Cleavage of cruciform DNA structures by an activity from *Saccharomyces cerevisiae* (DNA–protein interactions/inverted repeat sequences/Holliday junctions/mechloethamine)

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**ABSTRACT** Protein extracts from *Saccharomyces cerevisiae* have been fractionated to reveal a nuclease activity that cleaves cruciform structures in DNA. Negatively supercoiled plasmids that contain inverted repeats that are extruded into cruciform structures have been used as DNA substrates. The sites of cleavage of pColIR215 DNA are located within the extruded cruciform stems and are symmetrically opposed to each other across the cruciform junction. Neither relaxed duplex DNA nor single-stranded DNA serve as substrates. The native molecular weight of the activity was estimated to be ~200,000 by gel filtration.

Recent studies of plasmids that contain palindromes have shown that inverted repeat sequences are extruded in vitro to form cruciform structures and, thus, reduce the free energy of negative supercoiling (1–3). Two classes of enzymes have been shown to cleave plasmids that contain cruciform structures. The first includes a variety of single-strand nuclease, such as S1 nuclease, that nonspecifically cleave the single-stranded hairpin loops of the cruciform (see Fig. 1, path a) (4–6). The enzymes of the second class, which includes T4 endonuclease VII (7) and T7 endonuclease I (8, 9), show a more specific interaction and cleave diagonally across the cruciform junction (see Fig. 1, path b) (2, 10, 11).

The local DNA structure around a cruciform junction is indistinguishable from that of a Holliday junction (2, 10), a central intermediate in the process of genetic recombination (12–15). Therefore, plasmids that contain inverted repeat sequences may be useful as probes to assay cell extracts for enzymes that specifically recognize and cleave Holliday junctions.

In this paper, we describe studies with a partially purified fraction from *Saccharomyces cerevisiae* that cleaves cruciform junctions in vitro. The activity cannot be detected in crude extracts and only becomes apparent during protein fractionation. The sites of cleavage of plasmid DNA are shown to be located within inverted repeat sequences and occur at regions that are symmetrically opposed across the cruciform junction. As yet, we have been unable to purify the activity to homogeneity. However, since the activity appears to provide the first example of a eukaryotic nuclease that acts on cruciform junctions in duplex DNA, we feel that its demonstration is of sufficient interest to warrant this preliminary report.

**MATERIALS AND METHODS**

**Strains, Plasmids, and DNA.** Yeast strains S235 (a his3-11, -15 leu2-3, -112 can1) and SR154-6C (a ade2-1 trpl-1) were provided by S. Roeder. The plasmids pColIR215 (5) and pUC7 (16) were grown in *Escherichia coli* HB101 recA-. Covalently closed plasmid DNA was uniformly labeled with [methyl-3H]thymidine or [32P]P, and prepared as described (17). All DNA concentrations are expressed in moles of nucleotide residues.

**Enzymes, Materials, and Buffers.** Purified T4 endonuclease VII (200 units/μl) was generously provided by B. Kemper. Mechloethamine, phenylmethylsulfonyl fluoride, and phosphocellulose were obtained from Sigma, DEAE-Bio-Gel A and Bio-Gel A-0.5m were from Bio-Rad, Zymolyase-20T was from Miles, and single-stranded DNA (ss DNA)-agarose was from Bethesda Research Laboratories. Buffer R was 20 mM Tris-HCl, pH 7.5/1 mM EDTA/0.5 mM dithiothreitol/10% glycerol/0.1 mM phenylmethylsulfonyl fluoride. Buffer P was the same except Tris buffer was replaced with 20 mM sodium phosphate (pH 6.8).

**Purification of Cruciform Cleavage Activity.** Four liters of S235/SR154-6C diploid cells were grown in YPD (yeast extract/peptone/dextrose) broth with aeration to 1 × 10^7 cells per ml, mechloethamine was added at 20 μg/ml, and growth was continued for 3 hr. The cells were harvested by centrifugation, washed and resuspended in 100 ml of 50 mM Tris-HCl, pH 7.5/10% sucrose/1 mM EDTA, and stored at −80°C. When required, cells were thawed, and all subsequent procedures were performed at 0°C. For lysis, we added 6.2 ml of 5 M NaCl, 10 ml of Zymolase at 20 mg/ml, and 100 μl of mercaptoethanol and incubated at 0°C. After 30 min, 120 μl of 0.1 M phenylmethylsulfonyl fluoride and 1.2 ml of 10% Brij 58 were added, and incubation was continued for 20 min. Lysis was continued...
monitored by light microscopy. Cell debris was removed by centrifugation at 35,000 rpm in a Beckman 42.1 rotor.

