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T7 endonuclease I resolves Holliday junctions formed *in vitro* by RecA protein

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ABSTRACT

T7 endonuclease I is known to bind and cleave four-way junctions in DNA. Since these junctions serve as analogues of Holliday junctions that arise during genetic recombination, we have investigated the action of T7 endonuclease I on recombination intermediates containing Holliday junctions. We find that addition of T7 endonuclease I to strand exchange reactions catalysed by RecA protein of *Escherichia coli* leads to the formation of duplex products that correspond to 'patch' and 'splice' type recombinants. Resolution of the recombination intermediates occurs by the introduction of nicks at the site of the Holliday junction. The recombinant molecules contain 5'-phosphate and 3'-hydroxyl termini which may be ligated to restore the integrity of the DNA.

INTRODUCTION

The products of genes 3 (endonuclease I), 4 (primase), 5 (DNA polymerase) and 6 (exonuclease) of bacteriophage T7 are required for recombination and replication of T7 DNA [1–3] indicating that these two processes are closely linked during phage growth. Early studies with purified T7 endonuclease I showed that it has single- and double-stranded endonuclease activities with a strong preference for single-stranded over double-stranded DNA [4, 5]. Most probably, this endonucleolytic activity is responsible for degradation of host DNA which occurs after infection. In addition, the nuclease is specific for junction structures in DNA. A mutation in gene 3, the structural gene for T7 endonuclease I, leads to the accumulation of branched, rapidly sedimenting DNA which cannot be packaged [6]. The absence of mature DNA, and the defect in recombination observed in gene 3 mutants [7], indicate that T7 endonuclease I is required for the resolution of these branched intermediates by endonucleolytic cleavage.

The cloning of gene 3 [8–10], and over-production of its product has led to direct studies of the interaction of endonuclease I with junctions in DNA. Using cruciform structures and synthetic four-way junctions, de Massy *et al.* showed that T7 endonuclease I resolves branched DNA by the introduction of nicks in strands that are opposed across the branchpoint, to produce linear duplex DNA molecules [10, 11]. The specific interaction of T7 endonuclease I with junction DNA may be detected by gel retardation or filter binding assays, and footprinting studies of endonuclease I-junction complexes indicate that the nuclease binds all four DNA strands at the junction point [12]. Although these studies indicate that binding is structure-specific, it is known that the sites of cleavage about the junction are influenced by DNA sequence, with a preference for incision at the 5'-side of pyrimidine residues [13, 14].

The requirement for T7 endonuclease I for recombination, and the action of the nuclease on synthetic junctions (which are analogous to Holliday junctions which arise during recombination), has led us to the present study in which we have investigated the action of purified T7 endonuclease I on true Holliday junctions. To do this, we have used the recombination protein of *Escherichia coli*, RecA protein, which efficiently synthesizes Holliday junctions *in vitro*. We demonstrate that Holliday junctions formed by RecA protein are resolved by T7 endonuclease I to give rise to recombinant DNA products. The products of resolution contain ligatable nicks, thus allowing the restoration of the integrity of the recombinant DNA.

MATERIALS AND METHODS

Proteins and DNA Substrates

E. coli RecA protein was purified as described [15]. Concentrations in the text refer to moles of monomeric protein. T4 endonuclease VII (60,000 units/μl), a gift of Dr Börries Kemper (University of Cologne), was stored and diluted in 10 mM Tris-HCl (pH 7.5), 0.1 mM glutathione and 50% (v/v) glycerol. T7 endonuclease I was purified as described [10, 12] from strain BL21 (DE3) carrying pLysS and the T7 endonuclease I over-expression plasmid pAR2471 [16]. It was diluted in 20 mM potassium phosphate (pH 6.5), 1 mM dithiothreitol, 100 μg/ml BSA and 50% glycerol and stored at −20°C. Restriction endonucleases and T4 DNA ligase were obtained from New England Biolabs.

All DNA substrates were prepared as described [17]. DNA concentrations refer to moles of nucleotide residues.

Reaction conditions

Unless stated otherwise, complexes between 8.2 μM gDNA (circular duplex ΦX174 DNA containing a 162 nucleotide long single-stranded gap between the *Pst*I and *Ava*I restriction sites in the (−) strand) and 1.3 μM RecA protein were formed in 20 mM Tris-HCl (pH 7.5), 25 mM $MgCl_2$, 2 mM dithiothreitol,

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100 μg/ml bovine serum albumin and 2 mM ATP [17]. After 5 min at 37°C, strand exchange was initiated by addition of the linear 5042 bp *Pst*I-*Ava*II fragment of ΦX174 duplex DNA (3′-^{32}P end-labelled at the *Pst*I site) to a concentration of 3.8 μM. T7 endonuclease I was added immediately, and incubation was continued at 37°C. Reactions were stopped by addition of phenol, EDTA and sarcosyl to 8%, 20 mM and 0.25% respectively, and analysed by 0.7% agarose gel electrophoresis.

