Changes in site specificity of single-strand-specific endonucleases on supercoiled PM2 DNA with temperature and ionic environment

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ABSTRACT

Mung bean nuclease sites in supercoiled PM2 DNA at neutral pH were located by linearizing the singly-nicked circular DNA product with venom phosphodiesterase followed by restriction endonuclease mapping. The locations of the sites varied with small changes in temperature and in concentration of NaCl or magnesium ion. Different environmental changes which affect duplex stability in the same direction showed similar effects on the number of sites and in some cases resulted in identical cleavage patterns. Venom phosphodiesterase and P1 nuclease showed cleavage patterns similar to mung bean nuclease under the same environmental conditions and showed similar variations in cleavage patterns when environmental conditions were changed. Relaxed, closed-circular DNA was slowly cleaved at numerous sites whose locations did not vary with environment. Changes in site specificity are likely the result of environmental effects on the conformation of supercoiled DNA as opposed to effects on the single-strand-specific endonucleases themselves.

INTRODUCTION

Negative supercoiling has striking effects on the secondary structure of DNA. At sufficiently high negative superhelical density, torsional stress promotes untwisting of the helix (1,2). This renders supercoiled DNA highly reactive to reagents which prefer single-stranded over double-stranded DNA (1,3). Negative supercoiling is also important for biological function since, in prokaryotes, it affects DNA replication, transcription and recombination (4,5).

Since it is possible that the functional importance of negative supercoiling resides in the promotion of local untwisting at unique DNA sequences, the specificity of the sites cleaved by single-strand-specific endonucleases in viral and plasmid supercoiled DNAs has been of considerable interest. Two very different types of observations have been made. Some studies have shown that cleavage is random or at a large number of specific sites (6-8), suggesting that untwisting is distributed throughout the DNA molecule. Many other studies have clearly shown that cleavage occurs at a

limited number of specific sites (9-22), indicating that some portion of the untwisting is localized. Since some of these studies were performed with different supercoiled DNAs and single-strand-specific endonucleases, several factors might account for the different types of observations. For example, the DNA sequence (19) and the degree of negative supercoiling (25) can affect the site specificity of these enzymes. Also, the enzymes themselves may differ in specificity. It is not known whether different single-strand-specific endonucleases possess the same intrinsic specificity or not. This has not been directly tested since these enzymes are used under different environmental conditions and the environment itself might alter specificity (see below). Clearly, these variables must be controlled before any generalizations about the specificity of single-strand-specific endonucleases acting on supercoiled DNA can be made.

Conventionally, single-strand-specific endonucleases have been used to probe DNA conformation in environments optimal for enzyme activity. With S1 nuclease, most studies have used pH 4.5 and 1 millimolar zinc ion in addition to buffer and salt. Studies done with other single-strand-specific endonucleases have been done under conditions which differ from S1 nuclease conditions and from each other. Although it has not been characterized in detail, it is known that the site specificity of S1 nuclease (9,17,21,23), mung bean nuclease (24) and venom phosphodiesterase (12,14) on supercoiled DNA depends upon environmental conditions. The environmental conditions might affect site specificity by acting on DNA conformation, the enzyme itself, or both. Thus, the results of probing DNA conformation with different enzymes under their own optimal conditions may not be directly comparable.

In order to investigate relationships between the conformation and function of supercoiled DNA, it would be useful to probe DNA conformation under conditions where DNA can function in vitro. At a minimum, such conditions involve neutral pH. Mung bean nuclease (26) can be used to probe DNA conformation at neutral pH. While mung bean nuclease is a less efficient catalyst at neutral pH than at its acidic pH optimum, the enzyme specificity with respect to its relative cleavage rate of supercoiled vs. relaxed PM2 DNA is maintained (22). Also, it is desirable to reduce or eliminate the zinc ion required for enzyme stability at acid pH in order to probe for DNA conformational effects of other divalent cations required for DNA function in vitro. This is possible with mung bean nuclease since at neutral pH the enzyme is stable in the absence of added zinc ion (26).

