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Escherichia coli RuvC protein is an endonuclease that resolves the Holliday structure

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Genetic evidence suggests that the *Escherichia coli ruvC* gene is involved in DNA repair and in the late step of RecE and RecF pathway recombination. To study the biochemical properties of RuvC protein, we overproduced and highly purified the protein. By employing model substrates, we examined the possibility that RuvC protein is an endonuclease that resolves the Holliday structure, an intermediate in genetic recombination in which two double-stranded DNA molecules are linked by single-stranded crossover. RuvC protein cleaves cruciform junctions, which are formed by the extrusion of inverted repeat sequences from a supercoiled plasmid and which are structurally analogous to Holliday junctions, by introducing nicks into strands with the same polarity. The nicked ends are ligated by *E.coli* or T4 DNA ligases. Analysis of the cleavage sites suggests that DNA topology rather than a particular sequence determines the cleavage site. RuvC protein also cleaves Holliday junctions which are formed between gapped circular and linear duplex DNA by the function of RecA protein. However, it does not cleave a synthetic four-way junction that does not possess homology between arms. The active form of RuvC protein, as studied by gel filtration, is a dimer. This is mechanistically suited for an endonuclease involved in swapping DNA strands at the crossover junctions. From these properties of RuvC protein and the phenotypes of the *ruvC* mutants, we infer that RuvC protein is an endonuclease that resolves Holliday structures *in vivo*.

Key words: DNA repair/endonuclease/Holliday junction/recombination/RuvC protein

Introduction

Escherichia coli with *ruv* mutations are sensitive to DNA-damaging agents and are defective in homologous recombination in *recBC sbcA* and *recBC sbcBC* genetic backgrounds, although *ruv* mutations have only a minor effect on recombination in otherwise wild-type backgrounds (Otsuji *et al.*, 1974; Lloyd *et al.*, 1984, 1987). This genetic evidence suggests that the *ruv* locus is involved in recombination by the RecE and RecF pathways (for review, see Smith, 1988). The *ruvA* and *ruvB* genes constitute an SOS-regulated operon and are inducible by DNA-damaging agents (Shurvinton and Lloyd, 1982; Benson *et al.*, 1988; Shinagawa *et al.*, 1988). However, the separate *ruvC* gene, which is located upstream of the *ruvAB* operon, is not regulated by the SOS system (Sharples *et al.*, 1990; Takahagi *et al.*, 1991). The products of the *ruv* genes may be involved in the later stages of homologous recombination initiated by RecA protein (Benson *et al.*, 1991). RecA protein initiates pairing between homologous DNA strands and promotes strand exchange reactions leading to the formation of heteroduplex DNA (for review, see Radding, 1988 and Smith, 1988). An intermediate in the reaction is the so-called Holliday structure in which two homologous duplex molecules are linked by a single-stranded crossover (Holliday, 1964). To complete the recombination process, the Holliday structure has to be resolved by a specific endonuclease that cleaves the crossover junctions (for review, see West, 1989). Therefore, we suspected that the RuvA, RuvB or RuvC protein might be involved in resolving the Holliday structure.

The products of *ruvA* and *ruvB* have been purified and their biochemical properties have been studied (Iwasaki *et al.*, 1989a; Shinagawa *et al.*, 1991; Shiba *et al.*, 1991). RuvB protein is an ATPase whose activity is enhanced by RuvA protein bound to DNA. The RuvA–RuvB protein complex promotes re-adsorption of the cruciform structure containing inverted repeats that extrude from supercoiled DNA. However, the protein complex does not show an endonuclease activity that cleaves Holliday junctions. To examine whether RuvC protein possesses such an endonuclease activity, we overproduced RuvC protein in *E.coli* and purified it to near homogeneity. The purified RuvC protein showed properties expected for an endonuclease that was responsible for resolving the Holliday structure *in vivo*. Connolly *et al.* (1991) found that the endonuclease activity that cleaves Holliday junctions *in vitro* is absent in the extracts of *ruvC* mutants.

