Specificity of binding to four-way junctions in DNA by bacteriophage T7 endonuclease I

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Received May 23, 1990; Revised and Accepted July 5, 1990

ABSTRACT

T7 endonuclease I binds specifically to four-way junctions in duplex DNA and promotes their resolution into linear duplexes. Under conditions in which the nuclease activity is blocked by the absence of divalent cations, the enzyme forms a distinct protein-DNA complex with the junction, as detected by gel retardation and filter binding assays. The formation of this complex is structure-specific and contrasts with the short-lived binding complexes formed on linear duplex DNA. The binding complex between T7 endonuclease I and a synthetic Holliday junction analog has been probed with hydroxyl radicals. The results indicate that the nuclease binds all four strands about the junction point.

INTRODUCTION

Endonuclease I from bacteriophage T7 has two essential roles during phage growth: (i) it degrades cellular host DNA following phage infection, and (ii) it cleaves junctions in replicating phage DNA to produced unbranched linear duplexes that may subsequently be packaged into phage heads. Mutations in gene 3, which encodes the nuclease, result in mutant phage that are unable to degrade host DNA [1], and are defective in phage maturation [2] and genetic recombination [3–5].

Purified T7 endonuclease I catalyzes the endonucleolytic cleavage of both single- and double-stranded DNA [6]. Although the activity on duplex DNA is 100 times lower than that seen with single-stranded DNA, T7 endonuclease I is highly specific for branched DNA structures in the duplex DNA. Using synthetic X- and Y-junctions, or plasmids that contain cruciform DNA structures, it has been shown that the nuclease introduces highly specific cuts about the site of a junction [7–9]. Cleavage, to produce linear duplex DNA, occurs by the introduction of nicks at positions that are diametrically opposed across the junction point. Although recognition of a junction is thought to be structure-specific, the efficiency and site of cleavage is influenced by DNA sequence, and a preference for cleavage at the 5′-side of pyrimidine residues has been observed with junctions capable of branch migration [9, 10].

The specificity that T7 endonuclease I shows for branched DNA structures is of interest since such structures are analogous to Holliday junctions which are widely considered to be key intermediates in genetic recombination [11]. Holliday junctions are formed by a reciprocal exchange of strands between two duplex molecules, and need to be resolved by endonucleolytic cleavage to give rise to pairs of recombinant molecules. Studies with T7 endonuclease I and an analogous enzyme from bacteriophage T4, endonuclease VII [12–14], have provided our first insight into the mechanism of enzymatic resolution of Holliday junctions (for review see ref. 15). Moreover, studies with these enzymes have led to the development of assays that have enabled the identification of similar activities from yeast [16–18] and from animal cells [19].

The cloning of the genes that encode T7 endonuclease I [8, 20, 21] and T4 endonuclease VII [22], and over-production of their protein products, now allows a detailed investigation of the interactions of these proteins with DNA. In the present work, we have investigated the binding specificity of T7 endonuclease I to four-way junctions in DNA. Under conditions in which cleavage is blocked by the absence of divalent cations, we observe the formation of a stable protein-DNA complex in which the nuclease binds specifically to the junction. Hydroxyl radical footprinting experiments show that all four DNA strands at the junction point are protected by the nuclease.

MATERIALS AND METHODS

DNA Substrates

5′-^{32}P end-labelled junction DNA was prepared as described using oligos 1 (5′-GACGCTGCCGAATTCTGGCGTTAGGA-GATACCGATAAGCTTCGGCTTAA-3′), 2 (5′-CTTAAGCC-GAAGCTTATCGGTATCTTGCTTACGACGCTAGCAAG-TGATC-3′), 3 (5′-TGATCACTTGCTAGCGTCGTAAGCA-GCTCGTGCTGTCTAGAGACATCGA-3) and 4 (5′-ATCGA-TGTCTCTAGACAGCACGAGCCCTAACGCCAGAATTC-GGCAGCGT-3′) [23]. Linear duplex DNA was annealed from oligonucleotides 1 and 5 (5′-CTTAAGCCGAAGCTTATCGG-TATCTCCTAACGCCAGAATTCGGCAGCGT-3′). Unless stated otherwise, junctions and linear duplex DNA were labelled with ^{32}P at the 5′-terminus of oligonucleotide 1 using poly-nucleotide kinase.

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Purification of T7 endonuclease I

T7 endonuclease I was purified by modification of a published procedure [8] from strain BL21 (DE3) carrying pLysS and the T7 endonuclease I over-expression plasmid pAR2471 [24]. In brief, 5 litres of cells were grown at 37°C in M9ZB medium [24] containing 20 μg/ml ampicillin and 25 μg/ml chloramphenicol to OD_{600} = 0.6 without aeration. T7 endonuclease I expression was induced by addition of IPTG to 0.4 mM and growth was continued for 2 hours. The cells were collected by centrifugation, resuspended in 100 ml of 100 mM Tris-HCl, pH 8.0 and 2 mM EDTA and lysed by two cycles of freezing and thawing. To the viscous cell lysate, 1 ml of 1 M $MgSO_4$ was added to allow digestion of DNA by endogenous nucleases. After 20 min incubation at 30 °C, 0.1 volume of 4% sodium deoxycholate and 0.05 volume of 0.2 M EDTA were added, and the lysate was clarified by centrifugation.

