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The influence of an alternate template conformation on elongating phage T7 RNA polymerase

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ABSTRACT

We investigated the effect of left-handed Z-DNA on transcription by bacteriophage T7 RNA polymerase *in vitro* and, surprisingly, found that the enzyme can efficiently utilize a template containing a stretch of left-handed DNA close to the promoter. Analysis of transcription products revealed that only a small fraction of elongating polymerases abort transcription either at the promoter proximal or at the distal B-to-Z junction and, even less frequently, within the stretch of left-handed DNA. Our results indicate that, unlike *E.coli* RNA polymerase (ref. 1,2), T7 RNA polymerase can utilize a template with a CG stretch in an alternate conformation. In contrast, polymerases are completely blocked at the promoter proximal junction by a monoclonal antibody directed against Z-DNA. This blockage remains stable over a remarkable time, even when negative supercoiling is released by linearization of the template. Together with our recent finding of transcription-induced formation of Z-DNA (3), our data provide an example for a possible auto-regulatory mechanism that employs a change in DNA conformation.

INTRODUCTION

The concept of transcription-driven DNA supercoiling proposed by Liu and Wang (4) was recently supported by a number of studies using both pro- and eukaryotic systems (5–9). These findings raise the possibility that short-lived but nevertheless significant local changes in DNA supercoiling could play an important role in regulatory pathways by inducing structural alterations in DNA. These could include extrusion of cruciforms (10) or a transition from right-handed B- to left-handed Z-DNA (11,12), whereby both could be driven by the unfavorable free energy of negative (−) supercoiling generated behind a transcribing RNA polymerase (4). For example, once a transition from right- to left-handed DNA has occured, the alternative DNA structure could be stabilized by the rapid binding of Z-DNA specific proteins. Recently, such a scenario has been documented, using transcription of a topologically closed domain by phage T7 RNA polymerase (T7 RNP) *in vitro* . This induced a B-to-Z transition in a fraction of templates, which was then recognized and stabilized by a monoclonal antibody specifically directed against left-handed DNA (3).

In this context, we were interested in learning more about the effects of both an alternate left-handed DNA conformation and of Z-DNA-bound proteins on transcription. In previous studies the translocation of an elongating *E.coli* RNA polymerase *in vitro* was found to be completely blocked by a stretch of alternating CG residues in the left-handed conformation (1,2). There are, however, conflicting observations which indicate that both *E.coli* RNA polymerase and wheat germ RNA polymerase II can utilize the left-handed helical form of poly (dGdC) (13,14), albeit less efficiently than the right-handed conformation. We wondered about the generality of the potential of Z-DNA to prevent enzyme translocation and thus tested the DNA-dependent RNA polymerase encoded by bacteriophage T7. This enzyme differs in its composition from bacterial and eukaryotic RNA polymerases in that it consists only of a single polypeptide of approximately 98 kilo Dalton (15). Furthermore, it shows a very high specificity for the late T7 promoters (16), which makes this enzyme an ideal tool for *in vitro* and *in vivo* studies. We generated templates that contain a stretch of $d(CG)_{16}$, located within the transcription unit close to the T7 promoter. Such a tract of alternating purines and pyrimidines can adopt a left-handed conformation at relatively moderate levels of (−) supercoiling (17), which might in fact come close to the number of unconstrained (−) supercoils detectable within *E.coli* cells (18).

Our results show that, (i) T7 RNP can efficiently utilize such a stretch of left-handed DNA, (ii) a small fraction of polymerases abort transcription either at the two B-to-Z junctions or within the Z-stretch, (iii) polymerase passage is completely blocked by the binding of a monoclonal antibody directed against Z-DNA, and, (iiii) the antibody-mediated block of transcription is remarkably stable for one hour, even when the template is topologically relaxed by linearization after antibody binding.