Results

Overproduction and purification of RuvC protein

We could overproduce RuvC protein to levels that could be identified in cell lysates by SDS–PAGE by using a T7 expression system (Studier *et al.*, 1991). *E.coli* BL21(DE3)/pLysE harboring pHS641, which contains the *ruvC* gene under the control of the powerful $\phi 10$ promoter, produced an 18 kDa protein to ~30% of the total cellular protein after induction with isopropyl-β-D-thiogalactopyranoside (IPTG) (Figure 1A, lanes 3 and 4). Since this protein was induced in the strain harboring the *ruvC* plasmid, but not in the control strain that harbored the vector plasmid (lanes 1 and 2), and since the size of the protein agreed with that of the *ruvC* gene product identified by the maxicell method (Takahagi *et al.*, 1991), we thought the overproduced protein was RuvC protein.

The purification of RuvC protein was followed by SDS–PAGE (Figure 1B). Although only 20–30% of RuvC protein was recovered from the supernatant of the sonic

lysate of the RuvC-overexpressing cells, we started the purification with this fraction (lane 1). RuvC protein was ~70% pure after chromatography on an S-Sepharose column (lane 2), and an unknown 18 kDa protein which co-purified with RuvC protein in the crude lysates and showed the same mobility by SDS–PAGE was eluted slightly earlier than RuvC protein. This protein was completely separated from the RuvC fractions by subsequent chromatography on a phosphocellulose column. However, the RuvC peak fractions (lane 3) contained low levels of a non-specific DNase activity, whose peak was eluted from the column slightly later than the RuvC peak . The fractions with the DNase activity were eluted substantially earlier than the RuvC fractions from a hydroxylapatite column. The two contaminating proteins of ~30 kDa were separated from the RuvC fractions (lane 4) after affinity chromatography with a denatured DNA column. This purified RuvC protein (lane 5) was 99% pure as measured by densitometric scanning of the stained gel with a Beckman DU-88 spectrophotometer.

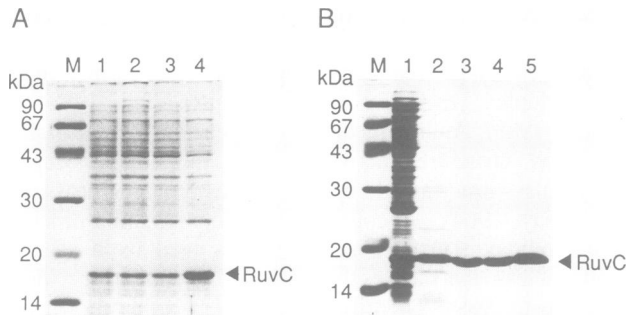

Fig. 1. Overproduction and purification of RuvC protein. Samples were separated by 15% SDS–PAGE and stained with Coomassie brilliant blue. The positions of RuvC protein are indicated by arrows. (**A**) Total cell extracts were analyzed. Lane 1, BL21(DE3)/pLysE/pET8-C. Lane 2, BL21(DE3)/pLysE/pET8-C induced with IPTG. Lane 3, BL21(DE3)/pLysE/pHS641. Lane 4, BL21(DE3)/pLysE/pHS641 induced with IPTG. (**B**) Successive fractions in the purification steps were analyzed. Lane 1, fraction 1 (40 μg). Lane 2, fraction 2 (6.0 μg). Lane 3, fraction 3 (5.0 μg). Lane 4, fraction 4 (4.5 μg). Lane 5, fraction 5 (4.5 μg). Molecular size markers (lane M in both panels) were phosphorylase B (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa) and α-lactalbumin (14 kDa).

The amino acid sequence of the purified RuvC protein was analyzed with an ABI 473A amino acid sequencer. The sequence of 41 amino acids from the amino terminus was AIILGIDPGSRVTGYGVIRQVGRQLSYLGS-GXIRTKVDDLP, which completely agrees with the sequence predicted from the nucleotide sequence of the *ruvC* gene (Takahagi *et al.*, 1991) except that the N-terminal methionine is removed from the purified RuvC protein.