All column chromatography was performed at room temperature. Soluble proteins were applied to a 10 ml phosphocellulose (Whatman P11) column equilibrated with buffer A (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5 mM dithiothreitol and 10% glycerol). The column was washed with buffer A and eluted with a 100 ml 0−1.1 M gradient of NH_4Cl in buffer A. Fractions containing T7 endonuclease I were identified by their ability to cleave ^{32}P-labelled junction DNA, as measured by denaturing PAGE. T7 endonuclease I, which eluted at approx 0.5 M NH_4Cl, was diluted 2-fold into buffer A and applied directly to a 2 ml column of SP-sephadex (Pharmacia) equilibrated with buffer A supplemented with 0.1 M NH_4Cl. The column was eluted with a 20 ml 0−1 M gradient of NH_4Cl in buffer A and fractions assayed for activity. Active fractions were pooled, diluted 2-fold and applied to a 2 ml hydroxylapatite (BioRad HPT) column. Following elution with a 0−1.3 M NH_4Cl gradient in buffer A, T7 endonuclease I peak fractions were located by SDS gel electrophoresis and coomassie blue staining. Active fractions were pooled and stored in column elution buffer at a concentration of 0.3 mg/ml. At the final step, the protein was >90% pure as judged by SDS gel electrophoresis. Prior to use, T7 endonuclease I was diluted in 20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol and 50 μg/ml bovine serum albumin.

DNA Binding Assays

To detect binding by gel retardation [25], varying amounts of T7 endonuclease I were incubated with 5′-^{32}P end-labelled junction or duplex DNA (approx 3 ng) for 15 min at room temperature in 20 μl of binding buffer (BB = 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 4% low ionic strength polyacrylamide gel (30:1 acrylamide:bisacrylamide). Electrophoresis was carried out at 4 °C at 160 V for 2.5 h with continuous circulation of the buffer (6.7 mM Tris-HCl, pH 8.1, 3.3 mM sodium acetate and 2 mM EDTA). Gels were dried and radiolabelled DNA detected by autoradiography.

Reactions (50 μl) for filter binding assays [26], contained approx 20 ng 5′-^{32}P end-labelled junction or duplex DNA and varying amounts of T7 endonuclease I in BB supplemented with 50 mM KCl. Incubation was for 15 min at 0 °C. Aliquots (20 μl) were applied to duplicate filters (Schleicher and Schuell BA85) and washed with 2 ml of iced BB + 50 mM KCl. Filters were dried and the amount of DNA bound by the filter was determined as a percentage of total radioactive counts. In these experiments the final levels of binding were variable from one experiment to another. However, a differential between junction and linear duplex DNA was always observed.

Cleavage Reactions

To determine the sites of cleavage, four uniquely 5′-^{32}P end-labelled junctions (approx 10,000 cpm) were incubated with 60 pg T7 endonuclease I for 15 min at 25 °C in 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 1 mM dithiothreitol, 50 mM NaCl, 5 mM spermidine and 100 μg/ml bovine serum albumin. The reactions (20 μl) were stopped, the DNA denatured and loaded onto a 12% polyacrylamide sequencing gel containing 7 M urea. Following electrophoresis, DNA fragments were detected by autoradiography. To assign the cleavage sites, 5′-^{32}P end-labelled oligonucleotides were sequenced using the chemical method [27]. An allowance of one to two bases was made to compensate for the nucleoside eliminated in the sequencing reaction.

Chemical Footprinting

Reactions (140 μl) containing 5′-^{32}P end-labelled junction DNA (approx 250,000 cpm: 10 ng DNA) were incubated for 7 min at room temperature in the absence or presence of T7 endonuclease I (in BB + 50 mM NaCl). Hydroxyl radicals were generated using hydrogen peroxide and iron-EDTA as described [28], and incubation was continued for 2 min before addition of thiourea. The DNA products were ethanol precipitated, denatured and electrophoresed on a 12% denaturing acrylamide gel.

Neutral Gel Electrophoresis

Reactions to be analyzed by neutral gel electrophoresis were stopped by addition of loading buffer and deproteinized by addition of SDS to 1%. Samples were incubated 5 min at 37 °C, and loaded onto a 10% polyacrylamide gel using a TBE buffer system (89 mM Tris-borate pH 8.3, 2 mM EDTA).

SDS Gel Electrophoresis

Samples were prepared and analyzed by SDS-PAGE through a 12% gel using a discontinuous buffer system [29]. Gels were stained with Coomassie blue R250.

RESULTS

Specific Binding of Junction DNA by T7 Endonuclease I

T7 endonuclease I was purified by a modification of a previously published protocol [8], using a plasmid containing the cloned T7 endonuclease I gene under control of the T7 RNA polymerase over-expression system [24]. The nuclease was purified by phosphocellulose, SP-sephadex and hydroxlapatite chromatography to greater than 90% homogeneity, as shown in Figure 1.

