ABSTRACT

In both eukaryotes and prokaryotes, negative supercoiling of chromosomal DNA acts locally to regulate a variety of cellular processes, such as transcription, replication, recombination and response to environmental stresses. While studying the interaction between the Hin recombinase and mutated versions of its cognate DNA-binding site, we identified a mutated DNA site that binds Hin only when the DNA is supercoiled. To understand the mechanism of this supercoiling-responsive DNA site, we used NMR spectroscopy and fluorescence resonance energy transfer to determine the solution structures and dynamics of three related DNA oligonucleotides. The supercoiling-responsive DNA site formed a partially unwound and stretched helix and showed significant flexibility and base pair opening kinetics. The single CAG/CTG triplet contained in this DNA sequence displayed the same characteristics as do multiple CAG/CTG repeats, which are associated with several hereditary neuromuscular diseases. It is known that short DNA sequence motifs that have either very high or low bending flexibility occur preferentially at supercoiling-sensitive bacterial and eukaryotic promoters. From our results and these previous data, we propose a model in which supercoiling utilizes the intrinsic flexibility of a short DNA site to switch the local DNA structure from an inefficient conformation for protein binding to an efficient one, or vice versa.

INTRODUCTION

The chromosomal DNA of both eukaryotes and prokaryotes is negatively supercoiled, either by the wrapping of DNA around histone proteins or by the topological constraints imposed upon closed circular DNA, respectively. Supercoiling plays an important role in a variety of cellular processes, including transcription, replication, recombination and response to environmental stresses (1,2). Global supercoiling changes are known to act locally and regulate the transcription of genes with promoters that are sensitive to supercoiling (~7% of the Escherichia coli genome) (3). Although the mechanism by which global supercoiling alters local DNA structure is not clearly understood, it is well known that many proteins bind preferentially to supercoiled rather than relaxed or linear DNA. In addition, it has been shown that changes in protein binding affinity and/or specificity induced by supercoiling are dependent on the local DNA sequence [e.g. (4)].

In our study of the DNA recombinase Hin from Salmonella typhimurium, we observed supercoiling-induced local structural changes in the Hin DNA-binding site. Hin catalyzes a site-specific DNA inversion between two 26 bp inverted sequences (hixL and hixR) that flank a 933 bp DNA segment. This invertible segment contains a promoter that directs the coordinate expression of the fljA and fljB genes, which encode H2 flagellin and a repressor of the H1 flagellin gene (fljC), respectively. By inverting this 933 bp segment, Hin regulates the expression of two major flagellar structural proteins, the H1 and H2 flagellins, which allow the bacteria to escape the host’s immune system (5). During the first stage of DNA inversion, Hin binds to each of the 26 bp hix DNA sites as a dimer with high affinity (Kd ~ 10^-10) (6). It has been suggested that the specificity of Hin binding results from direct or water-mediated sequence-specific contacts made by the Hin protein with the major groove at positions 9–13 and with the minor groove at positions 5–6 (numbering from the center of the inverted sequence, Figure 1A) (7,8). However, in the present study, we show that (i) a DNA mutation at the central positions (+1,–1) of the hix site also modulates Hin binding and (ii) the mutated hix site, which does not bind Hin when the DNA is relaxed, can bind Hin when the DNA is in a supercoiled state. The negative supercoiling is required for
formation of the complete Hin inversion complex (9,10); however, it is not necessary for the initial recognition of the hix site by Hin (6,11). Therefore, the supercoiling-induced Hin binding observed in our study is solely caused by the properties of the mutated DNA site and hence provides a model of DNA site that responds to supercoiling.

To characterize this supercoiling-responsive DNA site, we have determined the solution structures and dynamics of three hix-related DNA oligonucleotides by NMR spectroscopy and fluorescence resonance energy transfer (FRET). Our results show that the supercoiling-responsive mutant hix site has a partially unwound and stretched helix structure and shows significant flexibility and base pair opening kinetics. The single CAG/CTG triplet contained in the supercoiling-responsive hix site displayed the same characteristics as CAG/CTG repeats, which are associated with several hereditary neuromuscular diseases such as myotonic dystrophy and Huntington’s disease (12). Our results suggest that supercoiling affects predominantly the highly flexible DNA site and that such changes can switch the local DNA structure from an inefficient conformation for protein interaction to an efficient one, or vice versa.

