Probability of the Site Juxtaposition Determines the Rate of Protein-Mediated DNA Looping

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ABSTRACT Numerous biological processes are regulated by DNA elements that communicate with their targets over a distance via formation of protein-bridged DNA loops. One of the first questions arising in studies of DNA looping is whether the rate of loop formation is limited by diffusion of the DNA sites. We addressed this question by comparing the in vitro measured rates of transcription initiation in the NtrC-*glnAp2* enhancer-dependent transcription initiation system with predictions of two different theoretical models. The promoter and enhancer were in a 7.6-kb plasmid and separated by 2.5 kb. The measurements were performed for different values of the plasmid superhelix density, from 0 to −0.07. Earlier theoretical analysis, based on the Monte Carlo simulation of DNA conformations, showed that if the rate of loop formation is determined by the equilibrium probability of juxtaposition of the DNA sites, the rate should be ~100 times higher in supercoiled than in relaxed DNA. On the other hand, Brownian dynamics simulation showed that if the rate of loop formation is limited by the site diffusion, it should be nearly independent of DNA supercoiling. We found that efficiency of the transcription initiation increases by nearly two orders of magnitude as a result of the corresponding increase of the template supercoiling. This clearly shows that the rate of bridging in the enhancer-promoter system is not limited by diffusion of the DNA sites to one another. We argue that this conclusion derived for the specific system is likely to be valid for the great majority of biological processes involving protein-mediated DNA looping.

INTRODUCTION

Many processes of DNA metabolism involve interaction between or among two or more DNA sites widely separated along the molecule contour. Such processes require formation of loops by the DNA molecule (1). Loops are observed during DNA replication and repair (2), transcription regulation (3), genome rearrangements by site-specific recombinases (4), topological changes by topoisomerases (5), and action of many restriction enzymes (6). In at least some cases formation of the loops is a rate-limiting step for the entire process (7). Knowledge of the dynamic principles of DNA loop formation is important for better understanding of all these processes.

The process of loop formation can be divided into two steps: juxtaposition of two DNA sites and closing of the loop by a protein bridge. If the bridging is faster than separation of the juxtaposed sites by diffusion, the rate of loop formation is determined by the time of the first site juxtaposition, so the rate is limited by the rate of diffusion of the DNA sites to one another. Alternatively, if the separation of the juxtaposed sites by diffusion is faster than the protein bridging, many collisions of the sites precede stable bridging. In the latter case the rate of looping is proportional to the total fraction of time the sites are juxtaposed or to the equilibrium probability of the juxtaposition. Although many aspects of protein-mediated DNA looping have been studied both experimentally (see Halford et al. (6) for review) and theoretically (8–11), it has not been investigated which of two models better describes protein-mediated DNA looping.

The computer simulations showed that the equilibrium probability of juxtaposition of two selected sites in supercoiled DNA (scDNA) is ~100 times higher than in the relaxed molecule, whereas the average time of the first juxtaposition of the same sites is nearly unaffected by supercoiling (9,10). These simulation results enable us to discriminate between the two mechanisms of bridging by studying the rate of bridging as a function of DNA supercoiling. In this work we studied the dynamics of loop formation during distant, enhancer-dependent activation of prokaryotic transcription.

Transcriptional enhancers are relatively short (30–200 bp) DNA sequences usually composed of several binding sites for activator protein(s) and are capable of activating transcription initiation from the promoters, which are often located at considerable distances in vivo (up to 60 kb in Eukaryota and up to 15 kb in Prokaryota; see Blackwood and Kadonaga (12) and Bondarenko et al. (13) for review). This activation is independent of enhancer orientation on DNA and of its position upstream or downstream of the promoter. Proteins bound at the enhancer and promoter directly interact with each other, forming a DNA loop (14,15). One of the most extensively studied prokaryotic enhancers is