Synthetic four-way junctions in DNA were prepared using four oligonucleotides (1, 2, 3 and 4, see Materials and Methods) which anneal to form an X-structure with arms 25 base pairs in length [23]. Incubation of purified T7 endonuclease I with this Holliday junction analogue led to the formation of a single defined protein-DNA complex as determined by a gel retardation assay (Figure 2). The amount of complex formed during the binding reaction was directly proportional to the concentration of T7 endonuclease

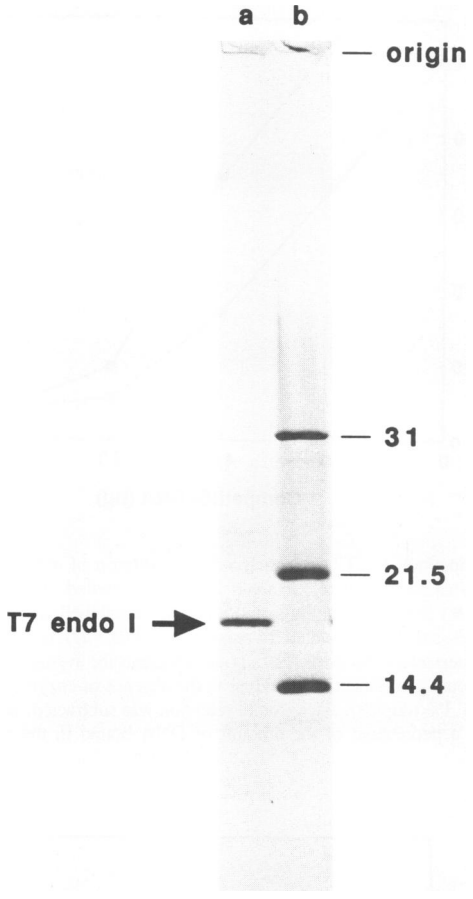

Figure 1. Gel electrophoresis of purified T7 endonuclease I. Lane a: 2.1 μg of T7 endonuclease I. Lane b, protein markers. The markers were carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa). Electrophoresis was through a 12% polyacrylamide mini-gel containing SDS. The arrow indicates the T7 endonuclease I polypeptide.

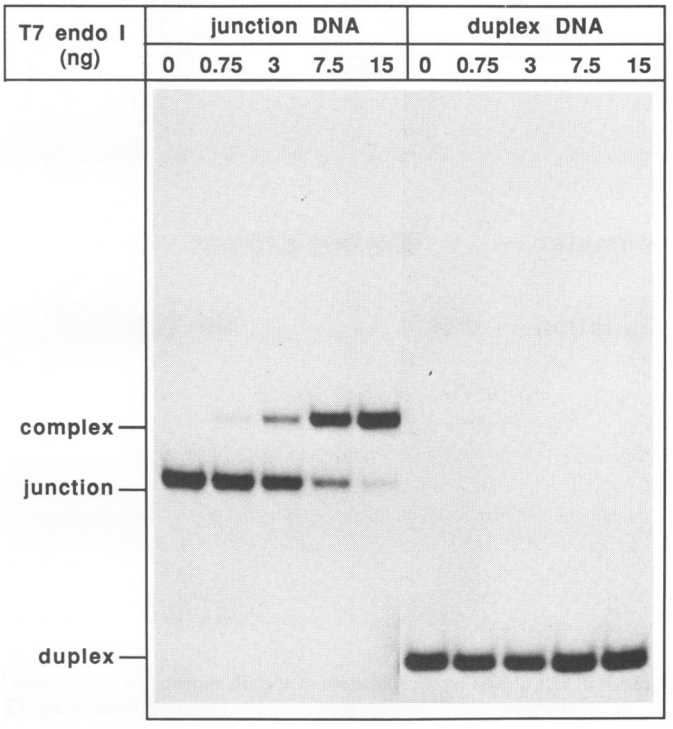

Figure 2. Specific binding of a synthetic Holliday junction by purified T7 endonuclease I . The indicated amounts of T7 endonuclease I were incubated with 2.5 ng 5′-^{32}P end-labelled junction (approx 5000 cpm) or linear duplex DNA (approx 100,000 cpm) as described in Materials and Methods. Complexes were separated on low ionic strength gels and radiolabelled DNA was detected by autoradiography. Due to the differences in specific activity of the two substrates, this photograph is composed of two different exposures of the same autoradiogram.

I. Binding to the four-way junction was inhibited by the presence of monovalent cations and little binding was observed at concentrations of KCl >100 mM (Figure 3).

Using the gel retardation assay, binding to linear duplex DNA was not observed (Figure 2). To investigate whether this apparent inability to bind was due to differential binding affinities for the X-structure and duplex DNA, we used nitrocellulose filter assays. Nitrocellulose filters provide a quick, sensitive assay for the formation of short-lived protein-DNA complexes [26]. When increasing amounts of T7 endonuclease I were added to reactions containing a constant amount of ^{32}P-labelled junction or duplex DNA, binding to both substrates was observed (Figure 4). The binding curves were similar showing a linear increase in the formation of protein-DNA complexes until a saturation level was reached. However, a greater affinity for the junction was observed.

To further examine the ability of T7 endonuclease I to bind preferentially to a junction, we performed two experiments: (i) using a limiting amount of enzyme, we investigated the effect of competition by unlabelled duplex DNA, and (ii) we measured the stability of the complex formed with either the junction or the linear duplex DNA. The competition assay is shown in Figure 5. Using 20 ng of ^{32}P-labelled junction or linear duplex DNA, it was found that the presence of a 4-fold excess of unlabelled linear duplex DNA abolished binding to either substrate. However, at lower levels of competitor, a significant difference in the ability to compete was observed, with T7 endonuclease I showing a preference for binding the ^{32}P-labelled junction. The ability of the endonuclease to bind linear duplex DNA, as seen in these experiments, may be a reflection of the way in which the nuclease needs to search long stretches of duplex DNA in order to locate the site of a junction.

