Elevated Unconstrained Supercoiling of Plasmid DNA Generated by Transcription and Translation of the Tetracycline Resistance Gene in Eubacteria†

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ABSTRACT: Our previous studies have indicated that the *leu-500* promoter of *Salmonella typhimurium* is activated by local supercoiling arising from the transcription of a divergent promoter (Chen et al., 1992). For this to occur on a plasmid, we have shown that the transcribing RNA polymerase must be anchored to the cell membrane by transcription, translation, and export of the *tetA* gene and that the cell background must be *topA*. In this study we have used $(AT)_n$ reporter sequences to analyze changes in unconstrained supercoiling of plasmid DNA under the circumstances in which the *leu-500* promoter becomes activated. $(AT)_n$ sequences undergo a structural transition to a cruciform at a threshold level of negative supercoiling that is determined by the length of the tract, and this can be detected in the cellular DNA by *in situ* chemical probing. These studies have shown that there is elevated unconstrained supercoiling in *tetA*-carrying plasmids in either *Escherichia coli* or *S. typhimurium* cells in exponential growth. This oversupercoiling depends on the function of the *tetA* gene in *cis* and the Δ*topA* cell background. These are exactly the conditions that lead to the activation of the *leu-500* promoter, supporting the proposed mechanism for the suppression of the *leu-500* mutation by *topA*. Use of $(AT)_n$ sequences of different lengths has permitted us to estimate the extent of oversupercoiling. When the *tetA* gene was initiated using the strong *tac* promoter, we were able to detect increased unconstrained DNA supercoiling even in *topA*$^+$ *E. coli* cells.

Transcription and DNA supercoiling are intimately connected. The interaction between RNA polymerase and DNA involves torsional, and probably flexural, changes in local DNA structure, and thus many promoters are affected by the prevailing level of superhelical stress [reviewed in Drlica (1984)]. In addition, the act of transcription may itself have topological consequences for the DNA template. Liu and Wang (1987) proposed that an elongating RNA polymerase might in some circumstances experience hindrance to rotation about the DNA, in which case there is a tendency to generate a domain of positive supercoiling ahead of the polymerase and one of negative supercoiling behind it. These domains will be subject to two principal mechanisms of relaxation. First, the superhelical tension may diffuse along the DNA, diluting the effective superhelix density; this may be particularly acute in a circular DNA molecule, where the domains of positive and negative supercoiling can migrate around the circle and undergo self-cancellation by rotation of the DNA about the duplex axis. Second, the domains will be subject to the action of cellular topoisomerases. In eubacteria, negative supercoiling may be relaxed by topoisomerase I, while positive supercoiling can be relaxed by DNA gyrase. It would be expected that the steady-state level of DNA supercoiling should reflect a balance between the rate of induction by transcription and the rate of relaxation. Perturbation of the activities of cellular topoisomerases may significantly distort the position of equilibrium and can lead to particularly marked changes in DNA supercoiling (Lockshon & Morris, 1983; Pruss & Drlica, 1986). There is now a wealth of observations in support of the twin domain of the supercoiling model (Liu & Wang, 1987; Wu et al., 1988; Tsao et al., 1989; Rahmouni & Wells, 1989, 1992; Cook et al., 1992; Dayn et al., 1992).

Thus, DNA supercoiling may influence transcription, and transcription can affect DNA supercoiling, and in principle both of these processes could operate simultaneously. We have described a system where this appears to be the case (Chen et al., 1992, 1993). The *leu-500* promoter of *Salmonella typhimurium* (Mukai & Margolin, 1963) is normally inactive, but may become activated in a *topA* background of either *S. typhimurium* (Dubnau & Margolin, 1972; Margolin et al., 1985) or *Escherichia coli* (Chen et al., 1994). We have shown (Chen et al., 1992, 1993) that for this to occur when the *leu-500* is present on a circular plasmid, transcription, translation, and membrane insertion of an adjacent tetracycline resistance gene *tetA* are required. Thus, activation of the plasmid-borne promoter requires the simultaneous presence of a *tetA* gene in *cis* and a *topA* background. We have proposed the following mechanism to account for these observations. Transcription of the *tetA* gene generates local negative supercoiling due to the effects described by the twin domain of supercoiling theory. In a *topA* background the negative supercoiling is poorly relaxed by enzyme action, but could be efficiently relaxed by superhelical diffusion. The role of the *tetA* gene is to provide a barrier against diffusion of supercoils, arising from anchorage of the RNA polymerase due to the coupled transcription, translation, and membrane insertion of the TetA protein. This mechanism is consistent with earlier experiments describing the importance of the *tetA* gene in generating increased negative linking differences in plasmids extracted from *topA* cells (Lodge et al., 1989; Pruss & Drlica, 1986).

While all the available data for the activation of the *leu-500* promoter are consistent with this model, we sought some more physical confirmation that the conditions leading to promoter activation were associated with increased local negative supercoiling. We had previously shown that a fraction of plasmid DNA with a very high negative linking difference was generated under the conditions where the *leu-500* promoter was active (Chen et al., 1992), but such measurements can

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Table 1: Strains and Plasmids Used in These Studies

strain	Bacterial Strains relevant genotype	references
Salmonella typhimurium		
LT2	*topA*$^+$	
CH582	Δ*topA 2762 leu-500 ara-9*	Richardson et al., 1984
Escherichia coli		
HB101	*topA*$^+$	
DM800	Δ(*topA-cysB*)*204 acrA13 gyrB225*	Di Nardo et al., 1982
SD108	DM800 Δ*trpE63 topA*$^+$ *cysB*$^+$ *pyrF287*	Di Nardo et al., 1982

plasmid	Plasmids markers	references
pXG540	Amp^r, $(AT)_{34}$	Greaves et al., 1985
pATn*tetA*	Tet^r, $(AT)_9$–$(AT)_{34}$	this work
pAT34*tetA*.P$_{tac}$	Amp^r, Tet^r (with *tac* promoter), $(AT)_{34}$	this work
p*I^Q.kan*	Kan^r, *lacI^q*	this work

only indicate the global supercoiling in the molecule and do not reveal any more specific local conditions. For this, we turned to the exploitation of supercoiling-dependent structural transitions by sequences placed at particular locations.

Any structural transition in DNA leading to a net negative change in helical twist will be relatively stabilized in a negatively supercoiled molecule, as has been demonstrated for cruciform structures (Gellert et al., 1979; Lilley, 1980; Panayotatos & Wells, 1981) and left-handed Z-DNA (Peck et al., 1982; Singleton et al., 1982). The underwound structure will have a stable existence in topoisomers more supercoiled than a critical threshold. This critical level of supercoiling occurs where the free energy of relaxation on formation (due to the local negative twist change) of the new structure is greater than the free energy of formation of that structure. At the threshold level of supercoiling, the molecule undergoes a conformational transition, with the formation of the new structure. Thus, the presence or absence of the structure can indicate whether or not the prevailing level of unconstrained negative supercoiling is greater than the threshold. In order for this to be useful for studies of the levels of DNA supercoiling inside the cell, it is necessary to have some way of detecting the new structure in the cellular DNA, and this is provided by the *in situ* chemical probing method (Boublikova & Palecek, 1989).

We have found that alternating adenine–thymine sequences ($(AT)_n$) are particularly good reporter sequences for measuring levels of DNA supercoiling inside bacterial cells (McClellan et al., 1990). Tracts of $(AT)_n$ can undergo cruciform extrusion above a critical level of DNA supercoiling (Greaves et al., 1985; Haniford & Pulleyblank, 1985; Panyutin et al., 1985). They do this with no detectable kinetic barrier (Greaves et al., 1985). Moreover, cruciform formation is an all-or-none process with a fixed free energy of formation, while the reduction in free energy of supercoiling due to cruciform formation depends on the length of the $(AT)_n$ tract. Thus, the critical threshold depends in a simple way on the size of the $(AT)_n$ sequence, and by using a series of $(AT)_n$ tracts we can estimate the effective level of negative supercoiling at a particular location. The cruciform can be detected in the cell using *in situ* probing with osmium tetraoxide/2,2′-bipyridine, because the formally single-stranded thymine bases of the cruciform loops are reactive to electrophilic addition at the 5,6 double bond. We have previously used this method to demonstrate oversupercoiling of cellular DNA in response to salt shock (McClellan et al., 1990).

