Site of Cleavage of Superhelical φX174 Replicative Form DNA by the Single Strand-specific Neurospora crassa Endonuclease*

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Experiments with the Neurospora crassa single strand-specific endonuclease have provided evidence for the existence of regions of partially single-stranded character in covalently closed superhelical replicative form DNA of φX174. The nuclease converts the superhelical molecules to either singly hit relaxed circular or doubly hit linear molecules. We show that the initial cleavage of φX174 superhelical DNA is a "nick" bounded by a 5'-phosphate and a 3'-hydroxyl; no nucleotides are excised as evidenced by the ability of T4-polynucleotide ligase to reform the phosphodiester bond. The nick can be found at any one of many possible locations in the genome. Thus, the regions in φX174 superhelical molecules that are sensitive to the N. crassa nuclease do not occur at highly specific sites in the genome.

Covalently closed circular DNAs are widespread in occurrence and are invariably found to contain tertiary super-twists when examined in vitro. Such molecules have many special properties due to the stress inherent in the superhelical structure (1). The property that concerns us here is that superhelical DNA exhibits regions of altered secondary structure when examined by physical, chemical, or enzymatic means (2-8).

A comparative study of covalently closed circular non-superhelical and superhelical φX174 RF DNA led to the conclusion that there exist weakly hydrogen-bonded regions in the superhelical forms because of the preferential sensitivity of these molecules to the single strand specific nuclease of Neurospora crassa (3). These enzymes have been used to produce specific fragments of DNA by digestion of unpaired regions in heteroduplex adenovirus DNA molecules (9) and for the biochemical mapping of mismatches or deletions (10).

We report here a further examination of the action of the N. crassa single strand specific enzymes on φX174 RF DNA and the implications it has for the structure of superhelical molecules. In a recent study (8) it was reported that specific cleavage sites could not be demonstrated in PM2 DNA by the mung bean or N. crassa nuclease, but no data were presented. We demonstrate in the experiments described below that the cleavage sites in φX174 RF DNA are not restricted to a few specific regions, such as the major palindromic sequences, and are either randomly distributed in the genome, or at least can occur at a large number of sites. This is in contrast with the limited number of sites in SV40 DNA available to the SI nuclease (5). We believe that apparent single-stranded regions in φX174 superhelical DNA are manifestations of minor distortions of the helix due to the torsional stress resulting from the tight folding of the duplex DNA and as such are not confined to a few specific sites in the genome.

Experimental Procedures

Viral DNA—[3H]Thymidine and uniformly 32P-labeled φX174 RF I DNAs were prepared in vivo as previously described (3, 11). 3H-Labeled single-stranded viral DNA was extracted with phenol from isolated phage particles and purified by neutral sucrose gradient centrifugation. All DNA preparations were stored at -20° in 50 mM Tris-HCl (pH 8.0) and had initial specific activities of 0.2 to 1.5 μCi/μg.

Neurospora crassa Nucleases—The endo- and exonuclease were both prepared from wild type conidia (strain ATCC 9279) by the methods previously described (12, 13). They were stored separately under sterile conditions at 4° in 20 mM potassium phosphate buffer, pH 6.5.

The enzyme preparations used in our studies were extremely specific for single-stranded polynucleotides. No degradation to acid-soluble material occurred using double-stranded substrates in reaction mixtures containing 10 times more enzyme than needed to completely digest an equal amount of denatured DNA or RNA. From an estimate of the number of phosphodiester bonds cleaved, one can calculate that the ratio of the activity on single-stranded DNA as compared with double-stranded DNA was greater than 60,000.

Enzyme Reactions—Superhelical RF I DNA was treated with nuclease in the same Tris MgCl2 buffer, with NaCl concentrations and other variables as indicated in the individual figure legends. Such reactions were carried out at DNA concentrations over 20 μg/ml, the preferred concentration being 50 μg/ml.