In this paper, I analyze the effects of temperature, NaCl and magnesium ion concentrations on the locations of mung bean nuclease cleavages in PM2 DNA

of constant superhelical density. I also determine if two other single-strand-specific endonucleases, venom phosphodiesterase (12) and P1 nuclease (27) recognize the same or different sites as mung bean nuclease under the same environmental conditions. As shown below, the locations of cleavages in supercoiled DNA exhibit complex variations depending on environment but not on the enzyme probe. In contrast, the locations of cleavages in relaxed, closed-circular DNA are not sensitive to environment. The results suggest that changes in site specificity result primarily from environmental effects on the conformation of supercoiled DNA as opposed to effects on the enzymes themselves. Two major cleavage sites map in regions of potential importance to PM2 DNA replication and transcription. The following paper reports the nucleotide sequences of these two regions of PM2 DNA and the locations of the mung bean nuclease cleavages within these sequences.

MATERIALS AND METHODS

Enzymes

Mung bean nuclease was isolated and purified to homogeneity as described by Kowalski et al. (26). Phosphodiesterase was isolated from the venom of Crotalus adamanteus and purified to homogeneity by Dr. H. Takamatsu as described (28). P1 nuclease prepared according to Fujimoto et al. (27) was from Yamasa Shoyu Co., Ltd., Choshi, Japan. Restriction endonucleases were supplied by New England Biolabs.

DNA

Bacteriophage PM2 DNA was isolated and purified as previously described (29). The titratable superhelical density of PM2 DNA (9550 bp) was -0.084 using the method and solution conditions of DeLeys and Jackson (30). Since the superhelical density of the DNA can vary in different preparations, the same preparation of PM2 DNA was used in all experiments.

Cleavage of Supercoiled DNA with Single-Strand-Specific Endonuclease

Reaction mixtures contained 10 mM Tris-HCl (pH 7.0), 2 μ g PM2 DNA and other components as indicated in figure legends in a volume of 18 μ l. After preincubation at the indicated temperature for 10 to 15 min, the reaction was initiated by addition of 2 μ l of the indicated enzyme dilution (in 10 mM Tris-HCl, pH 7.0, 0.005% Triton X-100, 0.1 mM serine, 1 μ M zinc acetate or in 10 mM Tris-HCl, pH 7.0, 0.005% Triton X-100 with no detectable difference in extent or specificity of cleavage). After incubating 30 min, the reaction was quenched by raising the pH to 9.2 with an equal volume of 40 mM Tris, 2 mM magnesium acetate, 0.01% Triton X-100, pH 11.7 and cooling on ice (22).

Linearization of Nicked-Circular DNA and Restriction Endonuclease
Fragmentation of Linear DNA

Cleavage opposite the single-strand-specific endonuclease nicks with venom phosphodiesterase was performed as previously described (22) except that sodium chloride and magnesium acetate were added to final concentrations of 91 mM and 0.91 mM, respectively, and the incubation time was 15 min. Since venom phosphodiesterase itself possesses single-strand endonuclease activity on supercoiled DNA (see Results and ref. 12), only samples free (or nearly free) of the supercoiled form were used. Linearization was generally 70 to 90% complete. The reaction was stopped and digestion of linear DNA with restriction endonucleases was performed as previously described (22).

Preparation of Relaxed, Closed-Circular PM2 DNA

Supercoiled PM2 DNA was relaxed using calf thymus DNA topoisomerase I (BRL). DNA (236 µg per ml) in a 500 µl solution containing 10 mM Tris-HCl (pH 7.0), 0.2 M KCl, 1 mM EDTA and 0.1 mg per ml bovine serum albumin was incubated at 37°C with 10 units enzyme for 3.5 hr and then 2 additional units for 0.5 hr. The enzyme was inactivated by heating at 65°C for 15 min. The DNA was desalted by two rounds of filtration through Sephadex G-50 equilibrated in 10 mM Tris-HCl (pH 7.0) using a spun-column procedure as described (31).

Gel Electrophoresis

DNA was electrophoresed through 22 cm long, horizontal slabs of 0.7% agarose prepared and submerged in 36 mM Tris, 30 mM NaH₂PO₄, 1 mM Na₂EDTA (pH 7.7). Electrophoresis was initiated at 50 V for 10 min and continued at 30-35 V for 16-20 hours. Staining of DNA in gels with ethidium bromide and photography of the fluorescence were previously described (29). Molecular weight values of DNA fragments were read from graphs of log molecular weight vs. mobility relative to linear PM2 DNA.

