

The RuvC protein dimer resolves Holliday junctions by a dual incision mechanism that involves base-specific contacts

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The *Escherichia coli* RuvC protein resolves DNA intermediates produced during genetic recombination. *In vitro*, RuvC binds specifically to Holliday junctions and resolves them by the introduction of nicks into two strands of like polarity. In contrast to junction recognition, which occurs without regard for DNA sequence, resolution occurs preferentially at sequences that exhibit the consensus 5'-A/TTT[↓]G/C-3' (where [↓] indicates the site of incision). Synthetic Holliday junctions containing modified cleavage sequences have been used to investigate the mechanism of cleavage. The results indicate that specific DNA sequences are required for the correct docking of DNA into the two active sites of the RuvC dimer. In addition, using chemically modified oligonucleotides to introduce a hydrolysis-resistant 3'-S-phosphorothiolate linkage at the cleavage site, it was found that, as long as the sequence requirements are fulfilled, the two incisions could be uncoupled from each other. These results indicate that RuvC protein resolves Holliday junctions by a mechanism similar to that exhibited by certain restriction enzymes.
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Introduction

Recombination plays an important role in the generation of genetic diversity during evolution. A key step in this process is the formation and processing of intermediates in which two homologous DNA molecules are linked by a crossover, or Holliday junction. Movement, or branch migration, of the junction results in the formation of heteroduplex DNA, whereas its endonucleolytic cleavage ends the recombination process and leads to separation of the mature recombinants. The latter step is catalysed by a Holliday junction-specific endonuclease.

The Ruv proteins of *Escherichia coli*, RuvA, RuvB and RuvC, promote the branch migration and resolution of Holliday junctions during genetic recombination and the recombinational repair of DNA damage (reviewed by Shinagawa and Iwasaki, 1996; West, 1996). Junction resolvases are ubiquitous in nature and fall into two classes: the first includes *E. coli* RuvC (Dunderdale *et al.*,

1991; Iwasaki *et al.*, 1991), RusA (Sharples *et al.*, 1994; Mahdi *et al.*, 1996) and yeast Cce1 (Kleff *et al.*, 1992; Lockshon *et al.*, 1995; Kupfer and Kemper, 1996; White and Lilley, 1996), and the second includes the bacteriophage resolvases T4 endonuclease VII and T7 endonuclease I (reviewed by West, 1993). Group I enzymes are characterized by a high level of selectivity for Holliday junctions (Dunderdale *et al.*, 1991; Bennett *et al.*, 1993; Benson and West, 1994; Takahagi *et al.*, 1994; Kupfer and Kemper, 1996; White and Lilley, 1996), exhibit sequence specificity at the resolution step (Shah *et al.*, 1994b; Shida *et al.*, 1996; White and Lilley, 1996), and are thought to play specialized roles in recombination and the recombinational repair of DNA damage. In contrast, group II enzymes show a broad substrate spectrum which includes Holliday junctions (Mizuuchi *et al.*, 1982; Duckett *et al.*, 1988; Parsons *et al.*, 1990), mismatches (Solaro *et al.*, 1993), heteroduplex loops (Kleff and Kemper, 1988) and lesions in DNA (Murchie and Lilley, 1993; Bertrand-Burggraf *et al.*, 1994). The phage resolvases exhibit little sequence specificity (Picksley *et al.*, 1990), and act in recombination and the processing of phage DNA before packaging. A mammalian resolvase activity has also been detected, but its substrate and DNA sequence specificities have yet to be determined in detail (Elborough and West, 1990; Hyde *et al.*, 1994). The primary amino acid sequence of RuvC is unrelated to that of Cce1 or the phage resolvases.

Significant progress has been made in understanding the mechanism of action of RuvC, and it serves as a paradigm to which other Holliday junction resolvases can be compared. The key points of its mechanism of action are illustrated in Figure 1. RuvC interacts specifically with a Holliday junction to form a complex in which the junction adopts a 2-fold symmetric open configuration (Bennett and West, 1995b). In the presence of divalent metal ions (Mg²⁺ or Mn²⁺), the junction is resolved to form nicked duplex DNA products that can be repaired by DNA ligase (Dunderdale *et al.*, 1991, 1994; Iwasaki *et al.*, 1991; Bennett *et al.*, 1993; Shah *et al.*, 1994a; Takahagi *et al.*, 1994). The nicks occur in two strands of like polarity, at the point where strands pass from one duplex to the other (Bennett and West, 1995a, 1996; Shida *et al.*, 1996). These strands are unpaired locally and assume the wide angles in the 2-fold symmetric open structure.

Using recombination intermediates, prepared *in vitro* using *E. coli* RecA protein, Shah *et al.* (1994b) demonstrated that RuvC preferentially cleaves Holliday junctions at specific sites. Twenty sites of cleavage were mapped over a region of 1 kb, and contained the consensus 5'-A/TTT[↓]G/C-3' (where [↓] indicates the site of incision). Sequence selectivity was relaxed when Mg²⁺ was replaced by Mn²⁺ (Shah *et al.*, 1994b). Since junction binding

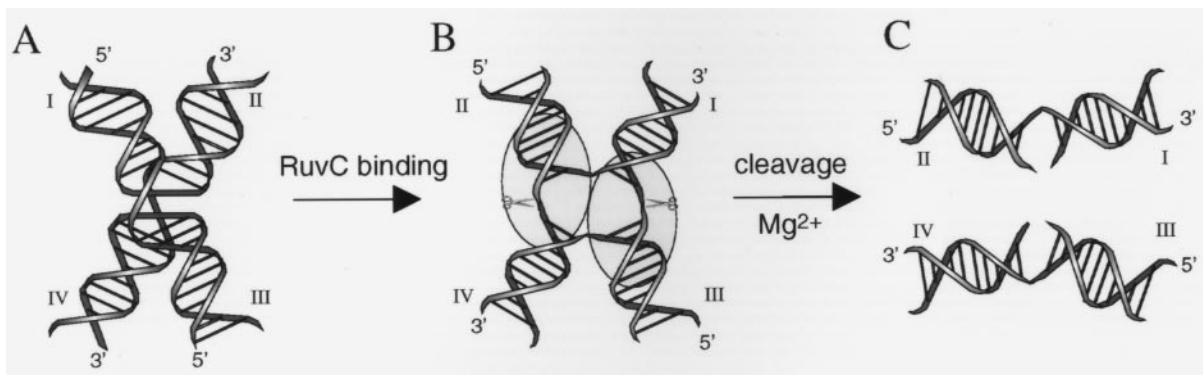


Fig. 1. Schematic model for the resolution of Holliday junctions by RuvC. (A) In the presence of divalent metal ions, protein-free Holliday junctions adopt a 2-fold symmetric stacked-X structure with the arms lying in an anti-parallel configuration (Duckett *et al.*, 1988). (B) Binding by RuvC unfolds the junction into a 2-fold symmetric open complex in which equivalent sections of DNA pass through each active site in the RuvC dimer. (C) In the presence of Mg^{2+} , resolution occurs by nicking of the two strands that constitute the wide angles in the open complex. In this diagram, the RuvC subunits are related by a dyad axis and the sites of incision are indicated by scissors. Adapted from Bennett and West (1996).