Formaldehyde-fixation of the RF I DNA was carried out in 20 µl of 10 mM Na2HPO4 (pH 8.0), 0.1 mM NaCl 8% HCHO, with temperature and time as indicated in the figure legends. The reaction was terminated by diluting the samples 30-fold in the ice-cold buffer containing 0.1 M Tris-HCl (pH 8.0), 10 mM MgCl2, and 0.1 M NaCl. The pH of the solution was checked at 37° and adjusted to pH 8.0 prior to addi-
tion of 2 units/ml of endonuclease. Enzyme digestion was carried out for 20 to 60 min at 37\(^\circ\)C in the Tris-MgCl\(_2\)-NaCl buffer indicated above.

**Centrifugation**—Low salt neutral sucrose solutions contained 10 mm Tris-HCl (pH 8.0) and 10 mm EDTA; high salt neutral sucrose solutions contained in addition 1.0 mm NaCl. Low salt alkaline sucrose solutions contained 0.1 M NaOH, 10 mm EDTA, and 0.01% sarkosyl. High salt alkaline sucrose solutions contained 0.2 M NaOH and 0.8 M NaCl, but were otherwise identical with the low salt solutions. All sucrose gradients were linear 5 to 20% gradients and were centrifuged at 4\(^\circ\)C for neutral gradients and at 14\(^\circ\)C for alkaline gradients in the Beckman L2-65B ultracentrifuge. Sucrose gradients were collected by volume via a hole punched in the bottom of the tube. Fractions of up to 0.5 ml could be collected directly into vials and counted using 10 to 15 ml of a toluene/Triton X-100/H\(_2\)O (6/3/1) solution containing 4 g of 2,5-diphenyloxazole (PPO) and 50 mg of 1,4-bis[2-(5-phenyl-1H-benzo[d][1,2,4]oxazol-5-yl)]benzene (POPOP) per liter. Fractions of alkaline gradients were first neutralized by the addition of one drop of glacial acetic acid.

**Kinase Reactions**—Singly nicked RF II [\(^3\)H]DNA in 0.01 Tris-HCl (pH 8.0) was treated with a total of 10 units/ml of *Escherichia coli* alkaline phosphatase for 30 min at 65\(^\circ\)C, the enzyme being added in two equal aliquots at 0 and 15 min. Either the DNA was repurified by neutral sucrose sedimentation or the phosphatase was inhibited by the addition of ethylene glycol bis(\(\beta\)-aminoethyl ether)N\(_2\)N\(_4\):tetraacetic acid (EGTA) to a final concentration of 6.5 mM and incubation for a further 10 min at 65\(^\circ\)C. A crude fraction of T4-induced polynucleotide kinase provided by Dr. A. Delaney (McGill University) was further purified by DEAE-chromatography and concentrated by vacuum dialysis. Neither enzyme had detectable nuclease activity when tested with RF I DNA. The 5'OH ends of the RF II molecules were phosphorylated using \([\gamma\text{-32P}]\text{ATP}\) (New England Nuclear, specific activity > 20 Ci/mmol). The reaction mixtures contained 0.05 M Tris-HCl (pH 8.0), 2 mM K\(_2\)HPO\(_4\), 10 mM each MgCl\(_2\) and \(\alpha\)-mercaptoethanol, and \([\gamma\text{-32P}]\text{ATP}\) in 100 to 300-fold molar excess over phosphorylatable material; the 32P-charged molecules were then purified by neutral sucrose gradient sedimentation.

**Nick Translation**—Singly-nicked RF II [\(^3\)H]DNA was labeled using *E. coli* DNA polymerase I (gift from G. McFadden, McGill University) at 4\(^\circ\)C for neutral gradients and 14\(^\circ\)C for alkaline gradients in the SW40 rotor at 14,000 rpm for 12 hours (panel a) and 15 hours (panel b). The top panel (a) shows a sucrose gradient analysis of the starting material; over 90% of the [\(^3\)H]RF is RF I. The RF I was exposed to 20 units/ml of enzyme for 20 min at 37\(^\circ\)C in a buffer containing high salt (0.2 M NaCl, 0.1 M Tris-HCl (pH 8.0) 10 mm MgCl\(_2\)) to maximize the stability of the duplex DNA. About 70% of the RF I was converted to RF II according to the sucrose gradient analysis presented in panel (b). The alkaline sucrose gradients presented in panel (c) illustrate that the RF II molecules were indeed only nicked once by this enzyme treatment since the proportions of circular and linear molecules were equivalent.