Under the conditions used in the previous experiments, T7 endonuclease I binds DNA rapidly, and complete binding was observed as soon as it was technically possible to sample a reaction (<15 secs; data not shown). We therefore determined whether the apparent low affinity of T7 endonuclease I for linear duplex DNA was due to a higher rate of dissociation from this substrate. To do this, endonuclease I was pre-incubated with ^{32}P-labelled junction or linear duplex DNA for 5 min. At this time, when equilibrium binding was attained, the mixture was diluted 50-fold (t=0) and aliquots were assayed at various times by filtration through nitrocellulose. Figure 6 shows that there was little significant dissociation from the junction within 10 min. In contrast, there was rapid dissociation of the complex formed with linear duplex DNA, and from this and other experiments (data not shown) we calculated a half-life of 90−120 secs. After this time, a plateau level was reached as a second equilibrium state was achieved under the new conditions. Together, the above results indicate that the complex formed between T7 endonuclease I and the four-way junction is more stable than a complex formed with linear duplex DNA.

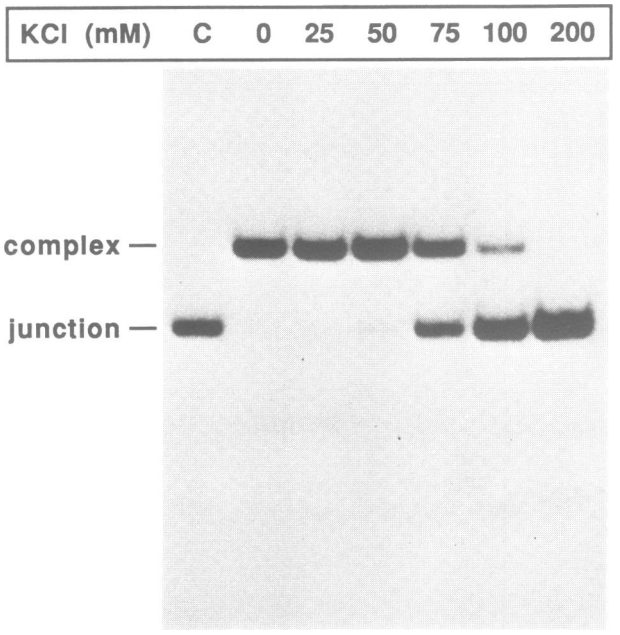

Figure 3. Effect of KCl on the formation of specific protein-DNA complexes. Binding assays were performed as described for Figure 1 using 6 ng T7 endonuclease I with 3 ng junction DNA. In each reaction, binding buffer (see Materials and Methods) was supplemented with the indicated concentrations of KCl. Lane C: control showing junction DNA in the absence of protein.

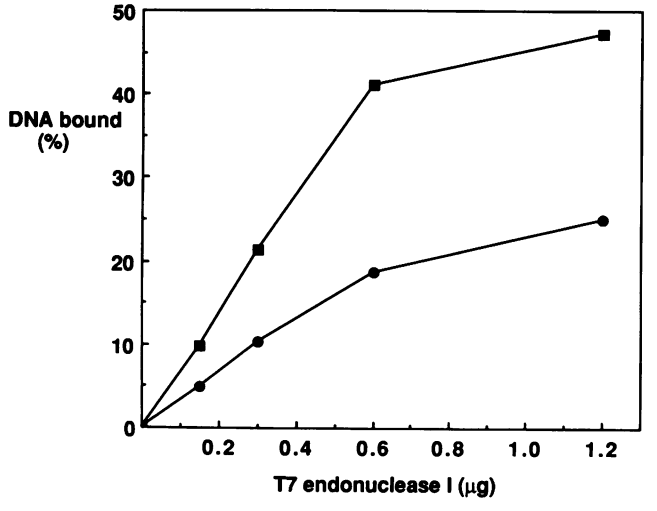

Figure 4. Binding of junction DNA or linear duplex DNA as a function of T7 endonuclease I concentration. Increasing amounts of T7 endonuclease I were included in reaction mixtures containing 20 ng ^{32}P-labelled junction (■) or duplex (●) DNA. Reaction conditions and nitrocellulose filter assay were as described in Materials and Methods. Each point represents the average of duplicate filters from one reaction mixture. The amount of radioactivity binding in the absence of enzyme was 5.15% (junction) and 1.6% (duplex). This value was subtracted when the percentage binding was calculated.

