Supercoil-induced unusual DNA structures as transcriptional block

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ABSTRACT

The transcriptional activity of pBR322 form V DNA template, a topologically unlinked, highly supercoiled molecule having unusual structures around or within coding regions was studied. Significant transcription was observed *in vitro* from this template despite high levels of supercoiling. An attenuated transcript, initiated accurately from the P4 promoter of *rep* gene, was observed which indicated pausing of *E.coli* RNA polymerase within the gene. This pausing could be removed by relieving the torsional stress implying that a supercoil induced structural alteration within the gene was acting as a transcriptional block. A stabilized unusual structure, most likely a cruciform, was found to be responsible for the elongation block. Absence of initiation from the tet^R gene was correlated with the unusual structure present within its promoter region in form V DNA. These *in vitro* studies show that structural alterations within natural DNA could act as transcriptional blocks both at the level of initiation and elongation.

INTRODUCTION

Expression of prokaryotic genes is known to be differentially regulated by the level of supercoiling (1,2). Although studies indicate that the supercoiled DNA in eukaryotic cells is more efficiently transcribed than linear DNA (3), the *in vivo* role of DNA topology and torsional stress on eukaryotic gene expression is much less understood (4). In prokaryotes, supercoiling is thought to modulate the strength of promoters and thereby regulate the level of gene expression (5–7). In circular plasmids, sequences having defined periodicity have been shown to undergo structural transitions under the influence of supercoiling (8,9). The supercoiling level at which different structural transitions occur is dependent on the nature of the sequence undergoing such transitions. In addition, structural transition in natural sequences may be achieved and stabilized by interaction with conformation specific proteins (10–12) and other *trans*-acting factors (13). Could such structural alterations in DNA have any role in the regulation of gene expression? Cloned DNA sequences with the potential to adopt Z-conformation such as $(TG)_{62}$ in the promoter region of tRNA gene (14), and $(CG)_{32}$ within the transcribed region of the *lacZ* gene (15) have been shown to decrease the extent of transcription. However the effect of structural alteration in natural sequences, around the promoters as well as within the gene, on transcriptional efficiency of genes is yet to be understood.

In vitro studies (16) with supercoiled DNA have shown that maximum transcription is observed at physiological superhelical density (−0.05). The fall in the level of transcription at lower superhelical density may be accounted for by the lack of unwound regions, occurrence of which has been shown to promote open complex formation (5). However, the reason for the observed decrease in the extent of transcription at high superhelical density, is not clear. The present work was initiated to understand the role of supercoiling and unusual DNA structures in modulating the various steps of the transcriptional process. An ideal system for such a study would be a template having high negative supercoiling with segments of unusual structures distributed in and around the genes. pBR322 form V DNA, the model system used in this study, is obtained by reannealing complementary single stranded DNA circles (17). Since in this molecule the linking number is zero, every right-handed helical turn has to be compensated by either a left-handed turn or a negative writhe. Thus, due to topological constraints, a large portion of the molecule (35–40%) is forced to adopt unusual DNA conformations such as Z-form, cruciform, single-stranded regions, etc. (17–19). Regions of pBR322 form V DNA having such altered conformations have been extensively probed and mapped using restriction endonucleases and modification methylases as structure sensitive probes (19,20,21).

The supercoil waves generated on a circular template during transcription (22, 23) could cause reversal of negative supercoil induced altered structures to B-form. However, in case of form V DNA reversal of such structure is energetically forbidden. Hence, form V DNA has been used as model system to understand the effect of stabilized unusual structures in RNA polymerase recognition and transcriptional elongation *in vitro*. Here we report that a stable unusual structure, most likely a cruciform, within the *rep* gene causes pausing of *E.coli* RNA polymerase. Occurrence of unusual conformation in the promoter region of tet^R gene can prevent initiation of transcription.

MATERIALS AND METHODS

E.coli RNA polymerase, Sau3AI, EcoRI and MspI methylase were from New England Biolabs. ^{3}H-UTP, α-^{32}P-UTP, α-^{32}P-

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dATP and α-^{32}P-CTP were from Amersham. 5'd-TTTGCCGGATCAAGAGC was synthesized on Applied Biosystems 380B synthesizer and was kindly supplied by Astra Research Centre, India. All other materials used were of analytical grade.