In these studies we have placed $(AT)_n$ sequences in the section of DNA upstream of the *tetA* gene in a circular plasmid, and tested for cruciform extrusion in *topA*$^+$ and *topA* cells. We find evidence for elevated unconstrained DNA supercoiling that depends on the presence of the *tetA* gene in *cis*, and a *topA* genetic background. These are precisely the conditions that lead to the activation of the *leu-500* promoter, strengthening our conviction that the *leu-500* promoter is activated by negative supercoiling generated by transcription.

MATERIALS AND METHODS

Bacterial Strains

The strains used during this study are summarized in Table 1. Bacteria were cultured in liquid LB media with aerobic growth at 37 °C or on 1.2% agar–LB plates. Antibiotics were added as required, with final concentrations as follows: 50 μg/mL carbenicillin, 150 μg/mL chloramphenicol, 50 μg/mL kanamycin, and 10 μg/mL tetracycline (except for strains related to *E. coli* DM800, which had only 2 μg/mL tetracycline). Transformation of plasmids into cells was performed using the calcium chloride procedure (Cohen et al., 1972).

Plasmids

The plasmids used in this study (Table 1) are based on pAT153 (Twigg & Sherratt, 1980) and were constructed as follows.

Construction of pAT34tetA. The *Eco*RI–*Pst*I fragment containing the $(AT)_{34}$ tract from pXG540 (Greaves et al., 1985) was cloned between the same sites in pAT153. This generated a plasmid conferring tetracycline resistance but lacking a complete *bla* gene (Figure 1B). Transformed cells were Tet^{r}Amp^s. The pATn*tetA* plasmids containing $(AT)_n$ tracts of lengths n = 9–25 were constructed by analogous procedures.

Construction of pAT34tetA.P$_{tac}$. In an earlier study (D.C., R.P.B., and D.M.J.L., unpublished results), a *tac* promoter (De Boer et al., 1983) was created upstream of the *tetA* gene of pLEU500Tc (Chen et al., 1992) by cloning the oligonucleotides 5′-AGCTCTGTTGACAATTAATCATCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAACAATTTCACACA-3′ and 5′-AGCTTGTGTGAAATTGTTATCCGCTCACAATTCCACACATTATACGAGCCGATGATTAATTGTCAACAG-3′ into the *Hin*dIII site, generating the plasmid pLEU500P$_{tac}$*tetA*. This plasmid was used as the source of the *tac* promoter in these studies. An oligonucleotide was cloned between the *Cla*I–*Hin*dIII sites of pAT153, generating the restriction sites *Cla*I–*Pst*I–*Eco*RV–*Hin*dIII (plasmid p153.PR). pLEU500P$_{tac}$*tetA*

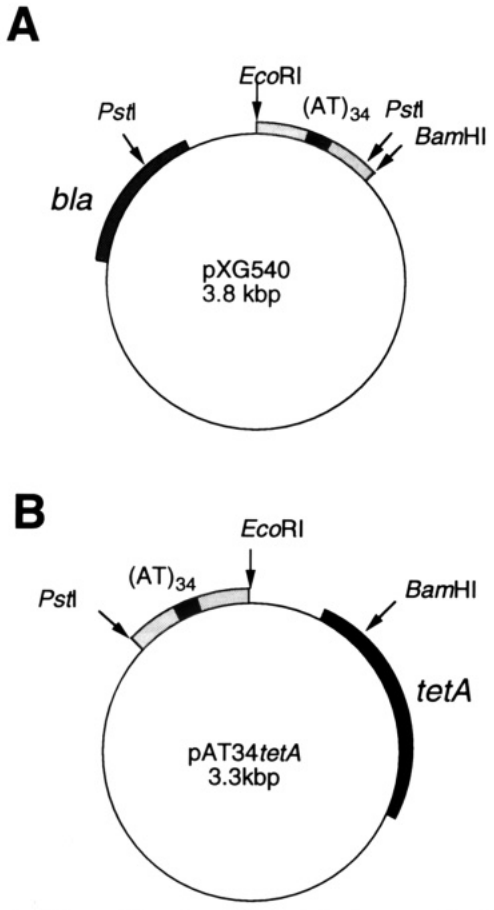

FIGURE 1: Maps of the plasmids used in these studies. (A) The construction of pXG540 has been described earlier (Greaves et al., 1985). It contains a 528-bp fragment of *Xenopus laevis* DNA, including the $(AT)_{34}$ repeat located between the *Eco*RI and *Bam*HI sites of pAT153, and thus lacks the N-terminal region of the *tetA* gene. (B) pAT34*tetA* contains a subfragment of the *Xenopus* fragment that includes the $(AT)_{34}$ sequence, located between the *Eco*RI and *Pst*I sites of pAT153. It contains a complete *tetA* gene, but an inactivated *bla* gene.

and p153.PR were each subjected to digestion with *Bam*HI and partial cleavage with *Eco*RV. The fragment of pLEU500P_{tac}*tetA* containing the *tac* promoter and the N-terminal section of *tetA* was ligated into p153.PR to generate the plasmid p153P_{tac}*tetA*. Finally, the *Pst*I–*Pst*I fragment from pXG540, containing the $(AT)_{34}$ sequence, was excised and ligated between the *Pst*I sites of p153P_{tac}*tetA* to generate pAT34*tetA*.P_{tac} (Figure 7A).

Construction of pI^Q*kan.* The fragment containing the kanamycin-resistance gene was excised from the plasmid pGP1-2 (Tabor & Richardson, 1985) by digestion with *Nhe*I and *Bam*HI and was cloned between the equivalent sites of pI^Q (Bellomy et al., 1988).

All plasmids were initially transformed into *E. coli* HB101, with subsequent transformation into strains DM800 and SD108. Plasmids from *E. coli* HB101 were also transformed into *Salmonella typhimurium* LT2 and CH582.

During our experiments with $(AT)_n$ tracts, we have encountered a relatively high frequency of deletions occurring specifically within the $(AT)_n$ tract (McClellan et al., 1990; R.P.B. and D.M.J.L., unpublished data). Thus, after each cloning step, the sequence within and surrounding the $(AT)_n$ tract was determined by dideoxy sequencing of DNA obtained by an alkaline lysis minipreparation (Birnboim & Doly, 1979).

Large-scale quantities of plasmid were purified from *E. coli* HB101 grown in M9 supplemented medium, with amplification for 16 h by chloramphenicol at 150 μg/mL. The DNA was isolated after lysis with lysozyme, SDS, and EDTA and purified by two cesium chloride density gradient ultracentrifugations in the presence of ethidium bromide. Supercoiled plasmid was isolated, and after extraction of the ethidium bromide with cold butan-1-ol, the solution was extensively dialyzed against 10 mM Tris·HCl (pH 7.5)/0.1 mM EDTA at 7 °C. Aliquots of solution were stored at −20 °C and thawed slowly on ice.

In Situ Chemical Modification

The *in situ* chemical modification procedure of McClellan et al. (1990) was altered in a number of respects to allow the procedure to be performed at the midlogarithmic growth phase. The times at which modification was performed varied to take into account the different growth rates of the various strains used; the following is a typical protocol: A 1-mL overnight culture was diluted into 50 mL of LB plus antibiotic and grown aerobically at 37 °C. At the midlogarithmic growth phase (A_{600} 0.6–0.7), the cells were harvested by centrifugation (10 000 rpm for 5 min) and resuspended in 8 mL of 250 mM potassium phosphate buffer (pH 7.2). To this was added 2 mL of a mixture of 10 mM OsO_4/10 mM 2,2′-bipyridine (final concentration of 2 mM OsO_4/2,2′-bipyridine in 200 mM phosphate buffer). After incubation at room temperature for 15–30 min, the reaction was terminated by two washes with 200 mL of cold 200 mM phosphate buffer, and the cells were recovered by centrifugation.