MATERIALS AND METHODS

Hin protein preparation

Wild-type and G102A mutant Hin proteins were expressed and purified as described previously (13).

Electrophoretic mobility shift assay (EMSA)

PCR-amplified, 200 bp, double-stranded DNA (dsDNA) fragments containing the hix-AT, hix-CG and hix-CG sites were end-labeled with T4 polynucleotide kinase and [γ-32P]ATP. Binding mixtures (20 μl) containing 0.8 nM of labeled DNA and 0–200 ng of Hin protein in binding buffer [0.2 M Tris–HCl (pH 7.5), 1 M NaCl, 10 mM EDTA, 100 mM DTT and 50 mM MgCl2] were incubated for 10 min at 25°C. The mixtures were subjected to electrophoresis on 5% polyacrylamide gels.

DNase I footprinting

Supercoiled plasmid DNA (20 nM) containing the hix-AT, hix-AG and hix-CG sites were pre-incubated with 0–50 ng of Hin protein in 50 μl of the binding buffer for 10 min at 25°C. After the addition of 1 U of DNase I, the incubation was continued for 2 min. The digested DNA was isolated by phenol/chloroform extraction and extended with a 32P-end-labeled primer. The extension products were separated on an 8% polyacrylamide/7 M urea gel.

NMR experiments

DNA oligonucleotides for NMR experiments were purchased from Bioneer Co., Ltd. (Daejeon, Korea). Buffer conditions for the NMR experiments were 10 mM sodium phosphate (pH 6.8) and 100 mM NaCl. All NMR spectra were obtained on a Varian Inova 600 MHz spectrometer except for the 1H-31P heteronuclear correlation spectra, which were acquired on a Bruker DRX 600 MHz spectrometer. The 2D NOE spectroscopy (NOESY) (τm = 180 ms) was carried out in 95% H2O/5% D2O at 4°C. The 2D NOESY (τm = 80, 160 and

Figure 1. Supercoiling-dependent Hin binding to hix-AG. (A) DNA-binding sites (hixL and hixR) of the Hin recombinase from S.typhmurium and the mutant hix sites used in this study (hixL, hix-CG and hix-AG). Residues involved in sequence-specific contacts with Hin are marked by horizontal lines. (B) EMSAs with the 200 bp PCR-amplified DNA containing mutant hix sites (0.8 nM) and 0–200 or 500 ng/ml of the Hin protein. (C) DNase I footprinting of supercoiled plasmids containing mutated hix sites in the presence of the Hin protein (0–50 ng/μl). Protected region from DNase I digestion is indicated by a vertical bar in the left.
RESULTS

The mutated Hin binding site, hix-AG, is recognized by Hin only if it is supercoiled

EMSAs using Hin and 200 bp linear dsDNA fragments showed that Hin binds to the symmetric hix site, which has AT at its central +1/-1 residues (hix-AT) (Figure 1A and B). Although the native hixL and hixR sites have AA sequences at their centers, the symmetric hix-AT site has been tested in in vitro DNA-binding assays, such as EMSAs and methylation protection assays, and found to bind Hin as well as the wild-type hix sites (25). Furthermore, the hix-AT sequence exhibits biological activity equivalent to that of the native hix site in inversionsome formation, and inversion reactions (13).

If the central AT sequence is changed to AG (hix-AG), Hin cannot recognize the hix site even though the other residues important for sequence-specific contacts between Hin and hix are preserved. However, DNA binding by Hin can be recovered by replacing the central AG with CG (hix-CG). Consistent with results obtained for linear DNA fragments, our DNase I footprinting experiments using supercoiled plasmid DNA showed that Hin binds to hix-AT and hix-CG DNAs. However, quite unexpectedly, Hin also bound to supercoiled hix-AG with comparable affinity (Figure 1C). A mutated version of the Hin protein (G102A), which does not bind to the hix site (26), also did not bind to supercoiled hix-AG. This confirms that the protection from DNase I digestion that was observed with wild-type Hin at the hix-AG site is not an artifact caused by experimental conditions and/or non-specific binding (data not shown).