**Restriction Enzyme Reactions**—Hind nuclease was purified from *Escherichia coli* strain BW12778 (13) and used as a control. The DNA was incubated with enzyme for 20 min at 37\(^\circ\)C in a buffer containing Tris-MgCl\(_2\)-NaCl buffer indicated above. The DNA was repurified by neutral sucrose sedimentation and dialyzed extensively against 10 mm Tris-HCl (pH 8.0) before electrophoresis.

**Preparative Electrophoresis**—Gels of 1.5% agarose were made in glass tubes (8 × 120 mm) and prerun at 40 volts for 30 min at room temperature in electrophoresis buffer containing 40 mm Tris-acetate/2 mm EDTA/20 mm CH\(_3\)COONa (pH 7.7). The electrophoresis apparatus used was designed by C. Hoers (McGill University) and will be described in a future publication. Samples of 50 to 200 ml containing 5 ml of bromphenol blue and 30% sucrose were layered onto the gels and subjected to electrophoresis at 50 volts for 20 hours at room temperature. Fractions of 0.8 ml were collected with a Gilson microfractionator in a cold box at 4\(^\circ\)C every 20 min. Aliquots were assayed for the radioactivity, and the appropriate fractions were pooled and concentrated by collodion bag vacuum dialysis against 10 mm Tris-HCl (pH 8.0).

**RESULTS**

**Formation of RF II and RF III by *Neurospora crassa* Nuclease**—Superhelical RF I DNA has been shown to be sensitive (under nondenaturing conditions) to the single strandspecific *N. crassa* endonuclease. The control molecules used in that study were covalently closed circular RF I molecules containing no super twists; these were synthesized in *vitro* from the superhelical molecules (3). To characterize the sensitive site in the RF I DNA, "singly-nicked" RF II was generated by the procedure described in Fig. 1. The top panel (a) shows a sucrose gradient analysis of the starting material; over 90% of the [\(^3\)H]RF is RF I. The RF I was exposed to 20 units/ml of enzyme for 20 min at 37\(^\circ\)C in a buffer containing high salt (0.2 M NaCl, 0.1 M Tris-HCl (pH 8.0) 10 mM MgCl\(_2\)) to maximize the stability of the duplex DNA. About 70% of the RF I was converted to RF II according to the sucrose gradient analysis presented in panel (b). The alkaline sucrose gradients presented in panel (c) illustrate that the RF II molecules were indeed only nicked once by this enzyme treatment since the proportions of circular and linear molecules were equivalent.
The first question investigated was the nature of the cleavage introduced by the *N. crassa* endonuclease into the RF I molecules. As shown in Fig. 1, the purified RF II molecules contained fully genome-length strands and no detectable small molecular weight material (in all cases the entire gradient was counted and is shown). However, this analysis did not indicate whether the enzyme put only a single nick into the DNA or acted in such a way as to leave a small gap. We therefore asked if the RF II molecules could be “ligased” using bacteriophage T4-induced polynucleotide ligase. The RF II DNA was reacted with ligase and the proportion of RF I determined by sedimentation in alkaline sucrose gradients containing 1 M NaCl (3). The results of a series of such experiments are summarized in Table I. Controls were performed using (a) RF II that was present in small amounts in the original RF I preparations, probably as the result of radiation damage (no reaction, compare Experiments 1 and 5); (b) RF II produced by DNase I treatment of RF I (60% conversion back to RF I, experiments 6 and 7); and (c) RF II produced by *N. crassa* endonuclease cleavage of superhelical RF I, then treated with bacterial alkaline phosphatase to remove the 5‘-terminal phosphate (no reaction, experiments 2 and 4). The data indicated that many of the RF II molecules produced from the superhelical RF I by treatment with the *N. crassa* endonuclease were a substrate for ligase and therefore contained a single phosphodiester bond break bounded by a 5‘ P and a 3‘ OH.