Experiments involving plasmids with the *tac* promoter (pAT34*tetA*.P_{tac}) were performed in exactly the same manner, except that IPTG was added to a final concentration of 1–2 mM before harvesting the cells.

Purification of OsO_4*-Modified DNA and Analysis of the Sites of Modification.* Plasmid DNA was isolated using a modified procedure of the boiling method (Holmes & Quigley, 1981). The cell lysis solution (0.7 mL) was extracted twice with phenol/chloroform (1:1 by volume) and then treated with 10 μL of 10 μg/mL DNase-free RNase A at 37 °C for 20 min. The DNA was precipitated by the addition of 0.7 vol of isopropyl alcohol, and the dried pellet was dissolved in 20 μL of 10 mM Tris (pH 7.5)/0.1 mM EDTA.

The sites of modification were observed at the nucleotide level after cleavage at the *Eco*RI site and radioactive 3′-^{32}P-labeling of the DNA using Klenow DNA polymerase. The DNA was ethanol precipitated from a 0.3 M sodium acetate solution and cleaved at the *Pst*I site, and the approximately 350-bp fragment was purified from a 1% agarose gel. The DNA was incubated with 1 M piperidine at 90 °C for 30 min to cleave at the sites of modification. After extensive lyophilization, the DNA was electrophoresed in a denaturing 6% polyacrylamide gel containing 7 M urea in 90 mM Tris–borate (pH 8.3)/1 mM EDTA (TBE buffer). Each gel also contained equivalent asymmetrically 3′-^{32}P-labeled fragments from pXG540 reacted *in vitro* with 1 mM OsO_4/2,2′-bipyridine (5 min at 20 °C in TBE buffer), to serve as a reference marker. Radioactive DNA fragments were observed by autoradiography of dried gels at −70 °C with intensifier screens or with storage phosphor screens and a 400S phosphorimager (Molecular Dynamics). Quantitation of the gels was performed directly on the phosphorimage.

Analysis of Changes in the Linking Number of Plasmids. The linking numbers of the plasmids used in this study were analyzed by electrophoresis on agarose gels containing chloroquine as described previously (Chen et al., 1992). Briefly, a 50-mL culture was grown to the midlogarithmic

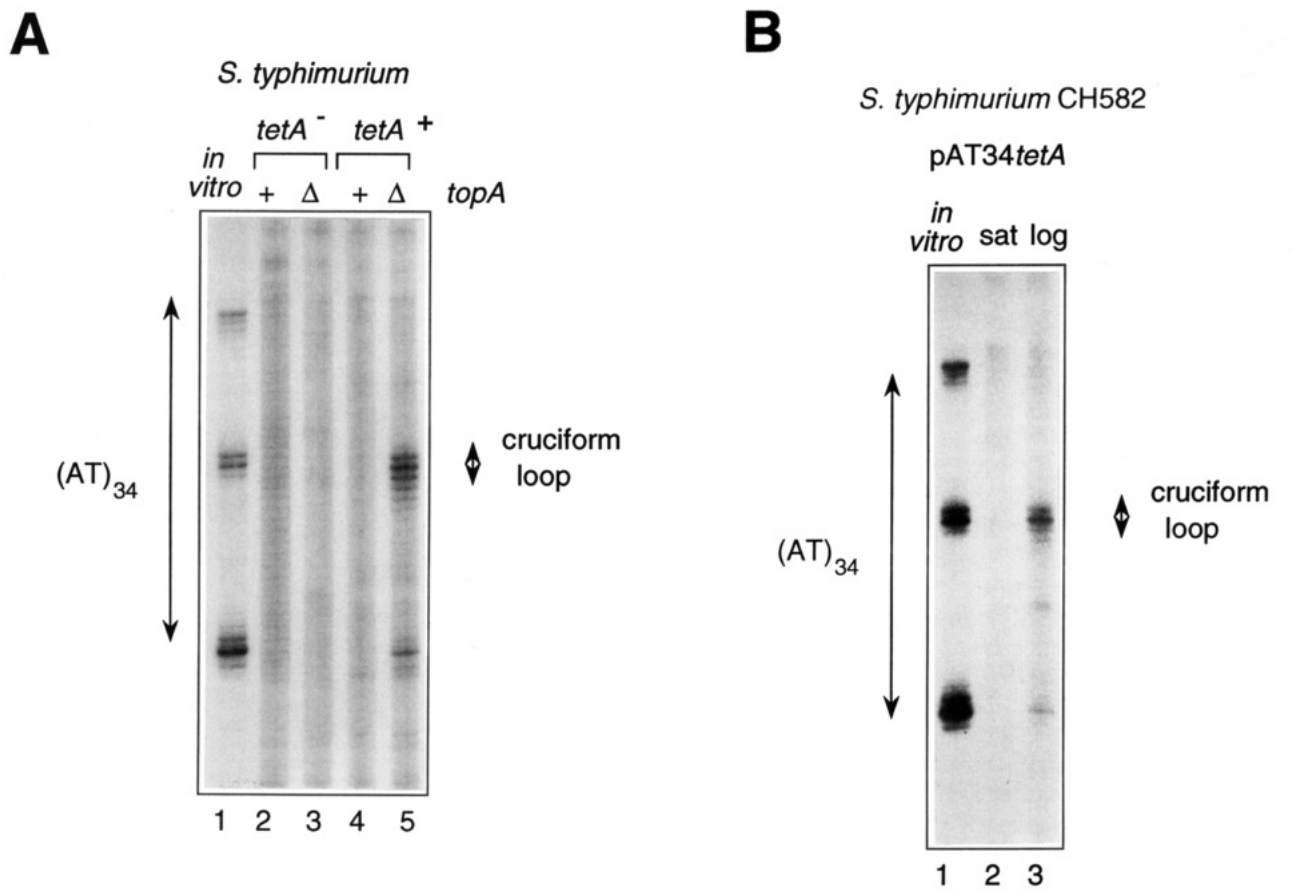

FIGURE 2: *In situ* chemical probing detects increased levels of unconstrained negative supercoiling in plasmids encoding TetA in Δ*topA Salmonella typhimurium*. (A) *In situ* modification with 2 mM OsO_4/2,2′-bipyridine was performed on wild-type (LT2) and Δ*topA* (CH582) *S. typhimurium* transformed with the plasmids pXG540 and pAT34*tetA*. Plasmid DNA was isolated, cleaved at the *Eco*RI site, and radioactively labeled with 3′-^{32}P. After further restriction cleavage with *Pst*I, and isolation of the 350-bp fragment containing the $(AT)_{34}$ tract, the DNA was cleaved at the position of osmium adducts by treatment with 1 M piperidine at 90 °C for 30 min. The positions of modification were revealed by sequencing gel electrophoresis and autoradiography. The gel also contained an equivalent asymmetrically 3′-^{32}P-labeled fragment from pXG540 reacted *in vitro* with 1 mM OsO_4/2,2′-bipyridine to serve as a reference marker. Note that under these conditions of modification, both the cruciform loop and junctions have been chemically modified, conveniently delineating the extent of the $(AT)_{34}$ tract (indicated on left). Track 1, *In vitro* modification of pXG540; track 2, *in situ* modification of pXG540 in LT2 (*topA*$^+$); track 3, *in situ* modification of pXG540 in CH582 (Δ*topA*); track 4, *in situ* modification of pAT34*tetA* in LT2 (*topA*$^+$); track 5, *in situ* modification of pAT34*tetA* in CH582 (Δ*topA*). Note that the only *in situ* reaction leading to significant modification of a cruciform loop is that of track 5, i.e., the *tetA*-carrying plasmid pAT34*tetA* transformed into the Δ*topA* strain. (B) *In situ* cruciform extrusion in pAT34*tetA* in Δ*topA S. typhimurium* depends on the physiological state of the cells. CH582 (Δ*topA*) carrying pAT34*tetA* was grown either to saturation (track 2) or to the midlogarithmic phase (track 3) before *in situ* modification with 2 mM OsO_4/2,2′-bipyridine. Plasmid DNA was isolated and analyzed as above. Track 1 contains the results of *in vitro* modification of pXG540. Note that the clear cruciform extrusion observed in exponential growth is not observed in the saturated culture.

phase, and the cells were harvested and lysed with alkali (Birnboim & Doly, 1979). After centrifugation, the supernatant was treated with DNase-free RNase A and extracted with phenol/chloroform, and the DNA was precipitated with ethanol. The DNA was electrophoresed in an agarose gel in TBE buffer with the indicated concentration of chloroquine and then stained with ethidium bromide. Photographs of the gels were taken under UV illumination through red and green filters to reduce background fluorescence. The negative was then densitometrically scanned, resulting in a negative image of dark bands of DNA on a light background.