hix-AT, hix-AG and hix-CG have different overall structures

In order to understand the structural basis of the sensitivity of hix-AG to supercoiling, we determined and compared the solution structures of three dodecamer DNAs (hix-AT, hix-AG and hix-CG) (Figure 2). The NOE connectivity and chemical shifts of imino protons in the D$_2$O and H$_2$O NOESY spectra showed that hix-AT, hix-AG and hix-CG have the expected right-handed helix structures. Also, nearly all of the residues of hix-AT and hix-CG showed H1'-H2' scalar couplings larger than 8–9 Hz, indicating that they have C2'-endo sugar puckering typical of B-form DNA. However, most of the residues in hix-AG showed smaller H1'-H2' scalar
couplings in a range of 6–8 Hz (data not shown), which suggests that hix-Ag undergoes a dynamic equilibrium between the C2'- and C3'-endo sugar puckers. Because typical A-form DNA, which has the C3'-endo sugar pucker, shows H1'-H2' scalar couplings of <2.0 Hz, hix-Ag appears to spend most of its time in a B-form-like structure rather than an A-form-like structure. A total of 350, 330 and 482 restraints, respectively, for the hix-Ag, hix-CG and hix-AG were derived from NMR data and were used for structure calculations to obtain well-converged ensemble structures (Table 1).

The major groove widths of hix-Ag, hix-CG and hix-AG were within a ±2 Å boundary of the major groove width of average B-form DNA (27). hix-Ag showed the largest major groove width (~14 Å), which is significantly larger than that of hix-Ag (~10 Å). The major grooves of hix-Ag and hix-CG had similar depths (~4 Å), but hix-Ag had a very shallow (~1.5 Å) depth in the middle of the sequence. hix-Ag had a narrow (width, ~10 Å) and deep (depth, ~5.3 Å) minor groove, which is similar to that of average B-form DNA, while hix-CG had a wide (width, ~10 Å) and shallow (depth, ~3.5 Å) minor groove, which is similar to that of average A-form DNA (27). The minor groove of hix-Ag was of intermediate width and depth, when compared with those of hix-Ag and hix-CG (Figure 2).

hix-Ag is partially unwound and stretched

The converged structures of hix-Ag, hix-CG and hix-Ag showed sequence-dependent structural differences in quantitative helical analyses. Because of the possible inaccuracy of the structure defined for the terminal residues, we assessed only the central 6 bp and their 5 bp steps for each DNA.

The accumulation of rises of central base pair steps from C4–C5 to G8–G9 was 18.1, 18.3 and 19.1 Å for hix-Ag, hix-CG and hix-AG, respectively (Figure 2). This increased helical rise could result from a twist. The sum of the twist angles of the base pair steps from C4–C5 to G8–G9 were 163.5° for the hix-Ag, which is less than that calculated for hix-Ag by 12.7° (Figure 2). The lack of a twist in hix-Ag compared with hix-Ag is consistent with previous analyses of the local helix parameters of high-resolution DNA crystal structures, in which the mean twist angles of AT, CG and AG base pair steps are 33.4 ± 3.5°, 31.1 ± 4.7° and 30.5 ± 4.9°, respectively (28). If we assume the standard A- or B-form DNA structures (27) keep their helical diameter constant upon unwinding, then a decrease in the twist angle of 12.7° could be transformed into an increase of 1.0–1.2 Å in the helical rise. Supporting this assumption, hix-Ag and hix-Ag had nearly identical helical diameters, in that the average interstrand P–P distances of base pairs from C4pC5 to G8pG9 were 18.0 ± 0.2 and 18.1 ± 0.2 Å, respectively. Similar mechanism of DNA stretching has been observed in

Figure 2. Superimposed overall structures of the hix sites. View into the minor groove of (A) hix-AT (25 structures), (B) hix-CG (15 structures) and (C) hix-AG (13 structures). Adenines are colored in red, guanines in blue, cytidines in cyan and thymidines in yellow. Rise and twist are shown for each of the central 5 bp steps.