Preparation of pBR322 form V DNA

Monomer plasmid pBR322 DNA from *recA*$^-$ strains of *E.coli* was purified by the procedure of Maniatis et al (24). Form V DNA was prepared following the procedure of Stettler et al (25) with certain modifications (17, 19, 20). Supercoiled DNA was digested with DNaseI in the presence of 0.3 mg/ml ethidium bromide to generate 80% form II DNA. Circular single-stranded molecules were separated from linear single stranded DNA on an alkaline sucrose density gradient (5−20%) at 35,000 rpm in SW41 rotor for 13hr at 15°C. Fractions containing single-stranded circular molecules were neutralized, pooled, and annealed at 60°C for 20 min to obtain double stranded form V molecules. Form V molecules were characterized by 1 and 2-dimensional agarose gel electrophoresis (17), Z-DNA antibody binding (18), characteristic CD spectra and UV melting profile (17, 20, 26). Electroeluted form V DNA was used for all transcriptional experiments.

Transcription assay

To compare template activity of pBR322 form I and form V DNA for *E.coli* RNA polymerase, transcription assays were carried out at 37°C in the presence of 1 μCi ^{3}H-UTP (10mCi/mMole) (Amersham), 0.5 μg DNA, 0.72 units RNA polymerase (New England Biolabs), 50 μg / ml BSA, 0.2 mM DTT, 100 mM KCl, 10 mM $MgCl_2$, 3% glycerol, human placental RNaseIn and 60 μM each of ATP, GTP, CTP. Reaction was stopped by 5% TCA. Unincorporated nucleotides were removed and the extent of ^{3}H-UTP incorporation was measured as described earlier (27). Relative concentration of form I, form II and form V DNA was determined by scanning the photographic negative of the DNA gel using Joyce Loebl microdensitometer. Analysis of DNA templates on 1% agarose gel at the end of the transcription assay showed that amount of pBR322 form II DNA present in supercoiled form I and form V template preparations used for transcription assays were approximately 10% of the total DNA. Correction for transcription from the form II template in form I and form V assay mixture was performed by substracting the extent of incorporation observed in an assay containing only form II template. It has been shown that the extent of transcription from relaxed pBR322 form II template is only 7% as compared to the supercoiled template under identical conditions (16). Although contribution of form II DNA towards total transcription is negligible (< 1%), this correction was considered to be necessary to normalize the form I and form V template concentrations in the samples. To determine the length of various transcripts, transcription assay was performed in the presence of α-^{32}P-UTP. The reaction was stopped by adding 15mM EDTA and 1% SDS. After phenol/chloroform extraction, the transcripts were ethanol precipitated in the presence of (20 μg) of tRNA and analysed on a 10% polyacrylamide gel containing 8.3M urea. To prevent reinitiation, rifampicin was added at a concentration of 40 μg/ml prior to the addition of the restriction enzyme. This concentration of rifampicin is sufficient to selectively inhibit the initiation of RNA synthesis and not the elongation of pre-initiated RNA by *E.coli* RNA polymerase (28,29).

