Single strand specific mung bean nuclease was used to probe for regions of altered secondary structure in supercoiled PM2 DNA. Supercoiled DNA is cleaved \( \geq 10,000 \) times faster than the relaxed topoisomerase. Catalytic quantities of enzyme convert supercoiled DNA to both nicked-circular and unit length linear forms at pH 5 but to predominantly the nicked-circular form near neutral pH. At the elevated enzyme concentrations required to cleave relaxed DNA, unit length linear DNA and smaller fragments are produced from pH 5 to 7. One nick per supercoiled DNA molecule is introduced at pH 6.6. The nicks are repairable by DNA ligase and are not strand-specific. Snake venom phosphodiesterase selectively cleaves the strand opposite the nicks, permitting restriction endonuclease mapping. The nicks occur at three specific sites. Sites at 0.75 and 0.76 map units are cleaved with equal frequency, while a site at 0.82 is cleaved less frequently. The former sites map near one of the eight known early denaturation regions of PM2 DNA, while the latter does not. Since most early denaturation sites are not cleaved, sites other than these DA + dT-rich regions may be the preferred locations of strand unwinding and separation in supercoiled PM2 DNA.

An endonuclease isolated from mung bean sprouts (1) preferentially cleaves denatured as opposed to native DNA (2) and thus has been called "single strand specific." Mung bean nuclease has been purified to homogeneity (3) and the activity on various regions of DNA characterized. Known single-stranded regions of DNA such as single-stranded tails (4) and internal single-stranded gaps (5) are preferentially cleaved, while nicks are relatively resistant. Transient, localized unwinding of duplex DNA structure may result in susceptible sites both within (2, 5, 6) and at the ends (7) of a linear molecule. Hydrolysis at these transiently single-stranded regions is slow relative to that of single-stranded DNA and is highly dependent on reaction conditions which affect the secondary structure of DNA (2, 5, 7). Model DNA heteroduplexes whose mismatched bases may be accommodated in a stacked helical structure are cleaved at extremely low efficiency when only one base is mismatched and at greater efficiency as the number of adjacent mismatched bases is increased (8).

Closed circular duplex DNA can be isolated in a supercoiled form which is highly reactive to enzymes, proteins, and chemical agents which show a preference for single-stranded DNA (for reviews, see Refs. 9 and 10). The single strand character of this DNA is a consequence of torsional strain which, at sufficiently high negative superhelical density, promotes unwinding of helical twists (10, 11). In the absence of strand breakage, the unwinding of one turn of the double helix allows the untwisting of one negative supercoil (12). Negative supercoiling of DNA in prokaryotes is essential for cell growth and may be required to promote the strand unwinding and separation which occur during DNA replication, transcription, and recombination (for reviews, see Refs. 13 and 14). Thus, it is likely that some sites in negatively supercoiled DNA which are recognized by single strand specific agents are of biological importance.

We wanted to assess the feasibility of using mung bean nuclease to probe the structure of negatively supercoiled DNA near neutral pH, away from the acidic pH optimum of the enzyme. Neutral pH is advantageous since it more closely approximates the pH that both DNA and proteins which interact with DNA experience in vivo. In addition, spontaneous nicking of DNA near neutral pH is negligible compared to that at acidic pH. At acidic pH, mung bean nuclease introduces a limited number of nonrandom cleavages in both supercoiled (15) and linear (5) forms of PM2 DNA. The locations of these sites were not determined.

In this paper, we demonstrate that mung bean nuclease is catalytically active on supercoiled PM2 DNA near neutral pH. We quantify the preference for the supercoiled over the relaxed topoisomerase as a function of pH and characterize the products formed. We map the nicks introduced into supercoiled PM2 DNA near neutral pH utilizing venom phosphodiesterase as a reagent to selectively cleave the strand opposite the nicks. Finally, we compare the locations of single strand character detected by mung bean nuclease to the locations of the known early melting sites in PM2 DNA (16).

**MATERIALS AND METHODS**

**Enzymes—** Mung bean nuclease was isolated and purified to homogeneity as described (3). DNA topoisomerase I was isolated from calf liver nuclei and purified to homogeneity as described. Phosphodiesterase was isolated from the venom of *Crotalus adamanteus* and purified to homogeneity by Dr. A. E. Pritchard as described (17). Restriction endonuclease *Hpa* II was purchased from Miles Laboratories, *Msp* I, the isoschizomer of *Hpa* II, as well as *Pst* I, *Eco* R I, and *Hind* IIII were purchased from New England Biolabs. *Msp* I was used interchangeably with *Hpa* II with identical results. *Escherichia coli* DNA ligase was purchased from P-L Biochemicals.