occurs in the absence of metal ions and without regard for DNA sequence (Bennett *et al.*, 1993; Takahagi *et al.*, 1994), it is assumed that sequence discrimination takes place at the cleavage step.

RuvC protein is a stable homodimer of 19 kDa subunits (Iwasaki *et al.*, 1991). The crystal structure has been determined at a resolution of 2.5 Å and indicates two subunits related by a dyad axis (Ariyoshi *et al.*, 1994). The catalytic centre of each subunit contains four acidic residues which lie at the bottom of a cleft and are involved in coordination of the metal ion (Saito *et al.*, 1995). Model-building studies suggest that each cleft contains DNA and that the two subunits act in concert to promote the dual incision reaction required for resolution (Ariyoshi *et al.*, 1994). The two clefts lie 30 Å apart, consistent with structural data indicating that the junction lies in an unfolded configuration (Bennett and West, 1995b).

In this paper, we have analysed the nature of the dual incision reaction. Using synthetic Holliday junctions containing modified cleavage sequences, we show that the DNA sequence affects the correct docking of DNA into the two active sites of the RuvC dimer. Proper positioning within each active site is required for resolution, a process that is likely to involve a coordinated conformational change involving both subunits. Additionally, using chemically modified Holliday junctions, we demonstrate that the two incisions can be uncoupled from each other. These results indicate that Holliday junction resolution by the RuvC dimer occurs by a dual incision reaction analogous to that exhibited by certain restriction enzymes.

Results

RuvC binds Holliday junctions as a dimer

Several pieces of evidence suggest that RuvC protein binds its DNA substrate as a dimer. These include: (i) its dimeric form during gel filtration (Iwasaki *et al.*, 1991); (ii) the dyad symmetry found in the RuvC crystal structure (Ariyoshi *et al.*, 1994) and the RuvC–Holliday junction complex (Bennett and West, 1995b); and (iii) the symmetry of the incisions made during resolution (Bennett *et al.*, 1993). However, direct evidence for the binding of a RuvC dimer to a Holliday junction has not been obtained. To achieve this we adopted a strategy similar to that used

for T4 endonuclease VII and Cce1 resolvase (Pöhler *et al.*, 1996; White and Lilley, 1996).

The *ruvC* gene was subcloned into the plasmid pMALc2 to allow expression of RuvC as a fusion protein with the *E. coli* maltose-binding protein (MBP). The 62.3 kDa MBP–RuvC fusion was then purified by chromatography on amylose and MonoQ resins (Figure 2A). Using gel retardation assays, it was found that RuvC and MBP–RuvC bound ^{32}P -labelled synthetic Holliday junctions (Figure 2B), to form distinct protein–DNA complexes that exhibited mobilities consistent with their relative sizes. Quantification of the gel data indicated that, at equimolar concentrations, the MBP–RuvC fusion bound the junction at ~60% the efficiency of RuvC. In the presence of Mg^{2+} , similar resolution efficiencies were observed with both proteins (Figure 2C). MBP alone failed to bind junction DNA (data not shown).

The binding of RuvC, with and without the MBP fusion, provided a means to investigate the subunit composition of the protein–DNA complexes (Figure 2D). The MBP–RuvC fusion contains a Factor Xa protease cleavage site between the MBP and RuvC domains. The protein was therefore incubated with varying concentrations of Factor Xa, to achieve partial cleavage, and the resulting protein mixtures were used in gel retardation assays with junction DNA (Figure 2D, lanes a–d). In addition to the major MBP–RuvC–junction complex, a second retarded band of intermediate mobility was observed at the higher protease concentrations (Figure 2D, lane d). Assuming that MBP–RuvC binds as a homodimer, this intermediate species would correspond to a heterodimer comprising one subunit of RuvC (produced by protease action) and one of MBP–RuvC. To confirm this, equimolar amounts of RuvC and MBP–RuvC were premixed and incubated with junction DNA (Figure 2D, lane g). Three bands were observed by gel electrophoresis, which corresponded to the expected mobility of a [MBP–RuvC]₂ homodimer, a MBP–RuvC/RuvC heterodimer and a [RuvC]₂ homodimer. The results confirm that RuvC protein binds Holliday junctions as a dimer.

Influence of DNA sequence on incision

Previously it was shown that Holliday junction cleavage by RuvC occurs at specific sequences, with the consensus