Ligation of resolution products

Strand exchange reactions (125 μl) contained gDNA (22.8 μM), RecA protein (3.8 μM) and uniformly ^{32}P-labelled ΦX174 duplex DNA linearized with *Pst*I (7.6 μM). After 5 min at 37°C, T7 endonuclease I was added to a concentration of 22 ng/ml and incubation was continued for 10 min. The products of the reaction were separated by electrophoresis through a 0.8% agarose gel and the nicked circular and linear dimer resolution products were excised and purified by electroelution. The DNA was concentrated by ethanol precipitation and resuspended in 80 μl of 50 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 2 mM dithiothreitol, 1 mM ATP. An aliquot (40 μl) was supplemented with 1 unit T4 DNA ligase and incubated for 30 min at 37°C. The reaction was stopped by addition of SDS and EDTA to 0.5% and 30 mM, respectively.

Analysis by gel electrophoresis

DNA samples were analyzed on agarose gels using 40 mM Tris-HCl (pH 7.9), 5 mM sodium acetate and 1 mM EDTA as the buffer system. For denaturing agarose gel electrophoresis, 50 mM NaOH and 2 mM EDTA was used as the buffer system. For analysis by denaturing polyacrylamide gel electrophoresis, reactions were stopped by addition of EDTA to 50 mM, extracted once with phenol/chloroform (1:1) and concentrated by ethanol precipitation in the presence of 30 μg/ml tRNA. The 5% denaturing polyacrylamide gels contained 7 M urea and were prepared using 89 mM Tris-borate (pH 8.3) and 2 mM EDTA as the buffer system. Following electrophoresis, they were fixed in 10% methanol and 10% acetic acid. All gels were dried and the DNA visualized by autoradiography on Fuji RX or Kodak XAR films.

RESULTS AND DISCUSSION

RecA protein-mediated strand exchange reactions were performed using the DNA substrates shown in Figure 1. First, nucleoprotein filaments were formed by incubation of RecA protein with circular ΦX174 duplex DNA containing a 162 nucleotide long single-stranded gap between the *Pst*I and the *Ava*I site in the (−) strand (gDNA). Strand exchange was then initiated by addition of a *Pst*I-*Ava*II duplex restriction fragment of ΦX174 DNA that was 3′-^{32}P end-labelled at the *Pst*I terminus. A time course of the strand exchange reaction (Figure 2, lanes a−f) shows the formation of the product which has the form of a σ-structure [18] over a period of 60 min. Previous results have shown that this reaction proceeds via the formation of recombination intermediates that contain Holliday junctions [17, 19, 20], as indicated in Figure 1.

To determine whether T7 endonuclease I can resolve Holliday junctions, an on-going RecA-mediated strand exchange reaction was supplemented with purified T7 endonuclease I. In this reaction, the formation of σ-structures was prevented, and instead we observed the formation of nicked circular and linear dimer

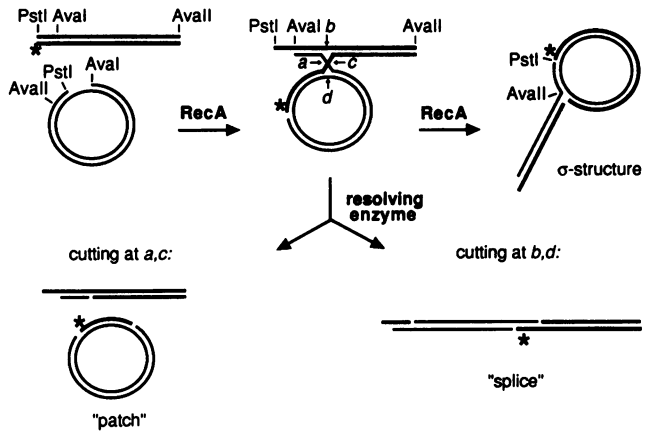

Figure 1. Schematic representation of substrates and products of strand exchange and resolution. Substrates of the RecA-mediated strand exchange reaction are gapped circular duplex ΦX174 DNA (gDNA) and linear duplex ΦX174 DNA (top, left). In the intermediate α-structure (top, centre), the DNA molecules are connected by a Holliday junction. The product of the strand exchange reaction with these substrates is a σ-structure (top, right). Resolution of the Holliday junction in orientation *a*−*c* leads to the formation of nicked linear and nicked circular DNA, the 'patch' recombinant products (bottom, left). Resolution in orientation *b*−*d* leads to the formation of nicked linear dimer DNA, the 'splice' recombinant product (bottom, right). The position of the ^{32}P-radiolabel used in the experiments shown in Figures 2 and 3 is indicated by the asterisk.

