Altered DNA conformations detected by mung bean nuclease occur in promoter and terminator regions of supercoiled pBR322 DNA

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ABSTRACT

Mung bean nuclease was used to probe for recognizable DNA unwinding and unpairing in the plasmid pBR322. In negatively supercoiled DNA, but not relaxed DNA, cleavages occurred preferentially in non-coding regions of the genome. The types of nucleotide sequences cleaved and which non-coding regions were cleaved depended upon environmental conditions. At 37°C, cleavages occurred in an 84 bp A+T-rich sequence in the terminator region of the ampicillin-resistance gene. Recognition is likely based on a novel DNA conformation which occurs in the longest, most dA+dT-rich region of pBR322. In the presence of 1 mM Mg²⁺, cleavages occurred in inverted repeated sequences in the promoter regions of the RNA primer for DNA replication and ampicillin- and tetracycline-resistance genes as well as the terminator of RNA-1. Potential loops of hairpin (cruciform) structures were cleaved. At 27°C, cleavages occurred near a promoter activated by cAMP receptor protein *in vitro* and in the 3' non-coding region of the tetracycline-resistance gene. Thus, in supercoiled pBR322 DNA, recognizable DNA unwinding and unpairing occurs preferentially in regulatory regions for transcription and DNA replication.

INTRODUCTION

Studies using nucleases and chemical reagents which can detect alterations in DNA conformation have revealed that genomic DNA from certain eukaryotes is punctuated with conformational information. Both micrococcal nuclease, which weakly favors single- over double-stranded DNA, and 1,10-phenanthroline-Cu²⁺, which intercalates in double-stranded DNA, preferentially cleave *Drosophila* DNA in non-transcribed as opposed to transcribed regions (1-3). In contrast, no such preference is seen with prokaryotic DNA (pBR322 and phage lambda). Mung bean nuclease, a single-strand-specific endonuclease, cleaves eukaryotic DNA (*Plasmodium*) before and after genes under partially denaturing conditions (4). The enzyme cleaves phage T7 DNA into gene-sized pieces (5) but the actual locations of the cuts with respect to genes are not known. Thus, it is possible that prokaryotic genomes are also organized with certain types of conformational information between genes.

Portions of prokaryotic genomes (6,7), and possibly eukaryotic genomes (8,9), are under torsional stress resulting from negative supercoiling of DNA. Supercoiling affects DNA replication and transcription by an uncertain mechanism which may involve helix unwinding and/or unpairing (10,11). The torsional stress of supercoiling might unwind or unpair DNA at sites involved in the regulation or initiation of DNA replication and transcription. In light of this possibility, the location and nature of sites cleaved by single-strand-specific endonucleases which can recognize DNA unwinding or unpairing are of considerable interest. Prokaryotic supercoiled DNA (plasmid and phage) is cleaved by S1 nuclease at inverted repeat sequences but a specific relationship between the cleavage sites and genes has not been found (12-14). In contrast, some eukaryotic genes inserted in supercoiled plasmids are cleaved by S1 at poly purine-poly pyrimidine sequences (16-19) in 5' non-coding regions of genes (15). The failure to detect a relationship between S1 nuclease sites and genes in prokaryotic DNA may be a reflection of the environmental conditions used to probe DNA since environmental conditions can affect the location of S1 sites in supercoiled DNA (15,16,20,21). Both the acid pH (22) and Zn^{2+} (23) used in S1 nuclease conditions may affect DNA conformation and site specificity.

Our laboratory has established the use of mung bean nuclease to probe DNA conformation under conditions where DNA can function in vitro (24,25). With phage PM2 DNA, we found that major sites occur in potential regulatory regions involved in DNA replication and transcription (26). An A+T-rich region or an inverted repeat sequence was cleaved, depending on environmental conditions. The altered DNA conformation we detected in the A+T-rich region had not been previously observed in supercoiled DNA. The significance of these findings in terms of the structure and function of PM2 DNA is limited by the paucity of information on both the genetic map and the nucleotide sequence. However, our findings underscore the importance of environmental conditions in shaping the conformation of supercoiled DNA and in understanding the relationships between DNA sequence, conformation and function.

We were interested in the specificity of mung bean nuclease for regulatory regions of other prokaryotic genomes. We were also interested in the nature of the nucleotide sequences cleaved and their altered DNA conformations. We chose to study the plasmid pBR322 (27) since the complete nucleotide sequence is known (28) and the genes and regulatory regions are reasonably well characterized. Also, the plasmid consists of three genetic regions derived from three different prokaryotic sources, adding to the

generality of any findings. Finally, pBR322 and its derivatives are cloning vectors, permitting one to probe the conformation of DNA inserts. In this paper, we demonstrate that mung bean nuclease sites preferentially occur in non-coding, regulatory regions of pBR322 DNA, depending upon environmental conditions and the presence of negative supercoiling. Cleavage occurs either in the longest, most A+T-rich span of the DNA which likely possesses a novel conformation or in certain inverted repeat sequences which likely form hairpins, depending on the presence of Mg^{2+} .

MATERIALS AND METHODS

Enzymes. Mung bean nuclease was isolated and purified to homogeneity as described by Kowalski et al. (29). Venom phosphodiesterase was a gift of Dr. H. Takamatsu and was purified as described (30). Enzymes from commercial suppliers were as follows: restriction endonucleases (New England Biolabs), bacterial alkaline phosphatase (Worthington Biochemicals), polynucleotide kinase (P-L Biochemicals).