MATERIALS AND METHODS

Plasmids

Transformations, plasmid isolations and cloning procedures were carried out by standard procedures. In pFP732 (19), the EcoRI-Hind III restriction fragment containing the 32 bp alternating CG

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stretch at the Bam HI sites (17) replaced the polylinker fragment of pTZ18R (Pharmacia) downstream from the T7 promoter (Fig. 1a and b). In p101, a 56 bp polylinker fragment was cloned into the Eco RI site of pFP732 (Fig. 1c).

Proteins

T7 RNA polymerase was either purified to homogeneity from an overproducer strain as described (20) or obtained from New England Biolabs. Purified calf thymus topoisomerase type I was a kind gift of Dr. R.Knippers. RNAsin was obtained from Promega. The generation and purification of monoclonal antibody Z-D11 has been described (21,22).

Preparation of topoisomers

Sets of topoisomer distributions of plasmids pTZ18R and pFP732, each characterized by a different average linking deficit, were obtained by reacting covalently closed superhelical DNA with calf thymus topoisomerase type I in the presence of ethidium bromide (0–120 ng/μg DNA) as described (23). After one hour at 37°C, the DNA was phenol/chloroform extracted, precipitated with ethanol and dissolved in TE buffer (10 mM-Tris.HCl (pH7.5); 1 mM-EDTA). Topoisomer distributions were displayed by one- and two-dimensional gel electrophoresis in TBE buffer (90 mM-Tris.borate (pH8.3); 2.5 mM-EDTA) containing various concentrations of chloroquine (Sigma). The average linking number difference for each topoisomer distribution was determined by the band counting method from ethidium bromide stained gels (23). The superhelical density (σ) for each set of topoisomers was calculated as the average linking number deficit divided by helical twist, taken as the the number of base pairs divided by 10.4. The given values of (−) supercoiling therefore correspond to the conditions used for electrophoresis. The DNA concentration of each set of topoisomers was determined in a DNA-fluorometer (Hoefer Scientific Instruments, San Francisco) using Hoechst dye 33258.

In vitro transcription assay

The transcription assays for the experiments presented in Figure 2 were carried out in a final volume of 50 μl buffer containing 20 mM-$NaPO_4$ (pH7.5); 5 mM-dithiothreitol (DTT); 10 mM-$MgCl_2$; 0.5 mM of each ATP, GTP and CTP; 0.025 mM-UTP with 0.5 μCi of [^{3}H]UTP (specific activity 13.5Ci/mMol; Amersham). The reaction mixtures were prepared on ice and 100 ng of each topoisomer population was preincubated with or without 150 ng of purified mAb Z-D11 for 30 minutes on ice. Transcription was initiated by the addition of 0.5 μg purified T7 RNA polymerase and stopped after 15 minutes incubation at 37°C with 4 volumes of trichloracetic acid (10% w/v). Acid insoluble [^{3}H] was determined as described (24).

To analyse *in vitro* transcription products, a typical transcription assay was carried out in 50 μl buffer containing 40 mM-Tris.HCl (pH 8.0); 8 mM-$MgCl_2$; 50 mM-NaCl; 25 mM-DTT; 2 mM-Spermidine; 10 U RNAsin; 6 U T7 RNA polymerase/ 0.2 μg of template DNA; 0.3 mM of each ATP, UTP, and CTP; and 0.03 mM of GTP with 1 μCi of α [^{32}P] GTP (Amersham). Incubation was as described in the text at 37°C and stopped by the addition of 1 volume of 4 M-NH_4.acetate (pH 4.5). After ethanol precipitation to remove unincorporated nucleoside triphosphates, the RNA was prepared for gel electrophoresis and analyzed on denaturing (8 M) urea gels which were run in TBE buffer (25).