Table 1. Structure determination statistics

<table>
<thead>
<tr>
<th></th>
<th>hix-AT</th>
<th>hix-CG</th>
<th>hix-AG</th>
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<tbody>
<tr>
<td>Total number of NOE distance restraints</td>
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<td>205</td>
<td>354</td>
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<tr>
<td>Intra-residue</td>
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<td>76</td>
<td>159</td>
</tr>
<tr>
<td>Sequential residue</td>
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<td>93</td>
<td>161</td>
</tr>
<tr>
<td>Interstrand</td>
<td>42</td>
<td>36</td>
<td>34</td>
</tr>
<tr>
<td>Dihedral restraints (β, γ, δ, ε and ζ)</td>
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<td>113</td>
<td>116</td>
</tr>
<tr>
<td>Base pair planarity restraints</td>
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</tr>
<tr>
<td>Total number of restraints</td>
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<td>330</td>
<td>482</td>
</tr>
<tr>
<td>Pairwise r.m.s.d. for all heavy atoms (Å)</td>
<td>0.56 ± 0.29</td>
<td>0.61 ± 0.34</td>
<td>0.92 ± 0.34</td>
</tr>
<tr>
<td>r.m.s.d. to the mean structure (Å)</td>
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<td>0.54</td>
<td>0.79</td>
</tr>
<tr>
<td>Average NOE violations (Å)</td>
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<td>0 (&gt;0.5Å)</td>
<td>0 (&gt;0.5Å)</td>
</tr>
<tr>
<td>Average dihedral angle violations (degrees)</td>
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<td>0 (&gt;5°)</td>
<td>0 (&gt;5°)</td>
</tr>
<tr>
<td>Mean deviation from covalent geometry</td>
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<td>0.007</td>
<td>0.007</td>
</tr>
<tr>
<td>Bond lengths (Å)</td>
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<td>0.9</td>
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</tr>
<tr>
<td>Improper angles (degrees)</td>
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<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>
several DNA complexes, such as those that contain catabolic activator protein (29) or TATA-box binding protein (30). Such DNA-binding proteins can make use of the natural coupling of twist and roll with slide and/or shift to stretch DNA at selected base pair steps.

The accumulated twist angle of hix-CG for residues from C4–C5 to G8–G9 was 154.4 base pair steps. twist and roll with slide and/or shift to stretch DNA at selected DNA-binding proteins can make use of the natural coupling of 258 Nucleic Acids Research, 2006, Vol. 34, No. 1

Because partially unwound DNA sites are easily bent (31,32), we sought to determine whether DNA bending differed among hix-AG, hix-AT and hix-CG by assessing their roll, tilt and twist angles (21). Because the distance constraints used in structural calculations cover a relatively short distance (<6 Å) and thus cannot define a long-range curvature accurately, we included only the central 5 bp steps in our DNA curvature calculations. Both hix-AT and hix-AG, which exhibited negative global rolls (−8 ± 5° and −10 ± 7°), were bent toward the minor groove by 8 ± 5° and 12 ± 6°, respectively. hix-CG, which had a positive roll (8 ± 6°), was bent toward the major groove by 9 ± 5°. Considering that the bending flexibility of generic B-DNA, which was estimated from Monte Carlo simulations using a static bend model, is 5° (33), our results indicate that all three oligonucleotides are only slightly bent and the magnitude of bending of the hix-AG is not remarkably different from that of the hix-AT or hix-CG.