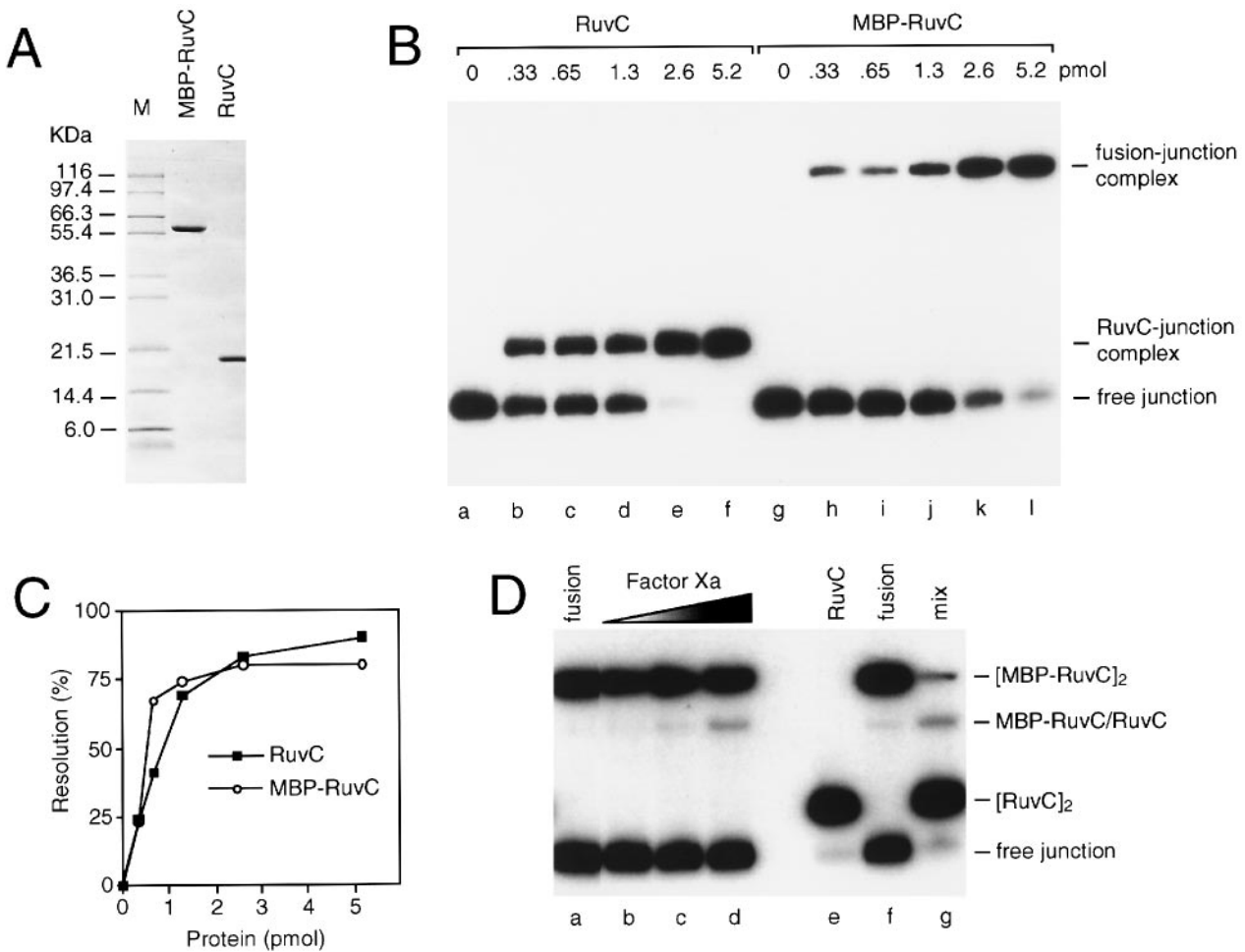


Fig. 2. Analysis of RuvC-Holliday junction complexes using the MBP-RuvC fusion protein. **(A)** SDS-PAGE of purified MBP-RuvC fusion (2 µg) and RuvC (2 µg). Lane M, molecular weight markers. **(B)** Band-shift assay of RuvC-Holliday junction complexes. 5'-³²P-labelled junctions were incubated with RuvC (lanes a-f) or MBP-RuvC (lanes g-l) as described in Materials and methods. Protein-DNA complexes were analysed by electrophoresis through 4% low-ionic strength gels, followed by phosphorimaging. The junction used in this experiment [and those described in (C) and (D)] was the Consensus junction. **(C)** Resolution of Holliday junctions by RuvC and MBP-RuvC. Reactions contained 5'-³²P-labelled junction and the indicated amounts of RuvC and MBP-RuvC in resolution buffer as described. Products were analysed by neutral gel electrophoresis. **(D)** RuvC binds Holliday junctions as a dimer. Lanes a-d: MBP-RuvC fusion protein (5.2 pmol) was incubated with 0, 20, 40 or 80 ng of Factor Xa protease as described, before addition of 5'-³²P-labelled Holliday junction DNA. Incubation was continued for a further 15 min and the complexes were analysed as in (B). Lanes e-g: RuvC (5.2 pmol) and MBP-RuvC (5.2 pmol) were incubated either alone (lanes e and f, respectively) or together (lane g) in binding buffer for 30 min at 4°C, before the addition of ³²P-labelled DNA. Reactions were then incubated for a further 15 min at 4°C and analysed as described for lanes a-d.

defined as 5'-^A/TT^G/C-3'. Single base changes within this sequence severely reduce the efficiency of cleavage. Figure 3 shows that RuvC cleaved a 5'-³²P-labelled Holliday junction (Consensus junction) containing a 5'-ATTG-3' consensus sequence (Figure 3a), whereas a similar junction in which the consensus was altered by a single base change to 5'-GTTG-3' (Mutant junction) was not resolved (Figure 3b).

The requirement for a specific DNA sequence was exploited to determine whether the two RuvC subunits act independently or in a coordinated fashion. A 'Hybrid' junction was constructed which contained the consensus sequence in strand 1 and the mutant sequence in strand 3 (Figure 3c). The sequences of strands 2 and 4 were altered accordingly to maintain full base pairing. Treatment of the Hybrid junction with RuvC resulted in a low level of resolution compared with the Consensus junction. The difference in resolution efficiency was determined in a

series of time-course experiments using constant amounts of RuvC. The maximal rate of cleavage of the Hybrid junction was found to be reduced ~10-fold relative to that of Consensus junction (data not shown). Control experiments indicated that all three junctions were bound comparably by RuvC, as determined by band-shift assays (data not shown). These results confirm that junction recognition and incision are biochemically separable events dependent on the sequence of the junction.