DNA. Plasmid pBR322 was grown in *Escherichia coli* HB101 and amplified using 150 μ g/ml chloramphenicol. DNA from cells lysed with lysozyme (0°C) was purified by two rounds of equilibrium centrifugation in cesium chloride density gradients containing ethidium bromide (31). The superhelical density, determined as described (33), was -0.046 (at 20°C in 40 mM Tris-acetate, 5 mM sodium acetate, 1 mM EDTA, pH 8.2). This preparation of DNA was used in almost all of the experiments reported here. DNA of superhelical density -0.067 was obtained from cells lysed by boiling in the presence of lysozyme (33), purified as above, and used in a few experiments where indicated. Relaxed, closed-circular DNA was prepared by treating supercoiled DNA with topoisomerase I (25).

Positional Specificity of Mung Bean Nuclease Nicks. Mung bean nuclease reaction mixtures contained 10 mM Tris-HCl (pH 7.0), 1.6 μ g of pBR322 DNA and other components, as indicated in Figure 1, in a volume of 18 μ l. After preincubation for 15 min at 37°C, 2 μ l of appropriate mung bean nuclease dilution (see figure legends) was added and the mixture incubated at 37°C. After 1 hr incubation, which was sufficient to cleave all of the supercoiled DNA, the reaction was quenched (25). Cleavage opposite the mung bean nuclease nicks with venom phosphodiesterase and restriction endonuclease digestions and gel electrophoresis of DNA were previously described (25). The number of base pairs in DNA fragments were read from graphs of log base pairs vs electrophoretic mobility relative to linear pBR322. The accuracy of this method using pBR322 DNA restriction fragments in the range of 1000 to 3300 bp was better than ± 20 base pairs.

DNA Sequencing Around Mung Bean Nuclease Nicks. The above mung bean nuclease reaction mixtures were scaled up 10 fold. When Mg^{2+} was present, the reaction mixture also contained 1.1 mM Mg^{2+} . After preincubation for 15 min at 37°C, 20 μ l of a mung bean nuclease dilution (25 units/ml) (24) was added and the mixture incubated at 37°C. After 1 hr incubation 0.1 M EDTA (22 μ l) was added and the mixture cooled to 0°C. The enzyme was removed by extraction with phenol (saturated with 10 mM Tris-HCl (pH 7.4), 1 mM EDTA). After restriction nuclease digestion, DNA fragments were ^{32}P labeled at the 5' ends, separated by polyacrylamide gel electrophoresis, and isolated as previously described (26). Singly, end-labeled restriction fragments were generated by cleaving end labeled DNA fragments with a second restriction enzyme, and were isolated. DNA fragments containing mung bean

nuclease nicks were denatured and electrophoresed along side the products of Maxam-Gilbert sequencing reactions (34) performed on the same fragment without nicks (26). The locations of the nicks within the nucleotide sequence were determined as previously described (26).

RESULTS

Effects of Environmental Conditions on Mung Bean Nuclease Specificity

Supercoiled pBR322 DNA was reacted with mung bean nuclease at neutral pH under a variety of environmental conditions to give predominantly nicked-circular DNA and a small proportion of linear DNA. After equalizing solution conditions, the nicked-circular DNA was linearized by cleavage opposite the nick with venom phosphodiesterase (25). The predominant product was unit-length linear DNA (4362 bp) indicating the presence of mainly one mung bean nuclease nick per molecule. The positional specificity of the nicks was determined by cleaving the linearized DNA at single restriction enzyme sites and separating the products by agarose gel electrophoresis. The products after Hind III cleavage are shown in Fig. 1. The discrete banding patterns indicate that the nicks occur at specific sites. Circular pBR322 DNA molecules cut once at a mung bean nuclease site and once at a restriction enzyme site will result in pairs of fragments whose lengths add up to 4362 bp (1.0 fractional genome length). Pairs of bands containing such fragments can be seen in Fig. 1. The relative intensity of one pair of bands to another reflects the relative frequency of mung bean nuclease cleavage.

As shown in Fig. 1, variations in temperature and in NaCl and Mg^{2+} concentrations in the mung bean nuclease reaction affect both the number and location of the cleavages in pBR322 DNA, as previously observed with PM2 DNA (25). A large number of specific sites were cleaved in the presence of NaCl (Fig. 1, lanes 5-7). These sites were not mapped in detail. Other solution conditions resulted in the identification of nine major sites (Fig. 2, A-I). Seven of these sites (A-G) were assigned unique positions by identifying pairs of Hind III fragments and then by determining which member of the pair was cut by Bam HI at its single recognition site. The two remaining major sites (H,I) which occur near the Hind III site were mapped by cutting the linearized DNA with Bam HI (shown later) followed by Hind III. The average nucleotide positions of the major sites are shown on the map of pBR322 DNA in Fig. 2. These values are overlined to indicate that they are average rather than exact positions. As shown in Fig. 2, at 27°C (half-filled triangles), 37°C (open triangles), and 37°C in the presence of 1 mM Mg^{2+} (filled triangles), the major cleavage sites for mung bean nuclease in

