{hpa} relieve this sensitivity. Such a relief appears to be promoter-specific and not to involve a generalized increase in promoter strength. In fact, the data in Figure 2 show that in exponentially growing cells, P*hisR*^{hpa} is slightly weaker than P*hisR*^{wt} (see below).

In order to better characterize the supercoiling responses of the three promoter variants, transcriptional activity was analysed *in vitro* as a function of template superhelical density. This study was carried out with derivatives of plasmids pKP1-13, pKP6-1 and pKP14 in which the *cat* gene is shortened by an *in vitro* deletion yielding a 'mini' transcript (300 nucleotides) that can be more easily detected in polyacrylamide gels. Populations of plasmid DNA topoisomers having mean negative superhelical densities (–σ) ranging from 0 (relaxed plasmid) to 0.1 (hypernegatively supercoiled plasmid) were prepared as described in Materials and methods and used as templates in single-run transcription assays. The amounts of RNA produced from the *hisR* promoter and its two variants were measured and normalized to RNA I, a transcript from the colE1 replication origin whose synthesis we found to be largely unaffected by supercoiling. Results shown in Figure 3A and B offer a revealing picture of the supercoiling responses of the three promoters. One can see that the activity of the wild-type promoter peaks at –σ values of ~0.08, and it is already down to 50% of the maximal rate at –σ = 0.05. In contrast, transcription from the –7 variant remains above 80% of the maximal rate within a broader range of superhelical densities (–σ = 0.05–0.08). Nonetheless, this promoter remains supercoiling-dependent as its activity drops significantly at –σ <0.03. Finally, the P*hisR*^{hpa} variant is almost insensitive to DNA supercoiling (Figure 3A and B). Therefore, these data confirm the *hisR* promoter response to negative supercoiling *in vivo* and show that the C/G→T/A change at –7 relieves this response while the multiple replacements in the 'hpa' variant nearly abolish it.

The *in vitro* transcription experiments described above were carried out under standard ionic strength conditions (150 mM KCl). We found that lowering the salt concentration to 10 mM KCl causes *hisR*^{wt} and *hisR*$^{-7}$ promoters to no longer require negative supercoiling for activity (data not shown). These findings suggest that the supercoiling requirement can be circumvented under conditions that favour promoter melting.

The transcriptional dependence on negative supercoiling correlates with a 'resistance' to promoter melting

DNA melting at promoters can be monitored by chemical probes such as potassium permanganate ($KMnO_4$) and chloro-acetaldehyde (CAA). $KMnO_4$ oxidizes preferentially unpaired pyrimidine residues (Sasse-Dwight and Gralla, 1989) whereas CAA reacts with unpaired adenine and cytosine residues (Dayn *et al.*, 1992). *In vivo*, this analysis often requires using rifampicin to allow accumulation of the otherwise transient open intermediate. Although the drug binds to RNA polymerase and inhibits the initial