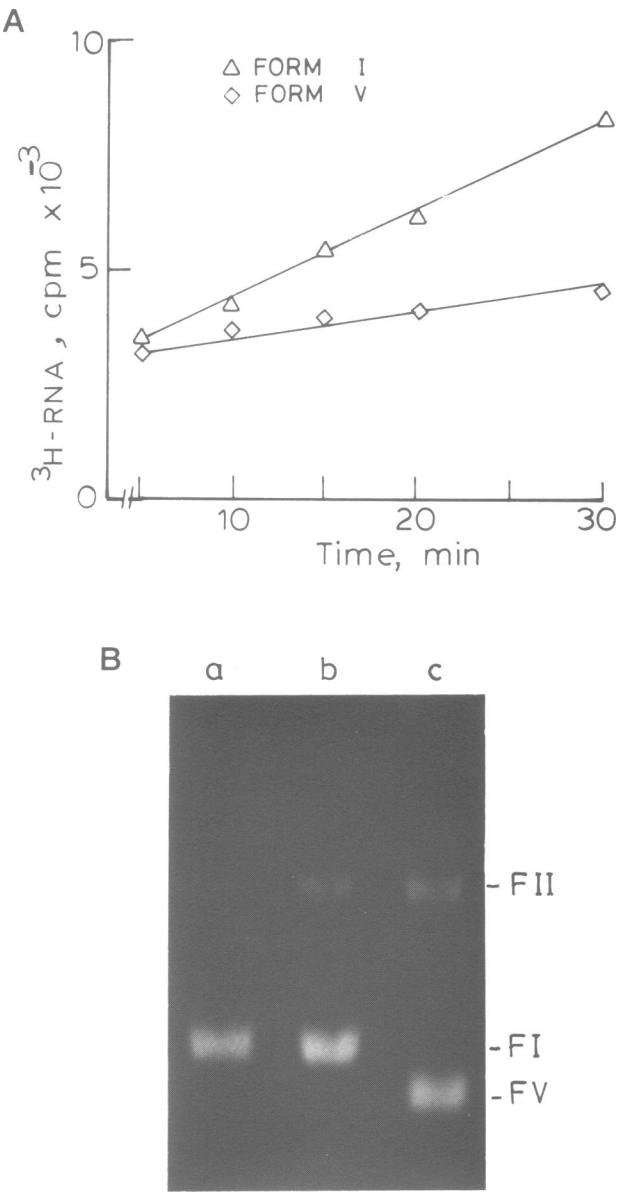

Figure 1. (A) Template activity of pBR322 form I and form V DNA for *E.coli* RNA polymerase. (B) Analysis of DNA templates on 1% agarose gel at the end of the transcription assay a) control pBR322 b) form I template and c) form V template.

Abortive initiation assay

Abortive initiation assay for *rep* gene of pBR322 was carried out in the presence of 1.6mM ATP, the initiating nucleotide (16), and α-^{32}P-CTP (3000Ci/mMole). Other conditions for the assay were similar as described above except that twice the amount of RNA polymerase was used for each reaction. Reaction was carried out for 30 min. Length of the aborted product was determined by electrophoresis on a 25% native polyacrylamide gel. To compare the extent of initiation the autoradiogram was scanned on a Joyce-Loebl microdensitometer.

Methylation sensitivity for mapping altered conformation

Both pBR322 form I and form V molecules were methylated using MspI methylase and S-adenosyl-L-methionine (SAM).

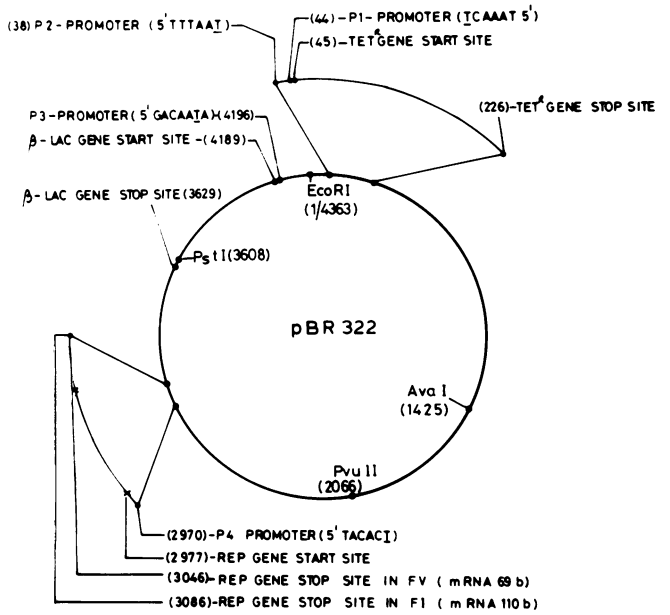

Figure 2. Map of pBR322 showing the location of various genes and length of respective transcripts.

Assay conditions were same as described earlier (19). Sau3AI digestion of the methylated DNA was carried out as specified by the vendor. Fragments were end labelled with α-^{32}P-dATP by *E.coli* DNA polymerase large fragment (Klenow) and separated on a 1.5% agarose gel in 1× TAE buffer at 2 V/cm. The gel was dried prior to autoradiography.

RESULTS

In vitro transcription assay of pBR322 form V DNA

The ability of pBR322 form V DNA to act as a template for *E.coli* RNA polymerase was tested. Significant transcription was observed from the pBR322 form V template (Fig. 1A) in spite of its very high negative superhelical density. However, the extent of transcription at longer time intervals for this template was lower than that of form I template. Form I and form V templates were found to be intact at the end of each transcription assay (Fig. 1B).