Neutral gel electrophoresis can only detect complete resolution events resulting from the incision of two DNA strands. To determine whether the presence of a mutant site (strand 3) altered the efficiency of incision at a consensus site (strand 1), denaturing PAGE was used to investigate the two incisions independently. Two Hybrid junctions were therefore prepared; one was 5'-³²P-labelled in strand 1 (consensus) and the other in strand 3 (mutant). As controls for the efficiency of cleavage in each strand

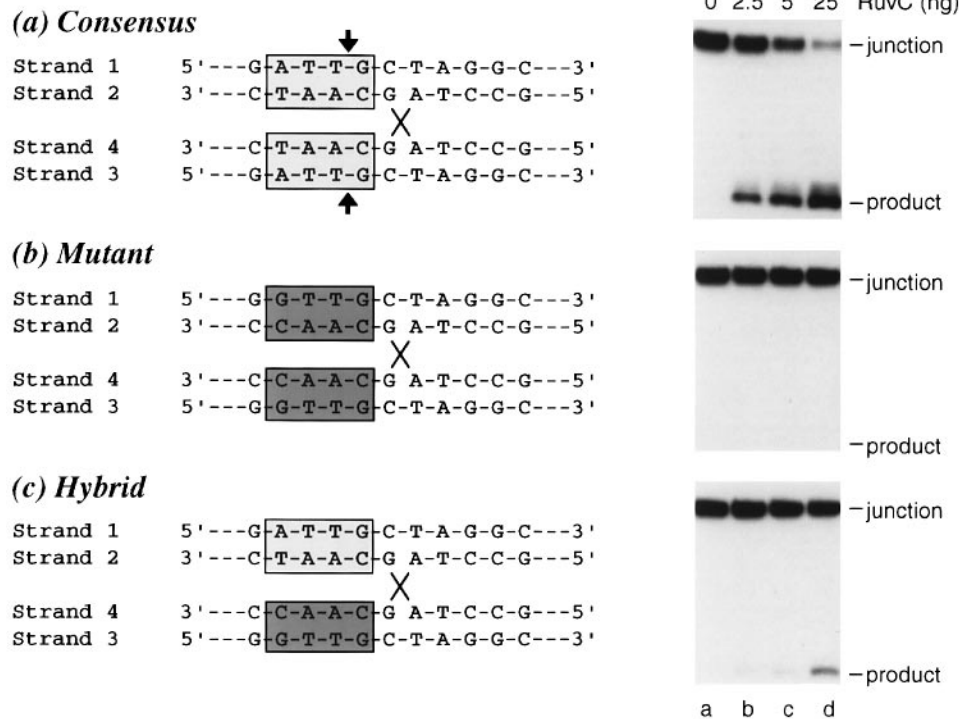


Fig. 3. Effect of DNA sequence on RuvC-mediated Holliday junction resolution. Reactions containing RuvC and 5'-³²P-labelled Consensus (panel a), Mutant (panel b) or Hybrid (panel c) Holliday junctions were incubated in resolution buffer. Products were analysed by 6% neutral PAGE. The 11 bp homologous core of each junction is shown. The consensus (5'-ATTG-3') and mutant (5'-GTTG-3') sequences are highlighted by light and dark shading, respectively. Sites of cleavage are indicated by arrows. For simplicity, the DNA is drawn folded and parallel; within the RuvC-junction complex the DNA will lie in an open configuration as shown in Figure 1B.

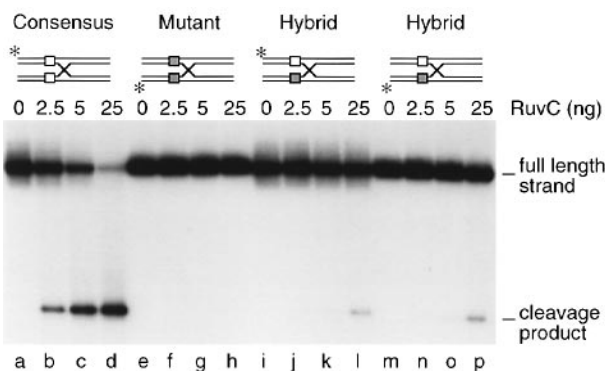


Fig. 4. Denaturing PAGE analysis of cleavage products. Resolution reactions containing 5'-³²P-labelled junction DNA and the indicated amounts of RuvC protein were carried out as described in Materials and methods, and the products were analysed by 12% denaturing PAGE. Each junction was uniquely 5'-³²P-labelled in the strand indicated with an asterisk (*). Lanes a–d, Consensus junction (strand 1 labelled); lanes e–h, Mutant junction (strand 3 labelled); lanes i–l, Hybrid junction (strand 1 labelled); lanes m–p, Hybrid junction (strand 3 labelled). Consensus (5'-ATTG-3') and Mutant (5'-GTTG-3') sequences are indicated by light and dark shading, respectively. For simplicity, the DNA is drawn folded and parallel; within the RuvC-junction complex the DNA will lie in an open configuration as shown in Figure 1B.

of the Hybrid junction, Consensus (strand 1 labelled) and Mutant (strand 3 labelled) junctions were also prepared (Figure 4).

All four junctions were treated with varying amounts of RuvC and the resolution products were analysed by denaturing gel electrophoresis (Figure 4). Analysis of the data with the Hybrid junction revealed that the incisions

in strand 1 (consensus; lanes i–l) and strand 3 (mutant; lanes m–p) were both severely reduced relative to those in the Consensus junction (lanes a–d). The level of cleavage in each strand corresponded to that observed in the non-denaturing PAGE assay (Figure 3c, lane d). These data show that the presence of a non-cleavable mutant site in one arm of DNA reduces cleavage of a consensus site positioned in the other arm. This result suggests that the presence of the mutant sequence within the active site of one RuvC subunit disrupts the correct positioning of the consensus sequence within the other subunit. Alternatively, the two incisions could occur via a concerted mechanism such that a block at one active site inhibits cleavage at the other.

The data presented in Figure 4 (lanes m–p) show additionally that strand 3 of the Hybrid junction was cut more efficiently than the same strand present in the junction containing two mutant arms (lanes e–h). This observation indicates some form of subunit interaction during resolution. In this case, incision of the mutant arm was enhanced by the presence of the consensus sequence within the other RuvC subunit.

Holliday junction resolution occurs independently of DNA homology

The Hybrid junction differs from other junctions studied previously in that the two arms are not perfectly homologous to each other. It was therefore necessary to determine whether homology (i.e. perfect symmetry) was required for efficient resolution by RuvC. To do this, a hybrid junction was constructed using arms that contained two consensus sites of different sequence (5'-ATTG-3'