RESULTS

Plasmids Containing Adenine–Thymine Reporter Sequences. In these studies, we have exploited the formation of cruciform structures inside the cell by sequences consisting of alternating adenine–thymine bases ($(AT)_n$ sequences). It has been demonstrated *in vitro* that when such sequences are supercoiled above a critical level, they undergo cruciform extrusion without any discernible kinetic barrier (Greaves et al., 1985). Upon formation of the cruciform, the formally single-stranded bases of the cruciform loop become reactive to certain chemical probes; for example, the thymine bases are reactive to attack by osmium tetraoxide, and this labilizes the DNA locally to subsequent cleavage by base. The osmium tetraoxide reaction can be performed on eubacterial cellular DNA *in situ* (Boublikova & Palecek, 1989), and this forms the basis for a method for measuring the level of unconstrained supercoiling of cellular DNA. Our earlier *in situ* chemical probing studies (McClellan et al., 1990) used the plasmid pXG540, which contains a section derived from a *Xenopus* αT1-globin gene, including a sequence of $(AT)_{34}$ (Greaves et al., 1985) (Figure 1).

Our studies of the activation of the *S. typhimurium leu-500* promoter (Chen et al., 1992, 1993) suggested that the promoter was responding to elevated levels of local negative supercoiling arising from transcription of the tetracycline resistance gene *tetA*. Our original plasmid pXG540 did not carry a functional *tetA* gene, as it was inactivated during the cloning of the *Xenopus* sequence. We therefore constructed a new plasmid in which the 350-bp *Eco*RI–*Pst*I fragment of pXG540, containing the $(AT)_{34}$ sequence, was cloned between

the *Eco*RI and *Pst*I sites of pAT153 (Figure 1). The resulting plasmid, pAT34*tetA*, contained the potential cruciform-forming sequence upstream of the complete functional *tetA* gene and was Tet^rAmp^s.

Increased Negative Supercoiling in tetA-Carrying Plasmids in ΔtopA S. typhimurium. Our studies of the *leu-500* promoter suggest that its activation is dependent on two factors: the presence of a functional *tetA* gene in *cis* and a *topA* genetic background. We therefore examined the level of unconstrained supercoiling in pXG540 and pAT34*tetA*, each in top^+ (LT2) and Δ*topA* (CH582) *S. typhimurium*, by means of *in situ* chemical probing.

Cells were grown to the midexponential growth phase, harvested, and incubated with 2 mM OsO_4/2,2′-bipyridine in 200 mM potassium phosphate (pH 7.2). The plasmid DNA was then isolated, digested with *Eco*RI, radioactively labeled with 3′-^{32}P, and digested with *Pst*I, and osmium adducts were cleaved by treatment with 1 M piperidine at 90 °C. The products were analyzed using sequence gel electrophoresis and autoradiography. From the analysis of the plasmids pXG540 and pAT34*tetA* in LT2 ($topA^+$) and CH582 (Δ*topA*) (Figure 2A), it is clear that the only conditions leading to the observation of extensive *in situ* modification at the center of the $(AT)_{34}$ tract (i.e., at the cruciform loop) arose when pAT34*tetA* was studied in CH582. Thus, the twin requirements for the observation of cruciform extrusion by the $(AT)_{34}$ sequence were the presence of the functional *tetA* gene and the absence of cellular topoisomerase I (i.e., Δ*topA* background). Under the conditions of the experiments, neither the presence of the *tetA* gene nor the absence of topoisomerase I was alone sufficient to permit cruciform extrusion. However, the combined effect of the functional *tetA* gene in a Δ*topA* background appears to generate a level of unconstrained negative supercoiling in the plasmid that permits significant cruciform extrusion to occur. It is significant that these twin requirements are exactly those required for the activation of the *leu-500* promoter (Chen et al., 1992), suggesting that supercoiling generated by transcription of the *tetA* gene is responsible for the modulation of its activity.

Increased Negative Supercoiling in tetA-Carrying Plasmids in ΔtopA S. typhimurium Is Not Observed in Stationary Phase Cultures. Previous studies of plasmids carrying *tetA* in Δ*topA* bacteria have shown that supercoiling is growth phase dependent. In both *E. coli* (Pruss & Drlica, 1986) and *S. typhimurium* (Chen et al., 1992), a proportion of plasmid DNA extracted from cells during exponential growth was found to have very high levels of negative supercoiling, but such a corresponding fraction could not be detected in DNA obtained from stationary phase cells.

Using the *in situ* probing approach, we analyzed the supercoiling of pAT34*tetA* in CH582 (Δ*topA*) at both midlogarithmic and stationary phase growth (Figure 2B). Cells were reacted with 2 mM OsO_4/2,2′-bipyridine, and the plasmid DNA was isolated and analyzed as described above. While extensive modification of thymine bases at the center of the $(AT)_{34}$ tract was observed for cells analyzed at midlogarithmic growth, no corresponding modification was found when cells at the stationary phase were analyzed. Thus, the level of unconstrained DNA supercoiling was not high enough in stationary phase CH582 to allow the $(AT)_{34}$ sequence to exist as a cruciform. We conclude that the oversupercoiling associated with the presence of the functional *tetA* gene depends upon the physiological state of the cells.

Increased Negative Supercoiling Is Also Observed in tetA-Carrying Plasmids in ΔtopA E. coli. All of the *in situ* chemical probing experiments discussed so far were performed on *S. typhimurium*. Viable *topA* strains of *E. coli* have been isolated (Di Nardo et al., 1982; Trucksis et al., 1981), although it has been shown that, unlike *S. typhimurium* (Richardson et al., 1984), these strains always carry compensatory mutations that tend to decrease negative supercoiling (Di Nardo et al., 1982; Pruss et al., 1982). We have recently shown that the *leu-500* promoter can be activated on a plasmid in Δ*topA E. coli* (Chen et al., 1994), suggesting that the same mechanism of topological promoter coupling operates in these different enteric bacteria. We have therefore used the $(AT)_n$ reporter sequences to examine unconstrained plasmid supercoiling in *tetA*-carrying plasmids in Δ*topA* and $topA^+$ *E. coli* strains.