Transcription assays were carried out in the presence of α-^{32}P-UTP and the transcripts were analyzed on a denaturing polyacrylamide gel. Four promoters control the expression of pBR322 gene (Fig. 2) wherein P4 promoter regulating the expression of *rep* gene is the strongest with weak P1,P2 and P3 promoters regulating the expression of *tet*^{R} and *amp*^{R} gene respectively (30). From the pBR322 template, as reported earlier (16), a *rep* gene product of 110 nucleotides (transcript A) was found to be the major transcript (Fig. 3). On the other hand, the only transcript obtained on using the pBR322 form V template was 69 residues long (transcript B) (Fig. 3). In particular the higher transcripts corresponding to *tet*^{R} and *amp*^{R} genes obtained from form I DNA template were absent from the form V template. To ensure efficent precipitation of smaller transcripts, if any, high concentration of cold tRNA was added prior to ethanol precipitation. Analysis of the transcriptional products on a sequencing gel showed absence of any transcript smaller than 69 bases from form V template (data not shown).

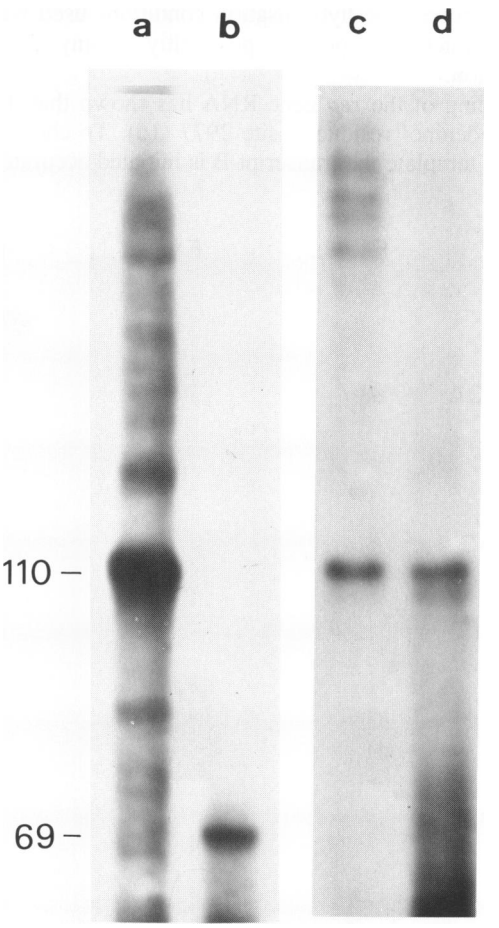

Figure 3. Analysis of α-^{32}P-UTP labelled RNA transcripts from a) form I and b) form V templates and subsequent to linearization with EcoRI c) form I and d) form V templates.

Characterization of transcript of form V template

Since only one transcript was observed from the form V DNA template, we examined the nature of the transcripts that resulted after the removal of topological stress. Transcription was initiated and rifampicin (40 μg/ml) was added to prevent fresh initiation after 15 min. An aliquot was removed to which EcoRI was added to linearize the template and transcription was allowed to continue for 15 min. The resulting products were analyzed as before (Fig. 3). When form I DNA was used as a template, the length of the resulting transcripts remained unchanged, whereas with form V DNA template a 110 nucleotide transcript A appeared with a concomitant disappearance of the 69 nucleotide transcript B. However, longer transcripts were still not observed even after the removal of torsional stress.

Since transcript A appeared with a corresponding disappearance of transcript B on the removal of topological constraints in form V DNA, hybridization studies were performed to determine whether transcript B was a sub-transcript of transcript A. A seventeen nucleotide long synthetic deoxyoligonucleotide, 5'd-TTT GCC GGA TCA AGA GC-3', extending from site 3036 to 3052 of the *rep* gene (Fig. 5) and complementary to the non-coding strand, was synthesized and used as a probe for dot blot analysis. Unlabelled transcripts A and B were eluted from the gel and were found to hybridize strongly with the ^{32}P-labelled

oligomer probe. The hybridisation conditions used were quite stringent which rule out the possibiltiy of any non-specific hybridisation.

Sequencing of the *rep* gene RNA has shown that it initiates with an 'adenine' residue at site 2977 (16). To check whether in form V template the transcript B is initiated accurately at the same nucleotide as transcript A, abortive initiation assay was perfomed in the presence of ATP and α-^{32}P-CTP. As expected, a major trinucleotide product (A*CA) was obtained from both form V and form I templates (Fig. 4A), though the extent of initiation of *rep* gene from form V template was lower than that of form I template (Fig. 4B). These results showed that following accurate initiation from the *rep* gene in form V DNA RNA polymerase paused to give 69 bases RNA transcript which could be elongated to give the full length transcript (110 bases) following the removal of torsional stress.