DNA was removed from the clear supernatant (fraction I) by passage through a 30-ml DEAE-Bio-Gel A column equilibrated with buffer R containing 0.3 M NaCl. The flow-through was precipitated with 0.5 g of ammonium sulfate per ml and dialyzed against buffer P to produce fraction II. Fraction II was loaded onto a 50-ml ss DNA-agarose column equilibrated with buffer P and was eluted with a 0–0.25 M NaCl gradient in buffer P. Fractions were immediately assayed for the cleavage of pColIR215 DNA. The activity specific for cruciform DNA usually was eluted between 0 and 50 mM NaCl and was dialyzed against buffer R containing 50 mM NaCl. Fraction III (50 ml) was then passed through a 5-ml column of phosphocellulose directly onto a 2-ml DEAE-Bio-Gel A column. The activity was eluted from the DEAE column with a 0.05–0.4 M NaCl gradient and active fractions, which eluted at about 0.25 M NaCl, were pooled and stored in small aliquots at −80°C (fraction IV). The protein concentration of fraction IV was too low to measure. Preparations have shown no loss of activity over a period of 1 year when stored at −80°C.

**Reactions and Assays.** Cleavage reactions (30 μl) generally contained 28 μM plasmid DNA in 50 mM Tris-HCl, pH 8.5/10 mM MgCl₂/1 mM dithiothreitol/100 μg of bovine serum albumin per ml/0.5 μl of fraction IV or 100 units of T4 endo VII. Reactions were incubated at 37°C for 60 or 90 min. They were stopped, and the products were deproteinized by the addition of EDTA and NaDodSO₄ to 50 mM and 1%, respectively. In experiments with pUC7, it was necessary to preincubate the plasmid at 60°C for 2 hr in 0.2 M NaCl to allow extrusion of the large cruciform (11). pColIR215 plasmid DNA did not require pretreatment.

The assay for single-strand nuclease activity measures the reduction in the number of infective centers obtained in transfection experiments after treatment of φX174 ss DNA with various dilutions of enzyme. φX174 ss DNA (28 μM) was incubated with fraction IV or S1 nuclease for 60 min at 37°C in the standard reaction buffer or 50 mM sodium acetate/10 mM zinc sulfate/150 mM sodium chloride for S1 nuclease. The reaction was stopped by the addition of excess EDTA, and proteins were inactivated by heating at 75°C for 10 min. Then 10-μl samples were added to 200 μl of CaCl₂-treated E. coli cells and incubated at 0°C for 30 min, followed by a heat pulse of 3 min at 42°C. The mixture was then returned to ice, diluted with Luria broth, and plated immediately to determine the number of infected cells.

The buffer system for neutral agarose gels was 40 mM Tris-HCl, pH 7.8/5 mM sodium acetate/1 mM EDTA and for polyacrylamide gels was 89 mM Tris base/89 mM boric acid/2 mM EDTA. In addition, the polyacrylamide sequencing gels contained 7M urea, and the DNA samples were denatured by heating at 95°C for 2 min in 95% formamide/20 mM EDTA.

**RESULTS**

**Preparation of Cruciform-Specific Fraction.** Protein extracts were prepared from exponentially growing diploid cultures of *S. cerevisiae* that had been treated with mechlorethamine (nitrogen mustard), a DNA-damaging agent that is known to increase the frequency of mitotic recombination (18). The DNA substrate in the cleavage assay was pColIR215 (5, 10), a recombinant plasmid that contains a 31-base-pair (bp) palindrome from ColE1 with 5 bp of nonrepeated sequence at its center. When negatively supercoiled, the plasmid extrudes this inverted repeat to form a cruciform structure.