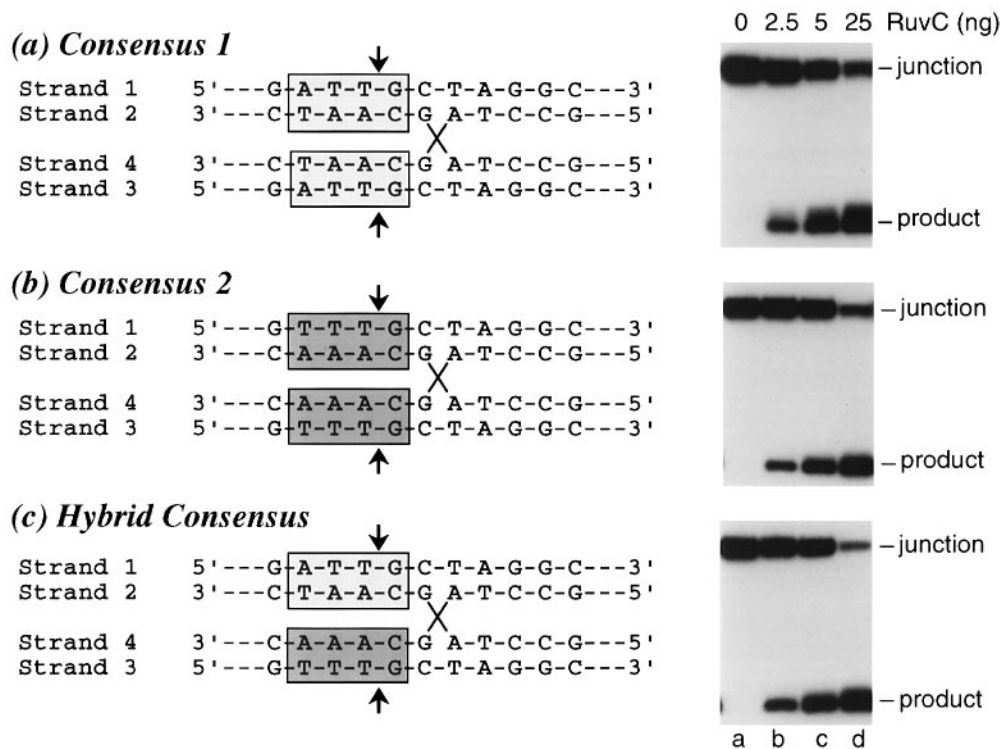


Fig. 5. Resolution of junctions with heterologous cleavage sites. Reactions contained 5'-³²P-labelled Consensus 1 (panel a), Consensus 2 (panel b) or a Hybrid Consensus (panel c) junction and the indicated amounts of RuvC. Incubation was carried out in resolution buffer and the products were analysed by 6% neutral PAGE. The Consensus 1 (5'-ATTG-3') and Consensus 2 (5'-TTTG-3') sequences are highlighted by light and dark shading. Sites of cleavage are indicated by arrows.

and 5'-TTTG-3'). RuvC was found to resolve this junction (Figure 5c) with an efficiency comparable with either of the related Consensus junctions (Figure 5a and b), despite the lack of perfect sequence homology. The results confirm that inefficient resolution of the hybrid junction (Figures 3 and 4) was due to the presence of a non-consensus cleavage site, rather than to a lack of sequence homology. Recent studies presented by Shida *et al.* (1996), using synthetic Holliday junctions containing specific DNA sequences related to the central core of the consensus junction, also indicate that DNA homology is not required for resolution.

Uncoupling the dual incisions using phosphorothiolates

The data presented above demonstrate that efficient dual incision requires the presence of cleavage sites in both arms of the junction. Because T4 endonuclease VII is known to resolve Holliday junctions by a nick and counter-nick mechanism (Pottmeyer and Kemper, 1992), we next investigated whether the two incisions made by RuvC could be uncoupled from each other. To do this, junctions were constructed in which one cleavage site was made resistant to cleavage by incorporating a hydrolysis-resistant 3'-S-phosphorothiolate (SP) linkage at the third (scissile) position of the RuvC cleavage site, i.e. 5'-ATT3'sG-3' (Figure 6).

Junctions were constructed with the SP linkage in strands 1 and 3 (SP/SP junction) or strand 3 only (SP hybrid junction), as shown in Figure 7. As a control, an identical but unmodified junction was used. All three junctions were incubated with RuvC in the presence of

Mg²⁺ and the products were analysed by non-denaturing PAGE (Figure 7A). Junctions with one (lane l) or both (lane h) modified consensus sequences were poorly resolved (3.4 and 7% respectively), compared with the unmodified junction (lane d; 41% resolution). These data show that RuvC is unable to efficiently hydrolyse the modified linkage. Control experiments showed that RuvC bound all three junctions similarly (Figure 7B), demonstrating that the presence of the SP linkage did not interfere with the ability of RuvC to either recognize or bind to its DNA substrate.

To determine whether incision of the SP hybrid junction was blocked in one or both DNA strands, resolution assays were performed on junctions that were 5'-³²P-labelled in either strand 1 (unmodified) or strand 3 (SP-modified). Denaturing PAGE showed that the non-SP strand was cleaved (Figure 8, lanes i-l), albeit at a level that was reduced to one-third of that observed with the unmodified junction (lanes a-d). Little or no cleavage occurred in the SP modified strand of the SP hybrid (lanes m-p). The conclusion that cleavage can be uncoupled, as seen by the different rates of incision at the two sites, was reinforced by time-course experiments using a constant amount of RuvC (Figure 8B).

Discussion

The results presented in this paper indicate that: (i) RuvC binds Holliday junctions as a dimer; (ii) resolution occurs by a dual incision mechanism; (iii) the two incisions can be uncoupled from each other; and (iv) although perfect DNA homology is not required for resolution, efficient