RESULTS

Effects of Temperature on Site Specificity

Supercoiled PM2 DNA was reacted with mung bean nuclease at neutral pH and various temperatures to give predominantly nicked-circular DNA and a small proportion of linear DNA (Fig. 1A). The nicked-circular DNA was linearized by cleavage opposite the nick using venom phosphodiesterase at 37°C (22). Unit-length linear DNA was produced (Fig. 1A) indicating that one venom phosphodiesterase-sensitive nick per DNA molecule was introduced by mung bean nuclease at each of the temperatures tested, as previously observed at 37°C (22). The site specificity of the nicks was determined by cleaving the linearized DNA at the single Msp I (Hpa II) site and sizing the products by

agarose gel electrophoresis. As shown in Figure 1B, the nicks occur at specific sites as indicated by the discrete banding patterns. The temperature of the reaction with mung bean nuclease has dramatic effects on the number and location of the sites. Since both mung bean nuclease (22) and Msp I (11) cut PM2 DNA once per molecule, pairs of fragments are produced. The sum of the fractional genome lengths (map units) is equal to 1.0. The relative intensity of one pair of bands to another reflects frequency of mung bean nuclease cleavage. At 37°C, two major cleavages lead to the fragment pairs 0.75+0.25 and 0.82+0.18 (Fig. 1B). Decreasing the temperature from 37°C results in a decrease in the frequency of cleavage at these sites and an increase in the frequency of cleavage at many new sites (Fig. 1B). The change in cleavage pattern is not a result of decreasing the rate of cleavage with temperature since decreasing the rate by lowering the enzyme concentration (as much as 500 fold) and increasing time (50 fold) at 37°C gave the same cleavage pattern seen at 37°C in Figure 1B (2 units per ml, 30 min). Increasing the temperature from 37°C results in an increase in the cleavage frequency at the site which leads to the pair at 0.82+0.18 as well as the appearance of new sites not present at 37°C or at lower temperatures. In contrast, the cleavage frequency at 0.75+0.25 shows little change.

To assign unique locations of the cleavages with respect to the Msp I site at position 1.0 or 0.0 on the PM2 map (32), the Msp I digests were treated with Pst I which cuts at 0.87 map units (22,33). If the mung bean nuclease site occurs between 0.87—>1.0 or 0.0—>0.50 on the map, the large member of the pair of fragments seen after Msp I digestion is expected to be cleaved by Pst I. Alternatively, if the site is between 0.50—>0.87 on the map, the small member of the pair of fragments would be cleaved. As shown in the right half of Figure 1B, Pst I does not cleave the large fragments of 0.82 and 0.75 map unit length, while their small fragment pairs at 0.18 and 0.25, respectively, are cleaved. Thus, for all temperatures tested, these mung bean nuclease sites are at map positions 0.82 and 0.75. Previous studies showed that at 37°C, the site at 0.75 actually consists of at least two closely-spaced cleavages at 0.75 and 0.76 (22).

The site at 0.75 maps with one of the eight early denaturation regions of PM2 DNA (34), while the site at 0.82 does not. The DNA sequence around the cleavage at 0.75 map units show that it is highly enriched in dA+dT (L. Sheflin and D. Kowalski, the following paper). Thus, the decreased cleavage frequency at 0.75 with decreasing temperature may be the result of stabilization of the duplex within the dA+dT-rich sequence. The basis for the increased frequency of

Figure 1. Effects of temperature on mung bean nuclease cleavage of PM2 DNA. **A)** Mung bean nuclease nicking (upper photograph) and venom phosphodiesterase linearization of mung bean nuclease-nicked DNA (lower photograph). PM2 DNA (100 μ g per ml) was incubated with mung bean nuclease in 10 mM Tris-HCl (pH 7.0, see Materials and Methods) at temperatures shown in the figure. Mung bean nuclease concentrations were 2 units per ml at 27°, 32° and 37°C and 0.2 units per ml at 42° and 47°C (units defined in ref. 26). The reactions