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NtrC(NRI)-dependent, σ^{54}-dependent transcriptional enhancer of *E. coli* that controls transcription from the *glnAp2* promoter (see reviews (13,16,17)). This enhancer, consisting of two high-affinity NtrC-binding sites, can strongly activate transcription when positioned up to at least 3.5 kb in vitro (18,19). NtrC is an activator that binds to the enhancer and, after phosphorylation mediated by NtrB(NRII) protein, interacts with the Eσ^{54} RNA polymerase (RNAP) holoenzyme, bound at the promoter in form of the closed, inactive initiation complex (RP_C) (Fig. 1). This enhancer-promoter (E-P) interaction activates conversion from the closed to the open (RP_O) complex (20–23). Phosphorylation of NtrC also activates its ATPase activity, which is required to stimulate the $RP_C \rightarrow RP_O$ transition. During E-P interaction, the spacer DNA forms a loop (24,25).

In this work, the efficiencies of protein-mediated DNA looping in plasmids supercoiled to different superhelix densities were measured in vitro. Comparing these measurements with predicted dependences of the looping efficiency on DNA supercoiling for the two models, we concluded that the rate of loop formation between enhancer and promoter is determined by the probability of enhancer and promoter juxtaposition rather than the rate of their first collision.

MATERIALS AND METHODS

DNA and proteins

Negatively supercoiled pLY10 DNA template was purified as described (26). The promoter and enhancer of the 7.6-kb plasmid are separated by 2.5 kb.

All proteins and protein complexes were purified according to previously described protocols (27). Chicken blood extract (CBE) containing strong topoisomerase I activity was prepared as described previously (28).

Preparation of plasmid DNA with different superhelix densities

Negatively scDNA templates, purified from XL1-Blue strain of *E. coli*, were relaxed by incubation at 50 μg/ml with CBE (0.07 μl of CBE per 1 μg of plasmid DNA) in 200 mM NaCl, 20 mM Tris-HCl (pH 8.0), 0.25 mM EDTA, and 5% glycerol for 45 min at 37°C. To obtain plasmids that are negatively supercoiled to various extents, the pLY10 plasmid was relaxed in the presence of the following concentrations of chloroquine (μg/ml): 50 ($\sigma = -0.01$), 100 ($\sigma = -0.022$), 200 ($\sigma = -0.035$), 300 ($\sigma = -0.046$), 500 ($\sigma = -0.056$), 700 ($\sigma = -0.069$), and 1000 ($\sigma = -0.074$). The relaxed and partially supercoiled plasmids were then purified after proteinase K treatment (150 μg/ml) for 2 h at 37°C in the presence of 1% SDS, followed by phenol/chloroform extraction, ethanol precipitation, and passage through a Sephadex G-50 spin column. The levels of DNA supercoiling (σ) were determined after separation of the purified pLY10 topoisomers in agarose gels containing various concentrations of chloroquine (29). The obtained sets of pLY10 topoisomers have average superhelical densities σ of 0, −0.01, −0.022, −0.035, −0.046, −0.056, −0.069, and −0.074.

In vitro transcription

Conditions for in vitro transcription were optimized for maximal utilization of the pLY10 templates. Transcription was conducted as described (27) for 1 min to guarantee that fewer than 20% of templates were activated in the experiments, and the number of transcripts was proportional to the rate of formation of the E-P complexes.

Computer simulation

The Monte Carlo simulations were used to compute the probability of juxtaposition of DNA sites at different degrees of DNA supercoiling (9). In these simulations a DNA molecule is modeled as a chain consisting of rigid cylinders with elastic bending potential. The length of each cylinder corresponds to 10 nm, one-fifth of the chain persistence length. The cylinders are not interpenetrable, and their diameter, d, accounts both for the geometrical diameter of the double helix and for electrostatic repulsion between charged DNA segments. The value of d, 5 nm, corresponded to physiological ionic conditions. The torsional rigidity of DNA was equal to $3 \cdot 10^{-19}$ erg·cm. The length of the model chain and separation between the analyzed sites corresponded to pLY10 plasmid. The time of the first collision of the two DNA sites was obtained by the Brownian dynamics (BD) simulations (10). Essentially the same discrete wormlike chain, with addition of the finite stretching rigidity, was used in the BD simulations. The hydrodynamic interaction between the model chain and solution and between the chain segments was also taken into account (10). All parameters of the model chain were identical for MC and BD simulations. Two chain segments were considered to be juxtaposed when the distance between their centers was <10 nm.