The stable binding of T7 endonuclease I to junction DNA was only observed in the absence of divalent cations. Figure 7 shows the effect of increasing concentrations of Mg^{2+}, in which the products were analyzed in two ways: (A) on a low ionic strength polyacrylamide gel that detects binding, and (B) following deproteinization and electrophoresis through a native

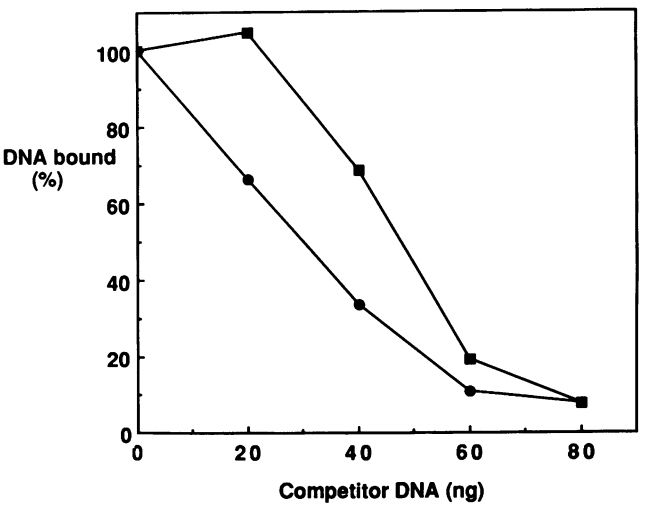

Figure 5. Competition for T7 endonuclease I by addition of unlabelled linear duplex DNA. Reaction mixture contained 20 ng ^{32}P-labelled junction (■) or linear duplex (●) DNA and the indicated amounts of unlabelled linear duplex DNA, plus a non-saturating amount of T7 endonuclease I (0.3 μg), under conditions described in Materials and Methods. Each point represents the average of duplicate filters. The amount of radioactivity binding in the absence of enzyme was 2.1% (junction) and 1.3% (duplex). Background retention was subtracted, and binding was plotted as a percentage of the amount of DNA bound in the absence of competitor.

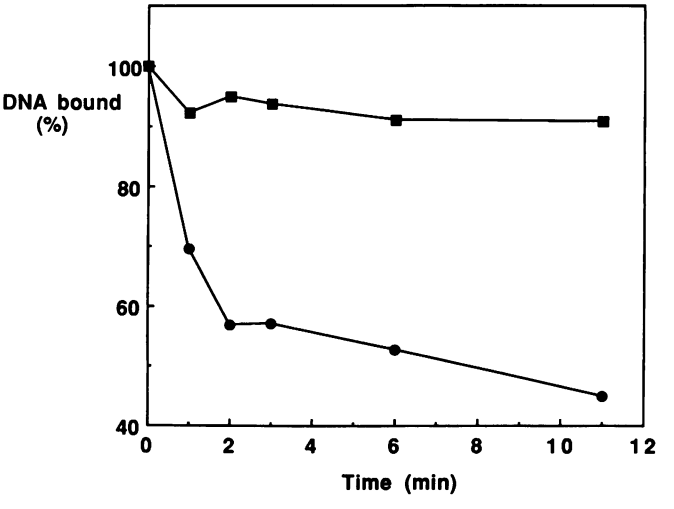

Figure 6. Dissociation of DNA-T7 endonuclease I complexes. T7 endonuclease I (3.6 μg) was incubated with 0.24 μg ^{32}P-labelled junction (■) or linear duplex (●) DNA, in binding buffer supplemented with 50 mM KCl (reaction volume 300 μl). After 5 min at 0 °C, the reactions were diluted 50-fold (time zero), and at the indicated times aliquots were assayed by nitrocellulose filter binding to determine the time course of dissociation. Background counts were subtracted and binding was expressed relative to the amount of DNA bound prior to dilution.

polyacrylamide gel which detects the products of cleavage. In reactions in which EDTA was omitted and replaced by Mg^{2+} at concentrations greater than 0.5 mM, binding by the gel retardation assay was not observed (Figure 7A). Under these conditions, T7 endonuclease I cleaved the four-way junction into products that comigrated with a linear duplex DNA marker. Analysis of the reaction products, as shown in Figure 7B, indicated that cleavage resulted in the formation of two linear duplexes, with slightly different mobilities.

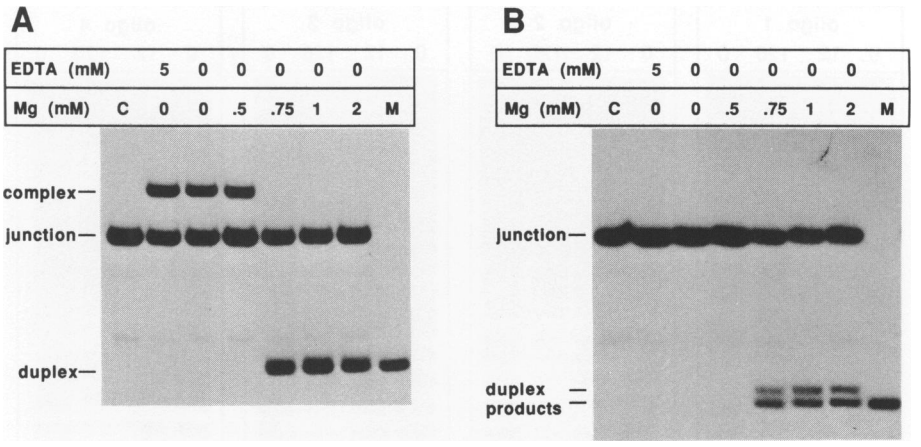

Figure 7. Binding or cleavage of junction DNA as a function of Mg^{2+} concentration. Binding assays were performed as in Figure 1 legend using 3 ng junction DNA and 0.6 ng T7 endonuclease I, in 50 mM Tris-HCl pH 8.0, 50 mM KCl, 1 mM dithiothreitol and the indicated concentrations of EDTA or $MgCl_2$. In this experiment the junction was ^{32}P-labelled at the 5′-terminus of oligonucleotide 4. Following incubation at room temperature for 15 min, 10 μl of sample loading buffer was added to each reaction, and aliquots (10 μl) were loaded immediately onto a low ionic strength polyacrylamide gel (A) to detect binding (Materials and Methods). The remaining 20 μl of the reactions were deproteinized by addition of $NaDodSO_4$ to 1% and incubation at 37 °C for 5 min. The samples were then loaded and run on a 10% native polyacrylamide gel using TBE as the buffer system (B). Lane C: control lane showing junction DNA in the absence of enzyme. Lane M: linear duplex DNA marker (oligo 1 annealed with oligo 5).