were quenched (see Materials and Methods) and 0.1 μ g DNA was removed from each reaction and electrophoresed through a 0.7% agarose gel shown in the upper photograph. The remaining DNA was linearized using venom phosphodiesterase (see Materials and Methods). DNA (0.1 μ g) was removed from each reaction and electrophoresed through a 0.7% agarose gel shown in the lower photograph. The rightmost two lanes in both photographs contain supercoiled and unit-length linear pBR322 DNA, respectively. B) Site specificity of mung bean nuclease nicks. The linearized DNA was cut either at the single Msp I site (left 5 lanes) or both the Msp I and single Pst I site (right 5 lanes). DNA (0.67 μ g in each of the left 5 lanes or 0.33 μ g in each of the right 5 lanes) was electrophoresed through a 0.7% agarose gel shown in the photograph. Map units refer to the fraction of the molecular weight of PM2 DNA (6.3×10^6). The center lane shows the following molecular weight (Mr) markers (all $\times 10^{-6}$): Msp I-PM2 DNA, 6.30 (52); Eco RI-lambda DNA, 4.79, 3.73, 3.59, 3.07, 2.18 (53); Hind III-PM2 DNA, 3.53, 1.42, 0.61 (52).

cleavage at 0.82 with increasing temperature (as well as at a site at 0.83) is unknown at present.

Effects of NaCl and Mg²⁺ on Site Specificity

Since the temperature affects the winding of the DNA helix (35,36), I tested the effects of NaCl and magnesium ion which also affect helical winding (37-39). Addition of NaCl (Fig. 2) or magnesium ion (Fig. 3) to the mung bean nuclease reaction (10 mM Tris-HCl, pH 7.0, 37°C) has a dramatic effect on the positional specificity of cleavage. As shown in Figure 2, addition of 20 to 100 mM NaCl results in a decrease in the frequency of cleavage leading to the pair 0.75+0.25 and in increase in cleavage frequency at many new sites. Cleavage leading to the pair 0.82+0.18 increases in frequency with addition of 5 mM NaCl, is maximal at 20 mM to 50 mM NaCl, and decreases at higher NaCl concentrations. A major site in 0.1M NaCl results in the pair 0.70+0.30. This site increases in cleavage frequency concomitant with the decrease in cleavage leading to the pair 0.75+0.25. Cleavages were assigned a unique position on the PM2 genome by secondary restriction endonuclease digestion with Pst I as described above. The major sites shown in Figure 2 map at 0.82, 0.75 and 0.70. As with decreasing temperature, increasing NaCl reduces cleavage frequency at the dA+dT-rich site at 0.75. The initial cleavage rate of supercoiled DNA is 10 to 20 fold lower in the presence of 50 mM NaCl than in the absence of NaCl (data not shown). However, the changes in cleavage position with NaCl are unlikely the result of NaCl inhibition of enzyme activity since when 13 fold excess enzyme is added to the reaction containing 50 mM NaCl, the same pattern shown in Figure 2 (50 mM NaCl) is obtained. The DNA sequence around the site at 0.70 map units indicates that cleavage occurs in a hyphenated inverted repeat sequence (L. Sheflin and D. Kowalski, the following paper).

The magnesium ion has a much greater affinity for DNA than does NaCl and binding is stoichiometric. Equivalence points occur around one magnesium ion

Figure 2. Effects of NaCl concentrations on mung bean nuclease specificity. After nicking with mung bean nuclease (2 units per ml) in 10 mM Tris-HCL (pH 7.0) at 37°C and the indicated NaCl concentration, DNA was cleaved opposite the nick with venom phosphodiesterase and subsequently cut at the Msp I site (see Materials and Methods). DNA (0.67 µg per lane) was electrophoresed through a 0.7% agarose gel shown in the photograph. Map units and Mr markers (right-most lane) are as described in Figure 1 legend.

per two phosphates (0.5 magnesium ion per DNA-P) and at one magnesium ion per phosphate (1.0 magnesium ion per DNA-P) (40,41). As shown in Figure 3, effects on the site specificity are seen at as low as 0.1 magnesium ion per DNA-P. This is equivalent to 25 µM magnesium ion at the DNA concentration used. Thus, magnesium ion is approximately 1000 times more effective than NaCl in bringing about comparable changes in site specificity. Increasing magnesium ion per DNA-P to 0.2 or 0.3 results in decreased cleavage frequency at the dA+dT-rich site at 0.75 and the site at 0.82 while cleavage at many new sites increases. From 0.3 to 0.6 magnesium ion per DNA-P, the cleavage patterns are almost identical. Further changes in the pattern are seen at 3.9 magnesium ion per DNA-P (Fig. 3). In the range of 0.1 to 0.5 magnesium ion per DNA-P, the ratio of magnesium ion to DNA-P and not the magnesium ion concentration is important since if magnesium ion concentration is held constant but the concentration of DNA changed, the digestion pattern changes (data not shown).