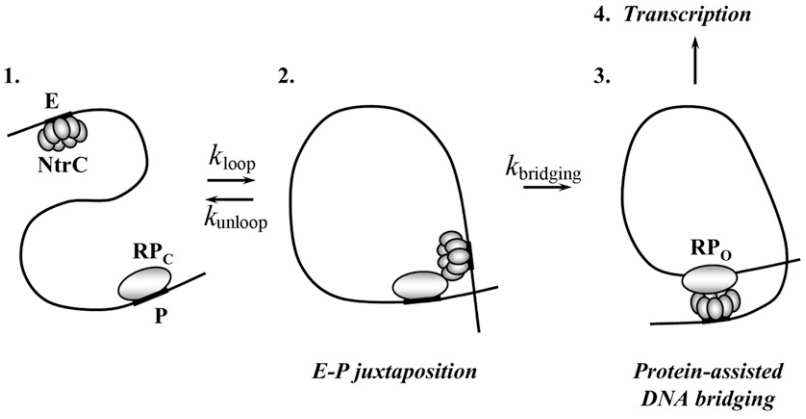

FIGURE 1 Diagram of the enhancer-dependent transcription activation of the *glnAp2* promoter. (*1*) Phosphorylated NtrC activator is pre-bound at the enhancer (E) and is able to interact with closed complexes RP_C preformed at the promoter (P). (*2*) Juxtaposition of the enhancer and promoter is accompanied by DNA looping and may result in (*3*) the E-P protein bridging; otherwise the two DNA-protein complexes may diffuse away from one another. The rate constant $k_{bridging}$ specifies the rate of bridging for DNA conformations with juxtaposed enhancer and promoter. Two models of the loop formation that are considered in the study correspond to the condition of $k_{unloop} \gg k_{bridging}$ and $k_{unloop} \ll k_{bridging}$. The bridging involves a specific E-P protein-protein interaction and results in the formation of the transcription-competent RP_O. It is important that under our experimental conditions this step is irreversible, so the number of protein-mediated E-P loops is equal to the number of RP_O (7). (*4*) Adding heparin and labeled NTPs allows transcription from RP_O. Because heparin prevents formation of new RP_O (preformed RP_O and the elongation complexes are resistant to heparin), the transcription is limited by one round. Thus, the number of transcripts is equal to the number of open complexes formed before addition of heparin (7).

RESULTS

Intrinsic activity of the *glnAp2* promoter does not depend on the level of DNA supercoiling

Supercoiling can change two features of DNA that, in turn, could affect the level of transcription. First, an increase of the level of negative supercoiling results in higher probability of basepair opening (30–32) that could modify the efficiency of transcription initiation from the *glnAp2* promoter. This local, promoter-specific effect of DNA supercoiling could considerably change the rate of transcription (33,34). Second, DNA supercoiling changes tertiary conformations of DNA molecules, causing formation of branched interwound superhelices (see review (35)). In a highly supercoiled form of the pLY10 plasmid, the probability of juxtaposition of specific DNA sites can be ~100 times higher than in the relaxed form (9), which could change the efficiency of E-P communication. Because in this study we wanted to address the second, global effect, the contribution of the local effect of DNA supercoiling on the transcription through the increased probability of basepair opening was evaluated first.