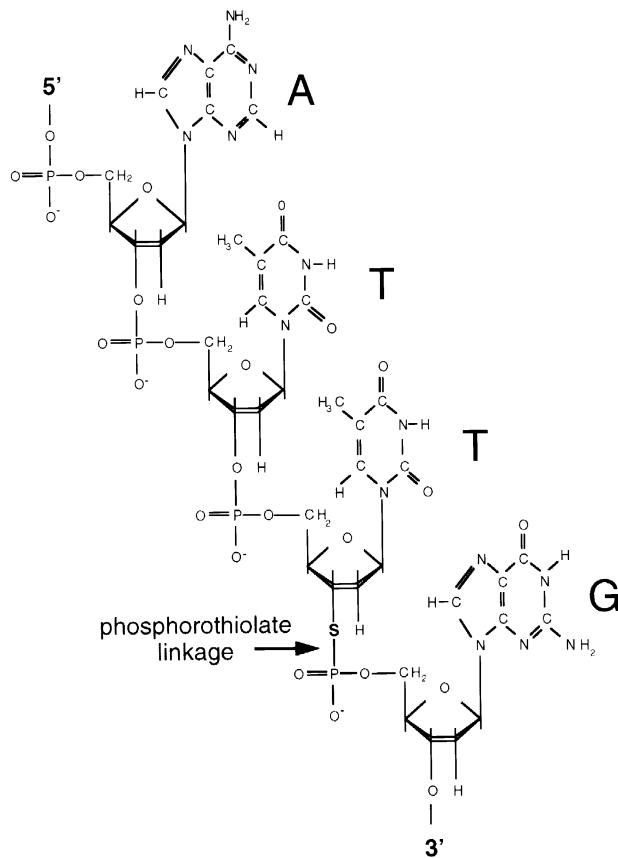


Fig. 6. Incorporation of an SP linkage at the RuvC cleavage site. A 3'-thiothymine link was incorporated at the third (scissile) position (5'-ATT3'sG-3') of the consensus sequence required for cleavage by RuvC. Oligonucleotides (strands 1 and 3) containing these non-cleavable SP linkages were hybridized with unmodified strands 2 and 4 to form synthetic Holliday junctions.

incision requires the presence of two consensus cleavage sequences, one in each subunit of the enzyme.

In vitro, RuvC protein can distinguish 5'-A/T^G/C-3' from all other sequences (Shah *et al.*, 1994b). The initial contacts involved in DNA binding do not require the presence of divalent metal ions, nor are they influenced by DNA sequence. Sequence discrimination must therefore occur at the cleavage step, as deviations from the consensus sequence affect the interface between key amino acid residues in the active site of the protein and the incision site. The presence of a non-cognate sequence may preclude correct alignment of the DNA within the protein such that the scissile phosphodiester bonds fail to come into close proximity with the catalytic residues. This rationale, however, does not address why deviation from the required sequence in one subunit effectively inhibits incision of a cognate sequence present within the other subunit. One way to understand this observation is to invoke a series of conformational changes to both the DNA and the protein that lead to the precise positioning of the catalytic functions (particularly the metal ions) against the scissile bond. Such conformational changes may not be possible unless suitable DNA sequences are present within each subunit.

The studies with non-cleavable (phosphorothiolate-modified) sequences presented here indicate that the two incisions can be uncoupled from each other (Figure 8).

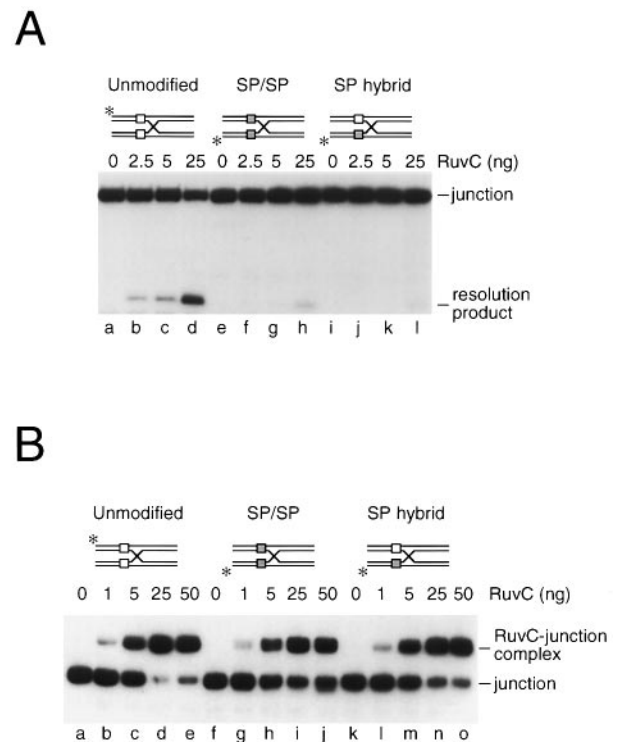


Fig. 7. Interaction of RuvC with Holliday junctions containing phosphorothiolate linkages at the cleavage site. **(A)** Resolution assay. Reactions containing RuvC and 5'-³²P-labelled Consensus (lanes a–d), SP/SP (lanes e–h) or SP hybrid (lanes i–l) junctions were incubated in resolution buffer as described and products were analysed by 6% neutral PAGE. **(B)** Band-shift assay. 5'-³²P-labelled Consensus (lanes a–e), SP/SP (lanes f–j) and SP hybrid junctions (lanes k–o) were incubated with RuvC in binding buffer. Protein–DNA complexes were analysed by 4% low-ionic strength neutral PAGE. Junctions are depicted diagrammatically above the gel. Consensus (5'-ATTG-3') and SP (5'-ATT3'sG-3') sites are highlighted by light and dark shading. ³²P-labelled strands are indicated by asterisks.

This result is important since it provides an explanation for the inhibitory effect imposed by a non-cognate sequence. We suggest that changing one base pair breaks the symmetry of the two recognition sites within the RuvC–junction complex, such that the two subunits of the dimer are unable to develop the same symmetric contacts. When the junction contains a phosphorothiolate-modified site, however, the two subunits are capable of inducing the same symmetric contacts, but in this case one bond is non-cleavable, resulting in the observed differential activation of the two catalytic centres.

The results obtained with RuvC are reminiscent of those observed with several restriction enzymes, and with *EcoRV* in particular. In the presence of Mg²⁺, type II restriction enzymes cleave DNA at their respective recognition sequence with very high specificities (Roberts and Halford, 1993). *EcoRV* is known to discriminate its cognate cleavage sequence during catalysis, rather than substrate binding, in a reaction that is dependent upon the presence of divalent metal ions (Vipond and Halford, 1995). *In vitro* studies have shown that *EcoRV* exhibits a high affinity for Mg²⁺ ions when bound to its cognate sequence, whereas it exhibits a very low affinity for Mg²⁺ when the cognate site is changed by a single base pair (Taylor and Halford, 1989). Co-crystallization of *EcoRV* with cognate and non-cognate DNA fragments, revealed that distortion

