Transcription regulation *in vitro* by an *E. coli* promoter containing a DNA cruciform in the '–35' region

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ABSTRACT

A promoter with the potential to adopt a 50 basepair (bp) cruciform spanning from –19 to –69 has been constructed in the plasmid pBR322 tetracycline resistance gene (*ter*) by forming an inverted repeat from '–35' sequences. Compared to a control promoter, the sequence of this cruciform promoter differs only by a 22 bp insertion between –48 and –69, upstream from the usual location of promoter sequences. The cruciform is extruded in a supercoil-dependent manner, and transcription from this promoter *in vitro* by RNA polymerase decreases as the negative supercoil density of the plasmid DNA increases. In contrast, transcription from the control promoter increases with negative supercoiling. Thus, DNA secondary structure in the '–35' region can affect promoter-polymerase interaction. The *ter* promoter cruciform also influences expression of the pBR322 β -lactamase gene (*bla*). This apparently results when extrusion of the cruciform reduces the superhelicity of the plasmid molecule to a level that is below the optimum for expression from the *bla* promoter, illustrating one mechanism for how DNA secondary structure may effect action-at-a-distance. Transcription from both promoters *in vivo* does not differ from controls, suggesting that this cruciform is not generated to a significant extent intracellularly, most probably as a result of the slow kinetics of extrusion.

INTRODUCTION

A promoter is the sequence of nucleotides at the beginning of a gene that serves as a signal for the initiation of transcription by RNA polymerase. *E. coli* promoters consist of two relatively conserved hexameric DNA sequences found at about –10 basepairs (TATAAT) and at about –35 basepairs (TTGACA) before the start site of transcription (1,2). How RNA polymerase identifies promoter sequences is not known, but the process could be mediated by the structure of DNA at this site. As a result, the potential contributions to gene regulation from alternative DNA structures have been a focus of much investigation. Several forms of DNA secondary structures have been shown to influence transcription in *E. coli*. For example, sequences with the potential to adopt Z-DNA structures block elongating *E. coli* RNA polymerase *in vitro* (3,4) and possibly also *in vivo* (5), and cruciform formation disrupts promoter function in *E. coli* both *in vivo* and *in vitro* (6,7). In particular, work from this laboratory has demonstrated that cruciform extrusion in the '–10' promoter region interrupts transcription in a manner dependent on DNA supercoiling (7). In this paper it is demonstrated that a cruciform in the '–35' region also has the potential to interfere with transcription by RNA polymerase. An additional finding is that cruciform extrusion affects transcription at the distant pBR322 *bla* promoter.

MATERIALS AND METHODS

Plasmid Constructions

The plasmid containing the promoter with the potential to extrude a cruciform, pX35, and the control plasmid, pConDown (8), were constructed from pBdEC (9). pBdEC was derived from pBR322 by deleting the 22 bp between the *Eco* RI and *Cla* I sites (enclosing the '–35' sequence of the *tet* promoter) and replacing this segment with a single *Eco* RI site. The following pair of 5'-phosphorylated complementary oligonucleotides, forming *Eco* RI sticky ends, was ligated into the *Eco* RI site of pBdEC, as described elsewhere (8):

The oligonucleotides contain a '–35' consensus sequence (overlined) spaced to align with the '–10' sequence of pBR322 *tet*. The products of the ligation reaction were used to transform competent cells, and several clones were characterized by dideoxy sequence analysis. pConDown contains a single copy of the above cassette inserted into the pBdEC *Eco* RI site, in the orientation as written. pX35 contains two copies of this cassette in head-to-head direction with the downstream copy in the orientation as written and the upstream copy in reverse. The plasmids were grown in strain DH5 (10) without chloramphenicol amplification and purified by alkaline lysis and CsCl-ethidium bromide density centrifugation (11).