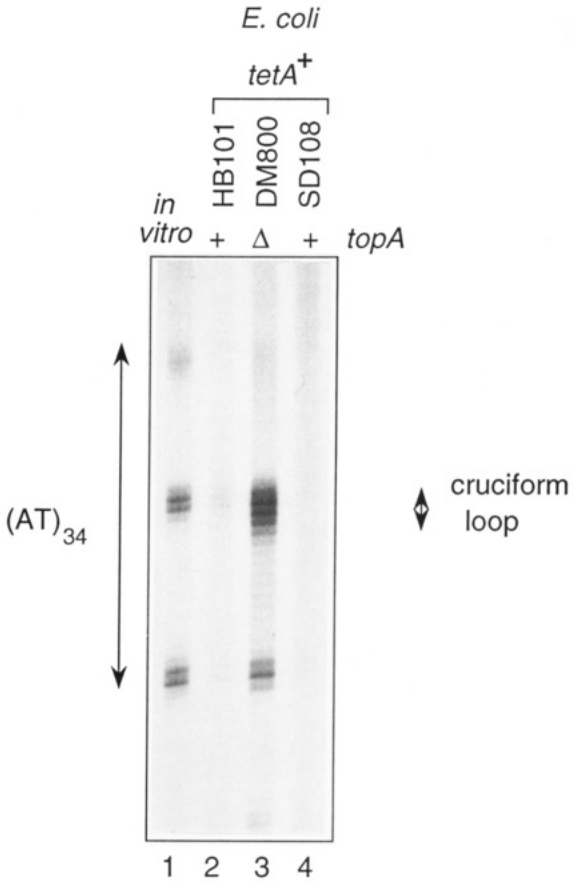

FIGURE 3: Increased levels of unconstrained negative supercoiling in plasmids encoding TetA, in Δ*topA E. coli*. pAT34*tetA* was transformed into the *E. coli* strains HB101 ($topA^+$) and the isogenic pair DM800 (Δ*topA*) and SD108 ($topA^+$) (Di Nardo et al., 1982). Cells in exponential growth were subjected to *in situ* chemical modification with 2 mM OsO_4/2,2′-bipyridine. The DNA was isolated and analyzed as described before. Track 1, *In vitro* modification of pXG540; track 2, *in situ* modification of pAT34*tetA* in HB101 ($topA^+$); track 3, *in situ* modification of pAT34*tetA* in DM800 (Δ*topA*); track 4, *in situ* modification of pAT34*tetA* in SD108 ($topA^+$). Note that the only strain leading to detectable cruciform extrusion *in situ*, i.e., oversupercoiling, is the Δ*topA* strain DM800.

The results of *in situ* OsO_4/2,2′-bipyridine modification of pAT34*tetA* transformed into *E. coli* HB101 (top^+), SD108 (top^+), and DM800 (Δ*topA*) are shown in Figure 3. No modification of the central thymines of the $(AT)_{34}$ sequence was observed in either of the $topA^+$ strains (HB101 and SD108). However, there was extensive reactivity in the DNA in the Δ*topA* strain (DM800), indicative of cruciform formation, i.e., oversupercoiling of plasmid DNA. When the plasmid pXG540 was subjected to similar *in situ* reactions in the same strains, no modifications were seen within the $(AT)_{34}$ tract (data not shown). Therefore, the requirements for

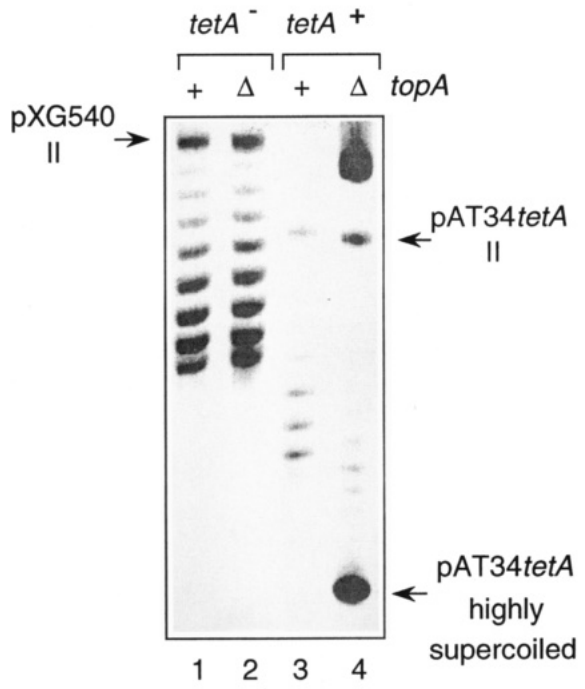

FIGURE 4: Analysis of plasmid linking number distributions in *E. coli*. Plasmid DNA was isolated from cells in exponential growth, and topoisomer distributions were resolved by electrophoresis in 1% agarose in 90 mM Tris–borate (pH 8.3)/1 mM EDTA containing 1.5 μg/mL chloroquine. Track 1, pXG540 plasmid DNA isolated from SD108 (*topA*$^+$); track 2, pXG540 isolated from DM800 (Δ*topA*); track 3, pAT34*tetA* isolated from SD108 (*topA*$^+$); track 4, pAT34*tetA* isolated from DM800 (Δ*topA*). Note the fraction of very highly supercoiled pAT34*tetA* isolated from the Δ*topA* strain indicated by the arrow at lower right. This arises only from the combination of a plasmid carrying the *tetA* gene in a *topA* background, i.e., the same conditions that lead to *in situ* detection of cruciform extrusion. The nicked circular forms (form II DNA) of pXG540 and pAT34*tetA* are indicated by the upper arrows; since pXG540 is larger than pAT34*tetA*, it migrates more slowly in the agarose gel.

cruciform formation in *E. coli* are the same as those in *S. typhimurium*, i.e., for a functional *tetA* in *cis* and a *topA* background.

Extremely high levels of negative supercoiling of plasmid DNA carrying the *tetA* gene extracted from Δ*topA E. coli* have been found by linking number measurements using agarose gel electrophoresis (Pruss, 1985). We also observed a fraction of highly supercoiled DNA of our *tetA*-carrying plasmid pLEU500Tc (Chen et al., 1992). We electrophoresed pXG540 and pAT34*tetA* purified from SD108 and DM800 on an agarose gel in the presence of chloroquine (Figure 4). All topoisomers were negatively supercoiled at the concentration of chloroquine used. In all samples there were topoisomers of superhelix density of approximately –0.058, but pAT34*tetA* isolated from DM800 (Δ*topA*) also exhibited a significant fraction of topoisomers with a negative superhelix density greater than 0.085 (calculated from two-dimensional gel electrophoresis, data not shown). It should be emphasized that these are the superhelix densities of extracted plasmids, and a proportion of the supercoiling may be constrained in some way inside the cell.

The results presented here are in agreement with earlier studies showing the formation of very highly supercoiled species of *tetA*-coding plasmids in Δ*topA E. coli*. They are also consistent with further experiments showing that the *leu-500* promoter may be activated on *tetA*-carrying plasmids transformed into Δ*topA E. coli* strains (Chen et al., 1994). The demonstrated difference in unconstrained DNA supercoiling between the *E. coli* strains has added significance, because SD108 was derived from DM800 by phage transduction to TopA$^+$ (Di Nardo et al., 1982), and they are therefore virtually isogenic strains. Both strains contain a compensatory mutation in *gyrB*, which clearly is not relevant to the observed supercoiling effects.

*Estimation of Plasmid Superhelix Density in Δ*topA *S. typhimurium and E. coli.* Formation of cruciform structures of the size employed in these studies is an all-or-none process, with a fixed free energy of formation that is independent of size [approximately 14 kcal mol^{-1} for $(AT)_n$ sequences (Greaves et al., 1985; McClellan et al., 1986)]. Stable cruciform formation occurs when the reduction in the free energy of supercoiling due to the local negative twist change is greater than the free energy of cruciform formation. Since the total free energy of supercoiling increases quadratically with linking difference, a threshold level of negative supercoiling exists, above which a given cruciform enjoys a stable existence. This critical level will be inversely proportional to the length of the $(AT)_n$ sequence undergoing the transition; thus, shorter $(AT)_n$ tracts require a higher level of negative supercoiling to support a stable cruciform structure. Thus, by studying a series of $(AT)_n$ sequences of different lengths, we can derive information on the prevailing level of unconstrained supercoiling. We have previously used a series of $(AT)_n$ sequences in *in situ* chemical reactions to estimate the effective superhelix density of plasmids inside *E. coli* cells following osmotic shock (McClellan et al., 1990).

During our studies with plasmids carrying $(AT)_n$ sequences, we have isolated a series of alternating tracts of a variety of lengths. These were cloned separately at the equivalent position of the $(AT)_{34}$ sequence in pAT34*tetA*, generating a set of molecules containing the *tetA* gene in front of reporter sequences sensitive to different levels of supercoiling. These plasmids were transformed into Δ*topA S. typhimurium* and *E. coli* and subjected to *in situ* probing as before. Cells containing plasmids with $(AT)_n$ repeats of $n = 9$, 11, 12, 15, 22, and 25 were grown to the midlogarithmic phase and reacted with 2 mM OsO_4/2,2′-bipyridine. The results for Δ*topA S. typhimurium* are shown in Figure 5A, and those for Δ*topA E. coli* are shown in Figure 5B. We observed a modification of the central thymines for all plasmids, but this clearly decreased with shorter $(AT)_n$ tracts. However, it is apparent that some cruciform extrusion occurred even in the shortest $(AT)_n$ tracts, indicating that transcription of the *tetA* gene leads to very high levels of unconstrained negative supercoiling. The similarity of the results in *S. typhimurium* and *E. coli* suggests that the mechanisms producing unconstrained supercoiling are the same in both eubacteria.