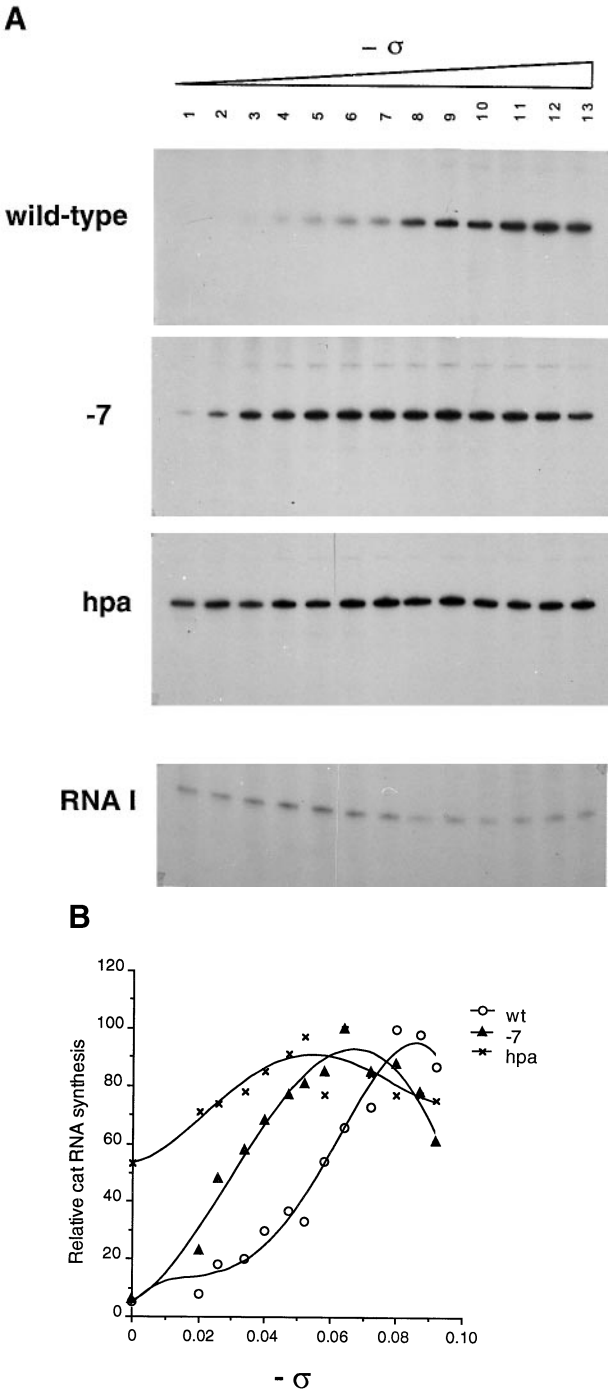

Fig. 3. Role of template superhelicity in the *in vitro* activity of *hisR* promoter and variants. (**A**) Populations of DNA topoisomers with mean negative superhelical density (–σ) ranging from 0 (lane 1) to 0.1 (lane 13) were prepared from the 'mini *cat*' derivatives of plasmids pKP1-13 (*hisR*^{wt}), pKP6-1 (*hisR*$^{-7}$) and pKP14 (*hisR*^{hpa}). The topoisomers preparations were used as templates in single-run transcription assays (ATP, CTP and GTP added before heparin challenge) in the presence of [α-^{32}P]UTP. Radiolabelled RNA was analysed on a denaturing 6% polyacrylamide gel. (**B**) The amount of mini *cat* transcript in each lane of (A) was quantified by phosphorimaging and normalized to the amount of RNA I (from the plasmid's origin of replication). 100% *cat* RNA synthesis refers to the highest expression level in each plasmid series.

stages of polymerization, it does not affect the enzyme's ability to recognize promoters and form stable open complexes. Thus, at most promoter sites, rifampicin treatment results in a substantial increase in chemical reactivity (Sasse-Dwight and Gralla, 1989).

To characterize further the *hisR* promoter and its derivatives, the plasmids carrying them were subjected to $KMnO_4$ and CAA probing *in vivo*. After treatment, plasmid DNA was extracted from cells and the presence of modified residues within the relevant region assessed by primer extension. Results in Figure 4 show that all three promoters are poorly reactive in the absence of rifampicin (Figure 4A and B, odd-numbered lanes). However, when open complex formation is probed in rifampicin-treated cells, clear reactivity differences are observed (Figure 4A and B, even-numbered lanes). While the wild-type profile does not change significantly in the presence of the drug (Figure 4A and B, lanes 2), some residues become accessible to the probes in the *hisR*$^{-7}$ promoter (Figure 4A and B, lanes 4) and this trend is accentuated in the P*hisR*^{hpa} variant where some positions are strongly modified (Figure 4A and B, lane 6). The modified bases are within the region between –12 and +2 which is known to be accessible to single-strand-specific chemical probes in open complexes (Sasse-Dwight and Gralla, 1989 and references therein). Thus, whereas the wild-type *hisR* promoter cannot remain open in the presence of rifampicin, the sequence changes in the '–7' and 'hpa' variants increasingly stabilize the melted configuration.

The transcriptional dependence on negative supercoiling correlates with a nucleotide requirement for formation of stable initiation complexes in vitro

Unlike typical *E.coli* promoters, the *rrnB* P1 promoter does not form a stable open complex with RNA polymerase *in vitro* unless the two initiating ribonucleotides are supplied (Gourse, 1988; Ohlsen and Gralla, 1992a,c). Formation of the first phosphodiester bond is apparently required to stabilize the open preinitiation intermediate. To test whether the same requirement existed with the *hisR* promoter and its variants, we assayed transcription *in vitro* in the presence of heparin which can disperse unstable interactions of RNA polymerase with the DNA. The assay involved adding the drug to complexes formed in the presence of incomplete nucleotide combinations and analysing the elongation proficiency of these complexes after addition of the missing nucleotide(s). From results in Figure 5, it is apparent that the initiating ribonucleotides are required to form a heparin-resistant complex at the wild-type promoter. The extent by which the different combinations overcome the heparin challenge closely reflects the hierarchy of initiation sites, with G–C being the most frequently used initiating pair followed by C–C (see Figure 1). A similar pattern is observed with the *hisR*$^{-7}$ promoter [Figure 5; the higher amount of the transcripts detected in this case simply reflects the different supercoiling sensitivities of the two promoters; the experiment was performed with plasmids at native superhelical density ($-\sigma = 0.06$) where P*hisR*$^{-7}$ activity is approximately twice that of wild-type]. For the P*hisR*^{hpa} variant, the initiating pair (A–A or A–C) is no longer required for heparin-resistant transcription (Figure 5, 'hpa' panel,