Figure 3. Effects of varying magnesium ion concentration on mung bean nuclease specificity. After nicking PM2 DNA (84 μ g per ml, 0.25 mM DNA-phosphate) with mung bean nuclease (2 units per ml) in 10 mM Tris-HCL (pH 7.0) at 37°C and the indicated ratio of magnesium ion to DNA-phosphate (magnesium ion per DNA-P), DNA was cleaved opposite the nick with venom phosphodiesterase and subsequently cut at the Msp I site (see Materials and Methods). DNA (0.67 μ g per lane) was electrophoresed through a 0.7% agarose gel shown in the photograph. Map units and Mr markers (right most lane) are as described in figure 1 legend.

Changes in Environment Similarly Affect the Site Specificity of Three Different Single-Strand Specific Endonucleases

In addition to using mung bean nuclease, I probed the structure of PM2 DNA with two other single-strand-specific endonucleases: P1 nuclease (27) and venom phosphodiesterase (12). As with mung bean nuclease, I used neutral pH conditions which are suboptimal for P1 nuclease (acid pH optimum) and venom phosphodiesterase (alkaline pH optimum). Figure 4 shows the results obtained for each of the three enzymes at 27°, 37° and 47° C in the presence of 0.1 M NaCl. The cleavage patterns for each of the enzymes change in a similar way with the temperature of the reaction. At a given temperature, the cleavage patterns are nearly identical indicating that the different enzymes recognize similar sites. Some differences in band intensities (cleavage frequency) are apparent. These may reflect differences in site preference.

Figure 4. Specificity of P1 nuclease (P1), mung bean nuclease (MB) or venom phosphodiesterase (VP) cleavages on PM2 DNA. Enzyme reactions were carried out in 10 mM Tris-HCl (pH 7.0) containing 0.1 M NaCl at 27°C (lanes 2, 5, 8), 37°C (lanes 3, 6, 9) and 47°C (lanes 4, 7, 10). Lane 1, 37°C, no enzyme. After 30 min incubation with P1 or mung bean nuclease at the specified temperature, the DNA was cleaved opposite the nick with venom phosphodiesterase (see Materials and Methods). The reaction of supercoiled DNA with venom phosphodiesterase was for 15 min at the specified temperature. Linearization of nicked-circular DNA in this case was achieved by adjusting pH to 9.2 (see Materials and Methods) and incubating at 37°C for 15 min without further addition of venom phosphodiesterase (see Materials and Methods). All linearized DNAs were subsequently cut at the Msp I site (see Materials and Methods) and 0.67 µg DNA per lane were electrophoresed through a 0.7% agarose gel shown in the photograph. Numbers shown at left are map units and those at right are $M_{rx}10^{-6}$ of markers in lane 11 (see Fig. 1 for identities of markers). P1 nuclease: 27°C, 1.0 µg per ml; 37°C, 0.33 µg per ml; 47°C, 0.033 µg per ml. Mung bean nuclease: 27°C, 10 units per ml; 37°C, 2 units per ml; 47°C, 0.2 units per ml. Venom phosphodiesterase: 27°C, 0.075 units per ml; 37°C, 0.015 units per ml; 47°C, 0.003 units per ml (units defined in ref. 29).

Without the addition of 0.1 M NaCl, the cleavage patterns for P1 nuclease and venom phosphodiesterase at 37°C and 47°C (data not shown) are also qualitatively similar to those for mung bean nuclease (Fig. 1B). Finally, the cleavage patterns seen with P1 nuclease at 37°C at various magnesium ion to DNA-P ratios (data not shown) are similar to those for mung bean nuclease (Fig. 3). Thus, the changes in site specificity with environment are independent of

