Interaction of a Four-way Junction in DNA with T4 Endonuclease VII*  

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The binding of a synthetic four-way junction in DNA by T4 endonuclease VII has been studied using gel retardation and footprint analysis. Two specific protein-DNA complexes have been observed, but only one is stable in the presence of moderate concentrations of salt. The footprint of T4 endonuclease VII in the salt-resistant complex has been probed using hydroxyl radicals generated by the reaction of iron(II)/EDTA with hydrogen peroxide. The hydroxyl radical cleavage pattern indicates protection of approximately 5 residues in two strands that are diametrically opposed across the junction point.

General genetic recombination (1-5) and many site-specific recombination events (6-9) take place by the formation and resolution of intermediates in which two DNA helices are interlinked by a crossover or Holliday junction. The structure of these four-way junctions in DNA and of complexes they form with enzymes is of central importance to our understanding of the process of genetic exchange. Endonucleases capable of processing four-way junctions to produce duplex helices have been isolated from Esherichia coli bacteriophages (10, 11) and from yeast (12-14). Here we demonstrate the binding of a four-way junction by the most extensively characterized enzyme of this class, T4 endonuclease VII (10, 15-19). Binding results in the formation of two specific protein-DNA complexes that show differential stability to the presence of low concentrations of monovalent cations. The structure of the most stable form has been probed by hydroxyl radical attack to reveal a footprint that occupies two of the four strands at the junction point.

Materials and Methods

Oligonucleotides—Junction DNA was made by annealing oligonucleotides 1(5'-GAGCCTGCGCAATTCTCGGTTAGAGA TACCGATAAGCTTCCGGCTTAA-3'), 2(5'-GTAAGCGAAGCTT ATCAGTGACCAAGGAGTGAAC-3'), 3(5'-TGATCATTGCTGATGATAGGCAGGCTTGCAGTCGTTCTAG TACCTGCA-3'), and 4(5'-ATGCTTGCTACAGTTGAAGAACCT CCGCTAAAGCGGATAGGCGGAGG-3'). Duplex DNA was annealed from oligonucleotides 1 and 5 (5'-CTTAAGCGGAAAG CAGTATCTGATGATAGGCAGGCTTGCAGTCGTTCTAG TACCTGCA-3'). All oligonucleotides were synthesized by phosphoramidite chemistry on an Applied Biosystems 380B DNA synthesizer and used fully unprotected. Following ethanol precipitation, oligonucleotides were resuspended in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA at a concentration of 1 µg/µl.

Construction of 3'-Labeled Substrates—Synthetic four-way junctions were prepared by a method similar to that described by Kellembach et al. (20). 9 µg of each of four complementary oligonucleotides were annealed in SSC buffer (150 mM NaCl, 15 mM sodium citrate) by incubation for 2 min at 95 °C, followed by 10 min at 65 °C, 10 min at 37 °C, and finally 10 min at room temperature (in a total volume of 40 µl). An aliquot of the annealed mixture (1 µl) was Klenow labeled at room temperature at one terminus using 40 µCi of [γ-32P]dCTP. Duplex DNA was made in a similar way by annealing oligonucleotides 1 and 5. Junction and duplex DNA were purified by electrophoresis through a 10%% native polyacrylamide gel using a Tris borate buffer. Interaction of a Four-way Junction in DNA with T4 Endonuclease VII  

Enzymes—T4 polynucleotide kinase and DNA polymerase I (Klenow fragment) were purchased from New England Biolabs. T4 endonuclease VII (60,000 units/µl) was purified to homogeneity by Dr. Hans Kosak from an overexpression vector (generously provided by Dr. W. Rüger) by a modification of a previously published procedure (10). It was stored and diluted in 10 mM Tris-HCl, pH 7.5, 0.1 mM glutathione, and 50% (v/v) glycerol. One unit of T4 endonuclease VII is defined as the amount of enzyme required to digest 50% of supercoiled plasmid DNA (0.2 µg) to form II and III DNA within 15 min at 37 °C in a total volume of 10 µl.