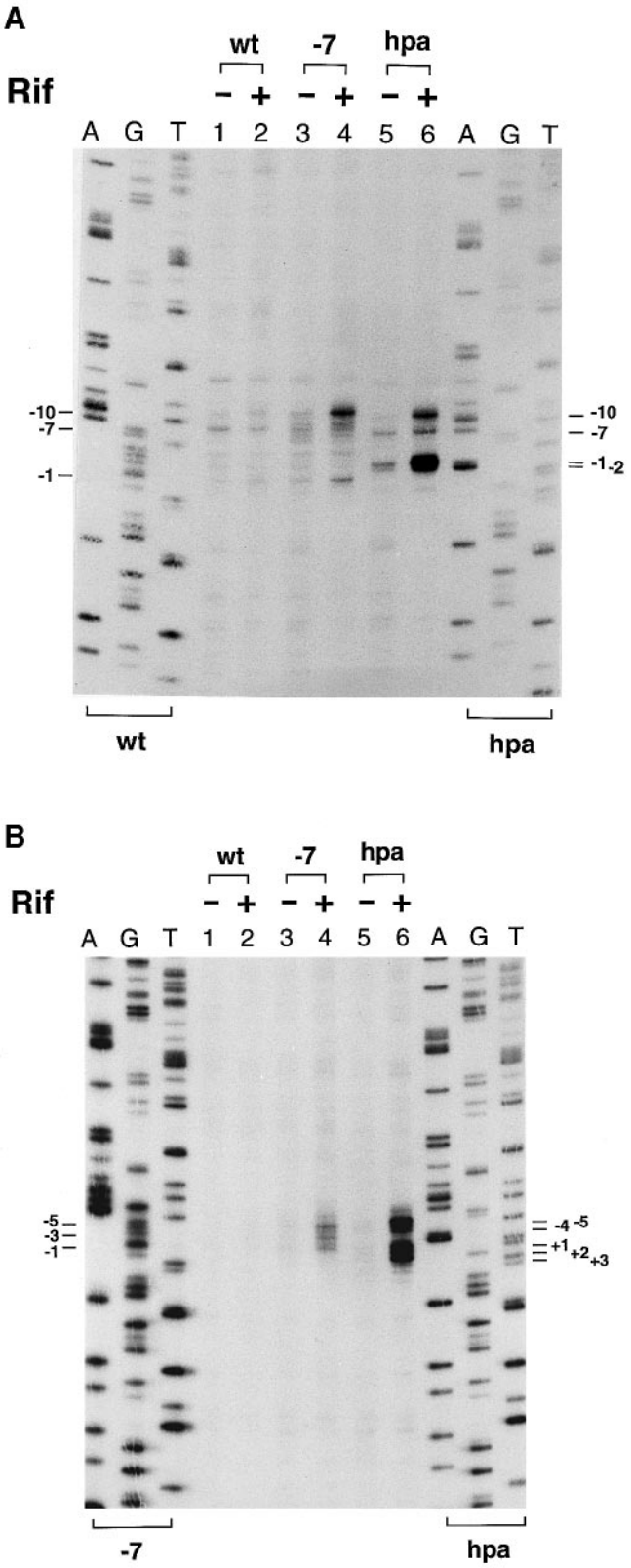

Fig. 4. Probing DNA melting at *hisR* promoter variants *in vivo*. Plasmid-harbouring cells (strain HB101) were treated with $KMnO_4$ (**A**) or CAA (**B**) as described in Materials and methods. Treatment was under unaltered transcription conditions (lanes 1, 3 and 5) or in the presence of rifampicin (lanes 2, 4 and 6). Modified bases within the relevant region of the non-template strand were revealed by primer extension and identified using a DNA sequence ladder as reference. Plasmids used were: pKP1-13 (*hisR*^{wt}), pKP6-1 (*hisR*$^{-7}$) and pKP14 (*hisR*^{hpa}).

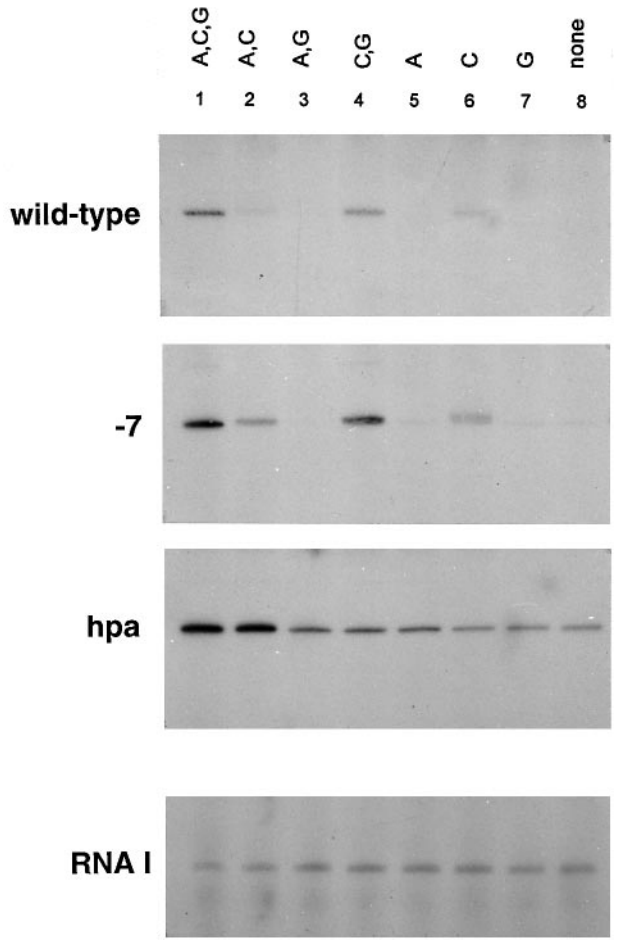

Fig. 5. Ribonucleotide requirement for formation of heparin-resistant transcription initiation complexes. Plasmid DNA (negative superhelical density, $-\sigma = 0.06$) was incubated with RNA polymerase in the presence of different NTP combinations as specified at the top of each lane. Following heparin challenge, the missing NTPs were added to allow transcription to take place. The *cat* transcript produced in each case was analysed on a 6% polyacrylamide gel. RNA I, from the plasmid's origin of replication, was used for normalization. Plasmids were the 'mini-*cat*' derivatives of pKP1-13 (*hisR*^{wt}), pKP6-1 (*hisR*$^{-7}$) and pKP14 (*hisR*^{hpa}).