Figure 1. Effects of temperature, NaCl and Mg^{2+} concentrations on mung bean nuclease specificity. Supercoiled pBR322 DNA (80 μ g/mL) in 10 mM Tris-HCl was nicked with mung bean nuclease, cleaved opposite the nicks with venom phosphodiesterase, cut at the Hind III site, and 0.53 μ g/lane was electrophoresed through the 1.0% agarose gel shown in the photograph. Mung bean nuclease reaction conditions corresponding to the lanes in the photograph were as follows (all 1hr): 1) 37°C, no enzyme. 2) 37°C, 2.5 units/mL. 3) 27°C, 25 units/mL. 4) 47°C, 0.25 units/mL. 5-7) 37°C, 25 units/mL, 20 mM, 40 mM and 60 mM NaCl, respectively. 8-10) 37°C, 2.5 units/mL, 0.025 mM, 0.12 mM and 1.0 mM Mg^{2+} , respectively. Lane 11, marker DNAs (28). Marker DNA sizes in base pairs are shown to the right of the figure. Numbers to the left of the figure are fractional genome lengths (fragment size/4362 bp).

supercoiled pBR322 DNA occur in non-coding, regulatory regions of the genome. The preference for these regulatory regions depends upon the environmental conditions since the frequency of cutting at many other regions increases in other environments such as in the presence of NaCl (Fig. 1, lanes 5-7).

Nucleotide Sequences Around Major Mung Bean Nuclease Nicks

We determined the nucleotide sequence around the nicks introduced into supercoiled DNA by preparing singly end labeled restriction fragments containing mung bean nuclease nicks and analyzing them on a DNA sequencing gel (26).

Figure 2. Map of pBR322 DNA showing major mung bean nuclease cleavages (top), genes and regulatory regions (middle) and sequencing strategies used to locate nicks (arrows at bottom). The cleavage map shows the average positions of major nicks mapped at the level of agarose gel resolution (sites A-I). The three mung bean nuclease reaction conditions examined are ∇ , 37°C; ∇ , 37°C, 1 mM Mg^{2+} ; ∇ , 27°C. On the genetic map, straight arrows represent protein-coding regions for ampicillin-resistance (28), tetracycline-resistance (67) and ROP (68) genes. Promoters for these genes are P₁ and P₃ (54,69), P₂ (54,69) and unknown, respectively. Wavy arrows represent RNA transcripts for the RNA primer for DNA replication (39) and RNA-1 (38). Promoters for these RNAs are P_p (48) and P_{RNA-1} (38), respectively. P₄ is a promoter activated by cyclic AMP receptor protein *in vitro* (55).

(1) Cleavage in the Terminator Region of the Ampicillin-resistance Gene.

Mung bean nuclease cleavage of supercoiled pBR322 DNA in 10 mM Tris-HCl (pH 7.0) at 37°C results in two major sites at the level of agarose gel resolution. The predominant site is at 3258 and the other site is nearby at 3223. A broad region of the sequence is susceptible to cleavage in the top strand (Fig. 3A, lane 1, bracketed area) and the bottom strand (Fig. 3B). Between 20-40 cleavage products are detectable. The intensity of the products varies, indicating that the frequency of cleavage at different positions within the sequence also varies. The locations and relative frequencies of the cleavages are shown in Fig. 4A. Cleavages occur within the same region on both strands. An approximately bimodal distribution of cleavages centered around positions 3220 and 3250 is seen in the top strand of the nucleotide sequence (Fig. 4A). These findings are in excellent agreement with the mapping of two sites at positions 3223 and 3258 at the level of agarose gel resolution. Also, the greater frequency and intensity of the cleavages around position 3250 as compared to those around position 3220 (Fig. 4A) are consistent with the greater intensity of the agarose gel bands for the site at 3258 compared to the site at 3223 (Fig. 1, lane 2, 0.26 vs. 0.27 fractional genome length). The 84 bp sequence spanning the cleavages has a A+T content

of 77%. This sequence is highly enriched in A+T compared to that of the pBR322 genome (46%).

(2) Cleavage in the RNA-1 Terminator and RNA Primer Promoter. Cleavage of the sites which map with the A+T-rich sequence in 10 mM Tris-HCl (pH 7.0) at 37 °C (Fig. 1, lane 2, bands around 0.74 and 0.26 fractional genome lengths) is greatly reduced when increasing concentrations of Mg^{2+} are added to the mung bean nuclease reaction mixture (Fig. 1, lanes 8-10). At 1mM Mg^{2+} (Fig. 1, lane 10), cleavage of the A+T-rich sites is nearly eliminated. This reduction of cleavage frequency can also be seen at the nucleotide sequence level as shown in the bracketed region of the lane 2 in Fig. 3A and lane 1 in Fig. 3B. The reduced cleavage frequency is not simply the result of inhibition of enzyme activity by Mg^{2+} since alternative sites are cleaved (Fig. 1, lane 10). The effect of Mg^{2+} is reversible by EDTA. If after preincubation of pBR322 DNA with Mg^{2+} excess EDTA was added prior to mung bean nuclease, a cleavage pattern identical to that observed in the absence of Mg^{2+} (Fig. 1, lane 2) was seen (data not shown).