DNA—PM2 DNA was isolated and purified as previously described (18). The supercoiled DNA was converted to the relaxed form using DNA topoisomerase I. Forty-two µl of topoisomerase I solution (16 units/ml in 1.0 M KCl, 10 mM Tris-HCl, pH 7.8, 0.5 mM NaEDTA, 0.006% Triton X-100) were added to a solution (0.42 ml) containing 40 µg of PM2 DNA in 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.8, 0.1 mM NaCl, 0.5 mM NaEDTA, 0.005% Triton X-100, and the mixture was kept at 37 °C for 30 min. One unit of activity converts 0.1 µg of PM2 DNA to the relaxed form in 10 min at 37 °C. Topoisomerase I was completely inactivated by heating the
solution at 65 °C for 15 min. Bacteriophage λ DNA was purchased from Miles Laboratories.

Reaction with Mung Bean Nuclease—Reaction mixtures containing 10 mM Tris or 10 mM sodium cacodylate adjusted to the appropriate pH with HCl or NaOH, respectively. Unless indicated otherwise, pH values refer to measurements made at 21 °C. Sodium chloride and additional Tris (≥1.3 mM each) were contributed to the reaction mixture by the DNA solution (10 mM Tris-HCl, pH 7.3, 10 mM NaCl). Mung bean nuclease units refer to the standard assay procedure (3) using denatured salmon sperm DNA. The mung bean nuclease diluent contained 10 mM Tris-HCl, pH 7.0, 0.1 mM serine, 1 mM zinc acetate, and 0.005% Triton X-100 (5). Two microliters of the appropriate enzyme dilution were added to the reaction mixture (18 µl) containing 0.4 µg of PM2 DNA (2.0 µg where indicated) in a 1.5-ml conical polystyrene tube at 37 °C. Incubation time was 10 min unless otherwise indicated. For fluorometric analysis, the reaction was terminated by transferring 0.1-0.2 ml of ethidium bromide solution (see under “Fluorometric Assay” below) from a cuvette containing 1.0 ml of ethidium bromide solution to the reaction tube and then transferring the mixture back to the same cuvette. The reaction mixture was then rinsed once with the DNA/ethidium bromide mixture, and the mixture was returned to the cuvette. For gel electrophoretic analysis, reactions were terminated as indicated either below or in the figure legends.

Linearization of Nicked-Circular DNA with Venom Phosphodiesterase—The mung bean nuclease reaction was terminated by placing the reaction mixture on ice. Conditions for phosphodiesterase digestion were established by addition of an equal volume of 40 mM Tris, 2 mM magnesium acetate, 0.01% Triton X-100, 11.7 pH (pH adjusted by addition of NaOH). The final pH is 9.2. One-ninth volume of phosphodiesterase (0.015 units of phosphodiesterase containing 12.5 µg of magnesium acetate, 0.00625% Triton X-100, pH 9.2) was added, and the mixture was incubated at 37 °C for 10 min. Control reactions without phosphodiesterase showed no further nicking of DNA by mung bean nuclease under these conditions. Phosphodiesterase was irreversibly inactivated by adding 1/6 volume 0.1 M NaOH, 10 mM EDTA (pH 7). The reaction mixture was then placed on ice and samples taken for electrophoresis.

Phosphodiesterase Assay—Phosphodiesterase was assayed using bis-p-nitrophenyl phosphate (5 mM bis-p-nitrophenyl phosphate, 10 mM MgCl₂, 0.10 M Tris-HCl, pH 9.0, 1.0-ml volume) as substrate. For digestion with Hpa II (Msp I) by lowering the pH to 6.0-6.5, the reaction was stopped with 0.1 M NaOH, 10 mM EDTA (pH 7). The reaction mixture was then placed on ice and samples taken for electrophoresis.