Preliminary experiments to search for a cruciform-specific activity revealed three problems. First, the extracts contained high levels of single-strand endonuclease activity that cleaved the substrate DNA. Second, a topoisomerase removed the negative supercoiling required for cruciform extrusion. Third, proteolytic activities affected protein stability. However, we did observe an activity specific for cruciform DNA after fractionation on a DNA-agarose column. This activity was further purified by using phosphocellulose and DEAE-Bio-Gel to produce fraction IV as described. Fraction IV did not contain any exonuclease activity as determined by using 5'- or 3'-end-labeled linear duplex DNA. For convenience, the endonuclease activity in fraction IV will be referred to as X nuclease.

In an identical preparation from which mechlorethamine treatment of the cells was omitted, the cruciform-specific activity was detected at a lower level. Therefore, all subsequent preparations were made from treated cultures.

Approximately 25% of supercoiled pColIR215 was converted to a linear form by incubation with fraction IV (Fig. 2, lane b). When relaxed by topoisomerase treatment, to generate topoisomers of low linking number that are incapable of cruciform extrusion, pColIR215 failed to be a substrate for the enzyme (Fig. 2, lane d). X nuclease did not degrade φX174 ss DNA (Fig. 2, lane f) or cut linear duplex DNA of pColIR215 (not shown), as analyzed by gel electrophoresis.

**Cruciform Structures Are the Targets for Cleavage.** The approximate site of cleavage of supercoiled pColIR215 DNA was determined by treating 32P-labeled plasmid with fraction IV, followed by restriction analysis using single-site enzymes. In each case, two major fragments were produced, indicating that X nuclease cleaved each plasmid molecule once (Fig. 3, lanes c–e). When we treated pColIR215 with T4 endonuclease VII and the same restriction enzymes, fragments of similar length were produced (Fig. 3, lanes g–i). Since T4 endonuclease VII cleaves pColIR215 at the site of the major inverted repeat (10, 19), these results indicate that X nuclease also cleaves at, or close to, this sequence. In addition to the major fragments, we also observed the formation of pairs of minor fragments, which result from cleavage by either X nuclease or T4 endonuclease VII at the minor inverted repeats of pColIR215, which are the natural palindrome of pBR322 (10, 18).

Other plasmids that contain cruciforms were also substrates for the nuclease activity in fraction IV. For example, pUC7 contains a perfect 48-bp cloned palindrome (16). When treated with fraction IV, we observed cleavage that was located by restriction mapping to this site (data not shown). Similar results were observed by using pBR322 DNA, which contains three palindromes 23 to 26 bp long (10) (data not shown). Although the inverted repeats in pColIR215, pUC7, and pBR322 are unrelated in sequence, they were cleaved with equal efficiency by

**Fig. 2.** Cleavage of supercoiled pColIR215 DNA by fraction IV. DNA was incubated with or without fraction IV as described, and the products were visualized by ethidium bromide staining of a 1% agarose gel. Lanes: a and b, supercoiled plasmid DNA; c and d, plasmid DNA relaxed by topoisomerase I; e and f, φX174 ss DNA. Fraction IV was present in the reactions of lanes b, d, and f.
fraction IV. This result indicates that the nuclease is specific for structure rather than for sequence. Moreover, since negative supercoiling is required for cruciform extrusion and for cleavage of these DNAs by fraction IV, the structure recognized by the nuclease activity is likely to be some feature of the cruciform.

Since inverted repeat sequences in supercoiled plasmids are strong sites for attack by single-strand nuclease (5), we compared the single-strand nuclease activities of fraction IV and S1 nuclease. As an assay, we used the loss of infectivity of φX174 ss DNA after the introduction of one nick. This extremely sensitive test showed that the amount of fraction IV (0.5 μl) that was sufficient to promote the maximal amount of cleavage of plasmid DNA that contained a cruciform, cleaved only 12% of phage ss DNA in 1 hr at 37°C. In a control experiment, we determined the amount of S1 nuclease required to linearize ~30% of supercoiled pColIR215. This concentration of S1 nuclease (0.1 unit) totally degraded the φX174 ss DNA, and no infective centers were observed.