RuvC protein specifically cleaves cruciform DNA in a supercoiled plasmid

As a model substrate for assay of an endonuclease specific for Holliday structures, we first employed a cruciform structure extruded from the supercoiled plasmid pUC4 which contains a 48 bp inverted repeat sequence (Vieira and Messing, 1982; Gellert *et al.*, 1983). As shown schematically in Figure 2, the majority of the pUC4 plasmid prepared from a *recA* strain is a regular supercoiled structure, which is converted to a form that extrudes a cruciform structure by intrastrand base-pairing after heating at 60°C for 2 h. The topology of the junction region of the cruciform is similar to that of the Holliday junction and such cruciforms have been used as a substrate for endonuclease VII of phage T4 (Mizuuchi *et al.*, 1982) and endonuclease I of phage T7 (de Massay *et al.*, 1987), both of which are considered to be responsible for resolving Holliday structures. Formation of the cruciform structure was assayed by *Pst*I digestion (Shiba *et al.*, 1991), since the *Pst*I site is located at the top of the extruded loop which makes the *Pst*I site single-stranded

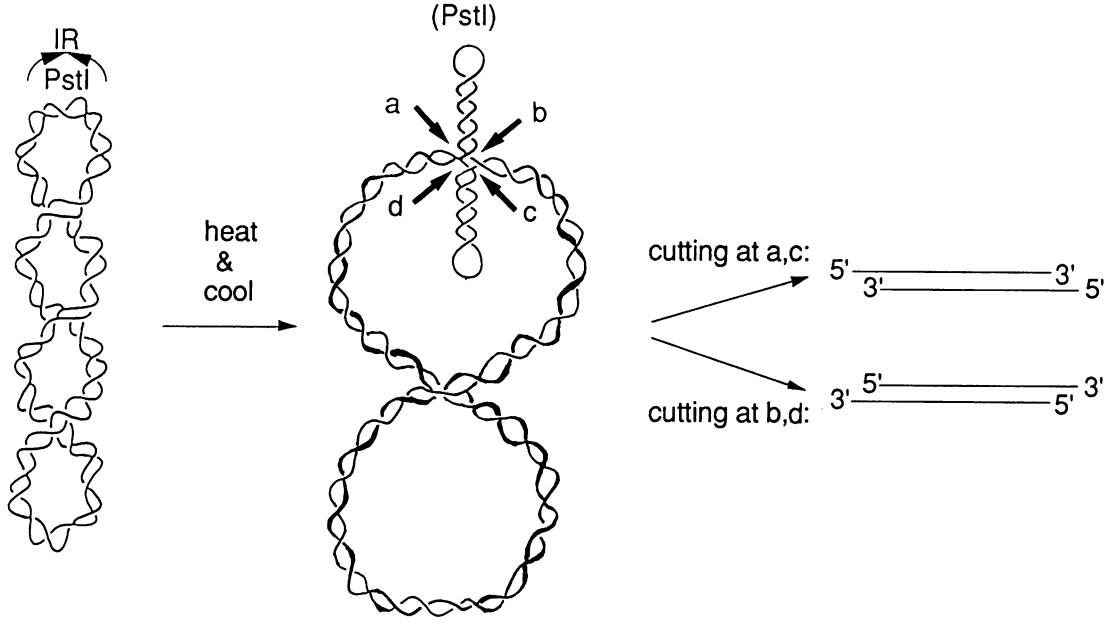

Fig. 2. Schematic diagram of formation and cleavage of cruciform structure in a supercoiled pUC4 DNA. IR indicates the 48 bp inverted repeat sequence.

and consequently resistant to *Pst*I digestion (Figure 3, compare lanes 4 and 5). The majority of pUC4 DNA became cleavable by RuvC protein only after it was heated (compare lanes 6 and 7), indicating that RuvC protein specifically cleaves the cruciform structure. Consistent with this interpretation, RuvC protein did not cleave pUC9 DNA (compare lanes 13 and 14), which is nearly identical to pUC4 except that it does not contain the inverted repeat sequence (Vieira and Messing, 1982) and therefore, does not form a cruciform structure upon heating, regardless of whether or not the pUC9 DNA was pre-heated (compare lanes 11 and 12).