Oversupercoiling Is Suppressed by Inhibition of Protein Synthesis. In earlier experiments, we showed that activation of the *leu-500* promoter in Δ*topA S. typhimurium* was dependent upon transcription and translation of the *tetA* gene (Chen et al., 1992, 1993), suggesting that these processes were essential for the generation of high levels of local negative supercoiling. We have obtained similar results in Δ*topA E. coli* (Chen et al., 1994). To examine the importance of protein synthesis on the generation of unconstrained plasmid DNA supercoiling, we performed *in situ* chemical modification on pAT34*tetA* in the presence of an inhibitor of protein synthesis. *E. coli* DM800 (Δ*topA*) carrying the plasmid was grown to the midlogarithmic phase and reacted with OsO_4/2,2′-bipyridine at various times after the addition of chloramphenicol (150 μg/mL). The plasmid DNA was then isolated and analyzed as before. The results are shown in Figure 6. Within 5 min after the addition of chloramphenicol the extent

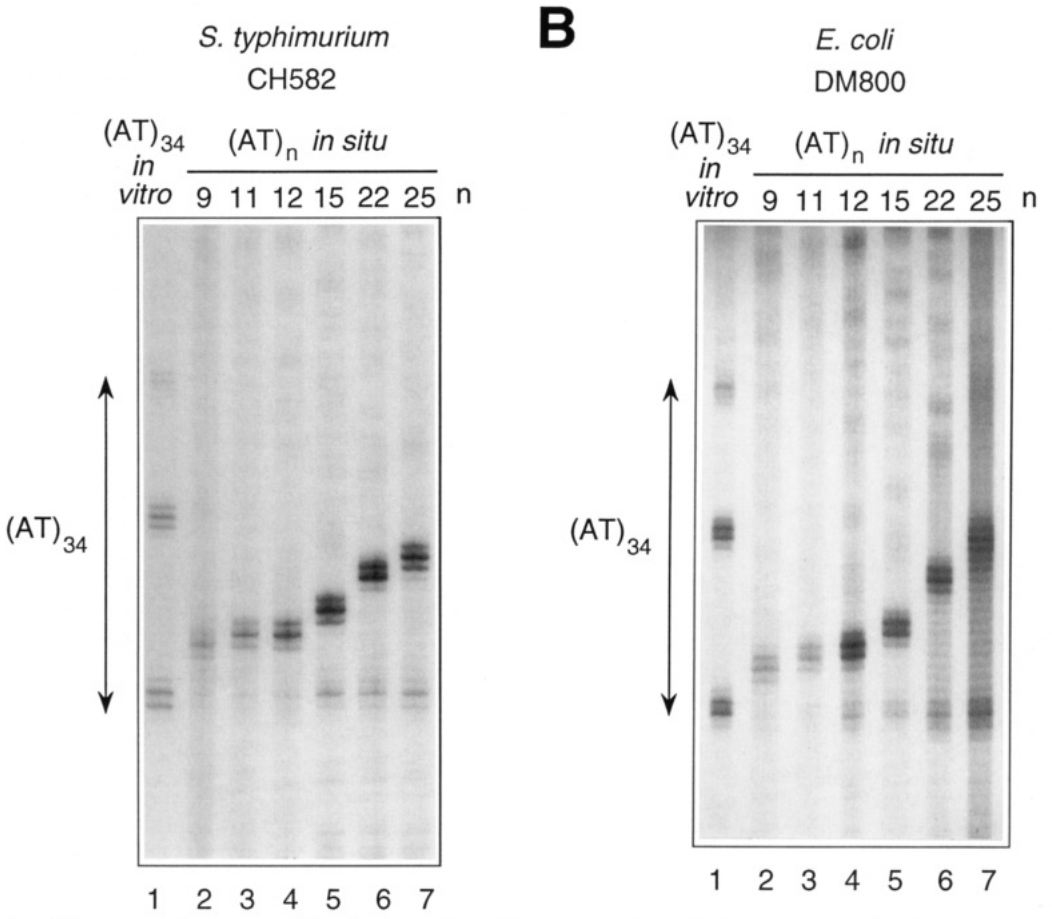

FIGURE 5: Estimation of the extent of oversupercoiling in Δ*topA S. typhimurium* and *E. coli* due to transcription of the *tetA* gene. Plasmids equivalent to pAT34*tetA* with $(AT)_n$ tract lengths $n = 9$–25 were transformed into (A) *S. typhimurium* CH582 and (B) *E. coli* DM800, and cruciform formation was analyzed by *in situ* chemical probing with 2 mM OsO_4/2,2′-bipyridine as before. Track 1, *In vitro* modification of pXG540; track 2, *in situ* modification of pAT9*tetA* (containing $(AT)_9$); track 3, *in situ* modification of pAT11*tetA* (containing $(AT)_{11}$); track 4, *in situ* modification of pAT12*tetA* (containing $(AT)_{12}$); track 5, *in situ* modification of pAT15*tetA* (containing $(AT)_{15}$); track 6, *in situ* modification of pAT22*tetA* (containing $(AT)_{22}$); track 7, *in situ* modification of pAT25*tetA* (containing $(AT)_{25}$).

of modification at the center of the $(AT)_{34}$ tract was greatly reduced, and after 15 min no reactivity could be detected (data not shown). We conclude that the level of unconstrained supercoiling is significantly reduced when protein synthesis is inhibited.

Observation of Oversupercoiling in topA⁺ E. coli. The *in situ* probing experiments indicated that transcription of *tetA* could not generate sufficient negative supercoiling to result in the formation of $(AT)_n$ cruciforms in *topA*⁺ bacteria in the plasmids studied. We wondered what the limits of topoisomerase I efficiency were in these systems and whether situations might exist where the enzymatic relaxation of negative supercoiling could be overwhelmed. This might occur if the rate of induction of negative supercoiling by transcription were increased above a certain level. We therefore decided to introduce a stronger promoter to initiate transcription of the *tetA* gene and to perform the *in situ* probing experiments in *E. coli* HB101 (*topA*⁺). The inducible *tac* promoter (De Boer et al., 1983) was cloned upstream of *tetA* in order to boost transcription of this gene; we have previously demonstrated that initiation at the *leu-500* promoter was increased between 5- and 10-fold when located adjacent to a *tac* promoter (Chen et al., 1993), albeit inserted at a different site. We also demonstrated that the activity of the *leu-500* promoter was dependent upon transcription of the *bla* gene of pBR322 in a leftward sense (i.e., locating the *leu-500* promoter in the region between the divergent *tetA* and *bla* transcription units). We therefore placed the $(AT)_{34}$ reporter sequence between the *tetA* gene (with the *tac* promoter) and the complete *bla* gene (pAT34*tetA*.P_{tac}, Figure 7A). In order to control the *tac* promoter, *lac* repressor was was provided in *trans* by transformation with a second compatible plasmid, p*lacI*^{Q}*.kan* (Table 1).

E. coli HB101 transformed with pAT34*tetA*.P_{tac} and p*lacI*^{Q}*.kan* was grown to the midlogarithmic phase, and transcription from the *tac* promoter was induced by the addition of 1 mM IPTG. The cells were harvested at various time intervals and reacted with 2 mM OsO_4/2,2′-bipyridine. The plasmid DNA was isolated and analyzed as before, and the results are shown in Figure 7B. Before the addition of IPTG there was a small amount of reactivity at the center of the $(AT)_{34}$ tract (possibly due to incomplete repression of the *tac* promoter), but the extent of modification increased very rapidly upon addition of the inducer. The amount of modification did not alter over the time period of 5–60 min after induction. We have obtained similar results in *S. typhimurium* LT2 (*topA*⁺) (data not shown).