These data show that fraction IV is substantially free of single-strand nuclease activity. Therefore, it is unlikely that the specific cleavage of cruciform DNA by fraction IV results from cutting of the single-stranded hairpin loops of the cruciform structure (position c-e in Fig. 1, path a).

Since it is possible that fraction IV might be specific for duplex DNA substrates that contain regions of local denaturation, we tested for cutting of relaxed plasmids that contained thymine dimers, psoralen adducts, or mismatched bases. However, we were unable to demonstrate cleavage of these substrates, which contain unpaired regions in duplex DNA (data not shown).

**Requirements for Cleavage.** Cleavage of cruciform DNA by fraction IV required the presence of magnesium as the divalent cation (data not shown). Concentrations between 5 and 10 mM MgCl2 were optimal. Neither Ca2+, Co2+, nor Mn2+ could substitute for Mg2+. We have not observed a requirement for any additional cofactors. Fraction IV was active over a broad range of pH, with pH 8.5 as optimum. The activity was stable during incubation in 50 mM Tris-HCl (pH 7.5) at 0°C or 37°C for 1 hr. However, activity was lost after heating at 65°C for 10 min or by treatment with 1 mg of proteinase K per ml and 1% NaDodSO4. The activity was insensitive to RNase treatment.

The protein concentration of fraction IV was too low to allow a determination of purity by NaDodSO4 gel electrophoresis. When analyzed by gel filtration through a Bio-Gel A-0.5m sizing column in R buffer supplemented with 100 mM NaCl, active fractions were eluted with a molecular weight of 200,000 compared with standard markers (thyroglobulin, 670,000; gamma globulin, 158,000; ovalbumin, 44,000; myoglobin, 17,000; cyanocobalamin, 1,350).

Fraction IV introduces staggered cuts in the Inverted Repeat Sequence. The results are consistent with the nuclease activity in fraction IV cutting cruciform DNA not only at positions a and c or b and d (Fig. 1), but also at positions a and d or b and c. To determine the relative frequency of cutting at these positions, pColIR215 was incubated with fraction IV and subsequently digested with Taq I, which cuts 90 bp from the center of the inverted repeat. The small DNA fragments produced in the reaction were analyzed by polyacrylamide gel electrophoresis. Sequence data (10) indicate that cleavage at positions a and d or b and c should, together with Taq I digestion, produce two new fragments of lengths 74 and 105 bp (i.e., differing in size by the length of the inverted repeat). In contrast, cleavage at a and c or b and d should produce only one new restriction fragment. Fig. 4, lane b, shows one new band, which migrated close to an 82-bp marker restriction fragment. This fragment was absent in control reactions from which fraction IV was omitted (data not shown). The other band visible in lane b of Fig. 4 was seen...
in control reactions and corresponds to an internal 141-bp Taq I fragment of pColIR215 (positions 1126–1267 of the pBR322 sequence; ref. 20). These data indicate that cleavages at a and d or b and c occur rarely under our conditions.

To determine the precise sites of cleavage relative to the cruciform structure, we treated pColIR215 DNA with fraction IV followed by EcoRI digestion. The EcoRI termini were 3'-end-labeled with 32P, and the DNA was denatured and run on a sequencing gel. To provide sequence markers, the 190-bp EcoRI-Taq I fragment of pColIR215 was purified, 3'-labeled at the EcoRI terminus, and subjected to Maxam–Gilbert chemical degradation (21). By taking into account that one nucleoside was chemically removed by the Maxam–Gilbert method, the results in Fig. 5 indicate that cutting took place within the C-T-A-G sequence on the 3' side of the inverted repeat. The major site of cleavage occurred between the C and T residues, and the second major site of cleavage occurred between T and A to produce a fragment one nucleotide shorter. Other, more minor, sites of cleavage were observed close to the center of the inverted repeat. No cleavage of significance was introduced on the 5' side of the inverted repeat, which would have produced fragments 20 nucleotides longer.

We also determined the site of cleavage in the other strand. pColIR215 that had been treated with fraction IV was cut with Taq I, and the Taq I terminus was 3'-end-labeled. As markers, we produced a Maxam–Gilbert sequencing ladder from a 190-bp EcoRI–Taq I fragment of pColIR215 that was 3'-labeled at the Taq I terminus. The sequencing gel of Fig. 6 shows that the sites of cleavage in this strand are similar to those observed with the first strand. Again, cleavage occurred predominantly in the C-T-A-G sequence, and the major site of cleavage was between the C and T residues. These results show that the major sites of cleavage by fraction IV occur in symmetrically related positions about the inverted repeat sequence.