The efficient cleavage of pre-heated pUC4 DNA by RuvC protein required Mg^{2+}, and substitution by other divalent metal ions such as Ca^{2+} and Zn^{2+} allowed little, if any, cleavage (data not shown). RuvC protein is not a single-strand specific endonuclease such as S1 nuclease, since it did not cleave single-stranded M13mp18 phage DNA nor a linear 49mer oligodeoxynucleotide (data not shown).

RuvC protein behaves as a dimer of 19 kDa subunits

We wanted to confirm that the cruciform-specific endonuclease activity is an intrinsic property of RuvC protein and to know whether RuvC protein becomes monomeric or oligomeric in solution. For these purposes, RuvC protein in fraction V was applied on a gel filtration column in buffer R containing 10 mM $MgCl_2$ and 150 mM NaCl and was eluted with the same buffer. By comparing the positions of the elution peaks of marker proteins analyzed in parallel, the molecular mass corresponding to the peak fraction of RuvC protein was calculated to be ~40 kDa, which is about twice the mononer size of RuvC protein (19 kDa) (Figure 4A). The cruciform cleaving activity of each fraction was measured in the same buffer as the one used for gel filtration chromatography. The endonuclease activity coincided with the protein peak (Figure 4B). The activity was also co-purified with RuvC protein peaks on hydroxylapatite and DNA affinity columns (data not shown). These results indicate that the cruciform-specific endonuclease activity is an intrinsic property of RuvC protein, and that the active form of RuvC protein is a dimer of 19 kDa subunits.

Identification of the RuvC protein cleavage site in the cruciform DNA.

First, we wanted to identify the approximate site of cleavage in pUC4 DNA. pUC4 DNA was cleaved with RuvC protein, deproteinized with phenol, and then digested with *Pvu*II or *Nde*I restriction enzymes which cleave pUC4 DNA near the sites flanking the inverted repeat as shown in Figure 5A. The 5′ ends of the cleaved DNAs were labeled with [γ-^{32}P]ATP and T4 DNA kinase, and the products were analyzed by electrophoresis. The sizes of the cleavage products were estimated by comparing their mobilities with those of the fragments obtained by the digestion of pUC4 DNA with restriction enzymes (Figure 5B). The cleavage patterns with RuvC+*Pvu*II and with RuvC+*Nde*I were indistinguishable from those with *Pst*I+*Pvu*II and with *Pst*I+*Nde*I, respectively. The shorter band of the RuvC+*Nde*I cleaved products migrated slightly slower than that of the *Eco*RI+*Nde*I cleaved products. These results show that the cleavage sites by RuvC protein are located very close to the *Eco*RI and *Pst*I cleavage sites, which are located at the junction and the top of the cruciform, respectively.

The cleavage sites were further analyzed by the primer-extension method for DNA sequencing using synthetic primers annealed with the regions just outside the inverted repeat sequences (Figure 6A). We employed *Taq* DNA polymerase to carry out the reaction at 60°C since we suspected that the template DNA would form a hairpin structure around the inverted repeat if RuvC protein nicks the strands at symmetrical positions across the junction at a−c or b−d in Figure 2. The major cleavage products were four nucleotides shorter than the *Eco*RI cleaved products analyzed in parallel (Figure 6B and C), suggesting that RuvC protein cleaves pUC4 DNA mainly between T and C in the *Eco*RI recognition sequence proximal to each primer. Several minor cleavage products with decreasing densities from the main cutting sites were also observed. The strong bands one nucleotide longer than the indicated bands were also observed in the sequencing gel. However, we think those bands were an artefact of the primer extension reaction with *Taq* DNA polymerase, since these extra strong bands were also observed in the primer extension experiments with the *Eco*RI