Two major sites cleaved at 37°C, 1mM Mg^{2+} correspond to the doublet bands around 0.71 and 0.29 fractional genome length (Fig. 1, lane 10) and map at 3117 and 3061. At the nucleotide sequence level, major cleavages were found within six nucleotides from these average positions (Fig. 3A, lane 2, positions 3122 and 3063). In contrast to the broad distribution of cleavages in the A+T-rich sequence, a single major cleavage (with only a few flanking minor cleavages) is seen at each site. Similar results were seen in the nucleotide sequence analysis of cleavages in the complementary strands (autoradiograms not shown). No cleavages are seen at 3123 and 3063 in the absence of Mg^{2+} (Fig. 3A, lane 1). At both sites, the major cleavages are flanked by inverted repeat sequences. When these sequences are drawn as hairpins or cruciform structures, the major single-strand-specific nuclease cleavages occur in the non-base-paired loops (Fig. 4B). Minor cleavages occur near the base of the stem of the hairpin at 3122 in Fig. 4B). The most prominent of the minor cleavages occurs at position 3132 (Fig. 3A, lane 2) which is in the loop of a potential hairpin structure (Fig. 4B). Formation of hairpins at 3123 and 3132 would be mutually exclusive since their stems share a common sequence.

(3) Cleavage in the Promoters for Ampicillin- and Tetracycline-resistance Genes. Two other major sites cleaved at 37°C in the presence of 1 mM Mg^{2+} correspond to the band at 0.94 fractional genome length (site H) and a portion of the band at 1.0 fractional genome length

Figure 3. Nucleotide sequence analysis around mung bean nuclease nicks in pBr322 promoter and terminator regions. Shown are autoradiograms after electrophoresis of ^{32}P -DNA in 10% polyacrylamide-8M urea gels. Supercoiled DNA was nicked by mung bean nuclease at 37°C in the presence or absence of Mg^{2+} . DNA fragments containing a single 5' ^{32}P label at

a restriction enzyme site were prepared, denatured and electrophoresed along side the products of Maxam-Gilbert sequencing reactions performed on fragments obtained from supercoiled DNA (lanes A (A>C), G, C and T (T+C)). Lanes 1 and 2 show the products of the mung bean nuclease reactions.

A. 5' ^{32}P at Alu I site (nucleotide 3035) in the top strand and 3'OH at Hinf I site (nucleotide 3362). Lane 1, no Mg^{2+} . Lane 2, Mg^{2+} .

B. 5' ^{32}P at Hinf I site (nucleotide 102) in the bottom strand and 3'OH at Alu I site (nucleotide 3036). Lane 1, Mg^{2+} . Lane 2, no Mg^{2+} .

C. 5' ^{32}P at Taq I site (nucleotide 4019) in the top strand and 3'OH at Hha I site (nucleotide 4259). Lane 1, no Mg^{2+} . Lane 2, Mg^{2+} .

D. 5' ^{32}P at Eco RI site (nucleotide 4360) in the top strand and 3'OH at Hha I site (nucleotide 102). Lane 1, Mg^{2+} .

Numbers along side autoradiograms are nucleotide positions at the 3'-OH of mung bean nuclease nicks. Nucleotides are numbered according to Sutcliffe (28). U, unit length fragment. N, native form of the unit length fragment.

(site I) (Fig 1, lane 10) and map at 4169 and 36, respectively (Fig. 2).

Both sites are clearly visible in the Bam HI digest which is shown below (Fig. 6D). Major cleavages were found at positions 4174 (Fig. 3C) and 24 (Fig. 3D) in the nucleotide sequence. These cleavages are also flanked by inverted repeat sequences (shown later in Fig. 6) suggesting that the cleavages occurred in the non-base-paired loops of potential hairpin structures. No cleavages are seen at position 4174 without Mg^{2+} addition (Figs. 3C, lane 1).

Effects of DNA Supercoiling on Mung Bean Nuclease Specificity

To examine the effects of increased negative supercoiling, we probed pBR322 of superhelical density -0.067 with mung bean nuclease and compared the site specificity to the pBR322 preparation of superhelical density -0.046 which was used for the nucleotide sequencing studies. At 37°C with no Mg^{2+} , the major site in the more negatively supercoiled plasmid (Fig. 5, lane 2) maps with the major site of the less negative supercoiled plasmid (Fig. 5, lane 1) in the A+T-rich region around position 3250. However, the A+T-rich site around 3220 (Fig. 5, lane 1) disappears with increased negative supercoiling (Fig. 5, lane 2). New bands appear at around 0.645 and 0.355 fractional genome lengths. Additionally, a minor band appears at 0.85 fractional genome length (the other member of the pair at 0.15 is not visible in the photograph). These new sites map at average positions 3200 and 4100, respectively. Thus, under these conditions, the level of negative supercoiling of pBR322 DNA has a small effect on the number and location of sites cleaved by mung bean nuclease.

At 37°C in the presence of 1 mM Mg^{2+} , the sites recognized at both superhelical densities are similar (Fig. 5); however, in the more negatively supercoiled plasmid, the intensity of the band around 0.38 fractional genome

A. **B.**

Figure 4. Nucleotide sequences around mung bean nuclease nicks.

A. Terminator region of ampicillin resistance gene. Mung bean nuclease reaction was performed in the absence of Mg^{2+} (Fig.3). Vertical arrows represent nicks, presumably one per DNA molecule (see Discussion). Increasing height of the arrows represents increasing cleavage frequencies within one strand, determined by inspection of band intensities.