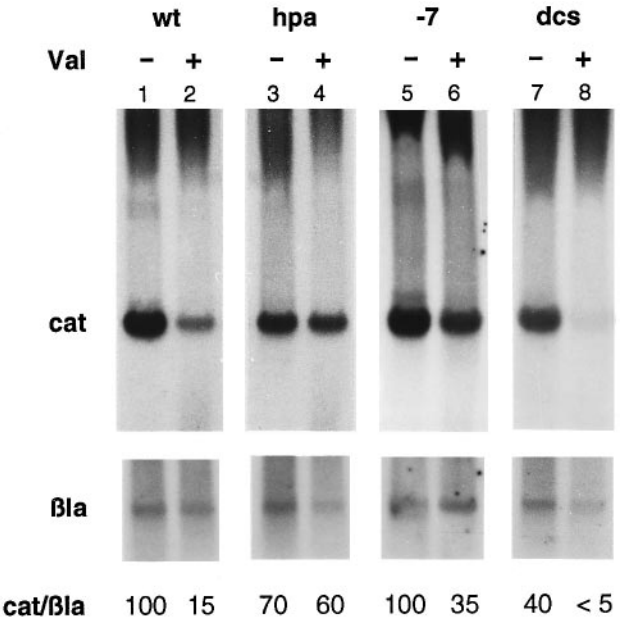

Fig. 6. The response of *hisR* promoter variants to stringent control. Exponentially growing cells (strain SU1675) carrying each of the plasmids listed below were starved for isoleucine by adding valine (see text). RNA was pulse-labelled with ^{32}P-phosphate in both valine-treated and untreated cells. RNA was extracted and separately hybridized to DNA from two M13 phages carrying *cat* and *bla* gene segments (mpKP-eco and mpKK-pst; template strand). Hybrid-selected transcripts were detected (by autoradiography) in an agarose gel where they co-migrate with the phage DNA. Plasmids used were: pKP1-13 (*hisR*^{wt}), pKP6-1 (*hisR*$^{-7}$), pKP14 (*hisR*^{hpa}) and pKP17 (*hisR*^{dcs}). The *cat:bla* ratio measured for pKP1-13 was defined as 100. The data presented are the most representative amid a set of three separate determinations in which variability of *cat/bla* ratios ranged between 5% (for the lower values) and 10% (for the higher values).

lanes 3–8) albeit contributing somewhat to the complex stability (compare lanes 1 and 2 with the remaining lanes). Thus, a correlation exists between the transcriptional dependence on supercoiling and the nucleotide requirement for formation of stable initiation complexes. Conceivably, this correlation reflects the high energetic cost to achieve melting at the *hisR* promoter.

The transcriptional dependence on negative supercoiling parallels the promoter responsiveness to the stringent control

The changes affecting the response to DNA supercoiling fall within the 'discriminator' region of the *hisR* promoter and lower the C–G content of this sequence. C–G-richness being the shared feature of stringently controlled promoters, we examined the effects of these mutations on the promoter susceptibility to such form of control. The stringent response was induced by exposing growing cells to valine which, by inhibiting both acetohydroxy acid synthetase isozymes of *E.coli* K12, causes cells rapidly to starve for isoleucine (Cashel *et al.*, 1996). The rate of incorporation of ^{32}P-labelled phosphate into *cat* RNA was measured in valine-treated cells as well as in untreated controls. Results in Figure 6 (lanes 1 and 2) show that the activity of the wild-type *hisR* promoter (normalized to that of the 'relaxed' *bla* promoter) drops >5-fold upon starvation. The changes in the P*hisR*^{hpa} variant render the promoter completely refractory to this control (Figure 6, lanes 3 and 4; the slight reduction in lane 4 reflects the general decline of ^{32}P incorporation in starving cells; Cashel *et al.*, 1996). Interestingly, the P*hisR*^{hpa} variant is actually weaker than the wild-type promoter (Figure 6, compare lanes 1 and 3; see also Figure 2), indicating that the loss of control does not originate from a generalized improvement of promoter performance. Again, the –7 variant shows an intermediate response since the promoter is inhibited to a lesser extent than the wild-type promoter (lanes 5 and 6). In summary, these results suggest that relieving the promoter dependence on negative supercoiling concomitantly diminishes its response to amino acid starvation.

The above data show that, although strongly inhibited, the *hisR*^{wt} promoter is not completely shut off upon starvation. Incomplete response can be partly ascribed to the plasmid-borne state of the promoter. Transcription of the corresponding locus on the *E.coli* chromosome (*argT*, which has a discriminator sequence identical to *hisR*'s) was reported >10-fold down upon starvation (Rowley *et al.*, 1993). An additional factor preventing a full response