Preparation of Plasmid Topoisomers of Defined Supercoil Density

Plasmids of the appropriate mean negative superhelical density $-\langle\sigma\rangle$ were prepared by relaxation of supercoiled DNA, previously purified as described above, in various concentrations of ethidium bromide (12). A 100 μ l reaction containing 4 μ g DNA, 50 mM tris-HCl, pH 7.6, 10 mM MgCl₂, 0.1 mM dithiothreitol, 2 mM spermidine, 4 units wheat germ topoisomerase I (purchased from Promega Biotech, Madison, WI), and the appropriate concentration of ethidium bromide (0, 2, 3, 4.5, 6, 8, 15, and 20 μ M for $-\bar{\sigma} = 0, 0.01, 0.025, 0.035, 0.045, 0.065, 0.10, \text{ and } 0.12$, respectively) was incubated at 29°C for 5 hrs. (The concentration of ethidium bromide was determined from Fig.2 of Ref. 12; our previous results have indicated that the expected $-\bar{\sigma}$ is in good agreement with that determined by the band counting method.) The reaction was terminated, the ethidium bromide removed, and the cruciform extruded by phenol (equilibrated in TE buffer (10 mM tris-HCl, pH 8.0, 1 mM EDTA), then CHCl₃ (mixed 24:1 with isoamyl alcohol)) extraction at room temperature followed by DNA precipitation with 2 volumes ethanol (in the presence of 250 mM NaAcetate, pH 5.2). The DNA pellet was resuspended in TE buffer by incubation for 20 hours at room temperature. All values of $-\bar{\sigma}$ are reported with respect to room temperature and the ionic conditions of the above topoisomerase reaction.

S1 Nuclease Mapping of the Cruciform

Phenol extraction of the DNA (to extrude the cruciform) was as described above. Digestion was performed in 20 μ l containing 3 μ g DNA, 30 mM NaAcetate, pH 4.6, 285 mM NaCl, 1 mM ZnSO₄, 20 μ g tRNA, and 6 units S1 nuclease (purchased from Boehringer-Mannheim, Indianapolis, IN). The reaction was incubated at 16°C for 30 min, terminated by phenol/CHCl₃ extraction, and the DNA was precipitated with ethanol. The site of cleavage of S1 nuclease was mapped by subsequent digestion with *Ava* I or *Pvu* II, and agarose gel electrophoresis with ethidium bromide staining. A '1 Kb DNA ladder'

Figure 1. A. The sequence of the cruciform *tet* promoter in plasmid pX35. B. The sequence of the control *tet* promoter in plasmid pConDown. For both plasmids, to the left, sequences resume with pBR322 sequence # 4355, and, to the right, pBR322 sequence # 48 (uncorrected pBR322 sequence numbering from Ref. 11).

(purchased from Bethesda Research Laboratories, Gaithersburg, MD) was used as a size standard.

Analysis of In vitro Transcription

σ^{70} RNA polymerase holoenzyme (a generous gift of Dr. R. Burgess, University of Wisconsin, Madison, WI) was purified (13,14) from *E. coli* K12 cells. The preparation was found to be approximately 60% active by promoter titration (15). Transcription was performed in 20 μ l containing 100 ng of the appropriately supercoiled plasmid DNA, 30 nM RNA polymerase (nominal concentration measured by A_{280}), 40 mM tris-HCl, pH 8.0, 10 mM $MgCl_2$, 150 mM KCl, 0.1 mM dithiothreitol, 0.1 mM EDTA, and 150 μ M each of unlabelled ATP, CTP, GTP, and TTP. The reaction was incubated at 37°C for 20 min and terminated by phenol/ $CHCl_3$ extraction and ethanol precipitation. The promoter-specific products were analyzed by quantitative cDNA reverse transcriptase primer extension analysis as previously described (using in a single reaction both the 5'- ^{32}P end-labelled P3 *bla*-specific 30-mer and the P2 *tet*-specific 21-mer from Ref. 8). Transcript levels were quantified by scanning densitometry of the resulting autoradiogram.

Analysis of In Vivo Transcription

Strain DH5, harboring either plasmid pX35 or pConDown, was grown from single colonies to late log phase ($OD_{550} = 0.8$) in 20 ml Luria-Bertani medium (11), supplemented with 0.1% glucose, in 250 ml flasks at 37°C with vigorous shaking (250 rpm). The RNA was purified by the method of hot phenol extraction (8), and promoter-specific initiation was quantified by primer extension (8).