B. RNA-1 terminator region (3063) and RNA primer promoter region (3122, 3133). The inverted repeat sequences are drawn as hairpin structures. Mung bean nuclease reaction was performed in the presence of 1 mM Mg^{2+} (Fig. 3). The numbers above and below the sequences indicate the nucleotide positions of the major nicks.

length is greatly reduced relative to the band at 0.62 (lane 2). This observation can be accounted for by an increase in the frequency of mung bean nuclease nicking at two or more sites in the same DNA molecule (unpublished results). Multiple nicking has been observed with other single-strand specific endonucleases (15,35,36). Thus, at 37°C in the presence of 1 mM Mg^{2+} the level of negative supercoiling appears to affect the frequency of

Figure 5. Effects of DNA supercoiling on mung bean nuclease site specificity in the absence and presence of Mg^{2+} . pBR322 DNAs of superhelical densities -0.046 (lanes 1) and -0.067 (lanes 2) were treated with mung bean nuclease at $37^{\circ}C$ in the absence and presence of Mg^{2+} . DNA was cleaved opposite the nicks with venom phosphodiesterase, cut at the single Bam HI site and $0.8 \mu g/lane$ was electrophoresed through the 1% agarose gel shown in the photograph. The numbers to the left of both photographs are the nucleotide positions of the cleavages in DNA sample 1 (see Fig.4). The numbers to the right are fractional genome lengths.

multiple mung bean nuclease nicking as opposed to the site specificity.

Negative supercoiling is required for the preferential cutting of pBR322 DNA in regulatory regions since covalently-closed, relaxed DNA is cleaved at many other regions (data not shown). Also, unlike supercoiled DNA, the site specificity on relaxed pBR322 DNA and PM2 DNA (25) is not affected by changing environmental conditions.

DISCUSSION

Our results show that mung bean nuclease preferentially cleaves non-coding regulatory regions of pBR322 DNA depending on environmental conditions and on the presence of negative supercoiling. Preferential cutting in regulatory regions is most striking for the antibiotic resistance genes where most of the DNA sequence codes for proteins (Fig. 2). Also, which regulatory regions are cleaved in pBR322 DNA depends on environmental conditions (Fig. 2). Our findings reveal an organization of altered DNA conformation with respect to genes in a prokaryotic DNA. Such an organization may be general for other prokaryotic DNAs since pBR322 was constructed from three independent genetic regions (27,28) all of which show preferential cutting in regulatory regions.

Structural Implications

At $37^{\circ}C$, an 84 bp A+T-rich sequence (77%) is susceptible to cleavage at multiple, preferred sites in each strand (Fig. 4A). Cleavage presumably

occurs at only one bond in each DNA molecule since under these digestion conditions a single nick relieves the torsional stress of supercoiling rendering the molecule resistant to further nicking. Our sequencing strategy examined only single-strand nicks and excluded double-strand breaks (26). No specific sequences can be identified at the nicks. The sequences in the region susceptible to cleavage differ from inverted repeat sequences (potential hairpins) (13), poly purine-poly pyrimidine sequences (16-19), and alternating purine-pyrimidine sequences (potential Z-DNA) (36,37) associated with S1 nuclease sites. Also, multiple, preferred cleavages over such a long stretch of DNA have not been observed using S1 nuclease (13,16-19). Our findings suggest that the altered DNA conformation detected by mung bean nuclease differs from those detected using S1 nuclease.

The type of sequence cleaved and the cleavage pattern in pBR322 DNA are similar to those observed after mung bean nuclease cleavage of supercoiled PM2 DNA under the same digestion conditions (26). In both DNAs, cleavages at runs of A and T are largely, but not completely, excluded (Fig. 4A; ref. 26). The results suggest that the altered DNA conformations detected in PM2 (26) and pBR322 DNAs are similar to each other. The available evidence suggests that this represents a novel DNA conformation associated with certain long, A+T-rich sequences.

We compared the nucleotide sequence of the A+T-rich sequence which is cleaved in pBR322 DNA with that of a second long, A+T-rich sequence in the molecule which is not cleaved. Both sequences contain runs of A and T (see above) indicating that these alone are unlikely the basis for the single-strand character. One major difference between the two sequences is the A+T content. The sequence cleaved is 77% A+T (positions 3199 to 3282) while the most A+T-rich region in a comparable length of the sequence not cleaved is 74% A+T (positions 4159 to 4242). Our results indicate that in 10 mM Tris-HCl (pH 7.0) at 37°C, mung bean nuclease preferentially recognizes the longest, most A+T-rich span in supercoiled pBR322 DNA.

The two A+T-rich sequences in pBR322 DNA described above correspond to the two early denaturation regions of the molecule (42). The all or none cleavage of the two regions which differ in A+T content is reminiscent of the cooperativity seen in melting of A+T-rich sequences under denaturing conditions. Regions melt-out cooperatively in order of decreasing A+T-content averaged over 50 to 200 bp (43,44). In our case, however, specific cleavage is detected under non-denaturing solution conditions under the torsional stress of negative supercoiling. The region encompassing the cleavages is

unlikely melted or completely single stranded since if it were, we expect that every bond would be cleaved to some extent as is seen with single-stranded DNA (M. Eddy and D. Kowalski, unpublished results). It is possible that the DNA is partially melted and only the sequences cleaved are single stranded. Alternatively, it is possible that the DNA is in a premelting conformation (46) and is not truly single-stranded. Mung bean nuclease may recognize distortions in the helix which are not single-stranded per se (45). Thus, it is not clear whether the nucleotide sequences cleaved are single-stranded or in a distorted B-DNA conformation (or both).