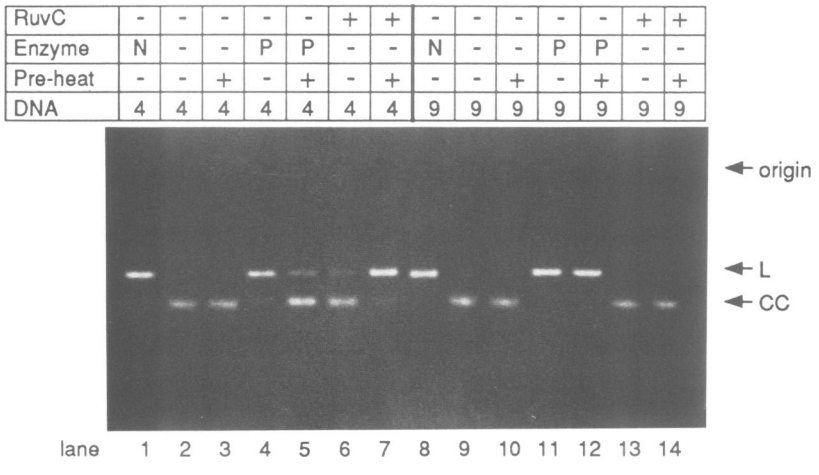

Fig. 3. Cleavage of supercoiled DNA with the cruciform structure by RuvC protein. Cleavage assays were performed as described in Materials and methods. Abbreviations: 4 and 9 indicate pUC4 and pUC9 DNA, respectively; N and P indicate *Nde*I and *Pst*I, respectively; L and CC indicate linear and closed circular plasmid DNA, respectively.

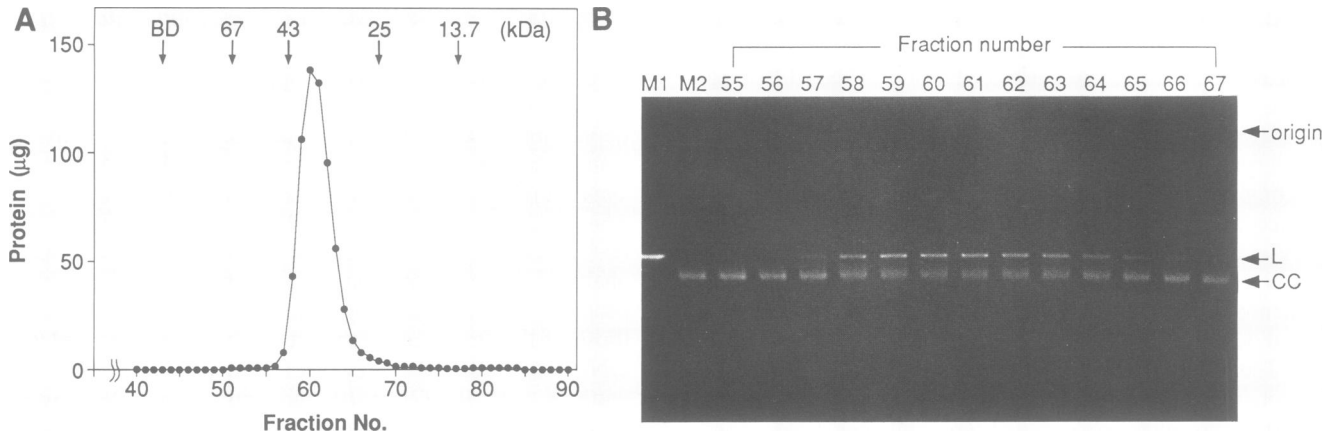

Fig. 4. Gel filtration analysis of RuvC protein. RuvC protein (700 μg) was applied to a Superdex 75 gel filtration column (FPLC) and eluted with buffer R containing 10 mM $MgCl_2$ and 150 mM NaCl at a flow rate of 1 ml/min. The protein concentration and cleavage activity of each fraction were assayed. (**A**) Gel filtration profile of RuvC protein. Molecular size markers were blue dextran (BD), bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotripsinogen A (25 kDa) and ribonuclease A (13.7 kDa). (**B**) Cleavage activity in the fractions. The activity in each fraction was assayed by incubating with pre-heated pUC4 DNA in buffer R containing 10 mM $MgCl_2$ and 150 mM NaCl at 37°C for 5 min. L and CC indicate the positions of linear duplex pUC4 DNA and closed circular pUC4 DNA, respectively.