RESULTS

Influence of template supercoiling and left-handed DNA on transcription by T7 RNA polymerase

Our initial experiments were aimed to quantitate the effects of both template supercoiling and the transition from right-handed B- to left-handed Z-DNA on T7 transcription. For this, we utilized two plasmids, designated pTZ18R and pFP732 (Fig. 1a and b), containing the T7 promoter consensus sequence immediately upstream of a polylinker sequence. In order to prevent any complications that might arise from transcription-driven DNA supercoiling (4), which in turn could affect the stability of Z-DNA, we placed a stretch of $d(CG)_{16}$ in pFP732 in close proximity to the transcriptional start site (Fig. 1b).

We started our analysis by generating sets of topoisomer distributions from both templates and measured the transcriptional activity as a function of template supercoiling. As shown in Figure 2A, transcription of control plasmid pTZ18R depends strongly on the level of (−) supercoiling and reaches an optimum at a superhelical density of about −0.07. This dependence of transcription on the superhelical state of the template is very similar to that found for *E.coli* RNP in a previous study (2) and agrees with the observation that the affinity of T7 RNP to its promoter is increased by (−) supercoiling of the substrate (26).

A different response to (−) supercoiling is observed when sets of pFP732 topoisomers are transcribed. It has been shown before (27) that the B-to-Z transition of an identical CG stretch occurs around σ −0.03, and this was confirmed for pFP732 using two-dimensional gel electrophoresis (data not shown). In the lower range of template supercoiling, transcription of pFP732 shows similar activity observed to pTZ18R. When template supercoiling exceeds the threshold for the B-to-Z transition in p101, transcription plateaus up to σ −0.07. This indicates that the presence of left-handed DNA at the very beginning of the transcription unit does not abolish transcription but instead shows a limited effect on the activity of T7 RNP. This effect, however, cannot simply be due to a reduction of (−) template supercoiling concomitant to the transition in DNA structure (which is equivalent to a change in s by about +0.02 for a stretch of $d(CG)_{16}$). In this case, transcriptional activity should resume its increase once s has reached a value around −0.05 in Figure 2A, and should, at higher levels of torsional strain, resemble that obtained with control plasmid pTZ18R.

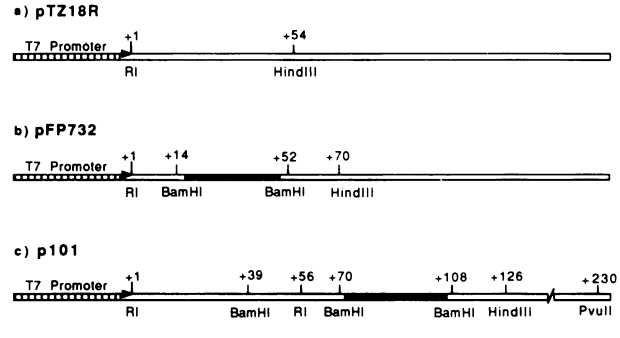

Figure 1. Plasmid constructions. The location of the start site of transcription in each plasmid is marked as (+1) and corresponds closely to the cleavage-position of restriction enzyme EcoRI (RI). The T7 promoter and the $d(CG)_{16}$ stretch, which is flanked by BamHI sites, are indicated by striped and stippled boxes, respectively. The numbers indicate the lengths of run-off transcripts terminating at the cleavage sites for the given restriction enzymes.

We were surprised to find only a moderate inhibition of T7 transcription by Z-DNA since in previous studies using *E.coli* polymerase transcription appears to be completely blocked by the presence of Z-DNA (1,2). In order to confirm that, under our assay conditions, the CG stretch has indeed undergone the B-to-Z transition at the expected level of σ, we used a monoclonal anti Z-DNA antibody (mAb), designated Z-D11 (19,22), as a Z-DNA specific probe. The same sets of pFP732 topoisomers were preincubated with saturating amounts of Z-D11 and transcription was measured as before. We observed that the antibody does not affect transcription at σ −0.02 but leads to inhibition around σ −0.04. This indicates that the conformational change in DNA structure has occured at the expected level of (−) supercoiling. Since stoichiometric amounts of antibodies block transcription, it indicates that this is a specific effect due to antibody binding (Fig. 2B). The residual transcriptional activity most likely results from a small amount of nicked template DNA to which the antibody cannot bind because of the lack of left-handed DNA.