At 37°C in the presence of 1mM Mg^{2+} , inverted repeat sequences are cleaved in preference to the A+T-rich sequence. No specific sequences are seen at the nicks. The simple cleavage patterns resemble S1 nuclease cleavage patterns in inverted repeat sequences with the potential to form a pair of hairpins or a cruciform (13). Consistent with recognition of a hairpin structure, the major cleavages occur in the potential non-base-paired loop as opposed to the base-paired stem. At sufficiently high sensitivity, minor mung bean nuclease cleavages were also detected at the top of the stem (Fig. 4B, top strands) and near the base of the stem (Fig. 4B, 3063, 3122). Minor cleavages may reflect fluctuations in hairpin structure involving DNA unwinding and/or unpairing.

To assess the requirements of hairpin structures for mung bean nuclease cleavage, the nucleotide sequence of pBR322 DNA was computer searched (47) for potential hairpin structures (loops of 3 to 10, stems > 4) and the locations were compared to the positions of prominent cleavages. The maximum stem sizes of the five potential hairpins cleaved range from six to eleven. The minimum loop sizes range from three to five. Only one potential hairpin (position 1319) was found which meets these criteria but is not a prominent cleavage site. Prominent cleavage at potential hairpins containing loops of six or greater or containing stems of five or fewer base pairs was not detected. There are no potential hairpins in pBR322 DNA with stems greater than eleven base pairs (loops of 3 to 10). Thus, prominent cleavages occurred at potential hairpins with longer stems and smaller loops. These represent the more stable hairpin structures since longer stems and smaller loops favor hairpin stability in supercoiled DNA (41).

Lilley (13) has suggested that limited G-T, A-C pairing is permitted in potential hairpins detected under S1 nuclease conditions. When such pairing is permitted and a computer search (47) of the pBR322 sequence is performed, many additional inverted repeat sequences can be found which conform to the

stem/loop sizes of the potential hairpins detected by mung bean nuclease; however, when examined at the nucleotide sequence level, cleavages were not detected at these sites (positions 2309, 2287, 3236, 3257, 4325) suggesting that limited G-T, A-C pairing are not favorable under our conditions.

Comparison of the potential hairpin structures cleaved by mung bean nuclease and by S1 nuclease reveals both similarities and differences in specificity. Potential hairpins at positions 3065 and 3123 are detected by both enzymes. Potential hairpins at positions 24, 3133 and 4174 are detected by mung bean nuclease but not by S1 nuclease (12). A potential hairpin at position 3220 (stem = 10, loop = 6) is detected by S1 nuclease (12) but not by mung bean nuclease. The differences in the number and type of hairpins detected may reflect differences in environmental conditions used with the two enzymes, intrinsic specificities of the enzymes or the level of DNA supercoiling.

How do changes in environment bring about changes in DNA conformation? With relaxed, closed circular DNA, it is known that decreasing temperature or addition of counter ions can increase the helical twist and result in negative supercoiling (49). Thus, one possibility is that with supercoiled DNA such environmental changes result in further increases in torsional tension which could alter DNA conformation. However, this explanation alone is not sufficient to account for our findings. While addition of 1mM Mg^{2+} has a dramatic effect on the locations of mung bean nuclease sites (compare Fig. 5, lanes 1), increased negative supercoiling (decreased linking number) over and above that which could be induced by changes in helical twist with Mg^{2+} (49) has little effect (Fig. 5, compare lanes 1 and 2). In addition to affecting the level of torsional stress, environmental conditions can affect the stability of unwound/unpaired regions or their associated altered secondary structures in supercoiled DNA. Mg^{2+} stabilizes linear DNA from melting (50) and stabilizes cruciforms in pBR322 DNA (position 3123) (51) and in perfectly repeated lac operator DNA (52). Thus, addition of Mg^{2+} may result in detectable changes in the conformation of supercoiled pBR322 DNA by destabilizing the altered secondary structure in the A+T-rich sequence and stabilizing cruciform structures. Theoretical aspects of such competitive conformational transitions in supercoiled DNA have been previously considered (53). In addition to effects on DNA secondary structure, the environment could directly affect the tertiary structure of supercoiled DNA which could lead to changes in DNA secondary structure (26). It is likely that all of the above possibilities are involved in establishing a minimum free energy

Figure 6. Major mung bean nuclease cleavages in inverted repeat sequences corresponding to promoter and terminator regions of pBR322. The vertical arrows show major nicks and the horizontal arrows indicate inverted repeat sequences. Transcription start sites (circled nucleotides) and direction (attached arrows) as well as consensus promoter sequences (-10 and -35 boxes) are shown. Translation start sites (ATG) are boxed.

conformation of supercoiled DNA in a given environment.

Functional Implications

The nucleotide sequences cleaved by mung bean nuclease occur in known and presumed regulatory regions of the pBR322 genome. The A+T-rich region cleaved (position 3199 to 3282) is at or near the terminator region for the ampicillin-resistance (amp-r) gene transcript (approx. position 3200; 54). Sequences cleaved at 1475 and 1522 occur in the 3' non-coding region of the tetracycline-resistance gene. The sequence cleaved at 2325 occurs near a promoter (P₄) activated by cyclic AMP receptor protein *in vitro*. The receptor protein binds to the -35 region of the promoter and protects nucleotides in the region of positions 2300 to 2322 (55). The inverted repeat sequences cleaved all occur in promoter or terminator regions as shown in Figure 6. The promoters for tetracycline-resistance (tet-r) and amp-r genes as well as for RNA priming of DNA replication are recognized. The terminator

for RNA-1 is also recognized. The only promoter in pBR322 at which prominent cleavage was not detected is the RNA-1 promoter. S1 nuclease cleavage in the inverted repeat sequences in the RNA primer promoter and in the RNA-1 terminator had been previously observed by others (12,13) but no correlation with these regulatory regions was established. Our results indicate that, in supercoiled pBR322 DNA, most of the nucleotide sequences involved in the regulation of transcription and some of the sequences involved in regulation of DNA replication can possess altered secondary structures.