and *Pst*I cleaved products. Essentially the same result was obtained when RuvC cleavage products were end-labeled, and analyzed by comparing with the Maxam−Gilbert DNA sequencing ladders of pUC4 DNA (data not shown). Therefore, RuvC protein cleaves the cruciform DNA by making single-stranded nicks across the junctions at symmetrical positions within the homologous arms (Figure 6A). This property of RuvC protein is expected for a Holliday junction-specific endonuclease (Mizuuchi *et al.*, 1982, West, 1989).

Ligation of RuvC-protein-cleaved pUC4 DNA.

We wanted to know whether a Holliday junction cleaved by RuvC protein could be sealed by DNA ligase since the last step of recombination *in vivo* is thought to be completed by sealing nicked DNA generated after the resolution. Pre-heated pUC4 DNA was cleaved by RuvC protein, and then treated with DNA ligase. The products were analyzed by agarose gel electrophoresis (Figure 7A). The mobilities of the DNAs treated with *E.coli* DNA ligase (lane 2) or with T4 DNA ligase (lane 3) and those of the linear DNAs cleaved by RuvC protein (lane 1) or *Pst*I (lane 4) were not distinguishable by a standard gel electrophoresis. By alkaline gel electrophoresis, however, the bands of the DNAs treated with DNA ligases migrated much more slowly (lanes 9 and 10) than those of the mononer-size linear DNAs produced by RuvC cleavage (lane 8) or *Pst*I digestion (lane 11) or than those of monomer-size double-stranded circular DNA (the fastest bands in lanes 12 and 13) produced by ligation of the *Pst*1-digested DNA or intact pUC4 DNA (lane 14). The DNA bands with slow mobility which migrate relatively slower in alkaline gel (lane 9 and 10) are dimer-length single-stranded circles (Figure 7B) (Mizuuchi *et al.*, 1982). Both products sealed by *E.coli* DNA ligase and T4 DNA ligase were identical as judged by electrophoresis. Therefore, we conclude that RuvC protein cleaves DNA leaving a 5′-terminal phosphate and a 3′-terminal hydroxyl group, which are ligatable by *E.coli* and T4 DNA ligases. This result also shows that RuvC protein makes nicks at exactly symmetrical positions within the homologous arms and only one nick in each strand of an individual pUC4 DNA molecule. Therefore, these properties of RuvC protein are also suitable for a specific endonuclease that resolves recombination intermediates *in vivo*.

Interaction of RuvC protein with other analogues of the Holliday structure

Although the cruciform extruded from supercoiled DNA is topologically similar to the Holliday junctions, it lacks some essential features of the Holliday structure. Since the RecA-mediated strand exchange reactions between gapped circular and linear duplex DNA would provide more ideal Holliday structures (Connolly and West, 1990; Müller *et al.*, 1990), we examined whether RuvC protein resolves such a Holliday structure. RuvC protein cleaved the intermediate of the strand-excange reaction and generated the products corresponding to the 'splice' and 'patch' recombinant products (data not shown). This was also reported by Connolly *et al.* (1991) with fractionated extracts of the *ruvC*$^+$ strains.