RESULTS*The tet Promoter of Plasmid pX35 Contains a Potential Cruciform.*

The *tet* promoter of plasmid pX35 (Fig. 1A) contains a 50 bp inverted repeat extending from -19 to -69, with respect to the start site of transcription initiation (vide infra). The center of the inverted repeat is located at -44. The repeat is constructed from two oppositely oriented 25 bp *Eco* RI restriction fragments. The more downstream of the two repeated units contains a hexameric consensus '-35' sequence in an orientation capable of complementing the pBR322 *tet* '-10' sequence. The inverted repeat is potentially able to adopt cruciform basepairing. The promoter in pX35 differs from the control promoter in plasmid pConDown (Fig. 1B) only by a 22 bp insertion between -48 and -69, upstream of the usual location of promoter sequences.

The Cruciform is Extruded by Helix Destabilizing Conditions.

The extrusion of the cruciform was assayed by sensitivity to S1 nuclease, a single strand specific nuclease known to cleave cruciforms at the two hairpin loops (reviewed in Ref. 16). The position of S1 cleavage can be mapped by subsequent digestion with *Ava* I, which uniquely cuts pX35 at 1447 bp downstream from the center of the inverted repeat (thereby releasing 1447 and 2944 bp fragments from the 4391 bp plasmid), or *Pvu* II, which uniquely cuts at 2303 bp upstream from the center of the inverted repeat (thereby releasing 2303 and 2088 bp fragments). Supercoiled pX35 plasmid DNA, as purified by alkaline lysis and CsCl-ethidium bromide density centrifugation ($-\bar{\sigma} \approx 0.065$), was not selectively sensitive to S1 nuclease (Fig. 2, lanes 1 and 2), indicating that it had not extruded the cruciform to levels detectable by this assay. Incubation of this plasmid DNA for 20 min at 37°C in 10 mM tris-HCl, pH 8.0, 10 mM $MgCl_2$, and 80 mM KCl (conditions known to favor cruciform extrusion (17)) prior to S1 digestion was not sufficient to increase nuclease

Figure 2. Analysis of cruciform extrusion by S1 nuclease digestion and restriction mapping. Lanes 1 and 2, DNA was as purified prior to S1 digestion; lanes 3 and 4, DNA was incubated at 37°C for 20 min in 10 mM tris-HCl, pH 8.0, 10 mM $MgCl_2$, and 80 mM KCl prior to S1 digestion; lanes 5 and 6, DNA was exposed to the helix destabilizing conditions of phenol/ $CHCl_3$ extraction and ethanol precipitation prior to S1 digestion. After S1 nuclease treatment the position of cleavage was mapped by digestion with *Ava* I (lanes 2, 4, and 6) and *Pvu* II (lanes 1, 3, and 5). mw, molecular weight standards.

sensitivity (Fig. 2, lanes 3 and 4), indicating that extrusion of the cruciform is insignificant within the extent of the *E. coli* generation time. However, when this plasmid DNA was exposed to the relatively helix destabilizing conditions (18) of phenol/CHCl₃ extraction and ethanol precipitation (performed as noted in Materials and Methods), the sensitivity to S1 nuclease was markedly increased (Fig. 2, lanes 5 and 6), demonstrating extrusion of the cruciform. Note that the mapping by restriction digestion confirms the expected location of the cruciform in the plasmid. The control plasmid pConDown and linear pX35 plasmid did not form cruciforms under similar conditions (data not shown). Therefore, this cruciform is extruded in a supercoil-dependent fashion, and there is an apparent kinetic barrier to its formation at near physiologic conditions.

The Cruciform Blocks Transcription in a Supercoil-Dependent Manner.