Variations in the conditions of *N. crassa* nuclease treatment of the superhelical RF I DNA resulted in the production of doubly hit linear RF III molecules. Fig. 2a shows the neutral sucrose sedimentation velocity pattern of a sample treated for 1 hour with 28 units/ml of enzyme. There was almost no remaining RF I and two-thirds of the DNA had been converted to RF III. The RF III molecules were purified by preparative agarose gel electrophoresis, which gave a better separation from RF II than centrifugation as shown in Fig. 2b.

A second, efficient procedure for obtaining RF III made use of formaldehyde to hydroxymethylate unpaired bases (the reason for using both of these methods to produce RF III will become apparent). Here, the DNA was treated with HCHO for 40 min at 26°, diluted, and then immediately reacted with endonuclease at an enzyme concentration so low that DNA which was not pretreated with HCHO was not affected. Although the hydroxymethylation reaction is a reversible one, single-stranded regions stabilized by reaction with HCHO remained available to the enzyme after dilution of the formaldehyde. The enzyme reaction was for 20 min using 2 units/ml of enzyme, and the initial products were the nicked circular RF II form (16 S) and the linear RF III form (14.5 S) (18); contained exposure to the enzyme caused a further shift of material from the 16 S to 14.5 S position. Analyses were then carried out by sedimentation in low salt alkaline sucrose gradients to determine whether or not the RF III DNA was internally nicked—i.e., contained any single strand breaks. Fig. 3a shows a control gradient of the starting preparation prior to HCHO or enzyme treatment. The denatured RF I sedimented to the bottom of the tube; the small amount of [3H]-RF II present initially was denatured and its two strands cosedimented with the 3P marker, circular (c, 18 S) and unit-length linear (1, 16 S) DNAs. Fig. 3b shows sedimentation of a 32P-labeled sample treated with HCHO and endonuclease as described above. The proportion of material present in linear strands greatly increased, as would be expected, but no smaller molecular weight material was detected. Since the RF III molecules yielded exclusively unit-length linear strands after denaturation we conclude

### Table I

**Ability of polynucleotide ligase to join termini produced by Neurospora crassa endonuclease**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>% RF I</th>
<th>% RF II</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Untreated RF I preparation</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>2. Singly nicked RF II (via N. crassa endonuclease nicking of RF I)</td>
<td>&lt;5</td>
<td>&gt;95</td>
</tr>
<tr>
<td>3. Singly nicked RF II (as No. 2) plus ligase</td>
<td>25-50</td>
<td>50-75</td>
</tr>
<tr>
<td>4. Singly nicked RF II (as No. 2) plus alkaline-phosphatase plus ligase</td>
<td>&lt;5</td>
<td>&gt;95</td>
</tr>
<tr>
<td>5. Untreated RF I preparation (as No. 1) plus ligase</td>
<td>80-85</td>
<td>15-20</td>
</tr>
<tr>
<td>6. DNase I produced RF II</td>
<td>5</td>
<td>95</td>
</tr>
<tr>
<td>7. DNase I produced RF II (as No. 6) plus ligase</td>
<td>60</td>
<td>40</td>
</tr>
</tbody>
</table>

Fig. 2. Analysis of double-stranded linear RF III DNA produced by *Neurospora crassa* endonuclease treatment of φX174 superhelical DNA. The reaction mixture contained 0.1 M Tris-HCl (pH 8.0), 0.1 M NaCl, 10 mM MgCl2, 20 to 50 μg/ml of [3H]-labeled φX174 RF I DNA, and was incubated for 1 hour at 37° with 20 units/ml of *N. crassa* single strand-specific endonuclease preparation. The reaction was terminated with EDTA and the samples were analyzed: a, low salt neutral sucrose sedimentation; 5 to 20% linear sucrose gradients were centrifuged for 17 hours at 38,000 rpm, 10° in the SW40 rotor. The arrows indicate the location in the gradients of marker 3P-labeled RF I, II and III. b, 1.5% agarose preparative gel electrophoresis performed as described under “Experimental Procedures.” The arrows indicate the positions of migration of 3P-labeled RF I, II, and III markers.
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6. . . . . ..- . . . . . . . . . I

oc.0 M Oc.0Oa 00 0QD-a.