Digestion of Linearized DNA with Restriction Endonucleases—After the phosphodiesterase treatment, reaction conditions were adjusted for digestion with Hpa II (Msp I) by lowering the pH to 7.4 with 0.17 M acetic acid and addition of magnesium acetate to 17 mM. Digestion of 2 µg of DNA in 60 µl was performed with 7.5 units (New England Biolabs) of enzyme at 37 °C for 2 h. A subsequent digestion with Pst I was performed by diluting 6 µl of Hpa II-restricted DNA (0.2 µg) with an equal volume of 36 mM Tris, 30 mM potassium phosphate, 1 mM Na₂EDTA, incubating with 1 unit of Pst I for 30 min at 37 °C, and keeping on ice for 30 min.

For HindIII digestions, pH and MgCl₂ were adjusted as for Hpa II digestion but, in addition, the solution was made 50 mM in NaCl. Digestion of 2 µg of DNA in 60 µl was performed at 37 °C for 3 h by adding 7.5 units of enzyme both at zero time and 1.5 h.

Fluorometric Assay for Endonuclease Activity on Covalently Closed Circular DNA—The assay monitors the conversion of covalently closed circular DNA (supercoiled or relaxed) to open forms (nicked-circular, linear). Covalently closed forms of DNA renature after heating is a measure of covalently closed circular DNA (18). For the pH 5.0 conditions, cleavages were corrected for those which occur in the absence of enzyme. No correction was necessary under pH 8.0 conditions.

RESULTS

Enzymatic Cleavage Rate of Supercoiled and Relaxed DNA as a Function of pH—A fluorometric assay for covalently closed circular DNA (18) was used to measure the rate of the first endonucleolytic cleavage (single or double strand break). Although the activity is optimal at acidic pH (7), cleavage of supercoiled DNA is readily measurable at neutral and alkaline pH values at elevated enzyme concentrations. Initial rate measurements with supercoiled DNA at pH 5 and pH 8 are shown in Fig. 1. The enzyme concentration at pH 8.0 (filled circles) is 75,000 times that at pH 5.0 (open circles). Providing less than 50% of the substrate was cleaved and chemical compounds which stabilize the activity at acidic pH values were present (see under “Materials and Methods”), the cleavage of closed circular DNA substrate was linear with time and enzyme concentration at all pH values tested.

The initial reaction velocity per enzyme molecule as a function of pH for supercoiled DNA and the same DNA converted to the relaxed form by topoisomerase I is shown in Fig. 2. The logarithm of activity declines as a relatively smooth and continuous function with increasing pH as expected for a single enzyme activity with acidic pH optimum. Supercoiled DNA is hydrolyzed at least 10,000-fold more rapidly than the
relaxed form (Table I). The maintenance of the highly preferential hydrolysis of supercoiled DNA suggests that the activity near neutral pH is due to the single strand specific nuclease itself and not a contaminating enzyme with neutral or alkaline pH optima. Even at pH 6.6, mung bean nuclease acts catalytically on supercoiled DNA, since 1 enzyme molecule cleaves 100 DNA molecules in 10 min (Fig. 2).

Hydrolysis Products of Supercoiled and Relaxed DNAs at Various pH Values—Following incubation of DNA with enzyme at 37 °C and pH values from 5 to 9, the hydrolysis products were analyzed by agarose gel electrophoresis. As shown in Fig. 3, with a supercoiled DNA substrate, the products consist of mainly nicked-circular DNA (II) and a low proportion of linear DNA (III). The proportion of linear DNA formed decreases with increasing pH values. At pH 7, supercoiled DNA is converted almost exclusively to nicked-circular DNA (gel 7), which is completely resistant to linearization by a 40-fold increase in enzyme concentration (gel 8). In contrast to these results with supercoiled DNA, at the ~10,000-fold higher enzyme concentrations required to hydrolyze relaxed DNA (gel 7), nicked-circular DNA does not accumulate as shown in Fig. 4. Instead, unit length linear DNA and smaller fragments are produced at pH 5 to pH 7.