T7 RNA polymerase can translocate through a CG stretch in negatively supercoiled DNA

In order to gain further insight into the behaviour of T7 RNP when it encounters a stretch of left-handed DNA, we performed an analysis of *in vitro* transcription products. To ensure that the polymerase has completely switched from the initiation to its elongation mode before encountering a stretch of left-handed DNA, we constructed a third template, p101, by inserting a 56 bp polylinker fragment into the EcoRI site of pFP732. This then displaces the GC stretch further downstream to position +70 (Fig. 1c).

The effects of template supercoiling and, consequently, the presence of left-handed DNA on transcription were assayed by comparing relaxed templates with those at a 'native' superhelical density (σ = −0.07). Equal amounts of both forms of p101 were incubated with T7 RNP under standard conditions and transcripts were labeled by the incorporation of α [^{32}P]GTP. At various times transcription was stopped and analyzed on a 10% denaturing gel. Run-off transcripts from linearized p101, which was first cut with HindIII, followed by a partial digest with BamHI (Fig. 1c), were used as position markers. As shown in Figure 3, two main obstacles appear in supercoiled p101. They are located at positions that correspond exactly to those of the two BamHI sites (+70 and +108, respectively) bordering the CG stretch (lanes M and 7 to 12). Although less pronounced, several polymerase stop sites within the GC stretch become visible after longer incubation times (lanes 10 to 12). Over this time course, none of these termination sites are detectable when relaxed p101 is used as template (lanes 1 to 6), indicating that transcriptional termination in (−) supercoiled p101 is not simply due to a sequence effect of the CG stretch. We conclude that, in contrast

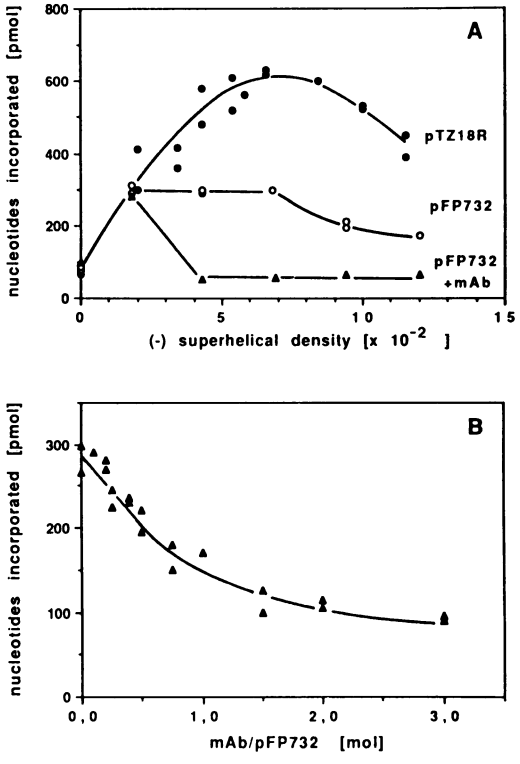

Figure 2. (A) The influence of template supercoiling and B to Z transition on transcription by T7 RNA polymerase *in vitro*. Equal amounts of sets of topoisomer distributions from pTZ18R and pFP732 were incubated with or without monoclonal antibody (mAb) Z-D11 as indicated. Transcription was started at 37°C and continued for 15 minutes. Incorporated [^{3}H] was determined and plotted against the average (−) superhelical density (σ) of each set of topoisomers. (B) Stoichiometry of the antibody mediated block of transcription. Appropriate amounts of mAb were preincubated with 150 ng of supercoiled pFP732 (σ = −0.064) for 30 minutes on ice. Transcription was initiated by the addition of T7 RNA polymerase and incubation at 37°C for 15 minutes.