To learn whether the intrinsic activity of the *glnAp2* promoter depends on the level of negative supercoiling, we explored the observation that in the absence of the enhancer *glnAp2* promoter has a weak basal activity (18). Enhancer-independent transcription is specific and activator-dependent because it requires the presence of all other key components of enhancer-dependent transcription, including the activator NtrC, the protein kinase NtrB, σ^{54}, and ATP (data not shown). The effect of negative DNA supercoiling on the intrinsic activity of the *glnAp2* promoter was lower than 1.5-fold within the experimental range of σ-values (from 0 to -0.075, Fig. 2). Because negative DNA supercoiling can change the efficiency of enhancer-dependent, distantly activated transcription up to 50-fold (see below), we concluded that in our system supercoiling predominantly affects the transcription efficiency by changing global conformations of the plasmid DNA.

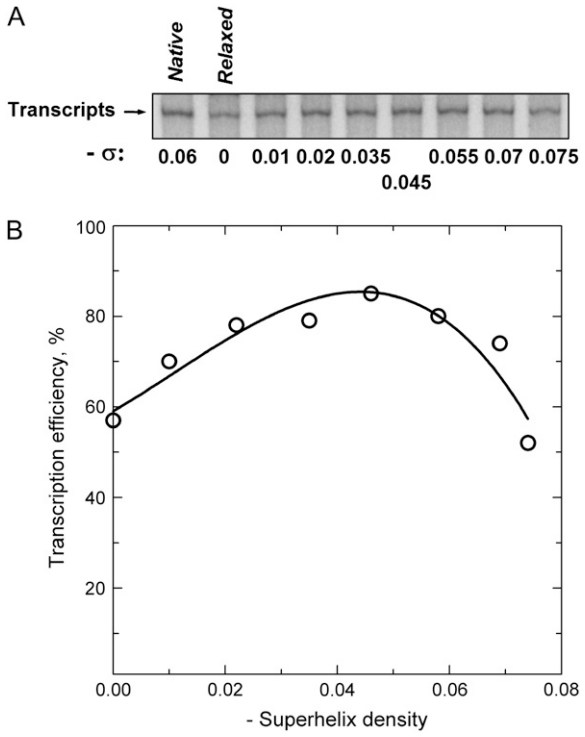

FIGURE 2 Supercoiling does not significantly impact the intrinsic activity of the *glnAp2* promoter. (*A*) Topoisomers of enhancerless pAN6 plasmid supercoiled to different extents were transcribed in the presence of an excess of NtrC protein. Transcripts were analyzed by denaturing polyacrylamide gel electrophoresis. (*B*) Intensities of the bands containing specific transcripts were quantified on a PhosphorImager and plotted as a function of the superhelical density of the plasmid DNA (the efficiency of transcription of native pAN6 ($\sigma = -0.06$) is 100%). All measurements were repeated twice; the standard deviation for the measured values of the promoter activity is close to 10%. The data show that the differences in supercoiling levels do not result in significant changes of the efficiency of enhancer-independent transcription from the *glnAp2* promoter.

DNA supercoiling strongly facilitates the efficiency of enhancer-dependent promoter activation

Next, the rates of protein-mediated DNA looping were measured using an enhancer-dependent transcription assay (Fig. 1). We prepared a set of samples of pLY10 plasmid with different values of negative superhelix density, σ (see Methods). Then the efficiencies of transcription of differently supercoiled pLY10 templates were compared in a single-round in vitro transcriptional assay (see Liu et al. (7) for details). The templates were preincubated with all components of the transcription machinery. This results in binding of NtrC activator to the enhancer and formation of the closed, inactive RP_C on the *glnAp2* promoter (18,36,37). The NtrC activator cannot interact with the RP_C and activate transcription unless it is phosphorylated by the NtrB protein, which is also present in the reaction but was inactive in the absence of ATP (24,25). Then ATP was added for a limited time (1 min) to provide the substrate for phosphorylation of the NtrC activator. NtrC phosphorylation makes possible E-P protein-mediated interaction and formation of the transcriptionally active RP_O. Subsequent addition of nucleotides together with heparin allows completion of initiated transcripts and prevents formation of new initiation complexes, resulting in a single-round transcription from each activated promoter. The majority of RNA polymerase molecules terminate after formation of 401-nt RNA transcript (7). Thus, the readout of the assay (the amount of 401-nt transcripts) reflects the number of the open initiation complexes formed, which is equivalent to the rate of the protein-mediated E-P looping (7). Of course, the equivalence is valid only under the condition that a small fraction of *glnAp2* promoters is activated during the experiments. Therefore, the experimental conditions were selected with a reaction condition to allow formation of open complexes on $<20\%$ of the templates.