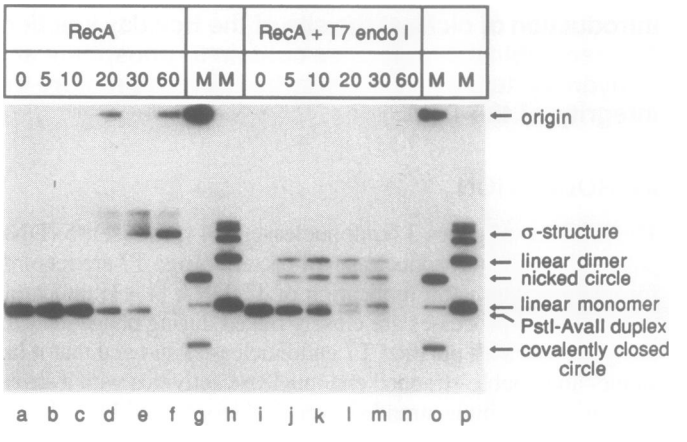

Figure 2. T7 endonuclease I resolves intermediates of the RecA-mediated strand exchange reaction. Strand exchange reactions (140 μl) between gDNA and ^{32}P end-labelled linear *Pst*I-*Ava*II fragment of ΦX174 duplex DNA were incubated as described in Materials and Methods. After the addition of the linear duplex the reaction was divided in two aliquots (70 μl). One aliquot was immediately supplemented with T7 endonuclease I (final concentration 27 ng/ml), the other was incubated without T7 endonuclease I. Samples (10 μl) of the two reactions were stopped at the times (min) indicated and the reaction products were analyzed by 0.7% agarose gel electrophoresis. Lanes a−f: time course of RecA-mediated strand exchange. Lanes i−n: time course of the strand exchange reaction supplemented with T7 endonuclease I. Lanes g and o: nicked circular, linear and covalently closed circular ΦX174 DNA. Lanes h and p: a ligation ladder of linear ΦX174 DNA.

DNA (Figure 2, lanes i−n). These products appeared much earlier (within 5 min) than the products of the strand exchange reaction (20−60 min), indicative of resolution during strand exchange. Prolonged incubation with T7 endonuclease I (lane n, 60 min) resulted in complete degradation of the DNA, probably due to the non-specific endonuclease activity of the protein [4,

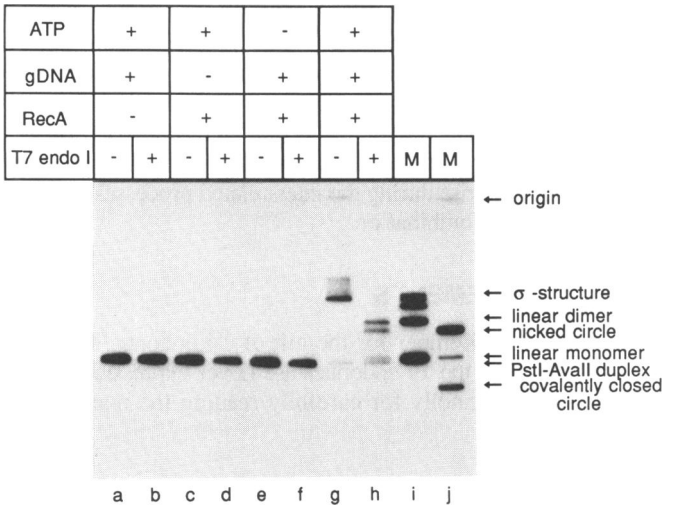

Figure 3. Product formation depends on RecA-mediated strand exchange. RecA protein, gDNA and ^{32}P end-labelled *PstI-AvaII* fragment of ΦX174 duplex DNA were incubated in four 30 μl reactions as described in Materials and Methods. Components were omitted as indicated on top of the Figure. 10 min after the addition of the linear DNA, 10 μl aliquots were removed from each reaction, supplemented with T7 endonuclease I (final concentration 27 ng/ml) and incubated for further 10 min. The remaining 20 μl were incubated for further 40 min to detect strand exchange. Reactions were stopped and the DNA products analyzed by 0.7% agarose gel electrophoresis. Lane i: ligation ladder of linear ΦX174 DNA. Lane j: nicked circular, linear and covalently closed circular ΦX174 DNA.