Characterization of the Nicked-Circular DNA Product—Having established the ability of mung bean nuclease to act catalytically on supercoiled DNA near neutral pH, we chose pH 7.0, 10 mM Tris-HCl (pH 6.6 at 37 °C) as a reaction buffer for further studies. The nicked-circular DNA produced by the action of two different levels of enzyme on supercoiled DNA was characterized. The number of nicks per molecule were determined by linearization of the nicked-circular DNA with venom phosphodiesterase which cleaves nicked-circular DNA 2000 times faster than covalently closed relaxed DNA (22). As shown in Fig. 5, nicked-circular DNA produced by mung bean nuclease at levels almost sufficient for complete hydrolysis of substrate (gel 1) and in 40-fold excess (gel 3) is converted to unit length linear molecules by phosphodiesterase (gels 2 and 4, respectively). No fragments of linear DNA are seen indicating that, even in the presence of excess mung bean nuclease, the nicked-circular DNA contains one nick per molecule. This conclusion was confirmed by alkali denaturation of the nicked-circular DNA prior to electrophoresis. The resulting single-stranded DNA separates into four bands of similar intensities as shown in Fig. 6, gel 2. The presence of 40-fold excess enzyme does not alter the band pattern (gel 4). Two of the

![Fig. 2. Initial reaction velocity per enzyme molecule as a function of pH for supercoiled and relaxed PM2 DNAs.](image)

![Fig. 3. Agarose gel electrophoresis of the digestion products of supercoiled PM2 DNA.](image)

![Fig. 4. Agarose gel electrophoresis of the digestion products of relaxed PM2 DNA.](image)
single-stranded DNA bands (bands I and 3, from top to bottom) correspond to the complementary strands of unit length linear PM2 DNA, since they co-migrate with authentic complementary linear strands produced by cleavage of PM2 DNA at the single Hpa II site and subsequent denaturation. The remaining two bands are the complementary single strand circles. The latter bands do not appear after nicked-circular DNA is linearized with phoshodiesterase prior to denaturation and electrophoresis (not shown). Since both sets of circular and linear complementary single strands are present in approximately equal proportions, the mung bean nuclease nicks are not strand-specific.

To confirm that the single strand cleavages are in fact nicks and not small gaps, the DNA samples applied to gels 2 and 4 in Fig. 6 were treated with E. coli DNA ligase prior to alkali denaturation and electrophoresis. Under the neutral pH conditions of electrophoresis, covalently closed circular DNA renatures, but open circular forms of DNA do not (18). As shown in gels 3 and 5, the bulk of the DNA forms a ladder of slower migrating bands, characteristic of the relaxed, covalently closed topoisomers produced by DNA ligase and nicked-circular PM2 DNA (23). Thus, the bulk of the mung bean nuclease cleavages are indeed nicks, and, consistent with the known specificity of mung bean nuclease (1) and the requirements of E. coli DNA ligase (24), the nicks contain 3'-OH and 5'-P termini.

**Positional Specificity of the Single Strand Cleavages—** After complete conversion of supercoiled DNA to the nicked-circular form by mung bean nuclease near neutral pH, the nicked-circular DNA was partially converted to unit length linear DNA using phosphodiesterase as shown above in Fig. 5. The conversion to the linear form was purposely limited to minimize the exonuclease degradation of the linear DNA by phosphodiesterase (22). Treatment of this PM2 DNA mixture with the single site specific restriction endonuclease Hpa II converts the residual nicked-circular DNA to the linear form and the phosphodiesterase-linearized DNA to a set of discrete fragments as shown in Fig. 7, gel 1. The appearance of discrete bands in the agarose gel indicates that the nicks produced by mung bean nuclease are at specific sites. Molecular weight analysis showed that the DNA in the four discrete bands corresponds to fractional genome lengths (PM2 map units) of 0.82, 0.755, 0.245, and 0.18 (barely visible in original). By quantitative densitometry of the photographic negative of the stained gel, the more abundant fragments 0.755 and 0.245 were found to be present in equimolar amounts, as were the less abundant fragments 0.82 and 0.18 (data not shown). Both by size and by relative intensity, the Hpa II fragments of sizes 0.755 and 0.245 clearly originate from the same linear DNA molecule, while the fragments of sizes 0.82 and 0.18 originate from a different linear DNA molecule. Band 0.245 is broad and actually consists of two bands of similar intensity as shown by higher resolution gel analysis (data not shown). The fractional genome lengths are 0.25 and 0.24. We infer that the 0.755 band consists of unresolved fragments of 0.75 and 0.76 map units. DNA molecules of these sizes are not resolved under our conditions of electrophoresis.