The results of the experiments show that efficiency of enhancer-dependent, distantly activated transcription greatly increases as the level of negative supercoiling of the pLY10 plasmid changes from 0 to −0.075 (Fig. 3). Over the entire interval of σ this increase constitutes nearly two orders of magnitude. Thus, the rate of the protein-mediated E-P looping (bridging) is dramatically increased by negative DNA supercoiling of the plasmid.

Probability of E-P juxtaposition determines the efficiency of transcription activation

We compared the experimental results on the efficiency of transcription activation with the theoretical predictions for two models of the protein-mediated DNA looping. The first model assumes that the rate of loop formation is not limited by diffusion of the promoter and enhancer into close proximity to each other. In this case many collisions of the promoter and enhancer precede formation of the loop, and the rate of looping is proportional to the equilibrium probability of the site juxtaposition. This probability strongly increases as a function of $|\sigma|$ (9). We recalculated the probability as function of σ for contour length and E-P separation in the pLY10 plasmid (Fig. 3 *B*, *solid line*). The second model assumes that the rate of loop formation is limited by the rate of diffusion of one site to another, so the first juxtaposition of the sites results in the loop formation. Brownian dynamics simulation shows that the average time of the first juxtaposition is nearly independent of $|\sigma|$ (10). This simulation was used to obtain the corresponding dependence for pLY10 plasmid (Fig. 3 *B*, *dashed line*). Clearly, the experimental data are consistent only with the first model suggesting that the rate of loop formation is not limited by the rate of the site diffusion.