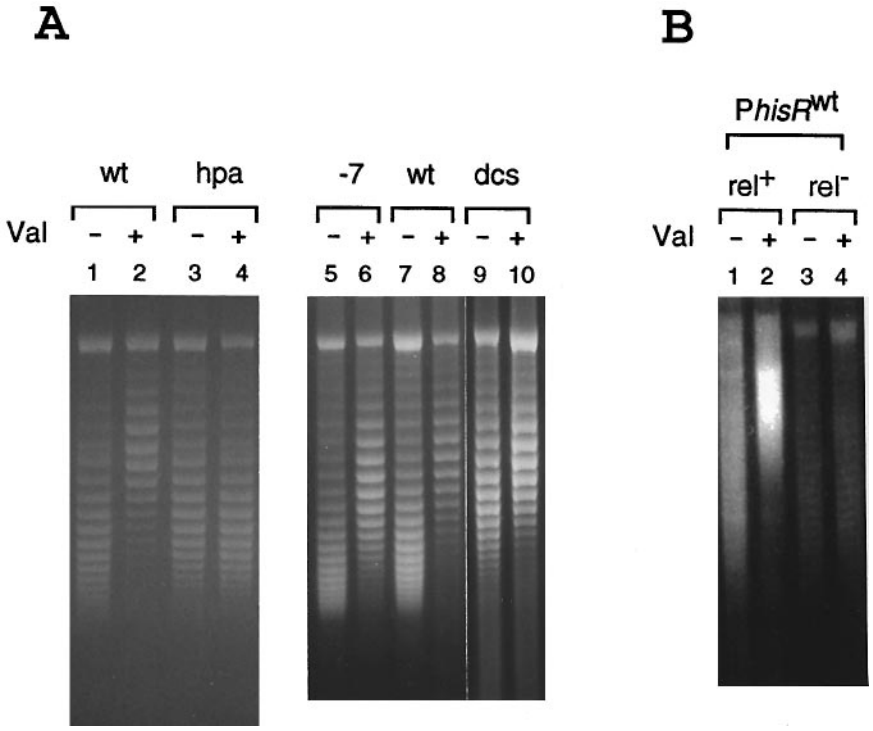

Fig. 7. Analysis of plasmid DNA linking number in cells undergoing the stringent response. (**A**) Plasmid DNA was extracted from cells (strain SU1675) before or 5 min after valine addition and subjected to electrophoresis for 30 h at 2 V/cm in a 1.2% agarose gel containing 22 μg/ml chloroquine. At this chloroquine concentration, DNA topoisomers with a higher linking deficit migrate faster in the gel. Plasmids used were: pKP1-13 (*hisR*^{wt}), pKP6-1 (*hisR*$^{-7}$), pKP14 (*hisR*^{hpa}) and pKP17 (*hisR*^{dcs}). (**B**) pKP1-13 DNA was extracted from strains KRE684 (*relA*$^{+}$, lanes 1 and 2) and KRE692 (*relA*$^{-}$, lanes 3 and 4) before and after valine treatment. Plasmid DNA was analysed as in (A), except that the chloroquine concentration was 35 μg/ml and electrophoresis lasted 48 h to allow the separation of topoisomers of the dimer plasmid population (the predominant form in these strains' background).

could be the divergence between the *hisR* promoter sequence and the canonical discriminator motif (Travers, 1984). To test this possibility, we constructed an additional variant in which two base-pair changes at positions –8 and –6 (C/G→G/C and T/A→G/C, respectively) create the GCGC discriminator consensus sequence motif (the 'dcs' variant). Results in lanes 7 and 8 of Figure 6 show that the two changes in P*hisR*^{dcs} tighten the control substantially, although they reduce overall promoter strength to less than half that of wild-type.

DNA supercoiling changes are the consequence, not the cause, of stringent regulation

The above data led us to entertain the idea that variations in negative DNA supercoiling could mediate stringent control. To test this hypothesis, we measured plasmid DNA linking number in cells undergoing isoleucine starvation. Results in Figure 7A show that significant relaxation of negative DNA supercoils can indeed occur in starved cells, but this is only observed in plasmids carrying a stringently controlled promoter such as P*hisR*^{wt} and P*hisR*$^{-7}$. No such relaxation is observed in the plasmid harbouring P*hisR*^{hpa} (lanes 3 and 4) nor in the cloning vector (data not shown). Thus, it seems unlikely that global changes in DNA supercoiling initiate the stringent response. Most probably, the relaxation observed results from the decrease in transcriptional activity on the plasmid. We showed previously that, *in vivo*, high-level transcription in a region of a plasmid causes the linking deficit of plasmid DNA to increase (Figueroa and Bossi, 1988). Apparently, this is a gyrase-mediated adjustment that counters the topological relaxation associated with transcriptional unwinding. The change is fully reversible as the excess negative supercoils are readily removed upon inhibiting transcription (Figueroa and Bossi, 1988; Spirito *et al.*, 1994). As a further test for the above interpretation, the supercoiling levels of plasmid pKP1-13 (P*hisR*^{wt}) were analysed in *relA* mutant cells. In the *relA*$^{+}$/*relA*$^{-}$ pair of strains used for this study, pKP1-13 mostly persisted as dimer, making topoisomer resolution difficult. Nonetheless, the results in Figure 7B allow one to say that the plasmid DNA globally relaxes following isoleucine starvation of *relA*$^{+}$ cells (lanes 1 and 2), whereas no changes in the topoisomer profile occur in *relA*$^{-}$ cells (lanes 3 and 4). Clearly, this is consistent with P*hisR*^{wt} no longer being inhibited by amino acid starvation in the *relA* mutant background.

In a way, the data in Figure 7A offer an independent ('topological') assessment of the different promoters' strength and regulation. From the positions of the centres of the topoisomer distributions, it can be inferred that the P*hisR*^{hpa} is not as strong as P*hisR*^{wt} (compare lanes 1 and 3), but is nonetheless totally insensitive to stringent control (lanes 3 and 4); and that P*hisR*$^{-7}$ is slightly less sensitive than P*hisR*^{wt} (4±1 linking increase as opposed to 5±1; lanes 3–8). Finally, the patterns in lanes 9 and 10 confirm the lower activity of the *hisR*^{dcs} promoter but also show the limitations of this type of analysis as it appears that high-level transcription is required for its effects on linking number to be noticeable.