The effects of DNA supercoiling on transcription initiation from the tet and RNA-1 promoters have been studied. Supercoiling strongly affects polymerase binding to the tet promoter (57) but not to the RNA-1 promoter (58) at 37°C. Our results indicate that supercoiling produces a radical change in DNA conformation at the tet promoter but not at the RNA-1 promoter under minimal conditions for in vitro transcription. It is possible that the potential cruciform which we detect at the tet promoter affects RNA polymerase binding.

In vitro expression of the tetracycline-resistance gene but not the ampicillin-resistance gene is dependent upon DNA supercoiling (66). The basis for this difference is not known. It is interesting in this regard that the location of altered DNA conformations (likely cruciforms) detected by mung bean nuclease in these promoters in vitro can be distinguished in detail. Cleavage in the tet-r gene promoter is upstream from the transcription start site while cleavage in both amp-r gene promoters is downstream from the start sites (Fig. 6).

Inverted repeat sequences frequently occur in regulatory regions (59) and in some cases are known to function at levels other than DNA secondary structure. For example, some inverted repeat sequences function in a B-DNA conformation as binding sites for multimeric proteins (40) and others function at the RNA level as hairpins in transcription termination (60). Thus, it is possible that the occurrence of altered DNA secondary structures in regulatory regions is coincidental and is of no functional significance. In the case of inverted repeat sequences in promoters, however, it seems likely that cruciform formation in DNA would at least affect initial binding by RNA polymerase in vitro (see above).

While it is clear that DNA supercoiling is important in vivo for transcription from certain promoters and for DNA replication (61), the question of whether altered secondary structures in supercoiled DNA are important for DNA function in vivo remains unanswered. It may be misleading to compare the lack of cruciform detection in certain studies

(62-65) to the present situation where natural, short palindromes present in non-coding, regulatory regions of DNA are being examined. We note that a potential cruciform in a protein-coding region of pBR322 (position 1319) is not a prominent recognition site under conditions where potential cruciforms in non-coding regions are cleaved by mung bean nuclease. It is likely that factors such as palindrome length, sequence (56) and flanking sequences affect detection of cruciforms both in vitro and in vivo.

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REFERENCES

1. Keene, M.A. and Elgin, S.C.R. (1981) *Cell* 27, 57-64.
2. Cartwright, I.L. and Elgin, S.C.R. (1982) *Nuc. Acids Res.* 10, 5835-5852.
3. Keene, M.A. and Elgin, S.C.R. (1984) *Cell* 36 121-129.
4. McCutchan, T.F., Hansen, J.L., Dane, J.B. and Mullins, J.A. (1984) *Science* 225, 625-628.
5. Kroeker, W.D. and Kowalski, D. (1978) *Biochemistry* 17, 3236-3243.
6. Sinden, R.R., Carlson, J.O. and Pettijohn, D.E. (1980) *Cell* 21, 773-783.
7. Pettijohn, D.E. and Pfenninger, O. (1980) *Proc. Natl. Acad. Sci. USA* 77, 1331-1335.
8. Ryoji, M. and Worcel, A. (1984) *Cell* 37 21-32.
9. Lilley, D.M.J. (1983) *Nature* 305, 276-277.
10. Gellert, M. (1981) *Ann. Rev. Biochem.* 50, 879-910.
11. Wells, R.D., Goodman, T.C., Hillen, W., Horn, G.T., Klein, R.D., Larson, J.E., Muller, U.R., Neuendorf, S.K., Grant, Panayotatos, N. and Stirdivant, S.M. (1980) *Prog. Nuc. Acid Res. Molec. Biol.* 24, 167-267.
12. Lilley, D.M.J. (1980) *Proc. Natl. Acad. Sci. USA* 77, 6468-6472.
13. Lilley, D.M.J. (1981) *Nucleic Acids Res.* 9, 1271-1289.
14. Panayotatos, N. and Wells, R.D. (1981) *Nature* 289, 466-470.
15. Larsen, A. and Weintraub, H. (1982) *Cell* 29, 609-622.
16. Hentschel, C.C. (1982) *Nature* 295, 714-716.
17. Schon, E., Evans, T., Welsh, J., and Efstradiadis, A. (1983) *Cell* 35, 837-848.
18. Nickol, J.M. and Felsenfeld, G. (1983) *Cell* 35, 467-477.
19. Shen, C.-K. J. (1983) *Nuc. Acids Res.* 11, 7899-7910.
20. Beard, P., Morrow, J.F. and Berg, P. (1973) *J. Virol.* 12, 1303-1313.
21. Shishido, K. (1979) *Agric. Biol. Chem.* 43, 1093-1102.
22. Dybvig, K., Clark, C.D., Aliperti, G. and Schlesinger, M.J. (1983) *Nuc. Acids Res.* 11, 8495-8508.
23. Shen, Y.A. and Eichkorn, G.L. (1968) *Biochem.* 7, 1026-1032.
24. Kowalski, D. and Sanford, J.P. (1982) *J. Biol. Chem.* 257, 7820-7825.
25. Kowalski, D. (1984) *Nuc. Acids Res.* 12, 7071-7086.
26. Sheflin, L.G. and Kowalski, D. (1984) *Nuc. Acids Res.* 12, 7087-7104.
27. Rodriguez, R. L., Bolivar, F., Goodman, H.M., Boyer, H.W. and Betlach, M. 1976. Construction and characterization of cloning vehicles. In *Molecular mechanisms in the control of gene expression* (ed. D.P. Nierlich et al.), p. 471. Academic Press, New York.