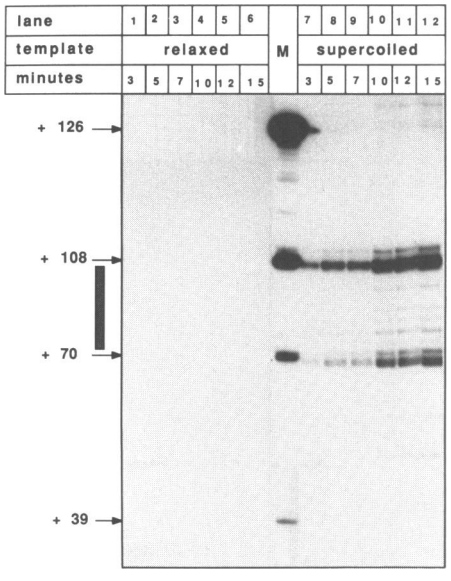

Figure 3. Identification of transcription stop sites in (−) supercoiled p101. *In vitro* transcription products were obtained by incubating relaxed (lanes 1 to 6) and (−) supercoiled p101 (σ = −0.07) (lanes 7 to 12) with T7 RNA polymerase for different time periods as indicated. Labeled transcripts were analyzed on a 10% denaturing urea gel. The numbers on the left side of the autoradiogram correspond to the position of run-off transcripts (lane M) obtained by a partial digest of p101 using HindIII and BamHI (see Fig. 1c). The position of transcripts terminated within the CG stretch is marked by the stippled vertical box positioned between +70 and +108. The BamHI site distal to the promoter appears to be a stronger obstacle for RNPs than the promoter proximal site. This is due to the label used in this experiment (α [^{32}P]GTP). The specific radioactivity of transcripts therefore increases when the polymerase reads through the CG stretch. In fact, by using a different label (α [^{32}P]UTP), the amount of transcripts terminating at each BamHI site is essentially the same. Thus, both regions exhibit a similar potential to affect transcription.

to *E.coli* polymerase (1), T7 RNP can enter and translocate through the CG stretch in a (−) supercoiled template.

A stable and complete transcriptional block at the B-to-Z junction is mediated by the binding of a monoclonal antibody directed against Z-DNA

From the data presented in Figure 2 it is evident that the binding of mAb Z-D11 to a (−) supercoiled template abolishes transcription. We wanted to map the termination sites for transcription when the antibody is bound to Z-DNA. Thus, p101 was preincubated with increasing amounts of Z-D11, transcribed, and transcription products analyzed. As shown in Figure 4, stoichiometric amounts of antibodies are sufficient for a strong transcriptional block (lanes 7 and 8). The position where T7 RNP terminates transcription correlates with the length of run-off transcripts generated at the BamHI site at position +70 (Fig. 1c). On the other hand, transcripts terminating at the promoter distal site (+108), as well as those terminating within the CG stretch are no longer detectable (lanes 7 and 8). In a control, no termination is seen when relaxed p101 was preincubated with mAb Z-D11, again confirming the specificity of antibody-binding (data not shown; ref. 4).

It is clear that compared to the strong antibody mediated block, left-handed DNA represents a very weak obstacle for T7 RNP. A rough quantitative analysis by densitometry revealed that the Z-DNA alone blocks at least 50 times more weakly than the antibody-Z-DNA complex (compare lanes 1 and 8 in Fig. 4).