DISCUSSION

The *in situ* modification of the thymine bases at the centers of the $(AT)_n$ tracts shows that a sufficiently high level of unconstrained DNA supercoiling can exist inside the cell in some situations, such that stable cruciform extrusion occurs. We have noted two requirements for the observation of oversupercoiling: the $(AT)_n$ reporter tracts must be located in a plasmid containing a functional tetracycline resistance gene,

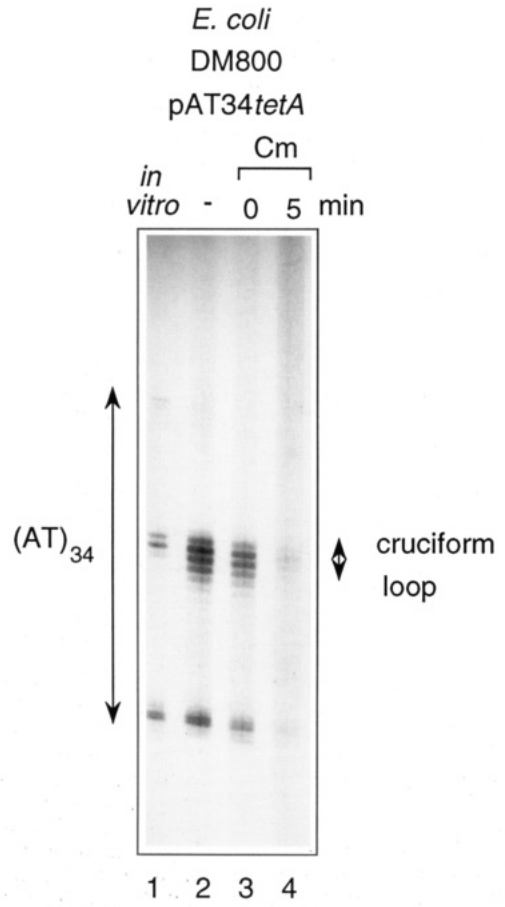

FIGURE 6: Plasmid oversupercoiling and protein synthesis in Δ*topA E. coli*; *in situ* chemical probing of plasmids encoding TetA after incubation with chloramphenicol. *In situ* modifications and analyses of DNA modification were carried out as before. Track 1, *In vitro* modification of pXG540; track 2, *in situ* modification of pAT34*tetA* without incubation with chloramphenicol; track 3, *in situ* modification of pAT34*tetA* with incubation with 150 μg/mL chloramphenicol (the sample was taken immediately at the point of addition of antibiotic); track 4, *in situ* modification of pAT34*tetA* after a 5-min incubation with 150 μg/mL chloramphenicol. Note that the oversupercoiling of pAT34*tetA*, as revealed by cruciform formation in the cellular plasmid, was not detectable in the cells in which protein synthesis had been inhibited.

tetA, and the genetic background of the host cell must be *topA*, i.e., the activity of the cellular topoisomerase I must be low.

These requirements are exactly those for the activation of the *leu-500* promoter of *S. typhimurium* on a plasmid, suggesting that similar mechanisms underlie both events. The observations are consistent with the generation of increased negative supercoiling due to transcription of the *tetA* gene. Transcription-induced supercoiling can potentially be relaxed either by the action of topoisomerases or by the diffusion and self-cancellation of supercoiling. The former is minimized in the *topA* cells, while the latter is reduced by the barrier generated by anchorage of the RNA polymerase transcribing *tetA* due to the coupled transcription, translation, and membrane insertion. We have demonstrated that activation of the *leu-500* promoter on a circular plasmid in either *S. typhimurium* (Chen et al., 1992, 1993) or *E. coli* (Chen et al., 1994) requires all three of these events, and the demonstration of unconstrained oversupercoiling in this situation strongly suggests that this is indeed the basis for the activation of the promoter.

Analysis of topoisomer distributions of the plasmids used in these studies shows that plasmids carrying the *tetA* gene

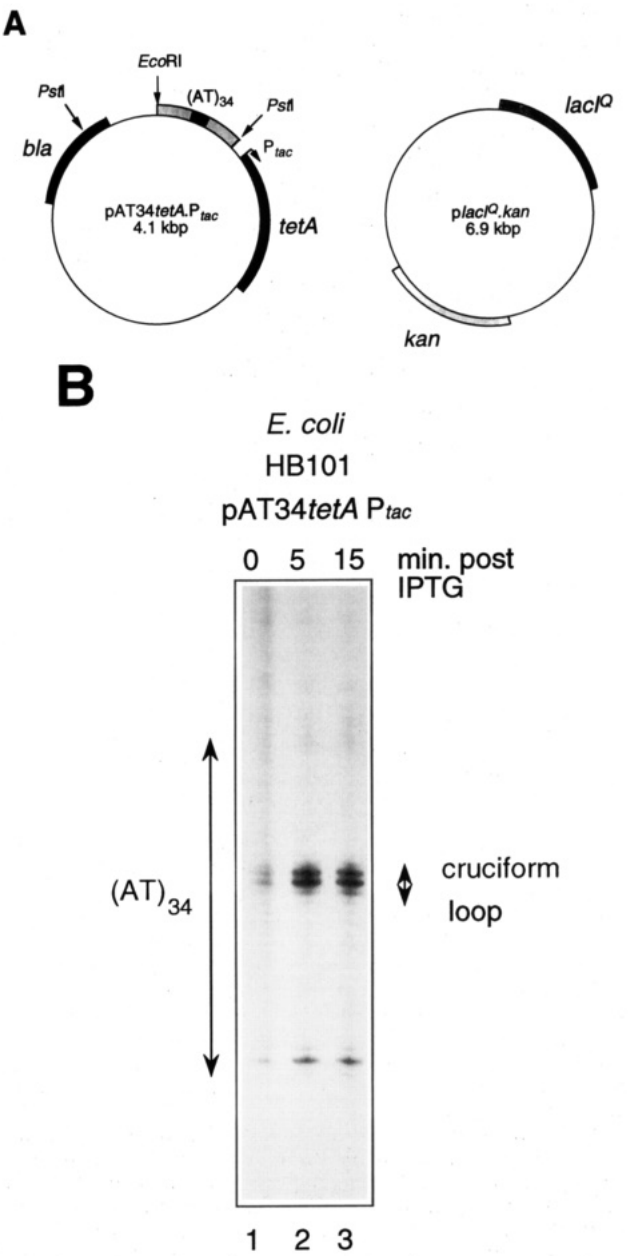

FIGURE 7: Detection of elevated levels of unconstrained DNA supercoiling in *topA*$^+$ *E. coli*. (A) Maps of the plasmids used in this experiment. pAT34*tetA*.P_{tac} contains the $(AT)_{34}$ reporter sequence between the *tetA* and *bla* genes. The natural promoter of *tetA* has been replaced by the strong *tac* promoter. *placI*^{Q}*.kan* is a compatible plasmid carrying the *lacI*^{Q} gene, which was used to provide *lac* repressor in *trans* in order to ensure control of the *tac* promoter. (B) Analysis of oversupercoiling in pAT34*tetA*.P_{tac} in *E. coli* HB101 (*topA*$^+$) detected by *in situ* chemical probing with 2 mM OsO_4/2,2′-bipyridine, 0 (track 1), 5 (track 2) and 15 min (track 3) after induction of the *tac* promoter with 1 mM IPTG. Plasmid DNA was isolated and analyzed as before. Note that despite the *topA*$^+$ background of the cells, significant oversupercoiling of plasmid DNA occurs with induction of the P_{tac}-*tetA*, as indicated by cruciform formation in the $(AT)_{34}$ reporter sequence.