In vitro transcription using *E. coli* σ^{70} RNA polymerase holoenzyme on plasmid DNA templates of varying mean negative superhelicity was performed in order to assess the effect of the cruciform. (The plasmid DNA was exposed to helix destabilizing conditions

Figure 3. A. Quantitative primer extension analysis of *in vitro* transcription. The 5' end of the RNA is inferred by the length of primer extension. The principal *tet* transcript extends the 21-mer primer by 35 nucleotides. The *bla* transcript extends the 30-mer primer by 70 nucleotides. B. Graphical presentation of above. *tet* transcription from the cruciform promoter plasmid pX35 (closed circles) or the control plasmid pConDown (open circles) is plotted as a function of $-\bar{\sigma}$. Transcription units are arbitrary. C. Graphical presentation of above. *bla* transcription from pX35 (closed circles) or the control plasmid pConDown (open circles) is plotted as a function of $-\bar{\sigma}$.

prior to adding it to the transcription reaction in order to ensure cruciform extrusion.) Transcription initiating from the *bla* P3 promoter and from the *tet* promoter of both the cruciform plasmid pX35 and the control plasmid pConDown was quantified by cDNA primer extension (Fig. 3A). This experiment reveals three things worth noting. First, the insertion unique to plasmid pX35 does not alter the start site of *tet* transcription, confirming it to be the same as the previously determined (8) start site for the control plasmid pConDown, as indicated in Fig. 1. Second, negative supercoiling activates transcription from the control *tet* promoter of pConDown (Fig. 3B). By contrast, the *tet* promoter of pX35 is initially activated by low levels of supercoiling, but is progressively inhibited as $-\bar{\sigma}$ increases. As supercoiling is known to energetically stabilize cruciform formation (19), the most probable explanation for these results is that as $\bar{\sigma}$ is increased, the cruciform is incrementally extruded and blocks promoter utilization by RNA polymerase. Third, the curve of promoter activity as a function of $\bar{\sigma}$ for the pX35 *bla* is shifted to the right in comparison to the same curve for pConDown *bla* (Fig. 3C). That is to say, the pX35 *bla* promoter has peak activity at a $-\bar{\sigma}$ greater than the pConDown *bla* promoter.

The Cruciform Has a Negligible Effect on In Vivo Transcription.

The *in vivo* level of *tet* and *bla* transcription in cells hosting plasmids pX35 and pConDown was measured (Fig. 4). There was only slightly less *tet* transcript detectable in cells harboring pX35, and *bla* transcript levels were equivalent among the two plasmids. By phenotype, there was no difference in the level of tetracycline resistance determined by these two plasmids (data not shown). The most likely interpretation of these results is that the cruciform is not extruded to a significant extent intracellularly. This interpretation is supported by the observation (data not shown) that the supercoil density of purified plasmid DNA was the same for both pX35 and pConDown; if the cruciform had been extruded, pX35 would be expected to be less negatively supercoiled (20). This result is not surprising in light of the observation of the slow kinetics of cruciform extrusion.

DISCUSSION

Cruciform extrusion has been studied in extensive detail (reviewed in Refs. 21,22), and there is now accumulating evidence that such structures may form intracellularly: (i) Inverted repeat sequences are prone to spontaneous deletion when they are of a length sufficient to stably extrude a cruciform at the levels of supercoiling that occur *in vivo* (23), presumably a result of a cruciform's resemblance to the Holliday junction intermediate of genetic recombination; (ii) Plasmid DNA containing inverted repeat sequences possess a less negative superhelical density than control plasmids when purified from *E. coli*, suggesting that cruciform extrusion relieves negative supercoiling *in vivo* (20); (iii) Cleavage at an inverted repeat sequence on a target plasmid has been observed in a host strain expressing a single strand specific nuclease at high levels, indicating that such a sequence may be in a cruciform conformation intracellularly (24); (iv) Cruciform-specific DNA binding proteins have been identified (for example, Ref. 25); (v) Transcription from promoters containing inverted repeat sequences varies as a function of *in vivo* DNA supercoiling (6,7). The role of cruciforms and other supercoil-dependent DNA secondary structures is highlighted by recent findings (26–28) that transcription generates substantial supercoiling, inducing negative supercoiling upstream from the RNA polymerase and positive supercoiling downstream from the polymerase (with respect to the nontemplate strand). The negative supercoiling is the greatest at the promoter, and this may be sufficient to drive DNA structural transitions (26–28), including cruciform extrusion.

Figure 4. Primer extension analysis of *in vivo* transcription.