In order to estimate the proportion of RNPs terminating transcription due to the presence of the alternate DNA conformation (Fig. 4, lane 1), we assume that the DNA-bound antibody prevents all translocating T7 RNPs from passage through the GC stretch and take the amount of transcripts terminated at +70 to represent the total amount of T7 RNPs engaged in transcription (Fig. 4, lane 8). We then estimate that only a few percent of RNPs cannot pass through the GC stretch in the absence of mAb Z-D11. Consistent with this, the vast majority of labeled transcripts is found in long readthrough transcripts, of which only a small portion is shown at the top of Figure 4. In contrats, this amount is drastically reduced when the antibody is bound to the Z-stretch because polymerase molecules are not

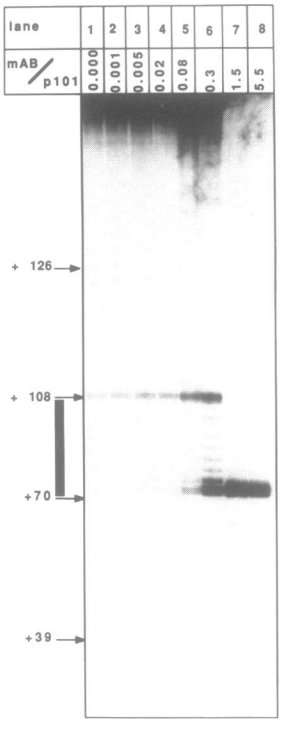

Figure 4. Complete blockage of T7 transcription at the promoter proximal B-to-Z junction requires stoichiometric amounts of antibody. Increasing amounts of monoclonal Z-D11, as indicated by the molar ratio of antibody to template (mAB/p101), were preincubated with (−) supercoiled p101, which was then transcribed by T7 RNA polymerase for 4 minutes at 37°C. Labeled transcripts were analyzed as described in the legend to Figure 3.

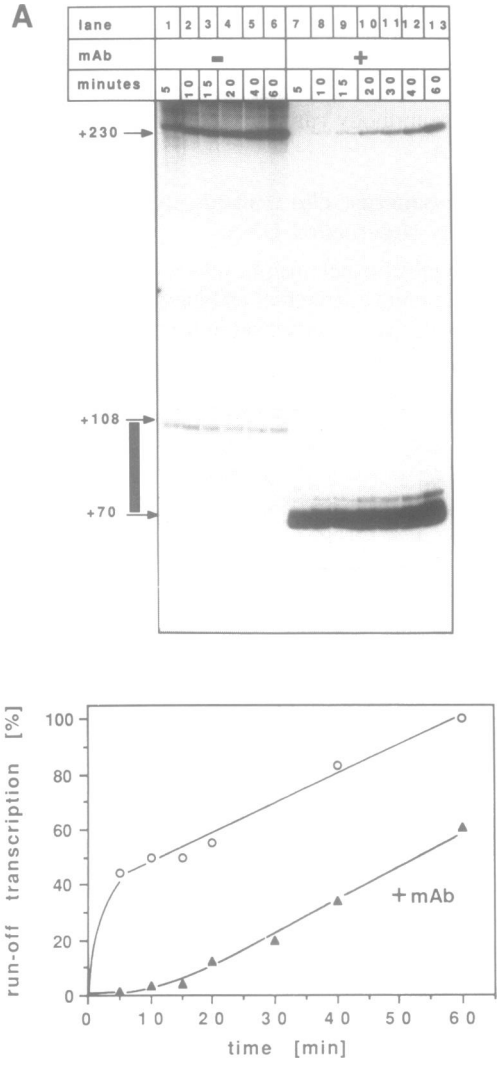

Figure 5. A) Stable antibody mediated block of transcription after the release of (−) supercoiling. Equal amounts of (−) supercoiled p101 were preincubated either with (lanes 7 to 13) or without (lanes 1 to 6) mAb Z-D11 on ice followed by the addition of restriction enzyme Pvu II and T7 RNP. Transcription was initiated by the addition of labeled NTPs at 37°C and aliquots for analysis of transcription products were taken at the time points indicated. The position of the CG stretch and the transcript lengths are indicated on the left side of the autoradiogram as before. B) Quantitation of the stability of the antibody-mediated transcriptional blockage. The bands corresponding to run-off transcripts generated at position +230 in Figure 5A in the presence (▲) or absence (○) of monoclonal antibody (mAb) were quantitated by densitometry. As 100% activity for run-off transcription, we took the signal obtained after 60 minutes in the absence of antibodies (Fig. 5A, lane 6), calculated the respective activities, and plotted them against the incubation time.

able to continue transcriptional elongation (compare the top of lanes 1 to 6 with lane 7 and 8).