Next we wanted to know whether RuvC protein specifically binds to and cleaves a synthetic four-way junction DNA without homologous arms as T4 endonuclease VII does (Parsons *et al.*, 1990). We prepared the four-way junction and duplex DNAs identical to those used by Parsons *et al.* (1990). We first examined specific binding of RuvC protein to the four-way junction DNA by a gel retardation assay. The mobility of the four-way junction DNA labeled with ^{32}P was slightly reduced at low concentrations of RuvC protein (Figure 8, lanes 8−10). At higher concentrations, the four-way junction DNA did not migrate at all (lanes 11−12). These results suggest that at low concentrations, RuvC protein binds preferentially to the junction, and at higher concentrations, it binds nonspecifically to any parts of the DNA. Consistent with this interpretation, corresponding slower band was not found with the duplex DNA incubated with RuvC protein, and the protein at higher concentrations completely blocked the migration of the duplex DNA in the gel (Figure 8, lanes 1−6). A 20-fold excess of cold duplex DNA did not affect the RuvC binding to the four-way junction (data not shown). However, RuvC protein did not cleave the four-way junction under the conditions that allowed efficient cleavage of the cruciform structure in pUC4 DNA (data not shown).

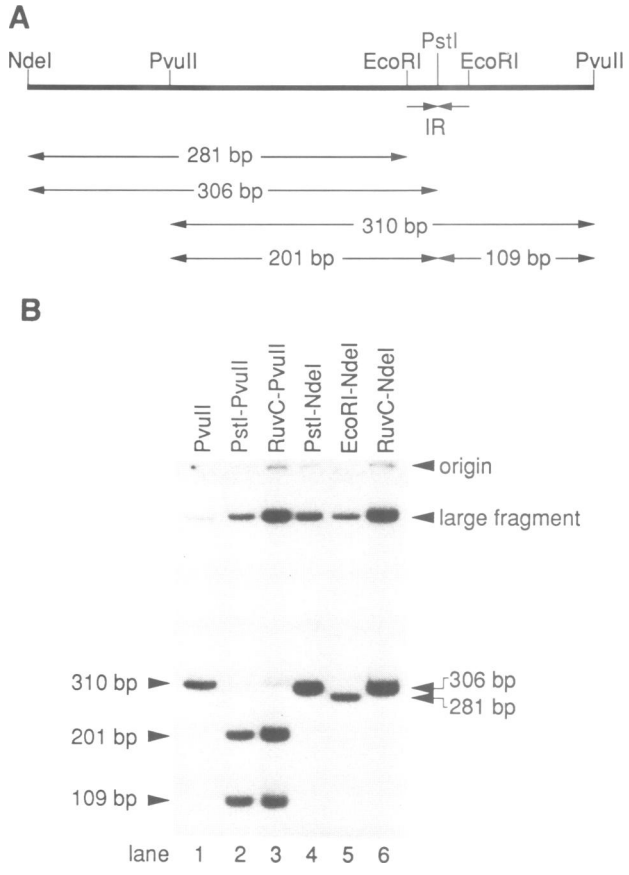

Fig. 5. Mapping of the RuvC cleavage sites in the cruciform of pUC4 DNA. (**A**) Physical map of the region including the inverted repeat sequence (IR) of pUC4 DNA, and the sizes of DNA fragments generated by restriction enzyme digestion. (**B**) Gel elctrophoresis of the fragments of pUC4 DNA generated by digestion with RuvC protein and restriction enzymes. Pre-heated pUC4 DNA was cleaved with RuvC protein and then digested with *Pvu*II (lane 3) or *Nde*I (lane 6). For the size markers, pUC4 DNA was digested with *Pvu*II (lane 1), *Pvu*II+*Pst*I (lane 2), *Nde*I+*Pst*1 (lane 4) or *Nde*I+*Eco*RI (lane 5). These DNA products were labeled at the 5′-end with [γ-^{32}P]ATP and T4 kinase, and analyzed by 6% polyacrylamide gel electrophoresis, followed by autoradiography.