28. Sutcliffe, J.G. (1978) Cold Spring Harbor Symp. Quant. Biol. 43, 77-90.
29. Kowalski, D., Kroeker, W.D. and Laskowski, M., Sr. (1976) Biochemistry 15, 4457-4463.
30. Laskowski, M., Sr. (1980) Meth. Enzymol. 65, 276-284.
31. Radloff, R. Bauer, W. and Vinograd, J. (1967) Proc. Natl. Acad. Sci. USA 57, 1514-1521.
32. DeLeys, R.J. and Jackson, D.A. (1976) Biochem. Biophys. Res. Commun. 69, 446-454.
33. Holmes, D.S. and Quigley, M. (1981) Anal. Biochem. 114, 193-197.
34. Maxam, A.M. and Gilbert, W. (1977) Proc. Natl. Acad. Sci. USA 74, 560-564.
35. Pritchard, A.E., Kowalski, D. and Laskowski, M., Sr. (1977) J. Biol. Chem. 252, 8652-8659.
36. Singleton, C.K., Klysik, J., Stirdivant, S.M. and Wells, R.D. (1982) Nature 299, 312-316.
37. Singleton, C.K., Kilpatrick, M.W. and Wells, R.D. (1984) J. Biol. Chem. 259, 1963-1967.
38. Morita, M. and Oka, A. (1979) Eur. J. Biochem. 97, 435-443.
39. Itoh, T. and Tomizawa, J.-I. (1980) Proc. Natl. Acad. Sci. USA 77, 2450-2454.
40. Pabo, C.O. and Sauer, R.T. (1984) Ann. Rev. Biochem. 53, 293-321.
41. Hshieh, T.-S. and Wang, J.C. (1975) Biochemistry 14, 527-535.
42. Perelroyzon, M.P., Lyamichev, V.I., Kalambet, Y.A., Lyubchenko, Y.L. and Vologodskii, A.V. (1981) Nuc. Acids Res. 9, 4043-4059.
43. Funnell, B.E. and Inman, R.B. (1979) J. Mol. Biol. 131, 331-340.
44. Tong, B.Y. and Battersby, S.J. (1979) Nuc. Acids Res. 6, 1073-1079.
45. Dodgson, J.B. and Wells, R.D. (1977) Biochemistry 16, 2374-2379.
46. Palecek, E. (1976) Progress in Nucleic Acids Research and Molecular Biology 18, 151-213.
47. Larsen, R. and Messing, J. (1982) Nuc. Acids Res. 10, 39-49.
48. Cesareni, G. (1982) J. Mol. Biol. 160, 123-126.
49. Anderson, P. and Bauer, W. (1978) Biochemistry 17, 594-601.
50. Dove, W.F. and Davidson, N. (1962) J. Mol. Biol. 5, 467-478.
51. Singleton, C.K. (1983) J. Biol. Chem. 258, 7661-7668.
52. Sinden, R.R. and Pettijohn, D.E. (1984), J. Biol. Chem. 259, 6593-6600
53. Benham, C.J. (1981) J. Mol. Biol. 150, 43-68.
54. Stuber, D. and Bujard, H. (1981) Proc. Natl. Acad. Sci. USA 78 167-171.
55. Queen, C. and Rosenberg, M. (1981) Nuc. Acids Res. 9, 3365-3377.
56. Lilley, D.M.J. (1985) Nuc. Acids Res 13, 1443-1465.
57. Bertrand-Burggraf, E., Schnarr, M., Lefevre, J.F. and Daune, M. (1984) Nuc. Acids Res. 12, 7741-7752.
58. Wood, D.C. and Lebowitz, J. (1984) J. Biol. Chem. 259, 11184-11187.
59. Muller, U.R. and Fitch, W.M. (1982) Nature 298, 582-585.
60. Rosenberg, M. and Court, D. (1979) Ann. Rev. Genet. 13, 319-353.
61. Drlica, K. (1984) Microbiol. Rev. 48, 273-289.
62. Sinden, R.R., Broyles, S.S. and Pettijohn, D.E. (1983) Proc. Natl. Acad. Sci. USA 80, 1797-1801.
63. Courey, A. J. and Wang, J.C., (1983) Cell 33, 817-829.
64. Gellert, M., O'Dea, M. H. and Mizuuchi, K. (1983) Proc. Natl. Acad. Sci. USA 80 5545-5549.
65. Lyamichov, V., Panyatin, I. and Mirkin, S. (1984) J. Biomol. Structure and Dynamics 2, 291-301.
66. Yang, H.-L., Heller, K., Gellert, M. and Zubay, G. (1979) Proc. Natl. Acad. Sci. USA 76, 3304-3308.
67. Peden, K. W. C. (1983) Gene 22, 277-280.
68. Cesareni, G., Muesing, M.A. and Polisky, B. (1982) Proc. Natl. Acad. Sci. USA 79, 6313-6317.
69. Brosius, J., Cate, R.L., Perlmutter, A.P. (1982) J. Biol. Chem. 257, 9205-9210.