Discussion

In the present work, we compared the initiation mechanism of the *hisR* promoter to that of mutant derivatives carrying sequence changes in the 'discriminator' region. The main characteristic of the wild-type promoter is its resistance to melting: the promoter cannot be trapped in open configuration *in vivo* and does not form heparin-resistant transcription complexes *in vitro* unless the initiating nucleotides are supplied to allow formation of the first dinucleotide bond. Both of these features, which are atypical for eubacterial sigma 70 promoters, can be relieved or suppressed by discriminator changes. In particular, the P*hisR*^{hpa} variant, where the C–G-rich stretch between –5 and +1 is replaced with an A–T-rich sequence, is readily found open in rifampicin-treated cells and shows no nucleotide requirements for formation of stable initiation complexes *in vitro*. These data suggest that the discriminator sequence can 'direct' the initiation programme, most likely by setting the energy required for promoter opening. In the case of P*hisR*^{wt}, the C–G-richness of this sequence might introduce a particularly high energetic barrier. The promoter response to negative supercoiling fits this interpretation. Negative torsional energy is known to drive DNA unwinding and is expected to contribute critically to overcoming the melting barrier. Consistent with this view, replacing a C–G bp with an A–T bp at position –7 of the *hisR* promoter lowers the value of negative superhelical density needed for optimal promoter activity. The multiple changes in the *hisR*^{hpa} variant further relieve the supercoiling requirement. The alternative possibility that supercoiling acts by correcting the angular separation between the –35 and –10 hexamers (raised for *rrnB* P1 and *tyrT* promoters where separation is suboptimal) does not apply in this case where the two regions are spaced by the optimal distance of 17 bp.

In spite of its intrinsic weakening features, P*hisR*^{wt} is consistently ~2-fold stronger than P*hisR*^{hpa} when assayed in fast-growing cells or *in vitro*, from fully negatively supercoiled templates. This observation might suggest that, under certain conditions, instability of the initiation complex can serve to favour promoter clearance and productive elongation (Straney and Crothers, 1987).

A major finding of this work is that the sensitivity of the different *hisR* promoter variants to negative DNA supercoiling closely parallels their responsiveness to stringent regulation. These results, together with the known supercoiling sensitivity of other stringently controlled promoters, led us to envisage that supercoiling changes might mediate the stringent response. To test this hypothesis, we monitored plasmid DNA linking number in cells undergoing the response. No significant changes were observed except when the plasmid carried a stringently controlled promoter. The relaxation that was observed in the latter could be more easily interpreted as the consequence rather than the determinant of the transcriptional shut-down (Figueroa and Bossi, 1988). Still, the possibility remains that the transcriptional response is triggered by a fall in torsional tension that occurs locally and does not manifest at the level of overall plasmid topology. This could be the case for local variations in nucleoid structure or in transcriptional rates. The latter scenario borrows from the notion that transcription generates DNA supercoils and contributes to the level of negative superhelical tension in the promoter region (Liu and Wang, 1987; Rahmouni and Wells, 1992). One could speculate that part of the negative superhelicity needed for the *hisR*^{wt} promoter to function efficiently derives from the promoter's own activity. In other words, the promoter might act autocatalytically and require proficient elongation for optimal activity. Albeit highly speculative, this model would explain the apparent paradox stemming from our *in vitro* data which show the *hisR*^{wt} promoter to be maximally active at a negative superhelical density, $-\sigma = 0.08$, much higher than the average inside *E.coli* ($-\sigma = 0.05$; Wang, 1984). Moreover, in hinting that supercoiling-requiring promoters should respond negatively to a slow-down of elongation, the model offers a new way of reconciling the claim that transcription elongation, as opposed to initiation, is the primary target of ppGpp-mediated control (Vogel *et al.*, 1992).

An alternative approach is to view the supercoiling dependence of stringently controlled promoters as a mere symptom of the physical properties that are at the basis of the regulatory mechanism. 'Resistance to melting' appears to be a common denominator of promoters susceptible to stringent regulation. The sensitivity to torsional energy might just be the natural consequence of the melting barrier and be irrelevant as far as regulation is concerned. Promoter melting was proposed to be the step of initiation affected by ppGpp (Ohlsen and Gralla, 1992a,b). One might speculate that ppGpp acts by hampering RNA polymerase's ability to melt the DNA helix. The interference could be exerted in such a way as to have no consequences for promoters that are easy to open, but result in the specific inhibition of hard-to-melt promoters. In fact, this mechanism could be tuned so as to allow different degrees of responsiveness as a function of the energy required to achieve melting at any particular promoter. Clearly, the hierarchy of responses of the *hisR* promoter variants studied here would be consistent with such a model. The model would also account for data in the literature and, in particular, explain why DNA melting-limiting conditions (relaxed DNA and high salt) are required for ppGpp-mediated control to be reproduced *in vitro* (Ohlsen and Gralla, 1992a,b). Finally, the role of ribonucleotide concentration (Gaal *et al.*, 1997) could be integrated in this scenario. Promoters that are refractory to melting are likely to remain in an unwound state for a very short time if NTP substrates are not immediately available to 'stabilize' the open complex. Once formed, such stressed intermediates might be rapidly channelled into productive elongation (Straney and Crothers, 1987).