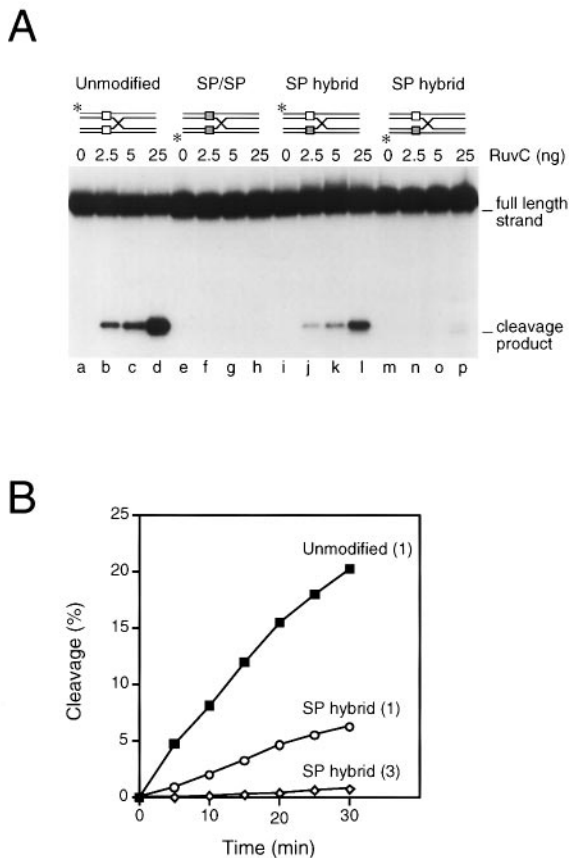


Fig. 8. Denaturing PAGE analysis of junctions containing a phosphorothiolate linkage in one arm. (A) Resolution reactions containing $5'$ - ^{32}P -labelled junction DNA and the indicated amounts of RuvC protein were carried out as described, and the products were analysed by 12% denaturing PAGE. Junctions are shown schematically and were uniquely $5'$ - ^{32}P -labelled as indicated by asterisks (*). Lanes a–d, unmodified Consensus junction (strand 1 labelled); lanes e–h, SP/SP junction (strand 3 labelled); lanes i–l, SP hybrid junction (strand 1 labelled); lanes m–p, SP hybrid junction (strand 3 labelled). Consensus ($5'$ -ATTG- $3'$) and SP ($5'$ -ATT $^{\text{s}}$ G- $3'$) sequences are indicated by light and dark shading. (B) Time-courses of specific strand cleavage of unmodified and phosphorothiolate-containing junctions. The junctions shown in (A) were incubated with RuvC (5 ng) in resolution buffer and at the indicated times samples were taken and analysed on 12% denaturing polyacrylamide gels. Junctions were $5'$ - ^{32}P -labelled in either strand 1 (unmodified) or strand 3 (SP-modified), as indicated in brackets.

takes place in the specific complex, to position the scissile phosphate near two aspartates in the active site (Winkler *et al.*, 1993). Indeed, the distortion not only positions the scissile phosphate, but also creates part of the Mg^{2+} binding site between the phosphate and the two aspartates. Without this distortion (i.e. with non-cognate DNA), the DNA lies distant from the active site.

A similar situation may exist with RuvC protein. In this case, the energy for DNA distortion, which must occur during the resolution step, is likely to be provided by interactions with charged amino acid residues present within a cleft in the active site. X-ray crystallography and mutation analysis has identified four acidic residues, Asp7, Glu66, Asp138 and Asp141, that are required for catalytic activity (Ariyoshi *et al.*, 1994; Saito *et al.*, 1995). The metal ion is bound by Asp7 and Asp141, but lies distant from Asp138, which is located away from the other three acidic residues. These observations suggest that a

conformational change occurs upon binding the cognate sequence, to bring Asp138 to the catalytic centre.

In conclusion, the results described here further define the mechanism by which Holliday junctions are resolved. Most importantly, they show that the dual incision reaction has defined structure and sequence requirements which must be satisfied for efficient nuclease activity. These requirements are biologically important since they serve as a control mechanism that prohibits the nuclease from cutting DNA at structures that could bear a physical resemblance to a Holliday structure. This specificity distinguishes RuvC from more general resolvases such as those encoded by bacteriophages. The results also indicate that RuvC shares mechanistic properties with certain restriction enzymes, suggestive of a common ancestral nuclease precursor. This study therefore extends observations on the crystal structure of RuvC (Ariyoshi *et al.*, 1994) which have identified physical similarities with RNase H1, the RNase H domain of HIV-1 reverse transcriptase, the catalytic domains of HIV-1 integrase and the phage MuA transposase.

Materials and methods

Enzymes

RuvC was purified as described (Dunderdale *et al.*, 1994). Protein concentrations were calculated using the theoretical extinction coefficients for RuvC ($\epsilon_{280\text{nm}} = 6400$) and MBP-RuvC ($\epsilon_{280\text{nm}} = 65\,430$). Proteins were stored at -70°C and diluted as required in 20 mM Tris-HCl pH 8.0, 1 mM EDTA, 1 mM dithiothreitol, 50 mM KCl and 10% (v/v) glycerol.

Oligonucleotide synthesis

All oligonucleotides were synthesized on an Applied Biosystems 380B DNA synthesizer and were gel-purified before use. Oligonucleotides containing an SP linkage were produced using a $3'$ -thiothymidine phosphoramidite as described (Cosstick and Vyle, 1989; Vyle *et al.*, 1992). To prevent chemical oxidation of the SP linkage, modified oligonucleotides and junctions were maintained in buffers containing 5 mM dithiothreitol. The presence and location of the $3'$ -thiothymidine link was confirmed by treatment with silver nitrate (Cosstick and Vyle, 1989).

Synthetic Holliday junctions

Synthetic Holliday junctions were prepared by annealing four oligonucleotides (Parsons *et al.*, 1990). Junctions defined as Consensus and Mutant have been described previously as Junctions A and B, respectively (Shah *et al.*, 1994b). The Consensus junction contains an 11 bp homologous core with a consensus cleavage site ($5'$ -ATT $^{\text{s}}$ G- $3'$) in strands 1 and 3. The Mutant junction is identical except that a non-cleavable site ($5'$ -GTTG- $3'$) replaces the $5'$ -ATTG- $3'$ sequence. Consensus 2 junction contains an alternative consensus sequence ($5'$ -TTT $^{\text{s}}$ G- $3'$) which is also cut efficiently. Hybrid junctions were made by annealing strands 1 and 2 of the Consensus junction with strands 3 and 4 of the Mutant junction. Similarly, the Consensus hybrid junction was made by annealing strands 1 and 2 from the Consensus junction with strands 3 and 4 from the Consensus 2 junction. The SP junctions were identical in sequence to the Consensus junction, but contained $3'$ -thiothymidine at the cleavage site ($5'$ -ATT $^{\text{s}}$ G- $3'$) in strands 1 and 3. SP hybrids were made by annealing Consensus strands 1, 2 and 4 with strand 3 containing a phosphorothiolate linkage.