Gel Binding Assay—Varying amounts of T4 endonuclease VII were incubated with 3'-32P-labeled junction or duplex DNA (10,000 cpm, approximately 0.1-0.4 ng of DNA) for 15 min at 16 °C in 20 µl of binding buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 1 mM dithiothreitol). To each sample, 5 µl of loading buffer (40 mM Tris-HCl, pH 7.5, 4 mM EDTA, 25% glycerol, and 400 µg/ml bovine serum albumin) was added and the mixtures immediately loaded onto a 1.5-mm-thick 10% polyacrylamide (29:1 acrylamide:bisacrylamide) gel. Electrophoresis was carried out at 4 °C at 160 V for 2.5 h with continuous circulation of the buffer (0.5% Tris-HCl, pH 8.1, 3.3 mM sodium acetate, and 2 mM EDTA). Gels were dried and radiolabeled DNA detected by autoradiography.

Cleavage Reactions—Reaction mixtures (20 µl) containing 3'-32P-labeled junction DNA (10,000 cpm, 0.1-0.4 ng of DNA) and T4 endonuclease VII were incubated for 15 min at 16 °C in cleavage buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 1 mM dithiothreitol) supplemented with the indicated concentrations of KCl. Reactions were stopped, deproteinized, and DNA products visualized by electrophoresis through a 10% polyacrylamide gel using a Tris borate buffer. To determine the sites of cleavage, four uniquely 5'-32P-labeled junction DNA (10,000 cpm, 0.1-0.4 ng of DNA) were annealed to 5 units of T4 endonuclease VII for 20 min at 25 °C in cleavage buffer containing 15 mM NaCl. The reactions (30 µl) were stopped, and the DNA was denatured and loaded onto a 13% polyacrylamide sequencing gel containing 7 M urea. Following electrophoresis, DNA fragments were detected by autoradiography. To assign the cleavage sites, 5'-32P-labeled oligonucleotides were sequenced using the chemical method (21). Allowances to compensate for the nucleoside eliminated in the sequencing reaction were made, and the sites of cleavage were confirmed by comparison with fragments produced by restriction enzymes.
digestion using both 5'- and 3'-³²P-labeled junctions.

Chemical Footprinting—Reactions (140 μl) containing 5'-³²P-labeled junction DNA (400,000 cpm, approximately 1 ng of DNA) were incubated for 8 min at room temperature in the absence or presence of T4 endonuclease VII (in binding buffer supplemented with 50 mM NaCl). Hydroxyl radicals were generated using hydrogen peroxide and iron/EDTA as described (22), and incubation was continued for 2 min before the addition of thiourea. The DNA products were ethanol-precipitated, denatured, and electrophoresed on a 12% denaturing acrylamide gel.

RESULTS AND DISCUSSION

To detect the binding of a DNA junction by purified T4 endonuclease VII, we used gel retardation assays (23–25). In binding reactions in which ³²P-labeled junction DNA was incubated with increasing amounts of T4 endonuclease VII, two bands of reduced mobility were observed (Fig. 1A, bands a and b). Band a, which migrated slightly behind unbound junction DNA (Fig. 1A, band X), was produced at low enzyme concentrations whereas band b was seen at higher enzyme levels. Under identical binding conditions and similar concentrations of enzyme, little binding was seen to a duplex control (Fig. 1A). The formation of band b was observed only in the absence of monovalent cation, and the inclusion of concentrations of KCl or NaCl greater than 25 mM resulted in the formation of band a exclusively (Fig. 1B). This salt-resistant protein-DNA complex was stable up to 300 mM NaCl (data not shown).

To prevent endonucleolytic cleavage of the junction, it was necessary to perform endonuclease VII binding experiments in the presence of EDTA. When EDTA was replaced by 10 mM Mg²⁺, the junction was cleaved to form fragments with gel mobility equivalent to that of duplex DNA molecules (Fig. 1C). In these reactions, the presence of monovalent cations had a stimulatory effect, leading to complete cleavage of the substrate.

It has been shown previously that cleavage of a synthetic four-way junction occurs by the introduction of symmetrical cuts about the junction point (26, 27). To determine the sites of cutting in the junction used in the experiments described here, four identical junctions were prepared in which one 5'-³²P-labeled oligonucleotide was annealed with three unlabeled strands. The junctions were treated with endonuclease VII, and the DNA was denatured and analyzed on polyacrylamide gels containing 7 M urea. As reference markers, 5'-³²P-labeled oligonucleotides were sequenced using the chemical method.
A. Four uniquely 5'-32P-labeled junctions were incubated with 5 units of T4 endonuclease VII in cleavage buffer containing 15 mM NaCl. The products of the reactions were denatured and run on 12% polyacrylamide sequencing gels. T+C and G+A sequence ladders flank the endonuclease VII cleavage reactions (lane eVII). The lane headings refer to the labeled oligonucleotide. B, central core of the junction indicating the major and minor (observed on long exposures of the same autoradiogram) sites of cleavage.