isolated from *topA* cells exhibit a markedly bimodal distribution of linking number, with a fraction of very highly negatively supercoiled DNA present. We suspect that the two fractions represent plasmids that were active (hence, oversupercoiled) and inactive in the transcription of *tetA* at the time of isolation of plasmid DNA. The use of $(AT)_n$ tracts of varying lengths allows us to place a lower limit on the extent of unconstrained negative supercoiling inside the cells. The threshold linking

difference for stable cruciform existence (ΔLk_c) is related to the free energy of cruciform formation (ΔG_x) by

$$\Delta G_x = \frac{1050RT}{N}(\Delta Lk_c^2 - (\Delta Lk_c - \Delta Tw)^2) \qquad (1)$$

where R is the gas constant, T is the absolute temperature, and N is the plasmid size (bp). ΔTw is the twist change brought about by the extrusion of the cruciform, given by

$$\Delta Tw = \frac{2n}{10.5} \qquad (2)$$

for an $(AT)_n$ tract of given n. Since ΔG_x is a constant value, independent of the size of the cruciform formed, ΔLk_c is simply related to n. We have estimated the extent of cruciform modification as a function of $(AT)_n$ tract length in *S. typhimurium* (Figure 5A) and analyzed the values by treating the supercoiling as if the entire plasmid were supercoiled to the extent experienced by the reporter $(AT)_n$ sequence, with a distribution of superhelix density populated according to Boltzmann statistics. Assuming that only topoisomers that are more supercoiled than the threshold required for a given tract length [calculated from eqs 1 and 2, assuming that ΔG_x has the value of 13.7 kcal mol^{-1} measured *in vitro* (Greaves et al., 1985)] react *in situ* with osmium tetraoxide, we obtain the fit shown in Figure 8B, calculated for a mean linking difference of –16 (topoisomer distribution shown in Figure 8A). This corresponds to a local unconstrained superhelix density of $-\sigma = 0.052$. However, the fit is not perfect, particularly at higher superhelix density, indicating that the distribution of superhelix density in the region of the reporter $(AT)_n$ tracts may not be well described by a Boltzmann population that would be calculated for the whole circular plasmid. Because the cruciform formation is responding to transcription-induced DNA supercoiling, this might be expected. Since we observe some cruciform formation *in situ* even for $(AT)_n$ tracts of $n = 9$ (i.e., inverted repeats of 18 bp in total length), this indicates that some topoisomers of the population have levels of unconstrained superhelix density that are more negative than $-\sigma = 0.075$. This is an extremely high level of negative supercoiling, representing an increase in the available free energy of supercoiling over the basal level [assumed to be –0.025 (Greaves et al., 1985; Bliska & Cozzarelli, 1987; Zacharias et al., 1988)] of approximately 10-fold.

The above treatment assumes that the supercoiling is distributed uniformly through the plasmid, without partition into domains separated by topological barriers. It is difficult to know to what extent the reporter sequences respond to the immediate local topological environment in the DNA, but it is quite possible that there is significant diffusion of DNA supercoiling through the circular DNA molecule during the course of the *in situ* reaction with osmium tetraoxide. Since the latter is reactive toward proteins, it is likely that the induction of supercoiling by transcription is halted at the start of intervention by the chemical probe. For these reasons, the values of unconstrained superhelix density measured in these experiments should be regarded only as lower estimates of the steady-state levels generated immediately upstream of the transcribing *tetA* gene.

It should be emphasized that the very high level of negative supercoiling was observed in a *topA* background, and that in cells containing a functional *topA* gene we should expect less variation in local levels of supercoiling. Nevertheless, we have shown that a degree of oversupercoiling is observable in *topA*$^+$ cells, when we increased the amount of transcription from the *tetA* gene by insertion of the strong *tac* promoter. This emphasizes the dynamic balance between the processes of induction and relaxation of negative supercoiling; apparently, the topoisomerase activity of the *topA*$^+$ cell is unable to cope with the induction of supercoiling by transcription when it is

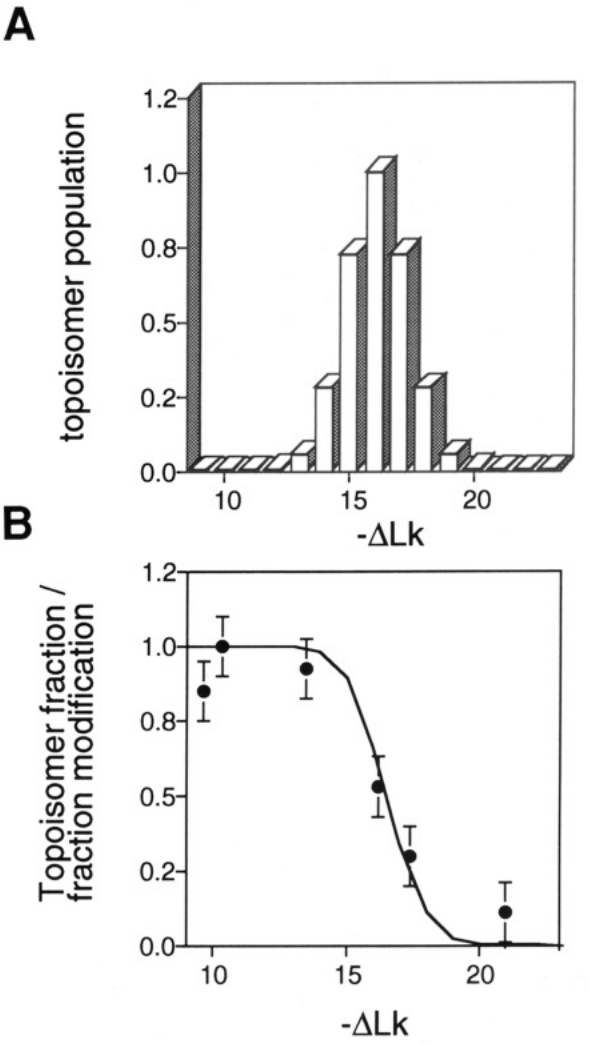

FIGURE 8: Estimation of unconstrained plasmid superhelix density in *tetA*-carrying plasmids in *topA S. typhimurium*. The relative extents of chemical modification of cruciforms in 3.25-kb plasmids containing $(AT)_n$ tracts of different lengths transformed into *S. typhimurium* CH582 (Δ*topA*) were used to estimate the apparent superhelix density. In order to fit the experimental data (quantified by phosphorimaging from Figure 5A), we adopted a simplified model in which supercoiling was assumed to be a global property of the entire plasmid and calculated Gaussian-shaped Boltzmann distributions of topoisomers from the quadratic dependence of supercoiling free energy on linking difference. We then assumed that the observed extent of cruciform modification would correlate with the proportion of topoisomers of the distribution that were more supercoiled than the calculated critical linking difference (eq 1), at which a given $(AT)_n$ tract would extrude a stable cruciform *in situ*. The best fit obtained is shown (B), which was calculated for a distribution of mean superhelix density of –0.052 (A). From this the proportion of topoisomers of superhelix density greater than a given value is plotted (B, line) and compared with the measured relative extent of cruciform modification for different $(AT)_n$ tract lengths (B, data points—each plotted at its calculated critical linking differences for cruciform formation). The maximum extent of cruciform modification was given a value of 1.0, and the error of quantitation was estimated as 0.1. Note that the significant level of cruciform modification that was observed for the plasmid containing an $(AT)_9$ tract indicates that the apparent topoisomer distribution may be broader than that calculated on the basis of thermal population, suggesting that this model may not be completely adequate for the calculation of local transcription-induced superhelix density.

boosted by a sufficiently strong promoter. This indicates that transcription-induced supercoiling could be a significant factor in cells with a normal topoisomerase background. Mirkin and colleagues also obtained evidence for oversupercoiling in *topA*$^+$ *E. coli* cells (Dayn et al., 1992). Membrane anchorage is important for the observation of oversupercoiling on the plasmid, although this seems to be less critical when the strong *tac* promoter is present (Dayn et al., 1992; R.P.B. and D.M.J.L., unpublished data). In any case, membrane anchorage may be less important on the chromosome, where the physical bulk and lack of simple circularity may reduce the importance of superhelical diffusion. Thus, twin supercoiled domain effects (Liu & Wang, 1987) may play a significant role in the genetic expression of wild-type cells, such as the topological coupling of promoters as exemplified by the suppression of the *leu-500* promoter mutation (Chen et al., 1992).

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