Materials and methods

Enzymes and chemicals

Restriction endonucleases were from New England Biolabs, Boehringer Mannheim or Promega. T4 DNA ligase, T4 polynucleotide kinase, *E.coli* RNA polymerase and RNase T_1 were purchased from Boehringer Mannheim. The 'Klenow' fragment of *E.coli* DNA polymerase I was from New England Biolabs or Boehringer Mannheim. MuLV reverse transcriptase was from Gibco-BRL. Calf thymus DNA topoisomerase I was a generous gift of M.Duguet, University of Paris. Most biochemicals were obtained from Sigma Chemical Co. Unlabeled nucleotides, deoxy- and dideoxynucleotides were from Boehringer Mannheim. Potassium permanganate and chloro-acetaldehyde (CAA) were from Fluka. CAA was double-distilled before use (boiling point 78–80°C). Inorganic

chemicals were from Sigma Chemical Co. or Merck. Radiochemicals were from Amersham. Oligonucleotides were synthesized by the phosphoramidite method using an Applied Biosystems DNA synthesizer.

Plasmids and bacteria

The *hisR* promoter sequences analysed in this study were initially isolated on M13 recombinant phages. The wild-type sequence (in phage mSt-546-12) and its '–7' variant (mutant allele *hisR10107*) originated from the chromosome of *S.typhimurium* wild-type and mutant strains (Figueroa *et al.*, 1991). The 'hpa' and 'dcs' variants were obtained *in vitro* by oligonucleotide-directed mutagenesis of mSt-546-12 (see below). The *hisR* promoter region (positions –293 to +28 relative to the main transcription start site) was subcloned into plasmid pKK232-8 as described (Figueroa *et al.*, 1991). In the resulting plasmids [pKP1-13 (*hisR*^{wt}), pKP6-1 (*hisR*$^{-7}$), pKP14 (*hisR*^{hpa}) and pKP17 (*hisR*^{dcs})] the *hisR* promoter and its variants drive transcription of the *cat* gene. Derivatives of pKP1-13, pKP6-1 and pKP14 carrying a shortened *cat* gene (the 'mini *cat*' series) were constructed by deleting a 900 bp *Hin*dIII–*Sty*I fragment. Finally, mpKP-eco and mpKK-pst are M13 derivatives carrying the N-terminal coding portions (~300 nucleotides) of *cat* and *bla* genes, respectively. They were constructed by cloning a 639 bp *Eco*RI fragment from pKP1-13 and a 951 bp *Pst*I fragment from pKK232-8 into M13 mp18 (at its *Eco*RI site and *Pst*I site, respectively) in the orientation that puts *cat* and *bla* template strands in the phage virion. All of the above constructions and the preparation of plasmid DNA were carried out in *E.coli* strains SU1675 (Figueroa and Bossi, 1988) or HB101 (Sambrook *et al.*, 1989) according to standard procedures. For DNA supercoiling studies, three isogenic *E.coli* strains were used: *gyr*$^+$ *topA*$^+$ strain JTT1 (Sternglanz *et al.*, 1981), its derivative RS2 carrying the *topA10* allele (Sternglanz *et al.*, 1981) and strain KD112 which harbours the *gyrB226* mutation (Hsieh *et al.*, 1991). These strains were a generous gift of Karl Drlica, Public Health Research Institute, New York. Strains KRE684 [(W3110) Δ*lac*] and KRE692 [(W3110) Δ*lac* Δ*relA251::kan*] are derivatives of strains CF1943 and CF1944 (Xiao *et al.*, 1991), respectively. They were constructed and kindly donated to us by Ken Rudd (NIH).

In vitro mutagenesis

Oligonucleotide-directed mutagenesis was carried out with the 'two-primer' method as described by Sambrook *et al.* (1989). Single-stranded DNA from phage mSt-546-12 (above) was used as the template. Mutagenic primers were 50 nucleotides long and contained the mismatching residues approximately in the middle of their sequence. The 'second primer' in the mutagenesis procedure was 20 nucleotides long and annealed in a region of mSt-546-12 ~1 kb away from the site of mutagenesis. Mutant clones were identified by plaque-hybridization using the mutagenic primers phosphorylated with [γ-^{32}P]ATP as probes. Presence of the desired sequence changes was verified by DNA sequencing (Sambrook *et al.*, 1989).

RNA extraction and quantification

Plasmid-harbouring cells were grown in Luria–Bertani broth (Sambrook *et al.*, 1989) to late-exponential phase (A_{600} = 0.8) and harvested by centrifugation. Cells deriving from 5 ml of culture were resuspended in 3 ml of 'protoplast' buffer (15 mM Tris–Cl pH 8.0, 0.45 M sucrose, 8 mM EDTA, 2 mg/ml lysozyme) and left on ice for 15 min. The cell suspensions were gently centrifuged and protoplasts lysed by homogenization in 1 ml guanidine thiocyanate buffer (50 mM Tris–Cl pH 7.5, 10 mM EDTA, 8% mercaptoethanol, 5 M guanidine thiocyanate). Lysates were mixed with cold 4 M LiCl (8 ml) and kept overnight on ice. Insoluble RNA was separated by centrifugation and resuspended in a phenol-saturated buffer (10 mM Tris–Cl pH 7.5, 1 mM EDTA, 0.1% SDS). RNA was extracted with phenol/chloroform and precipitated with ethanol. Pellets were resuspended in sterile water and after spectrophotometric quantification at 260 nm, RNA was used for cDNA synthesis with MuLV reverse transcriptase. Two ^{32}P-labelled primers were employed simultaneously: the 'cat' primer which anneals between positions +88 and +100 of the *cat* coding sequence and the 'bla' primer which anneals between positions +53 to +71 of the *bla* gene. Briefly, 1 pmol of each primer was incubated with 5 μg of RNA in a buffer containing 50 mM Tris–Cl pH 8.0, 75 mM KCl, 3 mM $MgCl_2$ and 10 mM DTT. The mixture was heated for 5 min at 65°C and allowed to cool slowly to 37°C. The extension reaction was started by adding 10 units of MuLV reverse transcriptase and dNTPs to a final concentration of 0.2 mM each. After 30 min incubation at 42°C, nucleic acids were precipitated with ethanol and analysed by electrophoresis on 6% denaturing polyacrylamide gels. Radioactive DNA was quantified by phosphorimaging (Molecular Dynamics phosphorimager using ImageQuant TM 3.0 software) and the *cat*-specific signal was normalized to that of *bla*.