T;

2

K 24

IS

0 20 40 60 SO I( 3

Fraction number

FIG. 3. Low salt alkaline sucrose gradient analysis of the cleavage products of HCHO-fixed φX174 RF I DNA treated with the endonuclease. The alkali-denatured DNA samples, containing 32P-labeled φX174 RF I and RF II DNA as marker, were layered onto 13-ml 5 to 20% sucrose gradients and centrifuged for 15 hours at 38,000 rpm, 10° in the Beckman SW40 rotor. The denatured RF I sediments to the bottom of the tube under these conditions, and the two peaks correspond to the 18 S circular (cl) and 16 S linear (c) single-stranded viral DNA. (a) Control: DNA exposed to HCHO (35°, 40 min) and then incubated without endonuclease. (b) DNA reacted with HCHO as in (a) and then incubated with 2 units/ml endonuclease at 37° for 20 min (see "Experimental Procedures"). (O) H-labeled RF DNA; (D) SzP-labeled marker RF DNA.

that the RF III molecules were not internally nicked.

Location of Nick Introduced into Superhelical DNA—The above experiments showed that the initial nick/cleavage was restricted to one site on any one molecule, but this site could have been in one, several, or many locations in the genome in a given population of molecules. Subsequent phosphodiester bond breaks were introduced at a very much lower rate because of the relaxed conformation of the DNA (3). In order to ascertain the location of the nuclease-sensitive region in the genome, RF II molecules were prepared as described in Fig. 1 to contain a single nick. The nicked molecules were labeled in the nicked region and then digested with Hind restriction enzyme to determine whether the nick was at one, a few, or many sites.

Two techniques were used to label the nick; one made use of T4 polynucleotide kinase, the second made use of E. coli DNA polymerase I. In the former case purified singly-nicked RF II molecules were first treated with E. coli alkaline phosphatase and then reacted with T4 polynucleotide kinase and [γ-32P]ATP to label the 5′ terminus at each nick. The DNA was purified by neutral sucrose velocity sedimentation, dialyzed against 10 mM Tris-HCl (pH 8.0), digested with Hind enzyme and subjected to electrophoresis in acrylamide-agarose gels. In the latter case purified singly nicked RF II molecules were "nick-translated" using [α-32P]dCTP and E. coli DNA polymerase I to incorporate radioactivity in a 5′ → 3′ direction on the nicked strand (14). The reaction was carried out for brief times, using limiting amounts of dXTP precursors. Between 30 and 300 nucleotides were incorporated per RF molecule. The DNA was then purified, dialyzed, digested with Hind enzyme and subjected to electrophoresis in acrylamide-agarose gels. The insert in Fig. 1 shows a autoradiogram of a Hind digestion of singly nicked [3H]RF II (produced as for Fig. 1, using 0.25 M NaCl during the N. crassa digestion) labeled with [α32P]dCTP using E. coli DNA polymerase I. (b) Hind digestion of linear [3H]RF III DNA (produced as for Fig. 2) subjected to co-electrophoresis on acrylamide-agarose gels as described under "Experimental Procedure." (a) Hind digestion of singly nicked [3H]RF II (produced as for Fig. 1, using 0.25 M NaCl during the N. crassa digestion) labeled with [α32P]dCTP using E. coli DNA polymerase I. (b) Hind digestion of linear [3H]RF III DNA (produced as for Fig. 2) subjected to co-electrophoresis on acrylamide-agarose gels as described under "Experimental Procedure." (a) Hind digestion of singly nicked [3H]RF II (produced as for Fig. 1, using 0.25 M NaCl during the N. crassa digestion) labeled with [α32P]dCTP using E. coli DNA polymerase I. (b) Hind digestion of linear [3H]RF III DNA (produced as for Fig. 2) subjected to co-electrophoresis on acrylamide-agarose gels as described under "Experimental Procedure."
the DNA fragments produced by Hind digestion of H-RF molecules which had been singly nicked with the N. crassa nuclease and then labeled with 32P using E. coli DNA polymerase I. The inset in b shows an autoradiogram of a kinase-labeled, Hind-digested RF II preparation produced by the N. crassa nuclease. In a series of such gels the distribution of 32P among the bands was similar to the 32P distribution among the bands generated from uniformly labeled molecules. In all cases, the 32P was distributed among the fragments approximately according to the mass of DNA present. From these results we conclude that in the population of singly nicked RF II molecules the nick could be found in a large number of different sites in the genome.