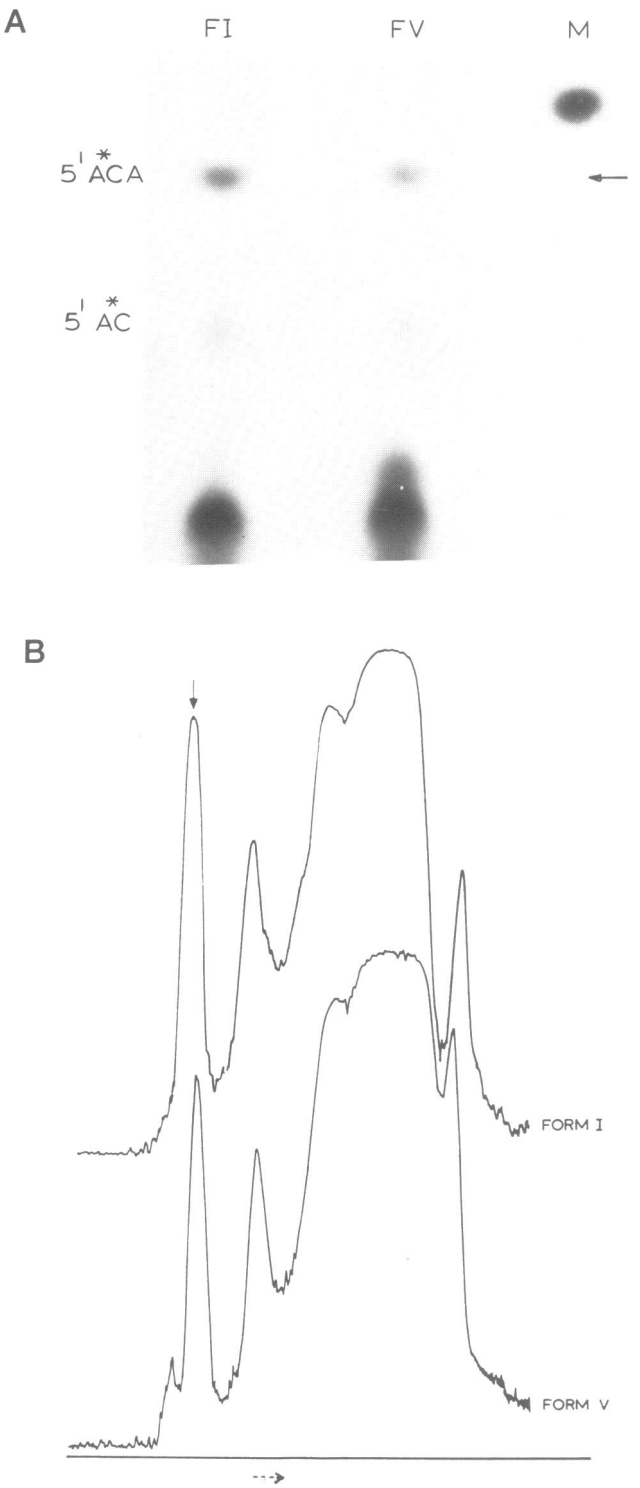

Figure 4. (A) Abortive initiation assay for *rep* gene. Lane (M) Tetramer marker, (FI)- Form I template, (FV)- Form V template. (→) indicates the position of aborted trimer product. (B) Densitometric scan of the autoradiogram.

Probing the nature of pause site

Since transcript B was initiating accurately at 2977, from its estimated length, it could be deduced that RNA polymerase paused near cytosine residue (3046) on pBR322 form V template. Sequence search showed the presence of an inverted repeat with the potential to form an extended stem length of 16 bp (Fig. 5) following this pause site. This inverted repeat sequence extrudes into a cruciform structure in pBR322 form I DNA at higher superhelical densities ($\sigma > -0.073$) as shown by the sensitivity of its loop region (3065) to SI nuclease (8). The pause site (3046) is at the junction region of this cruciform. The cytosine residue at 3045, before the pause site, is unique in pBR322 where Sau3AI (3042) site overlaps the mMsp I site (3045). Methylation of the cytosine residue (3045) by mMspI would render this site resistant to cleavage by Sau3AI. Methylation of MspI sites of pBR322 form I, form V and form III DNA were carried out using mMspI and S-adenosylmethionine. Methylated molecules were digested with Sau3AI and fragments were labelled using α-^{32}P-dATP. In case of form V, appearance of only 1376 bp fragment (Fig. 6A & B) implied the complete absence of methylation at this site due to structural alteration. In contrast, appearance of 1449 bp fragment in addition to 1376 bp fragment from form I DNA indicated partial methylation at this site. Similarly in case of form III DNA methylation at this site was also not complete