Figure 5. Specificity of mung bean nuclease on relaxed, closed-circular DNA in different environments. Supercoiled PM2 DNA was relaxed with DNA topoisomerase I (see Materials and Methods). Relaxed DNA (100 μ g per ml) was reacted with mung bean nuclease in 10 mM Tris-HCl (pH 7.0) under conditions favoring unit-length linear DNA as opposed to smaller fragment formation. The conditions corresponding to the lanes in the figure are: 1) 37°C, 1.5 hr; 2) 47°C, 10 min; 3) 37°C, 1 mM magnesium ion (3.3 magnesium ion per DNA-P), 1.5 hr; 4) 37°C, 50 mM NaCl, 16 hr. The enzyme concentration was 8 units per ml in 1-3 and 12 units per ml in 4. After quenching the reaction and adjusting the conditions for restriction endonuclease digestion (see Materials and Methods), the DNA was cut at the unique Msp I site. DNA (0.9 μ g per lane) was electrophoresed through a 0.7% agarose gel shown in the photograph. Fluorescence intensity of unit-length linear DNA and fragments varies depending upon the extent of mung bean nuclease linearization of the circular DNA. Lane 5 shows the following molecular weight markers (all $\times 10^{-6}$): MspI-PM2 DNA, 6.30 (38), EcoRI-lambda DNA (see Figure 1 legend) and a mixture of restriction endonuclease fragments of pBR322 DNA (54).

the three enzymes used to probe for single-stranded character in supercoiled DNA.

Changes in Environment Do Not Affect the Site Specificity on Relaxed DNA

Mung bean nuclease recognizes a limited number of sites in relaxed, linear PM2 DNA (42). The enzyme cleaves relaxed, closed-circular PM2 DNA 28,000 times more slowly than the supercoiled form (22). At the higher enzyme concentrations and longer incubation times required, relaxed, closed-circular DNA is converted to unit-length linear DNA and smaller fragments (22). The

specificity of the cleavages has not been previously determined.

Relaxed, closed-circular PM2 DNA was incubated with mung bean nuclease under several different environmental conditions. Enzyme concentration and incubation time were chosen to favor formation of unit-length linear DNA as opposed to smaller fragments. These limited digestions also contained unreacted relaxed, closed-circular DNA. The specificity of the mung bean nuclease cleavages was determined by cutting the DNA at the unique Msp I site and separating the products by agarose gel electrophoresis. As shown in Figure 5, no prominent sites such as those seen in supercoiled DNA are observed in relaxed DNA. Instead, numerous bands of similar intensities are seen. No differences in the banding pattern were observed when the mung bean nuclease reaction was carried out at 37°C (lane 1), 47°C (lane 2), 37°C, 1 mM magnesium ion (lane 3), 37°C, 50 mM NaCl (lane 4) or 37°C, 100 mM NaCl (not shown). When the DNA was cut at the unique Pst I site instead of the Msp I site, the banding pattern was also unaffected by the mung bean nuclease reaction conditions (data not shown). Thus, both the detection of prominent cleavage sites and the changes in location of those sites with environment are properties of supercoiled, but not relaxed, PM2 DNA.

DISCUSSION

I have shown that the site specificity of single-strand-specific endonucleases on supercoiled PM2 DNA can vary with small changes in temperature and ionic environment. Where tested, both the locations of the cleavages under the same environmental conditions and the variations in locations under different environmental conditions are largely independent of the three different single-strand-specific nucleases used. Also, the locations of numerous sites in relaxed DNA are not affected by changing environmental conditions. These and the following observations are consistent with the idea that the variation in site specificity is mainly the result of environmental effects on the conformation of supercoiled DNA and not on the enzymes themselves: 1) At 0.5 magnesium ion per DNA-P, the effects of magnesium ion are dependent upon the ratio of magnesium ion to DNA-P rather than the concentration of magnesium ion. 2) The effects of NaCl are exhibited in a concentration range where NaCl is known to maximally affect the stability of duplex DNA (50). 3) magnesium ion is 1000 times more effective than NaCl in stabilizing duplex DNA and in bringing about comparable changes in positional specificity. 4) At concentrations where changes in positional specificity are observed, magnesium ion and NaCl do not affect the enzymatic hydrolysis rate of

single-stranded DNA but strongly reduce the hydrolysis rate of supercoiled PM2 DNA (data not shown). 5) Different environmental changes known to affect duplex stability in the same direction can lead to identical cleavage patterns. For example, decreasing temperature from 37°C (Fig. 1B, 27°C) has the same effect as adding magnesium ion (Fig. 3, 37°C, 0.2 magnesium ion per DNA-P). 6) Different environmental changes which affect duplex stability in the same direction can be additive when performed in combination. For example, decreasing temperature from 37°C and adding NaCl (Fig. 4, lane 5, 27°C, 0.1 M NaCl) has a similar effect to adding excess magnesium ion (Fig. 3, 37°C, 3.9 magnesium ion per DNA-P).