Detailed studies have shown that the affinity of mAb Z-D11 to Z-DNA is very high. The association rate constant is about $2 \cdot 10^7$ $M^{-1} \cdot s^{-1}$ (19), and the dissociation rate constant is about $1 \cdot 10^{-5}$ s^{-1}. Furthermore, it was observed that the antibody remains stably bound to the CG stretch in the left-handed conformation for several hours even when (−) supercoiling was reduced or completely removed subsequent to antibody binding (F.M. Pohl, unpublished results). We were interested in testing whether a translocating polymerase might affect the stable antibody-binding after template linearization and performed the following experiment. Supercoiled p101 was preincubated with or without Z-D11, T7 RNP, and an excess of restriction enzyme Pvu II for 20 minutes on ice. This enzyme cuts p101 twice at positions −200 and +230 relative to the transcriptional start site (Fig. 1c), and digestion is found to be completed under our assay conditions within 5 minutes at 37°C (not shown). At various times after the initiation of transcription, aliquots were removed and transcripts analyzed. As shown in Figure 5A, lanes 1 to 6, in the absence of antibodies run-off transcripts accumulate at the Pvu II site (+ 230) by 5 minutes and continue to increase with time. The prebound antibody, on the other hand, drastically reduces the amount of RNPs reaching +230, while the amount of transcripts generated due to the antibody block at position +70 continues to increase (lanes 7 to 13). A quantitative analysis of run-off transcription to +230 (Fig. 5B) revealed that the antibody remarkably continues to block transcription of linearized templates for at least 30 minutes.

The result presented in Figure 5A also demonstrates that most, if not all, RNPs that stopped at the two junctions or within the CG stretch in supercoiled p101 in the absence of antibodies have either terminated or are not able to resume transcription. If so, the amount of these transcripts (lanes 1 to 6) would decrease with time because the Z-DNA has flipped back into the right-handed B-structure upon linearization within a few minutes, which in turn would allow the enzyme to continue with transcription. It is more likely therefore that the two junctions and the stretch of Z-DNA serve as a termination rather than a pausing site.

DISCUSSION

In this report we investigated the influence of left-handed DNA on transcription by T7 RNP. The results demonstrate that an alternating CG sequence in (−) supercoiled DNA can be efficiently utilized, and an analysis of transcription products revealed that only a small fraction of polymerases terminate elongation at positions corresponding to the region of the CG stretch.

What is the reason for termination and what are the mechanics of transcription when T7 RNP approaches a Z-stretch? Termination is most likely not the consequence of a sequence effect, since the DNA is transcribed normally when it is topologically relaxed and thus lacking the Z-stretch. From the data presented in Figure 3, we conclude that both structural junctions are the main impediments to elongating T7 RNPs and to an equal extent. It was demonstrated earlier that the DNA encompassing these junctions exhibits an unusual structure in (−) supercoiled DNA that might include a short region with single-stranded character (28,29). In this context it is interesting to note that elongating T7 RNP was recently found to transcribe short stretches of single-stranded DNA (30), and, in addition, is found capable to replicate specific RNA molecules (31). This might indicate that the enzyme is somewhat promiscuous with respect to template structure. Occasional termination of transcription at both junctions might therefore not only be a function of an unusual DNA structure with single-stranded character, but could also be due to a change of the sense of rotation of the DNA double helix from right- to left-handed (and *vice versa*) at the transition points. This interpretation is based on the assumption that the CG stretch remains in a left-handed form while the polymerase reads through it. We then speculate that once the polymerase has passed the first junction and adapted to the change in rotational sense of the template strands, transcription of the CG stretch is minimally affected until the enzyme reaches the second transition point where the sense of rotation changes again; now from left-handed back to right-handed. These changes in rotation, in conjunction with the presumed single-stranded character of the junctions, might slow T7 RNPs down to such an extent that a small fraction of enzymes falls off the template due to its weak DNA binding affinity (16,26). This would also help to explain why termination and not pausing is observed (Fig. 5A).