However, statistics of crystal structures of DNA and DNA–protein complexes have demonstrated that AG dinucleotide steps tend to undergo significant translational and tilt changes, while AT and CG dinucleotide steps have essentially no base pair displacement (34). Therefore, we suspected that hix-AG might have higher potential of deformation than hix-AT or hix-CG even though hix-AG did not appear to have significant intrinsic curvature. Because the DNA phosphate backbone is negatively charged, electrostatic interactions with monovalent or divalent cations are important in DNA bending, twisting, groove width variation and deformation (35). Furthermore, sequence-directed bending in DNA has been reported as an inducible, not a static, phenomenon (36,37). Thus we investigated the effect of salts on the deformation of 26 bp hix-AT and hix-AG DNAs, where the change of end-to-end distance was monitored by a change in FRET efficiency (38). Increasing the salt concentration of Na⁺, NH4⁺ and Mg²⁺ from 0.0 to 0.5 M augmented the FRET efficiency for all DNAs, hix-AT, hix-CG and hix-AG (Figure 3A–C). Salt-dependent changes in FRET efficiency may result not only from changes in the end-to-end distance, but also from changes in the fluorescence characteristics of the fluorophores; however, the absorbance and fluorescence of rhodamine used here are known to be very stable at least up to 0.5 M NaCl (24). In the case of Mg²⁺, FRET efficiencies of hix-AT, hix-CG and hix-AG increased so rapidly that no significant differences were observed between them. However, for both Na⁺ and NH4⁺, the FRET efficiency of hix-AG increased more rapidly than that of the hix-AT or hix-CG, suggesting that hix-AG is more flexible and, therefore, more easily deformed as the salt concentration increases.

**Base pair opening of hix-AG is very fast**

DNA deformation occurs concurrently with base pair opening, and the propensity of base pair opening is also related to the thermodynamics and kinetics of DNA deformation (21,30,39). In order to explore the differential dynamics of the three hix sites, we measured the base pair lifetimes of the common G8–C17 base pair using ammonia as a base catalyst (Figure 4A). In a stacked helix, the imino protons are protected from exchange with a base catalyst, but in the presence of higher concentrations of a base catalyst, exchange of imino protons may take place each time a base pair opens. When we consider that the typical base pair lifetimes of A–T and G–C base pairs are 0.5–7 and 4–50 ms, respectively (23), the lifetimes of G8–C20 base pairs measured at 17°C for hix-AT (5.0 ms), hix-CG (4.4 ms) and hix-AG (2.6 ms) and those values measured at 12°C for hix-AT (2.8 ± 3.0 ms), hix-CG (12.8 ± 4.6 ms) and hix-AG (7.0 ± 6.4 ms) imply that the central base pairs of hix-CG and hix-AG are rapidly opened and closed, with hix-AG undergoing the fastest local motion. It appears consistent with the previous observation that a

![Figure 3](https://academic.oup.com/nar/article-abstract/34/1/254/2401609/0.00)
G-C base pair within consecutive G-C base pairs has an unusually short base pair lifetime (40). The opening of the central G8–C17 base pair of hix-AG did not seem to follow the simple two-state model (23), and the measured exchange times were not extrapolated to a positive value at an infinite concentration of ammonia. However, we confirmed, by monitoring the central imino proton while increasing the temperature of samples at a given concentration of base catalyst, that hix-AG base pair exhibited faster base pair opening kinetics than did the hix-AT base pair (Figure 4B).

**DISCUSSION**

The three mutant hix sites (hix-AT, hix-CG and hix-AG) studied here are the same except for one or two base pairs. However, we have shown that their structural and dynamical properties are remarkably different, which may explain the differential binding of Hin to these sites in the presence or absence of supercoiling. Supercoiling confers topological constraints on the local DNA structure. As protein binding induces structural changes in the DNA-binding site, supercoiling should also induce structural changes in the local DNA site where flexibility of the DNA including and surrounding this site would be critical for a response to supercoiling. Supporting this idea, the partially unwound and stretched structure of hix-AG shows high potential for deformation and very fast kinetics of base pair opening. Furthermore, in the present study, we have revealed that a single CAG/CTG triplet sequence motif contained in hix-AG shows essentially the same characteristics as do multiple CAG/CTG repeats which are associated with several hereditary neuromuscular diseases, including myotonic dystrophy and Huntington’s disease; a block of multiple CAG/CTG repeats is present near or within genes associated with such diseases (12).

The gel mobility and cyclization kinetics of DNA that contains short tracts of CAG/CTG repeats revealed that the CAG/CTG repeats are intrinsically straight but extremely flexible (41). Also, a stretch of multiple CAG/CTG repeats shows an unusually high affinity for the histone octamer, forming a tight nucleosome (42) in which DNA wraps around a histone core in a left-handed configuration that produces a negative toroidal supercoiling. The free energy of supercoiling for the CAG/CTG repeats calculated by statistical mechanics is only 66% of that of random B-DNA at a length of 106bp (43).