RNA pulse-labelling and hybrid selection

Plasmid-harbouring cells were grown in MOPS-glucose medium (Neidhardt *et al.*, 1974), supplemented with vitamin B_1, all amino acids except glutamine, leucine, isoleucine and valine and 0.2 mM K_2HPO_4. At A_{600} = 0.3, the culture was divided into two and valine (0.5 mg/ml final concentration) added to one half. After 5 min, ^{32}P-phosphate (100 μCi) was added to 1 ml aliquots from both valine-treated and untreated cells and incubation continued for additional 5 min. RNA was rapidly extracted as previously described (Figueroa *et al.*, 1991). The amount of RNA originating from 0.1 ml of culture was hybridized to 2 μg of single-stranded M13 DNA carrying template-strand portions of *cat* and *bla* genes (mpKP-eco or mpKK-pst). Hybridization was for 30 min at 50°C in 10 μl of 50 mM Tris–Cl, 40 mM KCl. Samples were then treated with RNase T_1 (5 units for 30 min) and loaded directly on a 1.2% agarose gel where hybrid-selected *cat* and *bla* RNAs co-migrate with the phage DNA band. Radioactivity in bands was measured and the *cat* signal normalized to *bla*.

Preparation of plasmid DNA topoisomers

Populations of plasmid DNA topoisomers of increasing superhelical densities were prepared by relaxing plasmid DNA with calf thymus DNA topoisomerase I in the presence of increasing amounts of ethidium bromide (0–17 mM) as previously described (Albert *et al.*, 1994). Superhelical densities were measured upon resolving topoisomers on 1.5% agarose gels containing 0.8 μg/ml chloroquine.

In vitro transcription

Initiation complexes were formed by incubating 0.3 pmol of plasmid DNA with 1 pmol of RNA polymerase in 25 μl transcription buffer (40 mM Tris–Cl pH 8.0, 5 mM $MgCl_2$, 5 mM spermidine, 1 mM DTT, 150 mM KCl) in the presence of ATP, CTP and GTP (200 μM each) for 10 min at 37°C. The initiation complexes were then treated with heparin (50 μg/ml final concentration) for 30 s at 37°C. UTP (20 μM final concentration) and [α-^{32}P]UTP (0.5 μCi) were added and incubation continued for 15 min at 37°C. Reactions were terminated with SDS and EDTA (0.1% and 25 mM, respective final concentrations). RNAs were precipitated with ethanol and electrophoresed in a 6% denaturing polyacrylamide gel. When appropriate, initiation complexes were formed in the presence of incomplete combinations of ribonucleotide substrates and missing nucleotides added after the heparin challenge.

$KMnO_4$ and CAA probing

Plasmid-harbouring cells were grown at 37°C in M9 minimal medium supplemented with casaminoacids (2 mg/ml) and ampicillin (100 μg/ml). At A_{600} = 0.4–0.5, cells were exposed to rifampicin (200 μg/ml) for 5 min or left untreated. 200 μl of 0.3 M $KMnO_4$ were added to 10 ml of culture and incubation continued for 4 min. Cells were rapidly harvested and plasmid DNA isolated by the boiling method (Sambrook *et al.*, 1989). CAA probing and primer extension analyses were carried out as previously described (Guérin *et al.*, 1996).

Plasmid DNA extraction for supercoiling measurements

Cell growth conditions were the same as for RNA labelling (above) except that medium was supplemented with a standard concentration of phosphate (1.3 mM). At A_{600} = 0.4, cell cultures (50 ml) were exposed to valine (0.5 mg/ml final concentration) for 5 min or left untreated. Cultures were rapidly poured into centrifuge bottles containing 25 ml of frozen medium and shaken until completely thawed (typically 3–5 min). Cell suspensions were centrifuged at 8000 *g* for 3 min at 4°C and pellets immediately frozen by immersion in liquid nitrogen. The frozen pellet was thawed in 0.8 ml of 25 mM Tris–Cl pH 8.0, 50 mM glucose, 10 mM EDTA and the cell suspension mixed with 0.5 ml of a 10 mg/ml lysozyme solution in the same buffer. After 1 min on ice, cells were lysed with the alkaline method as described (Sambrook *et al.*, 1989). Nucleic acids were resuspended in 300 μl of 10 mM Tris–Cl pH 8.0, 1 mM EDTA and 20 μl of a 10 mg/ml solution of RNase A were added. The mixture was incubated at 37°C for 20 min. Samples were consecutively extracted with phenol, phenol/chloroform, chloroform and plasmid DNA recovered by ethanol precipitation. Pellets were resuspended in 25 μl of Tris–Cl pH 8.0, 1 mM EDTA and 15 μl loaded on a 1.2 % agarose gel (200 ml) containing 22 μg/ml chloroquine.

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