Other mechanisms are not excluded involving, for example, a localized enzyme mediated Z-to-B transition, possibly induced by the strand separation process of a translocating ternary complex. Such an enzyme mediated right-handed B-conformation adopted by the CG stretch could in principle be stable for a few minutes after polymerase passage and thus allowing following polymerases to translocate without hindrance before the track flips back into the left-handed conformation driven by (−) supercoiling (32). Continuous transcription through the CG stretch could further stabilize the B-conformation. In contrast, the Z-DNA-bound antibody might prevent such an enzyme mediated transition by stabilizing the left-handed conformation and thus blocking polymerase passage as observed in Figure 4.

If an enzyme mediated Z-to-B transition occurs, however, it is difficult to explain why RNPs terminate transcription at positions corresponding to the CG stretch and, even more pronounced, at the promoter distal junction (Fig. 3)—especially if the cooperative nature of such a transition is considered. Due to the relative slow relaxation times of up to 10 minutes observed for a non-enzyme mediated Z-to-B transition (19), we would also expect the promoter proximal junction to become the most prominent impediment in our experiments using T7 RNPs. Furthermore, one would expect polymerases to pause at the promotor proximal junction instead of terminating transcription. Recent data suggest that only about 3 base-pairs might be melted by both T7 RNP and *E.coli* RNP in a ternary complex (33,34). Surprisingly, *E.coli* RNP apparently cannot induce a Z-to-B transition when it encounters a Z-stretch but instead is stalled in front of the alternate DNA conformation until the Z-stretch is released by template linearization, after which the enzyme is able to transcribe through the CG stretch (1). Although our experiments do not distinguish between the various possibilities, we presently favour the scenario that the Z-stretch remains somehow in a left-handed conformation while the polymerase translocates through it.

We found that the Z-DNA-bound antibody presents an efficient roadblock for an elongating T7 RNP, thereby preventing the enzyme from reaching the second, promoter distal junction and to continue transcription of the remainder of the template (Fig. 4). The fact that the enzyme can effectively utilize left-handed DNA but is blocked by the stable binding of a Z-DNA specific protein might be used as a sensitive tool for the identification and characterization of other Z-DNA binding proteins, and (or) of

proteins that are capable of recognizing structural elements characteristic for B-to-Z junctions, such as single-strand specific DNA binding proteins (35).

We previously demonstrated that transcription-induced (−) supercoiling in a topologically closed domain can induce a B-to-Z transition in a stretch of $d(CG)_{16}$, which in turn is recognized by monoclonal Z-D11 (3). By stabilizing the alternate conformation in such a protein-DNA complex, structural information can be stored over considerable time (36). This could be the basis for an auto-regulatory pathway in which (−) supercoiling generated by transcription processes leads to structural transitions in the template which are recognized by specific factors. The presence of an alternate DNA conformation stored in a nucleoprotein complex could then be used as a regulatory element, just as shown in this study using mAb Z-D11 as a stable block for enzyme translocation. We think that such a system should in principle be capable of transiently memorizing whether and, possibly, to what extent a template has been utilized. Our findings represent a simple example of such a mechanism. However, these memory effects could also be of relevance in other pathways. Due to the elastic nature of DNA, the storage of structural information might be useful in the communication between different DNA segments separated from each other over large distances (37,38). This might play a role in the regulation of basic biological processes like gene expression, DNA replication, and recombination, or even in an interplay between these processes (39).

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