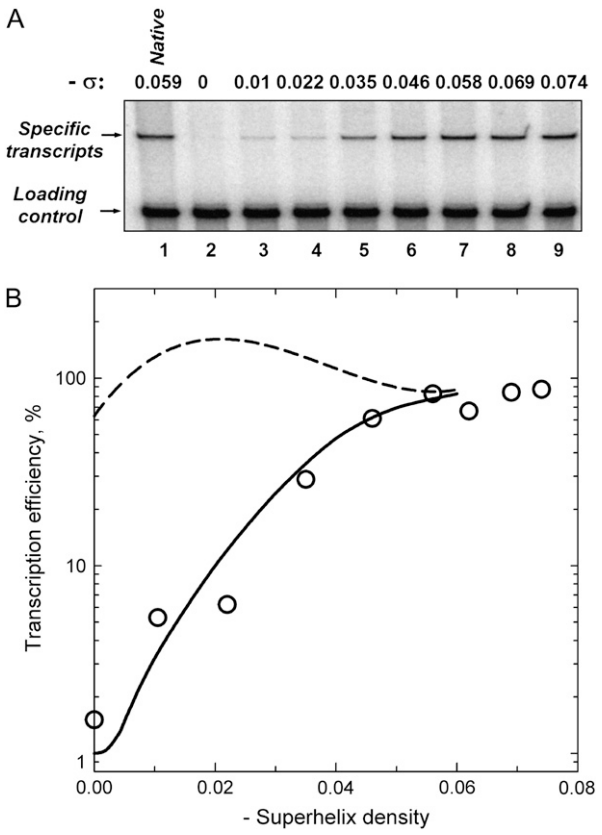

FIGURE 3 Effect of DNA supercoiling on the rate of enhancer-dependent transcription activation and its comparison with theoretical models. (*A*) The labeled transcripts, synthesized on supercoiled plasmid pLY10 with different values of σ. The transcripts were separated by denaturing polyacrylamide gel electrophoresis. (*B*) Intensities of the bands containing the specific 401-nt transcript were quantified on a PhosphorImager and are shown by open circles. The standard deviations for the measured values of the promoter activity are close to 20%. The solid line shows the dependence of the juxtaposition probability on σ; the rate of the first juxtaposition as a function of σ is shown by the dashed line. Both theoretical dependences are shown in arbitrarily chosen relative units, so only the changes of the corresponding values with σ should be compared with the experimental data. The standard deviations for both theoretical dependencies are close to 10%. The theoretical dependences were obtained for the plasmid pLY10 used in the experiments.

DISCUSSION

Supercoiled DNA molecules form interwound branched superhelices. In such a conformation, many pairs of sites, separated along the DNA contour, are positioned in close vicinity to each other. In other words, in each conformation of scDNA there are many pairs of juxtaposed sites. Typical conformation of relaxed DNA of the same size is characterized by a much smaller number of juxtaposed sites. Therefore, the probability of juxtaposition for any pair of sites is ~2 orders of magnitude higher in scDNA than in relaxed DNA (9). The corresponding data, obtained by the Monte Carlo simulations, perfectly matches the measured dependence of the transcription efficiency on DNA supercoiling (Fig. 3 *B*). On the other hand, it is known from the Brownian dynamics computer simulation that the average time of the first juxtaposition of two sites is hardly affected by DNA supercoiling (10). The latter result derives from the fact that supercoiling slows down the exchange between different DNA conformations (10). Comparison between the experimental and theoretical data clearly shows that formation of a DNA loop, mediated by the E-P interaction, is not a diffusion-limited process. The first collision of the specific sites does not result, typically, in protein bridging. The rate of bridging is proportional to the fraction of time the sites are juxtaposed or to the probability of their juxtaposition.

We studied here only one specific system of protein-mediated DNA looping. We think, however, that our conclusion should be valid for the great majority of such systems. Indeed, the conclusion means that reorientations and repeating collisions of the juxtaposed DNA sites, often bound with proteins, are not fast enough to provide the bridging during a single juxtaposition event before the sites diffuse away one from another. These relative rates of site reorientation and separation cannot be changed dramatically because both of

them are essentially specified by the internal flexibility of DNA molecules (this is not true for the juxtaposition probability, which is affected by many factors). The double helix is a very rigid molecule, and, therefore, any movement of the sites involves relatively large DNA segments. Thus, changing the size of bound proteins should not change essentially the size of the complex, which moves as a rigid body. Correspondingly, it should not change the rates of separation and reorientation of the juxtaposed DNA sites. For the same reason, the rates of separation and reorientation of the juxtaposed sites should not be affected by the spacing between them. The bridging of the juxtaposed sites could occur faster if it is mediated by very flexible proteins, attached to the DNA sites. In this case the protein bridging can occur for a wider range of the mutual orientations and distances between the sites because of faster motion of the protein interfaces. However, there are data that suggest that even increased flexibility of the bridging proteins can hardly make DNA looping a diffusion-limited process. It has been shown that protein-free DNA cyclization, which occurs by joining long cohesive ends, is not a diffusion-limited process (38), although the single-stranded cohesive ends are very flexible. Therefore, we believe that the conclusion, which was drawn for a particular E-P system, is valid in the general case of protein-mediated DNA looping in vitro. Our analysis, combined with other available data, strongly suggests that protein-mediated DNA looping is not a diffusion-limited process. Of course, inside the cell DNA is bound with chromatin proteins, which completely change DNA conformational properties, and our analysis cannot be extended to this case.

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