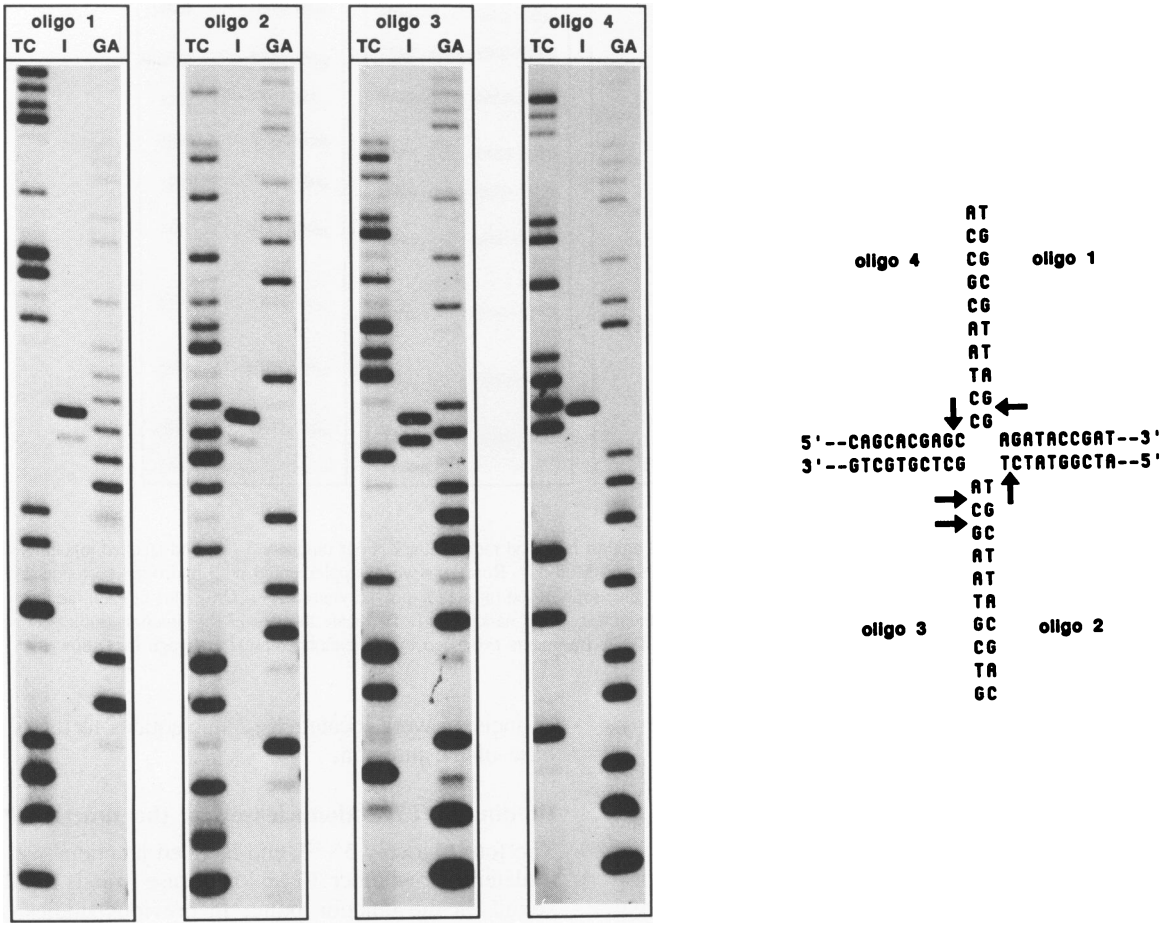

Figure 8. Sites of cleavage of the junction by T7 endonuclease I. Four uniquely 5′-^{32}P end-labelled junctions were incubated with T7 endonuclease I as described in Materials and Methods, and the products were analyzed by denaturing electrophoresis using 12% polyacrylamide gels containing 7 M urea. T+C and G+A sequence ladders flank the T7 endonuclease I cleavage reactions (lane I). The lane headings refer to the ^{32}P-labelled oligonucleotide. The cleavage sites are summarized in the diagram on the right. Only the central core of the junction is shown and major sites of cleavage are indicated by arrows.