![Fig. 5. Sites of cleavage of pColIR215 by fraction IV. Plasmid DNA, treated with fraction IV and digested with EcoRI, was 3'-labeled at the EcoRI termini. Fragments were run on an 8% sequencing gel flanked by chemical degradation ladders obtained from an EcoRI–Taq I pColIR215 fragment 3'-labeled at the EcoRI terminus. The sequences shown on the right were taken from ref. 10 and were in close agreement with those obtained here except for the region on the 5' side (upper) of the inverted repeat, where sequence reading is made difficult because the palindrome alters fragment mobility.](image1)

![Fig. 6. Sites of cleavage by fraction IV. Plasmid DNA treated with fraction IV were digested with Taq I and 3'-labeled at the Taq I termini. The fragments obtained were run on a sequencing gel flanked by chemical degradation ladders obtained from an EcoRI–Taq I pColIR215 fragment 3'-labeled at the Taq I terminus. The sequences shown on the right were taken from ref. 10 and were as described in the legend to Fig. 5.](image2)
In this paper we have demonstrated the presence of a novel endonuclease in a partially purified fraction from *S. cerevisiae*. Using plasmid substrates that contain inverted repeats that are extruded into cruciform structures, we have shown that the nuclease activity catalyzes cleavage close to the cruciform junction. Studies of the cleavage sites of pColIR215 indicate that nicks are introduced into the two extruded cruciform arms at the positions indicated in Fig. 7. The sites of cleavage of pColIR215 by T4 endonuclease VII have been characterized in detail (10). Lilley and Kemper showed that endonuclease VII introduced cleavages into the cruciform arms at positions two to three nucleotides from the base of the junction. Cleavages occurred in each strand at the 5' end of the inverted repeat. Using the same plasmid, we have shown that the cuts introduced by fraction IV also occur in the cruciform arms. However, in this case, cuts occur at positions four to six bases from the base of the junction, at the 3' side of the inverted repeat (Fig. 7). The major sites of cleavage within these regions occur between C and T residues (Figs. 5 and 6). The variability observed at the site of cleavage may be inherent to the nuclease activity or may be a consequence of incomplete extrusion of the cruciform. At the present time, we cannot distinguish between these or other possibilities.

The two regions of cleavage of pColIR215 DNA by fraction IV are symetrically opposed across the cruciform junction (Fig. 7). In this respect, the activity appears similar to T4 endonuclease VII and T7 endonuclease I (2, 10, 11). By contrast, other enzymes, such as S1 nuclease, that have been used *in vitro* as probes for cruciform structures cleave in the single-stranded regions of the hairpin loops (4, 6).

When this study was undertaken, our objective was to search for an enzyme capable of resolution of Holliday structures, a central intermediate in genetic recombination. We chose to use cruciform structures as DNA substrates for a number of reasons. (i) The DNA connections at the junction of a cruciform structure are geometrically equivalent to those of a Holliday structure (2, 10, 22). (ii) Phage enzymes that cleave cruciform structures also cleave Holliday structures *in vitro* (2, 10, 11) and are thought to be involved in the resolution of branched structures *in vivo* (2, 7, 23). (iii) The instability of plasmids that contain long inverted repeats, at least in *E. coli*, is suggestive of destructive processing by Holliday-resolving enzymes (5, 24, 25).

While fraction IV has been shown to cleave cruciform structures, the precise nature of the DNA structure recognized by the activity has not been determined. Indeed, although cleavage occurs within stem sequences, it is not at present clear whether a hairpin loop or the base of the cruciform is the structural feature recognized by the activity. While it may be tempting to speculate that the activity might be capable of resolving Holliday structures, and none of our present data rules out this possibility, caution must be exercised until the activity has been purified extensively and tested on other DNA substrates such as figure-8 DNA molecules made by RecA protein (17) and X-forms prepared by hybridizing specific DNA fragments (2).

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