For each pair of Hpa II fragments, there are two possible

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**Fig. 5 (left).** Electrophoresis of PM2 DNA on 0.7% agarose gels after nicking with mung bean nuclease and linearizing with venom phosphodiesterase. I, supercoiled DNA; II, nicked-circular DNA; III, unit length linear DNA. DNA (2 µg in 20 µl) in 10 mM Tris-HCl, pH 7.0, was incubated at 37 °C with mung bean nuclease at 0.05 units/ml of DNA (gel 1) and 0.20 units/ml (gels 2 and 3). After 10 min, reactions were stopped by boiling on ice. Two microliters of venom phosphodiesterase (200 units/ml of DNA) were added to each sample. Two microliters of 0.1 M Tris-HCl (pH 8.0), 20 mM magnesium acetate, 71 mM nicotinamide adenine dinucleotide, and 0.14 mg/ml of bovine serum albumin was added to each sample. Two microliters of E. coli DNA ligase (0.4 unit) were added to samples 2 and 3 while 2 µl of ligase diluent (10 mM Tris, pH 8.0, 5 mM magnesium acetate, 25 µM nicotinamide adenine dinucleotide, 50 µg/ml of serum albumin) was added to the remaining samples. After 20 min at 21 °C, Na2EDTA was added to 10 mM, and NaOH was added to 0.1 M. After 5 min at 21 °C, the samples (containing 125 ng of DNA) were cooled to ~0 °C, made 5% in glycerol and 0.01% bromphenol blue, and loaded under cold (4–8 °C) electrophoresis buffer (see under "Materials and Methods").

**Fig. 7.** Restriction endonuclease mapping of mung bean nuclease cleavage sites in supercoiled PM2 DNA. After nicking with 0.05 unit/ml of mung bean nuclease and linearization with venom phosphodiesterase as described in the legend to Fig. 5, the DNA was treated with restriction endonuclease Hpa II (gel 1) or Hpa II followed by Pst I (gel 2) as described under "Materials and Methods." DNA (200 ng) was electrophoresed through 0.7% agarose gels (see under "Materials and Methods"). Map units refer to the molecule weight ratio of the fragments to unit length linear PM2 DNA (6.3 × 106). Molecular weight markers (gel 3) are tll × 104: HindIII-PM2 DNA, 6.30 (21); Eco RI-ADNA, 4.79, 3.73, 3.59, 3.07, 2.18 (36); HindIII-PM2 DNA, 3.53, 1.42, 0.61, 0.265, 0.245, 0.18, 0.06 (21).
locations for the mung bean nuclease cleavage sites. In order to assign the locations of the mung bean nuclease cleavage sites on the PM2 genome, the Hpa II digest was treated with Pst I which cleaves at 0.87 map units (see under "Materials and Methods"). If mung bean nuclease cleaves at a site clock-wise from the Hpa II site (i.e. 0.18 or 0.245), the larger member of the Hpa II pair (fragments 0.82 or 0.755) would be cleaved by Pst I. Alternatively, if the cleavage site were counter-clockwise from the Hpa II site (i.e. 0.82 or 0.755), then the smaller member of the Hpa II pair (fragments 0.18 or 0.245) would be cleaved by Pst I (refer to diagram in Fig. 8 for clarification). As shown in Fig. 3c, fragments 0.245 and 0.18 are cleaved by Pst I while their counterpart fragments 0.755 and 0.82, respectively, are not cleaved. Therefore, the mung bean nuclease nicks occur at positions 0.755 and 0.82. The greater abundance of band 0.755 over band 0.82, along with the inference that the 0.755 fragment is a mixture of equal amounts of fragments 0.76 and 0.75 (see above), indicate that mung bean nuclease nicks PM2 DNA primarily at 0.76 and 0.75 map units and less frequently at 0.82 map units.

**DISCUSSION**

Though the optimal pH is acidic, catalytic quantities of mung bean nuclease efficiently cleave supercoiled PM2 DNA under neutral pH conditions. The reaction is highly preferential for supercoiled DNA, since 28,000 times more enzyme is required to cleave the relaxed topoisomerase at the same rate. Supercoiled DNA is converted to singly nicked circular DNA. The nicked-circular DNA is resistant to linearization in the presence of at least 40 times the amount of enzyme required for complete conversion. The efficiency and limited nature of this reaction makes mung bean nuclease a useful alternative to pancreatic DNase I for the preparation of singly nicked PM2 DNA molecules. Since the nicks possess 3'-OH and 5'-P termini, these molecules serve as substrates for DNA ligase (see under "Results"), exonuclease III, and DNA polymerase.