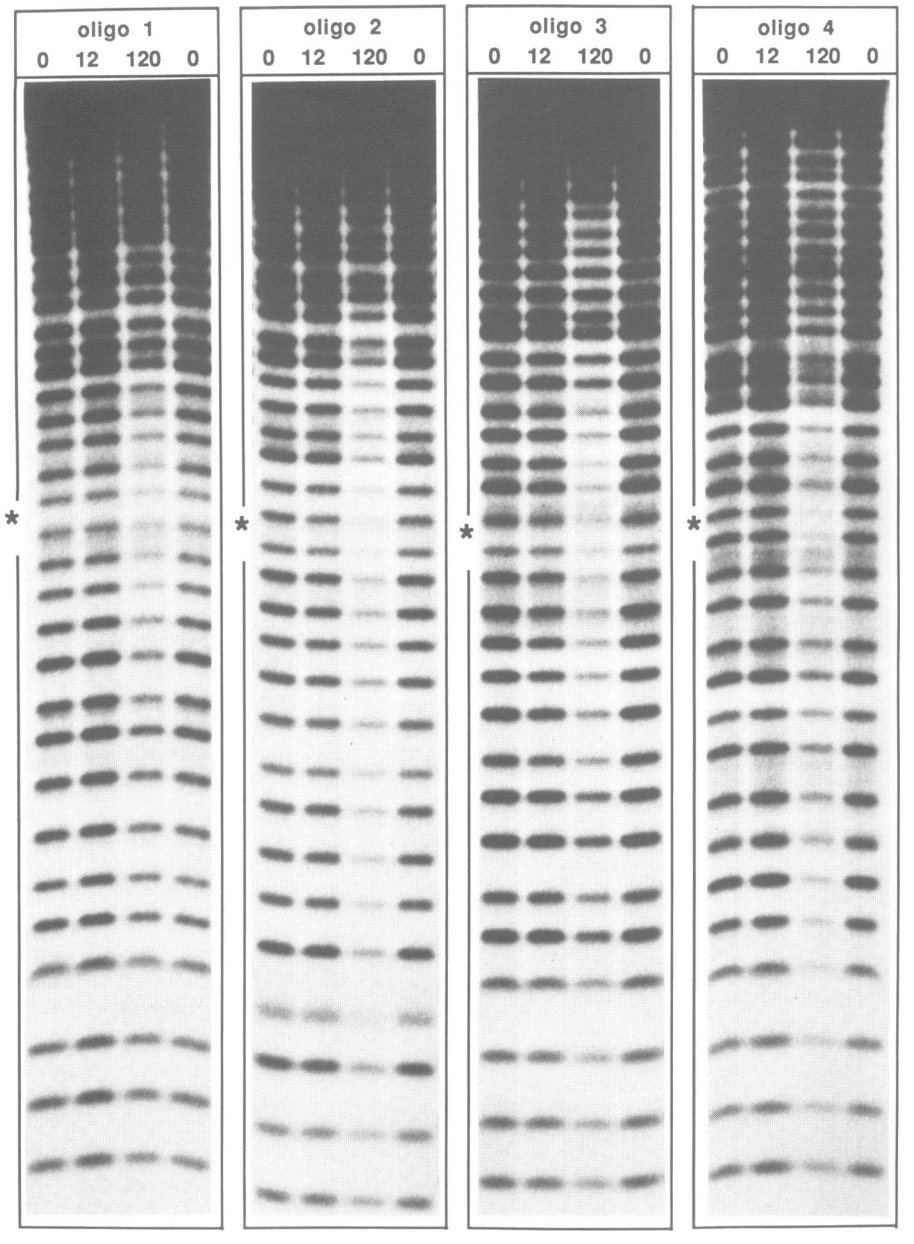

Figure 9. Protection of the synthetic Holliday junction by T7 endonuclease I against hydroxyl radical attack. Four uniquely 5′-^{32}P end-labelled junctions were incubated in the absence or presence of T7 endonuclease I as described in Materials and Methods. Reactions were supplemented with hydrogen peroxide and iron-EDTA, and incubation was continued for 2 min. The products were denatured and electrophoresed on a 12% polyacrylamide gel. Only part of each ladder is shown. Lane headings indicate the labelled oligonucleotide and the amounts of T7 endonuclease I (in nanograms). To locate the site of the junction (indicated with asterisks), hydroxyl radical cleavage ladders were run with sequencing ladders and DNA fragments produced by T7 endonuclease I cleavage (not shown).

Cleavage Occurs in Two Orientations

To determine whether the formation of two linear duplex products resulted from resolution of the substrate in either of the two possible orientations, four junctions were prepared in which one 5′-^{32}P end-labelled strand was annealed with three unlabeled strands. The junctions were then treated with T7 endonuclease I, and the DNA products were analyzed by denaturing gel electrophoresis. As reference markers, we used Maxam-Gilbert sequencing ladders produced by chemical degradation of 5′-^{32}P end-labelled oligonucleotides. The results shown in Figure 8 indicate that T7 endonuclease I cut the DNA in both orientations (by cleavage of strands 1 and 3, or strands 2 and 4). The sites of incision were located 1−2 nucleotides to the 5′-side of the base of the junction.

Binding of T7 Endonuclease I at the Junction Point

The four uniquely 5′-^{32}P end-labelled junctions were next used to determine whether T7 endonuclease I binds to all four DNA strands at the junction point. In previous studies, it has been observed that enzymatic probes such as DNase I are unable to gain access to the DNA sequences close to the junction point [23, 30, 31]. Instead, we used hydroxyl radicals generated by the reaction of iron(II)EDTA with hydrogen peroxide [28]. The advantage of hydroxyl radical cleavage is that it is a small

molecule that abstracts a hydrogen atom from deoxyribose, resulting in strand cleavage at the point of attack without any sequence specificity. However, it has the disadvantage that regions of weak protein binding are often accessible to chemical attack [32].