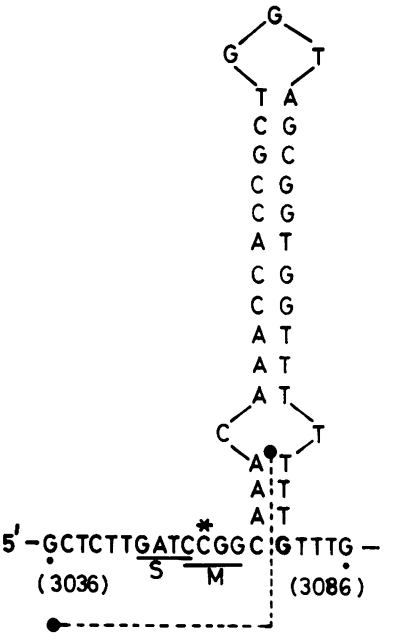

Figure 5. RNA polymerase pause site in *rep* gene of pBR322 (*). The cruciform structure responsible for transcriptional block within the *rep* gene. Complementary sequence of the 17mer oligonucleotide probe used for dot blot analysis (●---●). S: Sau3AI & M: mMsp I recognition sequence.

(data not shown). Even higher amounts of enzyme addition did not lead to 100% methylation at this site in both form I and form III DNA. Influence of flanking sequences on the differential cleavage of restriction sites by several enzymes has been well documented (21,31). This partial methylation of form I and form III DNA by mMspI could be attributed to the similar effect of flanking sequences. However, considering form III DNA as a control for the optimal activity of mMspI at this site, the extent of methylation observed in case of pBR322 form I is only 80%. Nonetheless, complete absence of methylation observed in case of form V DNA supports the presence of an altered conformation at the pause site. Thus, the cruciform nature of the RNA polymerase pause site in form V DNA is quite likely, considering the presence of an inverted repeat and inability of methylase to methylate the junction region. This is corroborated by the observed hypersensitivity to SI nuclease at site 3065 even in form I DNA at higher superhelical densities.

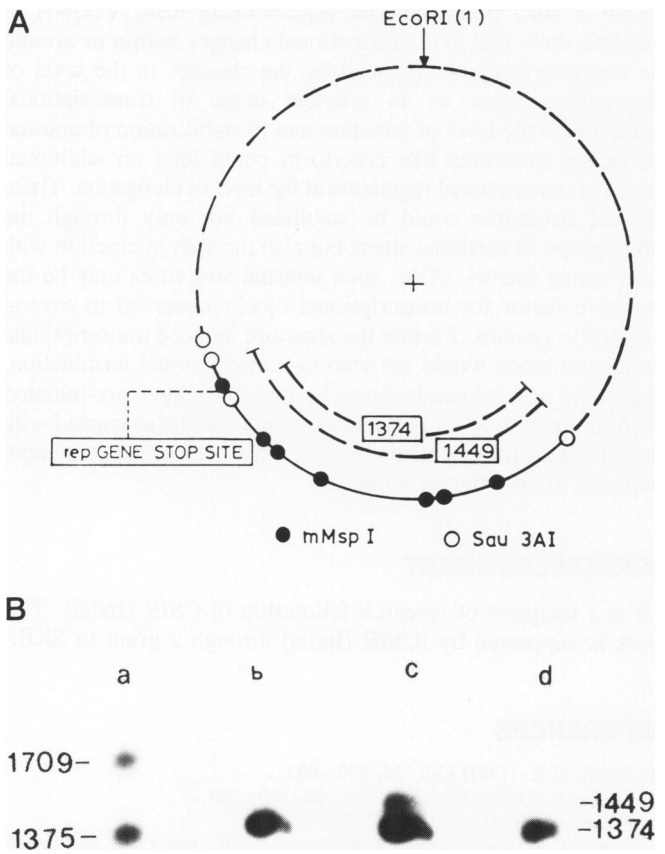

Figure 6. (A) Schematic representation of the methylation experiment to probe the nature of the pause site. (B) Sau3AI digestion of pBR322 form I and form V DNA after methylation with mMspI. Lanes (a) λ-EcoRI-HindIII Marker, (b) form I (unmethylated), (c) form I (methylated) & (d) form V (methylated).