Another approach to determining the location of the site attacked by the endonuclease in RF I DNA involved RF III molecules produced via N. crassa endonuclease cleavage of non-HCHO-treated RF I [32P]DNA. The H-RF III molecules were purified by preparative agarose gel electrophoresis as illustrated in Fig. 2b. They were then dialyzed against 10 mm Tris-HCl (pH 8.0) and co-digested with RF I [32P]DNA (uniformly-labeled) using the Hind restriction endonuclease. The resultant electrophoresis pattern of the DNA fragments produced by Hind is shown in Fig. 4b. The ratio of H to 32P for the individual fragments was fairly uniform, no peak showing more than a 2-fold enrichment of either label. If the ends of the linear molecules were unique, and therefore located within the same fragment in all molecules, it is likely that a given fragment would have been significantly depleted in H relative to the 32P, and two new, smaller fragments would have appeared. That this was not the case supports the conclusion that the site in RF I that is susceptible to the N. crassa endonuclease is not restricted to one or a few special sequences.

The question of the strand distribution of the nick was investigated by purifying the circular and linear single-stranded DNA components from denatured singly nicked RF II by sedimentation velocity centrifugation in alkaline sucrose gradients like those shown in Fig. 1c. The circular and linear molecules were centrifuged separately to equilibrium in alkaline CsCl buoyant density gradients with randomly nicked uniformly labeled 32P-RF II marker. Under these conditions, the viral and complementary strands of φX DNA band at different densities (19). Both the linear and circular single-stranded DNAs yielded identical density gradient profiles (data not shown), indicating the presence of comparable amounts of viral and complementary strands. These experiments showed (not surprisingly) that the initial nick introduced into the RF I molecules could be in either the viral or the complementary strand, with no apparent preference.

The above experiments did not rule out the possibility that the endonuclease itself bound to a region, which from the above experiments could have been found in many locations, and caused it to become susceptible to the enzyme. To test this, formaldehyde was used as described earlier in Fig. 3 to stabilize and fix the partially denatured regions present in the absence of nuclease. Linear RF III molecules were then produced by endonuclease treatment of HCHO-fixed RF I DNA and were purified by successive cycles of neutral sucrose velocity sedimentation. The purified RF III molecules were then tested to see if they represented a collection of unique or permuted molecules. They were denatured with alkali and renatured in 50% formamide (9, 10), and examined in the electron microscope. The renatured DNA consisted predominantly of circular molecules; some 20% were linear but most of these were longer than unit-length. Sedimentation velocity analyses of the renatured DNA in neutral sucrose gradients revealed no appreciable RF III; the majority of the material sedimented with or ahead of RF II. The control of attempting to circularize the RF III molecules by renaturing without prior denaturation was negative; N. crassa single strand-specific exonuclease digestion of the RF III prior to denaturation or subsequent to renaturing also did not affect these results. We conclude from these experiments that the RF III comprised a population of molecules with permuted sequences and that the cleavage by the N. crassa nuclease of hydroxymethylated RF I was not at a unique location.