Sequences with the potential to adopt cruciform structures have been found to occur with regular frequency in genetic regulatory regions (29). Work from this laboratory has previously demonstrated that a synthetic *E. coli* promoter containing a cruciform in the '-10' region may regulate transcription in a supercoil dependent manner (7). It is here demonstrated that a cruciform in the '-35' promoter region behaves similarly *in vitro*. Unlike the case for the '-10' promoter, this cruciform has little effect on *in vivo* function. This is consistent with the finding that extrusion of this cruciform is kinetically slow, with a half life of formation apparently much greater than the *E. coli* generation time of about 20 minutes. The rate of extrusion is known to be highly sequence dependent, varying particularly as a function of A + T content (21). It is significant then that the cruciform promoter constructed from '-10' sequences (7) is 88% A + T, whereas the '-35' cruciform described here consists of just 64% A + T. Despite the absence of an *in vivo* effect upon transcription, the general relevance of these results is that alternate DNA structures at the '-35' region may be capable of influencing promoter-RNA polymerase interactions.

The step in the transcription cycle at which the cruciform exerts its influence on *tet* promoter function is unknown. One promoter that behaves similarly with regard to supercoiling is the *E. coli* DNA gyrase promoter, in which transcription is also inhibited by negative supercoiling (30). A likely step for control of this promoter is the switch between abortive cycling and productive elongation (30). The sequences responsible for the

topological regulation of transcription have been located to the '-10' region, the start site, and the initially transcribed bases. It has been proposed that the initial portion of the nascent message is basepaired with the DNA template in an R-loop, and supercoil relaxation destabilizes the R-loop, thereby enabling the polymerase to escape from abortive cycling. This explanation may have bearing on the function of the cruciform promoter composed of '-10' elements that previously has been described (7). In that case, cruciform extrusion extends from +27 to -23. This proposal is unlikely to explain the activity of the cruciform promoter described here composed of '-35' elements, however, because the cruciform extends from -19 to -69 and so is upstream of both the unwound region and the start site of transcription initiation. It is more likely therefore to affect transcription by interfering with the initial binding of the promoter sequence by RNA polymerase. It is worth noting that inactivation of promoter sequence function by alternate DNA structures may provide a unique and complementary approach to the traditional method of kinetic analysis of promoters through inactivation by DNA sequence mutation.

The experiments described here also demonstrate that extrusion of the cruciform affects transcription of the distant pBR322 *bla* promoter. These results can be explained by proposing that as the cruciform is extruded, it reduces the supercoil density of the entire plasmid by an amount equal to the twist contained in the cruciform, thereby decreasing the apparent value of $-\bar{\sigma}$ at the *bla* promoter to a level below its optimum for function. The peak of *bla* activity in pX35 is shifted by a $\Delta \bar{\sigma} \approx 0.025$. Extrusion of the cruciform would be expected to produce only a $\Delta \bar{\sigma} = 0.011$ ($= (50 \text{ bp/h}) / (4391 \text{ bp/h})$, where $h = \text{bp/helical turn}$), however. The difference could be accounted for by transcription-driven supercoiling (26–28). The *tet* and *bla* genes are arranged divergently on the pBR322 plasmid. Transcription of *tet* therefore results in a transient negative supercoiling of both the *tet* and *bla* promoters. A reduction in *tet* transcription, in this case resulting from interference by the cruciform, would further diminish the negative supercoiling at the *bla* promoter (26,27). Thus, the cruciform at the *tet* promoter could reduce supercoiling at the *bla* promoter by two mechanisms, by both directly restraining negative supercoils as it is extruded and by indirectly decreasing transcription-driven supercoiling. Over-interpretation of the general significance of this result must be cautioned, however. For example, the finding that eukaryotic enhancer activity is independent of topological (but not covalent) linkage to the distant sites which it potentiates (31), suggests that a mechanism of this sort is an inadequate explanation for enhancer function. Nevertheless, the *E. coli* genome is divided into many discrete topological domains (32,33), so local changes in supercoiling could still be an important mechanism through which transcriptional information is communicated at distances along the length of the DNA molecule (34).

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