The results, shown in Fig. 2, A and B, indicate that endonuclease VII cut the DNA predominantly in one orientation by cleavage of a single phosphodiester bond in strand 1 and strand 3. The sites of incision were symmetrically opposed across the junction and were located two nucleotides to the 3'-side of the base of the junction. Minor sites of cleavage in the opposite orientation (strands 2 and 4) were observed only upon overexposure of the autoradiographs.

Our observation of unidirectional cleavage is consistent with previous experiments describing the cleavage of synthetic junction DNA by T4 endonuclease VII (26, 27) and is indicative of the junction adopting one of two potential isomeric forms. Choice of isomeric form is thought to be dependent upon stacking interactions which are governed by the base sequences at the junction point (26–28). The formation of one isomeric structure was advantageous and allowed us to investigate the footprint of T4 endonuclease VII on the junction. Initial studies in which enzyme-junction complexes, formed in the presence of Mg2+, were probed by DNase I (29) were unsuccessful since the base sequences close to the junction point were inaccessible to DNase I and because the enzyme levels necessary to observe binding resulted in excessive cleavage of the substrate DNA. The inaccessibility of junction sequences to DNase I has recently been reported by others (30).

However, the hydroxyl radical method of Tullius and Dombyrowski (22) or related chemical methods have been used successfully to investigate many protein-DNA interactions (31), including those of sequence- or structure-specific nucleases (32–36). The patterns of Fe(II)/EDTA/H2O2 cleavage on all four strands of the junction in the absence and presence of T4 endonuclease VII are shown in Fig. 3A. The reaction conditions chosen for footprint analysis include 50 mM NaCl in order to favor the formation of only the more stable of the two possible protein-DNA complexes observed in the earlier gel retardation experiments (Fig. 1, A and B, band a). In the absence of endonuclease VII, we observed that strands 1 and 3 showed reduced cleavage in 2 residues at the branch point, in agreement with the data of Churchill et al. (28). In the presence of the junction binding protein, this region of protection was extended to approximately 5 residues (Fig. 3A, brackets). Although it was necessary to carry out the hydroxyl radical cleavage reaction in the presence of EDTA, the high levels necessary to observe binding resulted in some cleavage of the substrate by the enzyme. The sites of cutting under these conditions were located within the protected region and were identical to those observed when EDTA was replaced by Mg2+ (data not shown). The presence of these cleavage sites served as internal markers, and comparison with Maxam-Gilbert sequencing ladders allowed the precise determination of the residues that were protected by endonuclease VII (Fig. 3B). These results indicate that the cleavage of the junction in one orientation (by cutting of diametrically opposed strands 1 and 3) is a consequence of the way in which endonuclease VII binds tightly to two of the four strands that comprise the junction.

In a series of experiments over a range of T4 endonuclease VII concentrations, we were unable to unequivocally demonstrate binding to strands 2 and 4. However, in some experiments, a weak footprints was observed about the junction point of strand 2 and at a region located approximately 8 residues to the 5'-side of the junction (Fig. 3A, broken lines). However, since this binding was weak and less reproducible than that observed in strands 1 and 3, it is possible that it may result from the formation of the second protein-DNA complex, as observed by the gel retardation assay (Fig. 1A, band b). Alternatively, it might reflect the formation of transient contacts that are unstable during the hydroxyl radical reaction.

The results in Fig. 3A are in agreement with the data of Churchill et al. (28), although it was necessary to carry out the hydroxyl radical cleavage reaction in the presence of EDTA, the high levels necessary to observe binding resulted in some cleavage of the substrate by the enzyme. The sites of cutting under these conditions were located within the protected region and were identical to those observed when EDTA was replaced by Mg2+ (data not shown). The presence of these cleavage sites served as internal markers, and comparison with Maxam-Gilbert sequencing ladders allowed the precise determination of the residues that were protected by endonuclease VII (Fig. 3B). These results indicate that the cleavage of the junction in one orientation (by cutting of diametrically opposed strands 1 and 3) is a consequence of the way in which endonuclease VII binds tightly to two of the four strands that comprise the junction.