How might the structural and dynamic properties of the CAG/CTG site explain the supercoiling-dependent interaction between Hin and hix-AG? There may be a few possible reasons for why hix-AG is not bound by Hin when the DNA is relaxed, but is bound by Hin when the DNA is supercoiled. First, the spacing between the half-sites in the hix sequence might be critical for DNA inversion, as demonstrated by a mutant hix site that contains AAA sequence rather than AA sequence at the center of an otherwise normal hix site (13). However, the Hin dimer is flexible enough to bind to both half-sites of hix sequences that contain a 2 (wild type), 3 or 5bp spacer between the half-sites, albeit the following inversion process is aborted (6). The same flexibility has been reported for the γ6 resolvase, which shares ~40% amino acid sequence identity with Hin and which binds to three res sites, each with a different spacer length (7, 10 and 16bp) between two half-sites (44). Therefore, the differential spacing or the helical rise may not be relevant at least with respect to Hin-hix-AG binding.

Second, the binding property of hix-AG may differ from that of hix-AT and hix-CG. Several studies on the homologous γ6 resolvase (44) and Gin invertase (45) and preliminary results of circular permutation assays on the Hin recombinase suggest that the hix site should be bent toward the major groove upon Hin binding (6). We showed that all three hix sites are similarly bent with a magnitude ranging from 8 to 12°. However, hix-AT and hix-AG are bent toward the minor groove, which is opposite to the expected orientation of the bending in the Hin-bound hix site. Also, kinetics of base pair opening and fluctuations of roll and tilt indicated that hix-AG is significantly more disordered than hix-AT. Therefore, a great entropy loss due to the constraints imposed by complex formation as well as additional energy required for inverting the orientation of bending may explain the unfavorable
binding between Hin and relaxed hix-AG site. Effect of supercoiling on a local DNA structure has been shown to be mimicked by a nick which is known to hardly alter the bending flexibility but to substantially increase twist flexibility (46,47). Our FRET data and the preferred occurrence of CAG/CTG triplet at the dyad of nucleosome suggest that CAG/CTG triplet has high twist and bending flexibility (48). Combined together with this intrinsic flexibility of CAG/CTG site, extrinsic twist flexibility provided by supercoiling would facilitate further unwinding of hix-AG, which shall increase roll and thus change the orientation of bending through strong negative correlation between twist and roll (28,34). This structural change will reduce the energetic cost of Hin binding. CAG/CTG site may gain flexibility by supercoiling because of its high susceptibility to deformation, on the other hand, surrounding DNA sites involved in the sequence-specific contacts between Hin and hix might become less flexible due to the structural constraints imposed by supercoiling, which reduces the overall entropy loss of binding between Hin and supercoiled hix-AG.

The strategy underlying the above explanations could be generalized as follows: supercoiling affects predominantly the DNA site having high intrinsic flexibility, and it switches the local DNA structure from an inefficient conformation for protein interaction to an efficient one, or vice versa. An example of this notion is the mdm2 promoter, which is responsive to changes in superhelicity (4). Unlike other binding sites for the tumor suppressor protein p53, the mdm2 promoter has two consensus p53 binding sites separated by a 17 bp spacer that contains a CAG/CTG in the middle of the sequence. Because a p53 tetramer binds to either a single consensus sequence or tandem sequences and stacks one on top of the other (49), the inhibitory effect of supercoiling on sequence-specific binding by p53 could be rationalized by flexibility of CAG/CTG site of the spacer that could determine the distance and relative orientation between the two consensus p53 binding sites.

Similar strategies can be found in supercoiling-sensitive E.coli promoters too, but they appear to adopt different sequence motifs for responding to changes of superhelicity (Supplementary Data). For eukaryotic promoters, analysis of a large set of human RNA polymerase II promoters has revealed a periodic occurrence of the CAG/CTG steps in the region of promoters downstream from the transcription start site (50). Because, in the polymerase–promoter complex, the polymerase topologically constrains 1–2 left-handed supercoiling (2), this suggests that the structural changes triggered at the periodic CAG/CTG steps by local supercoiling induce the initial open complex formation in a manner reminiscent of the DNA when it is packaged in a nucleosome.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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