T7 RNA polymerase cannot transcribe through a highly knotted DNA template

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ABSTRACT

The ability of T7 RNA polymerase to transcribe a plasmid DNA in vitro in its linear, supercoiled, relaxed and knotted forms was analysed. Similar levels of transcription were found on each template with the exception of plasmids showing varying degrees of knotting (obtained using stoichiometric amounts of yeast topoisomerase II). A purified fraction of knotted DNA with a high number of nodes (crosses) was found to be refractory to transcription. The unknotting of the knotted plasmids, using catalytic amounts of topoisomerase II, restored their capacity as templates for transcription to levels similar to those obtained for the other topological forms. These results demonstrate that highly knotted DNA is the only topological form of DNA that is not a template for transcription. We suggest that the regulation of transcription, which depends on the topological state of the template, might be related to the presence of knotted DNA with different number of nodes.

INTRODUCTION

It is widely accepted that the topological state of DNA has an outstanding role in processes such as recombination, replication, gene expression and DNA packaging (1–4). Since DNA is supercoiled inside living cells, any passage of segments of DNA within a topological domain by topoisomerases (5,6), site-specific recombinases such as Tn3 resolvase (7), or during the integrative recombination in phage lambda (1) leads to knotting or catenation of the polynucleotide. Indeed, both the natural requirement for powerful topoisomerases in living cells and for the packing of DNA in viruses results in the knotting of DNA (8). The arrangement of the tailless capsid of the bacteriophage P2 is such that it leads to the formation of a complex knot (8). DNA knotting obliterate chromatin assembly in vitro, though the presence of knotted template does not inhibit the assembly over the relaxed plasmid (9).

While several studies have examined the effects of knotted DNA on site specific recombination and DNA replication, there have been no studies which have analysed the effect of a knotted DNA substrate on transcription. Notwithstanding, large differences in transcription rates have been observed depending on the substrate topology, other than those of the knotted form, which suggests that the topology of DNA molecules has a fundamental role in the transcription process in vivo and in vitro (10–12). Circular plasmids appear to be more efficiently transcribed than linear ones, and transcription is substantially favoured by the torsional stress. Eukaryotic RNA polymerases prefer to elongate RNA on templates which are found under torsional stress, using a minimal set of transcription factors (13). There are reasons to believe that the sequence dependent flexure of DNA has little influence over the activity of the T7 promoter in Escherichia coli, but it has incidence on transcription with the E.coli polymerase. Moreover, the position of promoter sequences with respect to the confines of a nucleosome displays large effects in the initiation or elongation steps of transcription by the T7 RNA polymerase (10,14).

In this study, we have used a plasmid with different degrees of topological knotting (obtained by treatment with stoichiometric amounts of yeast topoisomerase II) and containing a T7 promoter and transcription stop signal, to gain new insights into the effect of this topological form on DNA transcription. The T7 RNA polymerase was chosen since, unlike the eukaryotic RNA polymerase II or the E.coli RNA polymerase, it is a single-subunit enzyme (15) that performs all the functions required for transcription, including promoter recognition, initiation and termination, without the need for auxiliary protein factors (15–17).

When highly-knotted plasmid, obtained by treatment of either supercoiled or relaxed plasmid DNA with stoichiometric amounts of topoisomerase II, was employed as a template for transcription in vitro by the T7 RNA polymerase, there was a total absence of transcript formation. However, when it was unknotted, by treatment with topoisomerase II, it recovered its capability of acting as a template for transcription.

MATERIALS AND METHODS

Enzymes and DNA

Topoisomerase II was purified from Saccharomyces cerevisiae as described elsewhere (18). The plasmid pET14b, which contains the T7 bacteriophage promoter and terminator signals, was purchased from Novagen. T7 RNA polymerase and ribonuclease inhibitor (RNasin) were purchased from Promega.

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Knotting and unknotting reactions

Knotting reactions were carried out as described previously (9) using a topoisomerase II/DNA mass ratio of 4. The final concentration of closed circular DNA (either relaxed or supercoiled) was around 10 ng/µl. The time reaction varied from 1 h to overnight and the reactions were stopped and deproteinized using proteinase K and phenol extraction. Unknotting/relaxing reactions with topoisomerase II were performed using smaller topoisomerase II to DNA ratios (9).

Isolation of highly knotted DNA

In order to obtain a homogeneous population of highly knotted DNA, ~25 µg of pET14b were knotted as described above, run for 2 h at 60 V on a 0.8% agarose gel, containing TBE Buffer [90 mM Tris-borate (pH 8.3), 2 mM EDTA]. The highly knotted DNA fraction was recovered (cut) from the gel and purified using the Gene clean kit (Bio101, Inc.).

In vitro transcription assays

Transcription was performed in a buffer containing 40 mM Tris–HCl (pH 7.5), 10 mM NaCl, 6 mM MgCl₂, 2 mM spermidine, 2 U RNAsin and 10 mM dithiothreitol; and 2.5 mM NTPs (500 µM each of CTP, UTP, GTP and 1 mM ATP), together with 50 ng of the different topological forms of plasmid pET14b, and 5 U T7 RNA polymerase (Promega). The samples were incubated at 37°C for 30 min and stopped by addition of phenol. The aqueous phase was ethanol precipitated and the template was sometimes subsequently linearized by treatment with HindIII.

To analyze the presence of short (aborted) transcripts, we performed additional experiments using radiolabelled RNA (20 µCi [α-32P]UTP in the transcription mix), and denaturing polyacrylamide gel electrophoresis (see below).