Synthetic four-way junctions, such as the one used in this study, have been used as models for Holliday junctions since the chemical structures of their junction points are analogous. However, in contrast to Holliday junctions, these synthetic substrates do not possess homologous arm sequences and this may have important structural consequences. Recently, it was shown that T4 endonuclease VII resolves Holliday junctions made in vitro by RecA protein, the recombination enzyme from E. coli (37). In these reactions, two DNA substrates were recombined such that homologous helices were joined at a single junction point. The presence of endonuclease VII resulted in resolution of the Holliday junctions made by RecA to give rise to the two expected types of recombiant product. The ability of the endonuclease to recognize a Holliday junction as it moves along DNA, either by branch migration or by unidirectional strand exchange promoted by RecA protein, indicates that the basis of recognition is DNA structure rather than sequence specificity. However, once bound to a junction, the sites of cleavage are influenced by the local DNA sequence about the junction point (17, 38). The model junctions used
FIG. 3. Hydroxyl radical protection of the four-way DNA junction by T4 endonuclease VII. A, four uniquely 5'-32P-labeled junctions were incubated in the absence or presence of T4 endonuclease VII as described under "Materials and Methods." Reactions were supplemented with hydrogen peroxide and iron/EDTA, and incubation was continued for 2 min. The DNA products were denatured and electrophoresed on a denaturing polyacrylamide gel. Only part of each ladder is shown. Lane headings indicate the labeled oligonucleotide and amounts of endonuclease VII (units, \( \times 10^{-4} \)). Regions of strong and weak protection are indicated by brackets (in strands 1 and 3) and broken lines (strand 2), respectively. Within the protected regions are the sites of cutting by endonuclease VII (intense bands). R, central core of the junction showing the regions (shaded boxes) protected from hydroxyl radical attack by the binding of T4 endonuclease VII, as determined from this and other experiments. Weak (less reproducible) regions of protection are not indicated. The location of the sites of protection was determined in comparison with sequence ladders (not shown).

in the present study may be best regarded as "frozen" Holliday junctions to which endonuclease VII can bind, and the observed protein-DNA contacts will be composed of the structural and sequence specificities of the nuclease.

Although the protein-DNA contacts detected in the present experiments were obtained in the presence of EDTA, the observed parallels between protection and cleavage are of interest with respect to the possible structures that a four-way junction may adopt. Fig. 4 shows three possible DNA configurations in which the cleavage and footprinting data are indicated. A tetragonal junction in which the four arms are unstacked and maximally extended in a square planar configuration is indicated in Fig. 4A. The adoption of such a structure, originally proposed to occur in the absence of Mg\(^{2+}\) (27), is inconsistent with recent evidence (39) and with the 2-fold symmetric patterns of cleavage and protection observed here. Two-fold symmetric DNA structures are shown in Fig. 4, B and C. In the Sigal-Alberts junction (40) in which helical arms are aligned in parallel, the symmetrical axis of the complex is parallel to the helical axis (Fig. 4B). In contrast, the antiparallel alignment of arm sequences (27) has 2-fold symmetry perpendicular to the plane of the helical axis (Fig. 4C).

Gel filtration experiments (10) indicate that T4 endonuclease VII is a multimeric protein (relative \( M_r \), 43,000) composed of two identical peptide subunits (\( M_r \), 18,000 (41)). The hydroxyl radical protection data indicating that endonuclease VII protects 5 deoxyribose residues, in strands that are diagnostically opposed, is consistent with the binding of a protein (or proteins) of this size. However, at the present time, the stoichiometry of the enzyme-junction complex is unknown. Parallel helices of the type shown in Fig. 4B would require that each of the crossover strands binds one enzyme unit (i.e. a monomer or a dimer), as shown in Fig. 4E. In contrast, cleavage of antiparallel helices could be accomplished by two enzyme units or by a single dimeric protein (Fig. 4F). In either case, it is likely that the structural distortion of the DNA at the crossover point will be the recognition target for the nuclease.
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In vivo, it is unlikely that T4 endonuclease VII is restricted solely to a role in recombination. Indeed, its ability to cleave four-way junctions (15–17), Y junctions (18), and heteroduplex loops (19) indicates that the primary role of the nuclease may be to remove any branches in duplex DNA generated by recombination-dependent initiation of replication. The way in which it recognizes and binds such structures is unlikely to be unique and is therefore of interest with regard to the mechanism of Holliday junction resolution by cellular proteins.

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