Absence of initiation at tet^R gene promoter

In vitro transcription assay of pBR322 form I DNA yielded a 180 nucleotide transcript which corresponds to tet^R gene (Fig. 3) (16). However in case of form V template, the tet^R gene as well as higher transcripts corresponding to amp^R gene were not obtained. In addition, absence of these higher transcripts even after the removal of torsional stress from form V DNA implied the lack of initiation from these promoters.

DISCUSSION

Using pBR322 form V DNA as a template for *in vitro* transcription, we have shown here that supercoil stabilized unusual DNA structures could prevent both transcriptional initiation and elongation. Since *E.coli* RNA polymerase is able to recognise accurately the promoter region and can transcribe *rep* gene in form V DNA it implies that the zero link in the template per se has not affected the transcriptional process. Occurrence of an altered conformation within the *rep* gene effectively blocked transcriptional elongation resulting in the formation of an attenuated transcript which could be elongated following removal of torsional stress. Thus, structural alteration within the template, as a consequence of high negative supercoiling, is responsible for pausing of RNA polymerase. The exact site of RNA polymerase pausing on form V template was confirmed by ruling out non-specific initiation using abortive initiation assay for *rep* gene.

DNA structure sensitivity of MspI methylase was used as a probe to confirm the presence of unmethylatable non-B conformation. Complete absence of methylation of MspI site (3045) in form V DNA in the junction region of potential cruciform structure suggests that a stable cruciform structure in the form V DNA template could be responsible for RNA polymerase pausing. However, partial methylation observed for the same site in form I DNA could be due to flanking sequence effect. Although partial stabilization of a cruciform structure at physiological superhelical density ($\sigma \approx -0.05$) could not be ruled out. Since this region has been shown to extrude into a cruciform structure in pBR322 form I DNA at high superhelical density ($\sigma \approx -0.073$) as shown by the sensitivity of its loop region to SI nuclease. Presence of an inverted repeat, inability of MspI methylase to methylate the junction region and the occurrence of a SI nuclease hypersensitive site suggests a stabilized cruciform structure as transcriptional block within the *rep* gene in form V template for *E.coli* RNA polymerase. Elimination of cruciform structure through deletion could not be undertaken as this sequence is essential for plasmid replication. In fact the corresponding hairpin in the RNA helps in the regulation of replication cycle. Nevertheless the desired transition of cruciform to linear form was achieved by releasing the torsional stress through restriction enzyme cleavage of form V DNA, after prior

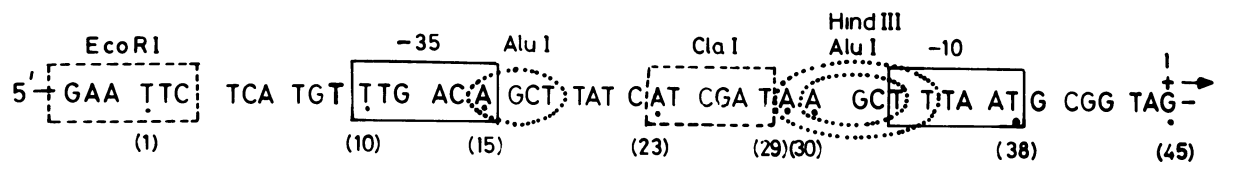

Figure 7. Structure of the regulatory region of tet^R gene in pBR322 form V DNA. Hind III and AluI sites adopt non-B conformation, EcoRI and ClaI sites adopt B-conformation.

initiation. Elongation of the attenuated RNA to the expected 110 bases transcript supports this observation.