Gel electrophoresis and quantitative analysis

The different topological forms obtained using topoisomerase II (see above) were resolved on 0.8% agarose gels in the absence of ethidium bromide. The products of the transcription in vitro were analyzed on 2% agarose gels in TBE buffer. The gel and the running buffer contained 0.5 µg/ml ethidium bromide. All the gels were photographed under a UV light using Polaroid 665 film. Radioactive transcripts were heated at 90°C for 2 min prior to electrophoresis on 6% polyacrylamide gels containing 7 M Urea. Gels were subjected to autoradiography at 70°C with an intensifying screen.

Densitometric scans of the negatives were carried out in a Molecular Dynamics densitometer to produce profiles from which the relative intensity of the full length transcript was quantified.

RESULTS AND DISCUSSION

In this article we assess the relationship between the topoisomerase II-mediated knotting of plasmid pET14b (which contains initiation and termination signals for the phage T7 RNA polymerase) and its usefulness as a template for transcription. Figure 1 shows that the supercoiled form of the plasmid incubated with stoichiometric amounts of yeast topoisomerase II became knotted. Each band of the extended ladder (lane 2) corresponded to a population of knots with a different number of crosses (nodes), in which the upper band of the knotted series was the trefoil, the simplest knot.

Figure 1. Topoisomerase II produces knots in plasmid DNA with different degrees of compactness. Supercoiled pET14b was knotted using topoisomerase II at an enzyme to DNA mass ratio of four. In unknotting experiments, the knotted DNA was treated with a smaller amount of enzyme (catalytic conditions). The figure displays a 0.8% agarose gel stained with ethidium bromide. Lane 1, 0.1 µg supercoiled pET14b plasmid; lane 2, 0.3 µg knotted DNA; lane 3, 0.3 µg unknotted DNA; lane 4, 0.3 µg relaxed pET14b DNA; lane 5, 50 ng knotted DNA digested with HindIII. Forms I, II and III represent supercoiled, relaxed and linear DNA respectively. Lane 2 shows that the knotted species run electrophoretically displaying a broad range of mobility according to its compactness.

On the other hand, the knotted DNA can be unknotted (relaxed) by using smaller amounts of topoisomerase than those used in the initial knotting experiments (9). Here, any relaxed DNA originating from a previously knotted substrate is described as ‘unknotted’, since the unknotted species run as a typically relaxed DNA during electrophoresis (cf. lanes 3 and 4 in Fig. 1). As with other topological forms, knots can show varying levels of compactness. Thus, a minimum of three crosses were required to produce the simplest knot (trefoil), and as the number of nodes increases the compactness is progressively enhanced (6,9,19). For the sake of comparison, Figure 1 displays the linear (form III), supercoiled (form I) and relaxed (form II) forms of pET14b. The linear form was obtained by treatment of a knotted sample with HindIII.

Figure 2 shows the full transcripts (191 nucleotides long) produced by the T7 RNA polymerase using pET14b as a template, under the different topological states displayed in Figure 1. The knotted template, Figure 2 lane 3, generated a marked decrease in the RNA synthesis, while qualitatively similar amounts of transcript were observed for the other ‘topological templates’. These results suggest that knotted DNA acts as an impediment to transcription, even for a highly processive enzyme such as the T7 RNA polymerase (16). The different templates were also visible at the top of the agarose gels.

Since this partial inhibition of the transcription procedure could be related to the compactness of the template, we sought to gain further insights into the inhibition of transcription by the phage RNA polymerase by analyzing the transcription procedure using a highly knotted template. Therefore, highly knotted plasmid (Fig. 3, lane 4) was isolated from a preparative agarose gel as described above (see also Material and Methods). The most highly knotted fraction of the heterogeneous population (indicated by a
The bracket in Fig. 3 exhibited the highest electrophoretic mobility concomitant with its dense compactness (9). Lane 5 in Figure 3 with the templates displayed in Figure 3. It is evident, even to the unaided eye, that highly knotted DNA was totally refractory to transcription, rather than well-phased ones. Therefore, aborted transcripts of well-determined size did not accumulate. The results in Figures 2, 4 and 5 clearly suggest that highly-compacted knots in DNA are able to form effective blocks that impede transcription, regardless of whether they are a dynamic rather than a well-positioned obstacle. A quantitative comparison of the relative amounts of the 191 nt transcript is displayed in Figure 6. It shows that in both experiments using knotted and highly-knotted DNA, the supercoiled, relaxed or linear DNA produced almost the same quantities of transcript (cf. Figs 2 and 4).
RNA polymerase. In fact, this enzyme is quite small (15) compared to the larger eukaryotic transcription systems, which are known to have a size and multiprotein complexity (22). Nevertheless, topoisomerases I and II, are needed for the various topological manipulations of DNA in vivo. It is worth mentioning that one of them, the yeast topoisomerase II, is able to produce knotted plasmids with equivalent efficiency, using either supercoiled or relaxed DNA. The knotted DNA can be unknotted with the same enzyme, though under different experimental conditions, but while the knotted template does not transcribe efficiently, depending on the level of knotting (Figs 2 and 4), the unknotted template is transcribed. An equivalent effect of the knotting on chromatin assembly has been described (9). In summary, our results show how the presence of knots in DNA might decrease and even abolish transcription from strong promoters, and how their formation can strongly affect transcription from more complex systems, such as eukaryotic RNA polymerases which are large protein complexes (22).

In general, gene activation/repression is understood as being controlled by proteins which work as trans-acting factors. Therefore, repressors, activators and nucleosome displacement among other events (12,22–24) have been well characterized. Nevertheless, knotting/unknotting appears to be able to exercise fine tuning over biological processes as replication, site-specific recombination or DNA package (1–4), and the control of transcription depending on the density (amount) of knots, the other topological templates retain the same capacity of acting as templates in any experiment.

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