All substrates were $5'$ - ^{32}P -labelled in strand 1 or 3 before annealing, using T4 polynucleotide kinase (Pharmacia) and [γ - ^{32}P]ATP (Amersham Corporation). Annealed DNA substrates were purified by gel electrophoresis and their concentrations determined by calculation of specific activity using the DE81 filter binding method (Sambrook *et al.*, 1989).

Plasmid pMALc2-RuvC construction

The *ruvC* gene was amplified from plasmid pFB512 by PCR using two primers: $5'$ oligonucleotide, $5'$ -GGCCTGCTAGAATTCAAACGGAG-ACGCGTGATG- $3'$; $3'$ oligonucleotide, $5'$ -GGAGTGGAAAAGCTT-

CAGCCGG-3'. The primers introduce restriction sites at either end of the amplified gene that facilitated subcloning into the *EcoRI* and *HindIII* sites of the pMALc2 expression vector (New England Biolabs) to produce the plasmid pMALc2-RuvC. Cloned sequences were verified by dideoxy sequencing of double-stranded plasmid DNA using the PRISM™ Ready Reaction Dye-Deoxy™ Terminator Cycle Sequencing Kit (Perkin-Elmer Corporation) and an ABI Model 373 automated DNA sequencer.

Purification of MBP-RuvC fusion

RuvC was expressed as a fusion protein with *E. coli* MBP from the plasmid pMALc2-RuvC in *E. coli* strain DH5 α F'1^q (Bethesda Research Laboratories). Two 1-l cultures were grown at 37°C with aeration in LB medium containing 100 μ g/ml ampicillin and 0.2% glucose to an OD₅₉₅ of 0.5–0.6. MBP-RuvC was induced by addition of isopropyl β -D-thiogalactoside (IPTG) to 0.5 mM and growth was continued at 30°C for a further 2.5 h. Cells were harvested by centrifugation and resuspended in 100 ml buffer A (20 mM Tris-HCl pH 7.4, 1 mM EDTA, 1 mM dithiothreitol) supplemented with 200 mM NaCl. Lysozyme was added to 1 mg/ml and the cells were incubated on ice for 30 min, before lysis by sonication (4 \times 45 s bursts on ice). The lysate was cleared by centrifugation (30 000 g, 30 min at 4°C) and applied to a 10 ml amylose column (New England Biolabs) pre-equilibrated with buffer A containing 200 mM NaCl. After extensive washing, the fusion protein was eluted with 2 column volumes of buffer A containing 200 mM NaCl and 10 mM maltose. Fractions (2 ml) were collected and analysed by SDS-PAGE. Peak fractions were pooled, diluted 1:1 with buffer A and applied to a MonoQ HR5/5 FPLC column (Pharmacia) pre-equilibrated with buffer A containing 50 mM NaCl. The column was eluted with a 15 ml gradient of 0.05–1.0 M NaCl in buffer A. The fusion protein eluted between 250 and 290 mM NaCl and appeared as a single band when analysed by SDS-PAGE (Figure 2). Proteins were analysed according to standard procedures using 13% gels and visualized by staining with Coomassie brilliant blue. Molecular weight standards were purchased from Novex. The yield of pure MBP-RuvC was ~4 mg from 2 l of culture. The protein was stored at -70°C after addition of glycerol to 50%.

Factor Xa cleavage of MBP-RuvC

Purified MBP-RuvC fusion protein was incubated with Factor Xa protease (New England Biolabs) for 60 min on ice, in a 20 μ l reaction containing 50 mM Tris-HCl pH 8.0, 1 mM CaCl₂, 1 mM dithiothreitol and 100 μ g/ml bovine serum albumin (BSA). In general, cleavage at this site was inefficient but it was not possible to use harsher proteolysis conditions due to the presence of a cryptic Factor Xa cleavage site within the RuvC domain. Reactions were stopped by addition of 5 mM EDTA. For DNA binding assays, 5'-³²P-labelled junction DNA was added directly to the protein mixture.

Gel retardation assays

Binding reactions (20 μ l) containing 5'-³²P-labelled junction DNA (1 ng) and RuvC (or MBP-RuvC) were incubated on ice for 15 min in binding buffer (50 mM Tris-HCl pH 8.0, 5 mM EDTA, 1 mM dithiothreitol, 100 μ g/ml BSA). Reactions were supplemented with 5 μ l loading buffer [50% glycerol, 0.1% (w/v) bromophenol blue and 0.1% (w/v) xylene cyanol], and equal amounts of radioactivity were loaded onto 4% low-ionic strength polyacrylamide gels (acrylamide:bisacrylamide, 29:1). The gel and buffer system contained 6.7 mM Tris-HCl pH 8.1, 3.3 mM sodium acetate and 2 mM EDTA and the buffer was recirculated (Parsons *et al.*, 1992). Electrophoresis was carried out at 150 V (9 V/cm) for 3 h at 4°C.

Resolution assays

Reaction mixtures (20 μ l) containing 5'-³²P-labelled synthetic junction DNA (1 ng) were incubated with RuvC (or MBP-RuvC) at 37°C for 15 min. Unless otherwise stated, the standard cleavage buffer was 50 mM Tris-HCl pH 8.0, 10 mM MgCl₂, 1 mM dithiothreitol and 100 μ g/ml BSA. Reactions were stopped by addition of EDTA (25 mM) and SDS (0.5%). For neutral gels, 5 μ l of loading buffer was added and equal amounts of radioactivity were analysed by electrophoresis through 6% polyacrylamide gels using TBE buffer (89 mM Tris-borate, 2 mM EDTA). Electrophoresis was carried out at 200 V (12 V/cm) for 2.5 h at room temperature. For denaturing PAGE, samples were ethanol-precipitated and resuspended in 80% formamide (v/v), 0.1% (w/v) bromophenol blue and 0.1% (w/v) xylene cyanol. The DNA was denatured by heating at 95°C for 3 min, and equal amounts of radioactivity were loaded onto 12% polyacrylamide gels containing 7 M urea.

All gels were dried (except for denaturing PAGE) and the ³²P-labelled DNA visualized by autoradiography on Kodak XAR film and/or analysed using a Molecular Dynamics Model 425E phosphorimager with ImageQuant software.

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