During the process of transcription the positive supercoil generated ahead of the RNA polymerase−RNA complex, as proposed recently (22,23), could lead to reversal of the metastable cruciform structure to transcribable B-form in form I DNA. However, the positive supercoil generated during the process of transcription in form V template is not enough to reverse the stable cruciform block. Since the maximum level of supercoiling has already been attained in the form V molecule any structural transition leading to an increase in negative supercoiling is energetically forbidden. Thus, a stabilized cruciform conformation in topologically constrained form V DNA could act as a stable transcriptional elongation block. The physiological significance of this observation lies in the fact that if such unusual structures could be stabilized through DNA supercoiling or protein factor binding, the transcriptional process could be regulated. It is tempting to suggest that such stabilized structures could function as cis-acting elements in modulating the transcriptional elongation even *in vivo*. *Trans*-acting factors stabilizing cruciform structures have been reported recently (32). Transcriptional attenuation resulting in the formation of a premature transcript observed in cases of *his, trp, leu* and *phe* operons among prokaryotes is suggested to be due to the presence of a G-C rich hairpin and a series of U-residues at the 3'end of the RNA product (33). However, no role for the DNA sequences have been implicated in these cases. Even in eukaryotes, premature transcriptional termination has been observed in case of *hsp70, c-myc, c-myb, c-fos*, SV40 and MVM genes (34−37) . In case of *hsp70*, a putative heat shock factor has been implicated in premature termination of transcription in uninduced cells (36). Deletion analysis has shown that in case of *c-myc* a 95 bp region containing a potential cruciform may be responsible for attenuation (37). But due to instability of the truncated RNA it could not be concluded whether attenuation involves recognition sequences or structures within the RNA transcript or template DNA (37). Results presented here suggest a possible role of structural variabilty of natural DNA sequences in regulation of transcriptional elongation. Abundance of potential cruciform sequences within coding regions could reflect biological significance of such secondary structures (38). Form V DNA is only a model system in our endeavour to unravel the constituents of the transcriptional control elements. Nevertheless, in the absence of concrete evidences for the exact level of supercoiling during the process of transcription *in vivo*, a localized generation of very high levels of supercoiling seems not improbable (39,40).

It was interesting to note that in form V DNA the transcriptional initiation from P4 promoter (*rep*) was not affected significantly whereas no initiation occurred from P1, P3 (amp^R) and P2 (tet^R) promoters. Promoters for amp^R gene have been shown to be sensitive to high levels of supercoiling even in form I DNA (41). The structure of the promoter region of tet^R gene in form V DNA has been probed earlier by taking advantage of the sensitivity of restriction endonucleases and methylases to DNA conformation (19,20,21). As shown in Fig 7, both EcoRI and ClaI recognition sites in form V DNA are sensitive to cleavage whereas HindIII site shows resistance to cleavage. In addition, AluI sites at position 15 and 30 were unmethylatable by AluI methylase. Thus, promoter region of tet^R gene in form V DNA comprises of alternating B- and non-B conformations. The absence of any transcript from P2 promoter of tet^R gene in form V DNA even after the removal of torsional stress could be attributed to the presence of this unusual DNA conformation in the promoter region (19,20). B and non-B (Z-form) conformations alternating after every 6 bp has been shown to be stabilized even under the physiological superhelical density (42). Such unwound structures are expected to facilitate open complex formation. On the contrary, in form V DNA the alternating B and non-B conformation in the region of −10 and −35 sequence of the tet^R promoter seems to have prevented the recognition by RNA polymerase. Previous studies carried out by cloning the synthetic segments, e.g. alternating T/G (14) or palindromic sequences(43), have a major limitation in that there is a change in the distance due to change in the number of nucleotides between the contact points of RNA polymerase. On the other hand, in the present study inspite of maintaining the same number of nucleotides in the promoter region the RNA polymerase contact points could be differentially disposed due to the presence of unusual DNA structure in this region.

Our *in vitro* transcriptional studies using form V DNA as template show that a) conformational changes within or around the regulatory region, induced by the changes in the level of supercoiling serve as an efficient mode of transcriptional regulation at the level of initiation and b) stabilization of unusual secondary structures like cruciform could lend an additional means of trancriptional regulation at the level of elongation. These unusual structures could be stabilized not only through the introduction of torsional stress but also through interaction with *trans*-acting factors. Also, such unusual structures may be the causative factor for transcriptional blocks observed in several eukaryotic systems. Further the structure induced transcriptional elongation block would not lead to transcriptional termination. This could be used to advantage by the cell to have pre-initiated RNA along with RNA polymerase which can be elongated with the arrival of the necessary signal. This could lead to a rapid response to an external stimulus.

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