The striking variations in site specificity of single-strand-specific nucleases on supercoiled PM2 DNA with environment suggest that re-interpretation of earlier studies where the environment was not the primary concern may be warranted. For example, since similar site specificities were found for mung bean nuclease and venom phosphodiesterase when compared under the same reaction conditions (Fig. 4), the earlier observations of different specificities on PM2 DNA were likely the result of the different reaction conditions used (14,22) rather than differences in intrinsic specificities of the enzymes. In addition to affecting site specificity, environmental conditions can affect the number of nucleases cleavage sites. Decreasing temperature from 37°C (Fig. 1B) or adding NaCl (Fig. 2) or magnesium ion (Fig. 3) increases the number of locations cleaved (at a level of one cleavage per DNA molecule). It is possible that previous findings of single-strand-specific endonuclease cleavage at a large number of specific sites (6-8) vs. a limited number of sites (9-22) can be reconciled by differences in environmental conditions. However, since in most of the studies the superhelical density of the DNA was not reported and since the intrinsic specificities of two of the enzymes used (S1 nuclease and N. crassa nuclease) have not been compared to each other or the enzymes used in the present study, no conclusion about the effects of differences in environmental conditions on the number of cleavage sites observed in previous studies can be drawn.

The torsional stress associated with negative supercoiling facilitates untwisting of DNA strands and enhances the reactivity toward single-strand-specific reagents. Supercoiled PM2 DNA is cleaved by mung bean nuclease 28,000 times more rapidly than the relaxed form (22). The results show that the increased cleavage rate of supercoiled DNA is not a result of increased extent or frequency of untwisting at all the sites observed in relaxed DNA. Relaxed DNA is cut at numerous sites, each at about the same frequency. By comparison, supercoiled DNA is cut at fewer, more prominent sites at different

frequencies. While the locations of sites in supercoiled PM2 DNA are extremely sensitive to changes in temperature and ionic environment the locations of sites in relaxed DNA are not. This finding is not unique to highly supercoiled PM2 DNA (superhelical density = -0.084 , see Materials and Methods) since the locations of cleavages in DNAs of lower superhelical density such as pBR322 (-0.067) and SV40 (-0.05) are also sensitive to changes in environment (M. Fowler, L. Iacono, L. Wilson, unpublished results from this laboratory). Thus, the environmental sensitivity of DNA conformation detected by single-strand-specific nucleases seems to be a property of torsionally-stressed DNA. Other studies done with S1 nuclease at acid pH have arrived at a similar conclusion (23).

In torsionally-stressed DNA, alterations in DNA conformation by temperature and ionic environment are partitioned between helical twist (secondary structure) and supercoiling (tertiary structure) in an unknown manner. Thus, it is possible that the primary effects of environmental changes which lead to alternative cleavage sites occur at the level of secondary structure, tertiary structure or both. It is known that the tertiary structure of torsionally-stressed DNA can change with ionic strength at values in the range of NaCl concentrations studied here (43,44). However, it is not known how local changes in tertiary structure affect secondary structure and accessibility of DNA sequences. The conformation of DNA at the level of secondary structure is better understood. Unusual secondary structures such as cruciforms and Z-DNA are favored in negatively supercoiled DNA (45-47). The untwisting of DNA strands associated with these and possibly other structures can be recognized by single-strand-specific nucleases (18,20,25). Changes in temperature and ionic environment can alter the stability of cruciforms (48), Z-DNA (49) and possibly other structures in supercoiled DNA. Changes in stability of these structures could alter their recognition by single-strand-specific nucleases. Structures at different sites may compete for existence depending upon the environment (51). The present study can not distinguish whether the stability or the detectability (or both) of these structures changes with environment. Structures that are detectable under some conditions might be present but not detectable under other conditions. Such structures might go undetected as result of inaccessibility to the enzyme probe. In addition, since a single nick in supercoiled DNA results in loss of both torsional stress and structures dependent upon torsional stress, only the most rapidly-cleaved structures are detected. Thus, the detectability of a structure also depends on its cleavage rate relative to those of other structures present. Using

single-strand-specific nucleases in a variety of environments allows one to scan different subsets of altered secondary structures, the full complement of which is not detectable in any one environment. The following paper presents the nucleotide sequences around two major sites (0.70 and 0.75 map units) whose cleavage depends on the ionic environment of supercoiled PM2 DNA.

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