When complexes formed between T7 endonuclease I and the four 5′-^{32}P end-labelled junctions were probed with hydroxyl radicals, and the products were analyzed by denaturing gel electrophoresis, regions of protection about the junction point were observed (Figure 9, third lane of each set). Away from the junction point, the pattern of protection diminished, indicative of weak binding. Due to variation from one experiment to another we are unable to state the size of the protected region with any certainty. The observed binding is indicative of the formation of specific contacts with all four DNA strands at the junction point. However, since the DNA was not completely protected against hydroxyl radical attack, we can not exclude the possibility that the enzyme binds two two arms only (in the two possible orientations), and that the protection pattern results from an average of the two bound structures.

DISCUSSION

Initial studies of the activities of T7 endonuclease I indicated a single-stranded DNA-specific endonuclease with limited ability to cleave double-stranded DNA substrates [1, 6]. The ability of T7 endonuclease I to cleave single-stranded DNA at physiological pH led to its use as a probe for cruciform structures in supercoiled plasmid DNA. However, these studies assumed that cleavage occurred at the single-stranded hairpin loops [33], whereas subsequent detailed mapping of the cleavage sites showed that cleavage occurred exclusively at the base of the cruciform junction, and established that the nuclease was structure-specific [7, 8]. It has yet to be shown whether the ability of endonuclease I to cleave single-stranded DNA is also a reflection of its ability to recognize unusual DNA structures, this time in the form of folded regions of secondary structure.

The results presented in this paper provide a basis for the observed nucleolytic specificity of T7 endonuclease I for junction structures in duplex DNA. We have shown that the endonuclease binds specifically to a synthetic Holliday junction, as seen by the formation of a defined protein-DNA complex in band shift assays. In comparison, binding to a similar sized linear duplex molecule (which shared DNA sequences with the junction) was weak and could only be detected by a sensitive filter binding assay. Competition studies, together with experiments that investigated the rate of dissociation, showed that complexes formed with linear duplex DNA were short-lived in comparison with those formed with a junction structure. The ability to form transient contacts with linear duplex DNA is presumably required to locate junction structures as the primary targets for cleavage. During the phage life cycle, DNA replication and recombination lead to the formation of multiply branched DNA [2, 4], and the rapid location of junction structures is an essential function of this enzyme at the time of phage maturation and packaging.

Superficially, the action of T7 endonuclease I on a four-way junction appears highly analogous to that observed using T4 endonuclease VII. However, we did not find this to be the case. Since the junction used in the present experiments was also used in binding and cleavage studies with T4 endonuclease VII [23], we are now able to directly compare the two enzymes. T7 endonuclease I cut this substrate by the introduction of diametrically opposed nicks at positions located one to two nucleotides to the 5′-side of the junction. Cleavage occurred with approximately equal efficiency in the two possible orientations. Under conditions in which cleavage was inhibited by the absence of divalent cations, the hydroxyl radical protection patterns were consistent with binding to all four strands at the junction point. In contrast, T4 endonuclease VII cleaved the junction in one orientation only, at sites located two residues to the 3′-side of the junction. In this case, footprinting studies showed that T4 endonuclease VII bound tightly to only two DNA strands at the junction point (those in which cleavage occurred).

The present results are of interest with regard to the way in which these junction-resolving proteins interact with DNA. Both enzymes are small and are likely to bind junctions as a dimers or tetramers. In the absence of DNA, T4 endonuclease VII is known to be dimeric (M_r 43,000; [34]) and is composed of two identical 18 kDa subunits [22]. Similarly, T7 endonuclease I is also dimeric with a M_r of 40,000 as determined by gel filtration (our unpublished observations), and is made up from two identical 17 kDa subunits [35]. It is possible that T4 endonuclease VII binds the junction as a dimer whereas binding by T7 endonuclease I involves the assembly of two dimeric proteins at the junction point. Such a proposal would be consistent with the mobility of the protein-DNA complexes observed by gel retardation and with hydroxyl radical footprinting experiments [23]. However, further experiments will be required to determine the stoichiometry of binding.

Recent results indicate that synthetic four-way junctions form structures in which pairs of arms are coaxially stacked to produce a two-fold symmetrical structure with two sets of non-equivalent strands [36–39]. How T7 endonuclease I might promote cleavage of apparently non-equivalent strands remains to be determined. However, it is possible that the geometric structure of the junction may be changed as the protein accepts the DNA into its active site in readiness for cleavage. In this regard, it is of interest that T4 endonuclease VII and T7 endonuclease I are both capable of resolving true Holliday junctions made *in vitro* by the RecA protein of *E. coli* ([40]; B. Müller, C.A. Jones and S.C.W. in preparation). Future studies of the interaction of the T7- and T4-encoded junction-resolving proteins with synthetic and natural Holliday junctions, together with further studies of the resolving proteins from yeast [41, 42] and animal cells [19], will lead to a more complete understanding of the process of enzymatic resolution.

ACKNOWLEDGEMENTS

We thank Dr. William Studier for the generous gift of the T7 endonuclease I overproduction plasmid system and for communication of unpublished results. The experiments described could not have been performed without these overproducer strains. We are grateful to Iain Goldsmith's unit for preparation of the oligonucleotides and to John Nicholson for photography.

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