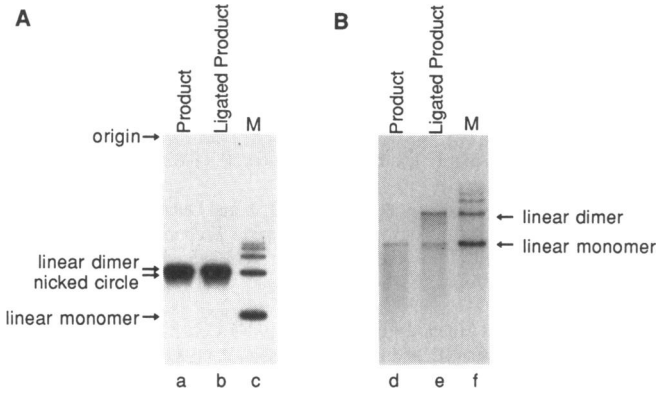

Figure 4. T7 endonuclease I resolves recombination intermediates by introducing ligatable nicks. Strand exchange reactions were performed with gDNA and *PstI*-linearized uniformly ^{32}P-labelled ΦX174 duplex DNA. Resolution products formed by T7 endonuclease I were isolated and ligated as described in Materials and Methods. The DNA was analyzed by 0.8% native (A) and denaturing (B) agarose gel electrophoresis, and ^{32}P-labelled DNA was visualised by autoradiography. Lanes a, d: purified nicked circular and linear dimer resolution products; lanes b, e: resolution products after ligation with T4 DNA ligase; lanes c, f: ligation ladder of linear ΦX174 DNA as markers. The position of nicked circular ΦX174 DNA (as determined in parallel reactions) is indicated.

8]. In previous studies using endonuclease VII, an analogous enzyme from bacteriophage T4, we also observed the formation of nicked circular and linear dimer DNA [17]. In these experiments, the products were characterised and found to correspond to 'patch' and 'splice' recombinants that were produced by Holliday junction-resolution (Figure 1). The similarity between the reactions catalysed by T4 endonuclease VII and T7 endonuclease I therefore indicate that T7 endonuclease

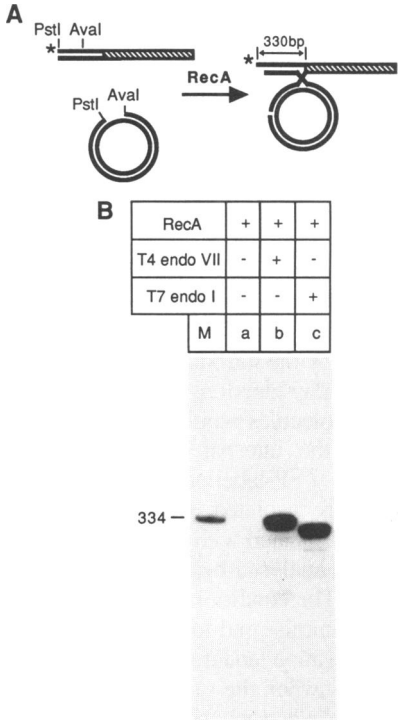

Figure 5. T7 endonuclease I resolves recombination intermediates by cleavage of the Holliday junction. (A) Schematic representation of the substrates (gDNA and linear pCJ10 DNA; left) and the product of strand exchange (right). Heterologous sequences in pCJ10 are indicated by the cross-hatched regions. The assay detects cleavage in the labelled strand as indicated by the asterisk. (B) gDNA (21 μM) was incubated with RecA protein (10 μM) for 5 min in 120 μl. Strand exchange was started by adding 5′-^{32}P end-labelled *PstI*-linearized pCJ10 DNA (final concentration: 15 μM). After 10 min, three 20 μl aliquots were taken and incubation was continued for a further 10 min under different conditions. The reaction products were analysed by 5% denaturing polyacrylamide electrophoresis. Lane a: no additions; lane b: T4 endonuclease VII added (50 units); lane c: T7 endonuclease I added (final concentration 14 ng/ml). Lane M: 334 nucleotide marker (3′-^{32}P end-labelled *PstI-DraI* fragment of ΦX174 DNA).

I can resolve intermediates of strand exchange containing Holliday junctions.