It is interesting that the single strand specific endonuclease activity of venom phosphodiesterase shows a similar preference (10,000-fold) for supercoiled over relaxed PM2 DNA (22). In contrast to mung bean nuclease hydrolysis of supercoiled DNA, however, the nicked-circular DNA produced does not accumulate but is rapidly linearized, presumably facilitated by gap formation by the associated 3' → 5' exonuclease activity. Since efficient linearization occurs whether the initial nick is generated by the enzyme itself, by DNase I (22), or by mung bean nuclease, venom phosphodiesterase appears to be an excellent reagent for specific cleavage of the strand opposite nicks containing 3'-OH and 5'-P termini in duplex DNA.

The mung bean nuclease nicks in supercoiled PM2 DNA occur at three specific sites at a frequency at one nick per DNA molecule with no apparent strand specificity. Since several previous studies with supercoiled DNA showed a correlation between sites reactive to single strand specific agents and sites of early denaturation (16, 25-27), it was interesting to compare the locations of the mung bean nuclease cleavages to the locations of the early denaturation sites determined by Brack et al. (16). As illustrated in Fig. 8, mung bean nuclease prefers two sites (filled arrows) which map closely to each other (0.75 and 0.76 map units) and to one of the eight early denaturation sites (open circles). No cleavages are detected at the remaining seven early denaturation sites. A minor site for nicking occurs at 0.82 (Fig. 8, open arrowhead) which does not correspond to any known early denaturation site. Under different reaction conditions than those used here, the single strand specific endonuclease activity of venom phosphodiesterase (22) cleaves PM2 DNA at three sites (0.15, 0.62, and 0.78) which correlate with early denaturation sites and at two sites (0.72 and 0.85) which do not (27). Preliminary results with mung bean nuclease indicate that the positional specificity of cleavage is extremely sensitive to reaction conditions. Thus, the lack of correspondence of the sites cleaved by the two enzymes may reflect the differences in reaction conditions as well as possible differences in enzyme specificity. In any case, these studies indicate that the property of early denaturation alone is not a sufficient criterion to predict the sites recognized by these single strand specific endonucleases in supercoiled PM2 DNA. In contrast to these results obtained using enzymatic probes, single strand DNA binding protein (bacteriophage T4, gene 32 product) sites map exclusively at each of the eight early melting regions of PM2 DNA (16). Since mung bean nuclease preferentially cleaves DNA at dA + pN and dT ↓ pN (1), and since early denaturation sites are presumed to be rich in dA + dT regions (28, 29), the base preference of the enzyme can not explain why most early denaturation sites are not cleaved. The discrimination between early melting sites by single strand specific endonucleases and the cleavage of additional sites not recognized by single strand DNA binding protein suggest that properties other than or in addition to early denaturation are involved in the recognition of supercoiled DNA by single strand specific endonucleases. Thus, sites other than dA + dT-rich regions may be the preferred locations of strand unwinding and separation in supercoiled PM2 DNA under our conditions.

Other sites of single strand character in negatively supercoiled DNA are possible. Recently, single strand specific endonucleases S1 (30-32) and the T7 gene 3 product (32) have been found to cleave some negatively supercoiled DNAs in the nonbase-paired loops of potential cruciforms. Thus, inverted repeat sequences in DNA are possible recognition sites for mung bean nuclease. Another possible site is in unwound regions which might occur at junctions between right- and left-handed DNA helices. One type of left-handed DNA called Z-DNA can form in sequences of alternating purines and pyrimidines (33-35). Finally, kinks or bends at regions in highly supercoiled molecules where the DNA helix doubles back on itself may occur in specific regions and may be suggested as possible cleavage sites (15). DNA sequencing studies in progress will help us distinguish between some of these possibilities.

\[ J. F. Burke, M. J. Evans, and J. A. Huberman, personal communication. \]

\[ D. Kowalski, and J. P. Sanford, unpublished experiments. \]
Single-stranded Regions in Supercoiled PM2 DNA

Acknowledgments—We thank Joel Huberman and Lowell Sheflin for helpful discussions.

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J. Biol. Chem. 1982, 257:7820-7825.

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