**DISCUSSION**

We have shown in the experiments above that the cleavage of φX174 superhelical DNA by the Neurospora crassa single strand-specific nuclease is not localized to a few specific sites on the genome. The evidence for this included the demonstration that all of the fragments generated from RF II by digestion with a Hind nuclease preparation contained radioactivity inserted by either polynucleotide kinase or DNA polymerase I roughly in proportion to the size of the fragment. The S1 nuclease, on the other hand, has been reported to hit SV40 DNA within two regions, each encompassing some 10% of the genome (6). The suggested interpretation is that SV40 DNA contains two AT-rich regions that are preferentially hit whereas superhelical φX RF does not contain such regions.

A priori, there are a number of reasonable explanations for the sensitivity of a double-stranded superhelical DNA molecule to a single strand-specific nuclease. One is the existence of AT-rich, and thus easily denaturable, regions. Another more interesting explanation, and a reason for initiating this work, is the existence of a palindromic sequence, complete or hyphenated, that could form a cruciform structure (20) possibly sensitive to the nuclease. The stress inherent in the superhelical conformation would be reduced by the formation of cruciforms.

We have shown elsewhere (21) that φX174 DNA contains at least two specifically located hairpin loops and thus the existence of a cruciform structure is possible. However, our results reported here on the absence of site specificity for N. crassa nuclease and our results, reported elsewhere (21), on the resistance of the hairpin loops in single-stranded DNA to the N. crassa enzyme indicate that if a loop out exists it is neither observed to be nor necessarily expected to be a site of action for the N. crassa nuclease. Palindromic sequences have not to date been demonstrated to form cruciform structures in DNA.

What then is a possible common feature of the sites in superhelical φX RF I that are sensitive to the single strand-specific nuclease? Given the strong dependence of the initial rate of nicking on the superhelix density (Ref. 8 and Footnote 1), it seems probable that the "kinks" or "elbows" in highly twisted superhelical DNA (superhelical density > 0.03) disturb the normal base pairing and provide the sites we detect in our experiments. Such nuclease susceptible bonds, which become especially evident at superhelix densities above 0.03, can presumably occur at many locations in the molecule, and when the first phosphodiester bond is broken the molecule im-

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mediately relaxes, loses these distorted regions, and becomes resistant to the nuclease. Only when high concentrations of enzyme are employed, or a distorted region is stabilized by hydroxymethylation, is a double strand break made. Our data do not permit the conclusion that these breaks occur randomly.

The experiments with HCHO-fixed DNA are important because they suggest that the distorted regions preexist and are not induced by the nuclease; however, we cannot exclude a cooperative effect, especially at higher enzyme concentrations. The precise conformation of the partially denatured region will be difficult to elucidate, especially since its structure may be influenced by the methodology used to study it. Our results do not exclude the possibility that φX174 RF molecules with other superhelix densities (particularly below 0.031) might exhibit specific regions with enhanced sensitivity to nuclease. Furthermore, the distribution of A-T base pairs within any given molecule may influence the locations of the distortions due to the superhelical twists. Whatever the nature of these distortions, it seems logical to expect them to be most evident in A-T rich regions of the DNA. Although Roizes (22) described a preparation of N. crassa nuclease that he believed showed a new activity specific for A-T rich regions, we have not seen any evidence for this in any of our preparations. We think that the activity he described is compatible with the lower Tᵅ of A-T rich regions.

The formation of superhelical structures in vivo could be a means of storing or translating energy in the duplex. The positive free energy contained therein would facilitate interactions with proteins that unwind the duplex, perhaps initiated because of the exposure of the non-hydrogen bonded nucleotides revealed in our studies reported here. Whether DNA is superhelical (in the sense of this paper) in vivo will require further investigation.

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Site of cleavage of superhelical phiX174 replicative form DNA by the single strand-specific Neurospora crassa endonuclease.
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