The presence of a unique radiolabel at the *PstI* terminus of the linear DNA substrate (Figure 1) allowed us to compare directly the frequency of resolution in the two possible orientations (cleavage at $a-c$ or $b-d$). The results presented in Figure 2 (lanes j and k) indicate a slight preference for resolution in orientation $b-d$ to form the linear dimer product. At later times during the reaction, we observed the formation of full length linear ΦX174 DNA as a third product (Figure 2, lanes l and m). This may be produced by cutting of the two uninterrupted strands at the Y-junction of the σ-structure, consistent with previous observations of cleavage of Y-junctions by T7 endonuclease I [10, 13].

To demonstrate that the formation of resolution products was dependent on RecA-mediated strand exchange, a series of reactions were performed in which various components were omitted. The omission of RecA protein (Figure 3, lane b), gDNA (lane d) or ATP (lane f) prevented the formation of resolution products. These were only observed in the complete reaction (lane h), thus demonstrating that resolution depends on a functional strand exchange reaction.

While the production of resolution products was dependent

upon the RecA-mediated formation of intermediates containing Holliday junctions, in further experiments we have observed no requirement for the continued presence of RecA protein. In reactions in which RecA protein was removed from the DNA by ADP dissociation, efficient resolution of the junction has also been observed (data not shown). The role of RecA protein is therefore limited to the production of the junction, and it is thought to play no role in the subsequent resolution by T7 endonuclease I.

Studies of the endonuclease activity of T7 endonuclease I have shown that the nuclease produces breaks with 5'-phosphates and 3'-hydroxyl termini [4]. If resolution of recombination intermediates occurs by the introduction of symmetrically related nicks across the Holliday junction, then it would be expected that the product DNA molecules would contain ligatable nicks. To determine whether the internal nicks could be ligated, the resolution products (^{32}P-labelled linear dimer and nicked circular DNA) were isolated by preparative agarose gel electrophoresis and incubated with T4 DNA ligase. The products of ligation were then analysed by native and denaturing agarose gel electrophoresis. The conditions chosen for the ligation were pre-determined to minimise end-to-end ligation, as demonstrated by the absence of a ligation ladder on a native agarose gel (Figure 4, lane b). However, when the same samples were analysed by denaturing agarose gel electrophoresis, we observed the formation of dimer length single-strands (lane e). The ability of DNA ligase to seal the internal nicks produced by T7 endonuclease I indicates that resolution occurs by the introduction of symmetrically related nicks in the two duplexes connected by a Holliday junction.

To show that resolution by T7 endonuclease I occurs at the site of the Holliday junction, we used partially homologous DNA molecules as substrates for strand exchange. Using these substrates, the Holliday junction is driven by RecA protein up to a heterologous block (Figure 5A). Previous experiments have shown that addition of T4 endonuclease VII leads to resolution at the site of the stalled Holliday junction [17]. To demonstrate cleavage at the site of the Holliday junction by T7 endonuclease I, 5'-^{32}P end-labelled pCJ10 DNA linearized with *Pst*I was reacted with gDNA. This linear DNA molecule contains 330 bp of ΦX174 DNA and 4149 bp of pBR322 DNA (which acts as a block to strand exchange). After 5 min of incubation, the mixture was divided into 3 separate reactions. The first reaction was incubated unchanged for another 10 min. To the second reaction, we added T4 endonuclease VII and continued the incubation, and to the third we added T7 endonuclease I. The reaction products were then analysed on a denaturing polyacrylamide gel which allows single base resolution. The T4 endonuclease VII reaction provided a marker for cleavage at the site of the stalled Holliday junction (Figure 5, lane b). In previous studies, this site of clevage was determined and found to be located two nucleotides into the heterologous sequences [17]. In the reaction with T7 endonuclease I, we observed that more than 95% of the cleavage occurred at a single site which was located 3 nucleotides to the 5'-side of the T4 endonuclease VII cleavage site (lane c). This site therefore corresponds to cleavage at the 5'-side of the junction, at a position 1 nucleotide away from the border between homology and heterology. These results indicate that T7 endonuclease I resolves recombination intermediates by nucleolytic cleavage of the Holliday junction.

In summary, we have demonstrated that T7 endonuclease I resolves recombination intermediates containing Holliday junctions to form genetically sensible products. Resolution occurs by the introduction of symmetric nicks at the site of the Holliday junction. The products contain nicks with 5'-phosphates and 3' hydroxyl termini which may subsequently be ligated to restore the integrity of the DNA. Our observations with true Holliday junctions therefore support the view that the role of T7 endonuclease I in phage maturation is to resolve recombination intermediates that arise during the inter-related processes of DNA replication and recombination.

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