Discussion

RuvC protein cleaves the cruciform structure in supercoiled DNA by nicking two strands with the same polarity at sites symmetrically opposed at the junction in the homologous arms and leaves a 5′-terminal phosphate and a 3′-terminal hydroxyl group. The protein also resolves the *in vitro* recombination intermediate formed by RecA protein in two directions that produce the 'splice' and 'patch' recombinants. These are some of the expected properties for the Holliday junction-specific endonuclease (West, 1989) and taking the phenotypes of *ruvC* mutants into consideration, we conclude that RuvC protein has a role in resolving recombination intermediates *in vivo*.

Connolly and West (1990) identified an endonuclease activity in the fractionated extract of a *recBC sbcBC* strain of *E.coli* that resolves Holliday junctions formed by RecA protein. The elution profile of the activity from a phosphocellulose column is similar to that of RuvC protein, and the properties of the endonuclease so far analyzed are indistinguishable from those of RuvC protein that we analyzed. Therefore, the endonuclease they identified in the *E.coli* extract is very probably RuvC protein, and in fact, the resolvase activity is absent in the fractionated extracts of *ruvC* mutants and is enhanced in the extract of cells carrying a multicopy plasmid with the *ruvC*$^+$ gene (Connolly *et al.*, 1991).

Although the present work as well as the results of Connolly *et al.* (1991) support that the *ruvC* gene encodes a Holliday junction resolvase, the *ruvC* mutants in a wild type background are nearly as proficient in recombination as the wild type strain. The recombination defective phenotype of *ruvC* mutants is seen only in *recBC sbcA* or *recBC sbcBC* genetic backgrounds. This suggests that there is one or more endonucleases that resolve Holliday junctions, or there is a recombination pathway that does not involve the formation and resolution of Holliday junctions.

Some of the properties of the RuvC endonuclease revealed here are similar to those of T4 endonuclease VII and T7 endonuclease IV, which are well characterized resolvases of the Holliday structure (West, 1989). However, their amino acid sequences do not show statistically significant similarity to that of RuvC protein (Takahagi *et al.*, 1991). Although RuvC protein can bind preferentially to the synthetic four-way junction DNA that has no homology between the arms, it cannot cleave the junction which is a known substrate of the phage-encoded endonucleases (Dickie *et al.*, 1987; Parsons *et al.*, 1990). However, the *E.coli* resolvase activity identified by Connolly *et al.* (1991), which requires the *ruvC* function, cleaves a synthetic four-way junction containing a central core of homology sequence. Therefore, the endonuclease activity of RuvC protein requires a central core of homology in the junction while the phage-encoded resolvases do not. Therefore, RuvC endonuclease appears to have more stringent requirement for substrate specificity than the phage endonucleases and to be 'true resolvase'.

Analysis of RuvC protein by gel filtration suggests that the active form of RuvC protein is a dimer (Figure 4). However, Connolly *et al.* (1991) reported, based on a gel filtration experiment, that the resolvase activity in the extract of the *ruvC*$^+$ strains was eluted at the position corresponding to ~20 kDa protein in gel filtration chromatography, which corresponded to the monomer size of the protein. This discrepancy probably results from the difference in ionic strengths of the buffers for the gel filtration experiments. Since the resolvase activity of the RuvC protein is severely inhibited by NaCl when its concentration is >200 mM (data not shown), RuvC subunits appear to form dimers at physiological ionic strngths (~150 mM) and to be dissociated into monomers at a higher ionic strength (500 mM), which was employed by Connolly *et al.* (1991) for the gel filtration experiment.

That the RuvC protein makes nicks in only one pair of strands at the cruciform junctions in pUC4 DNA may be due to the configuration of the cruciform which may only allow one pair of strands with the same polarity at the crossover accessible to RuvC endonuclease attack. The dimeric nature of RuvC protein may mechanistically ensure that two strands with the same polarity are nicked at the equivalent phosphodiester bonds; this property of RuvC protein might be important for completing genetic recombination without causing insertion and deletion mutations.

We interpret the results shown in Figure 6 to indicate that RuvC protein makes nicks in DNA at the cruciform junction points, and the relative frequencies of the nicked sites by RuvC protein reflect the relative stabilities of the

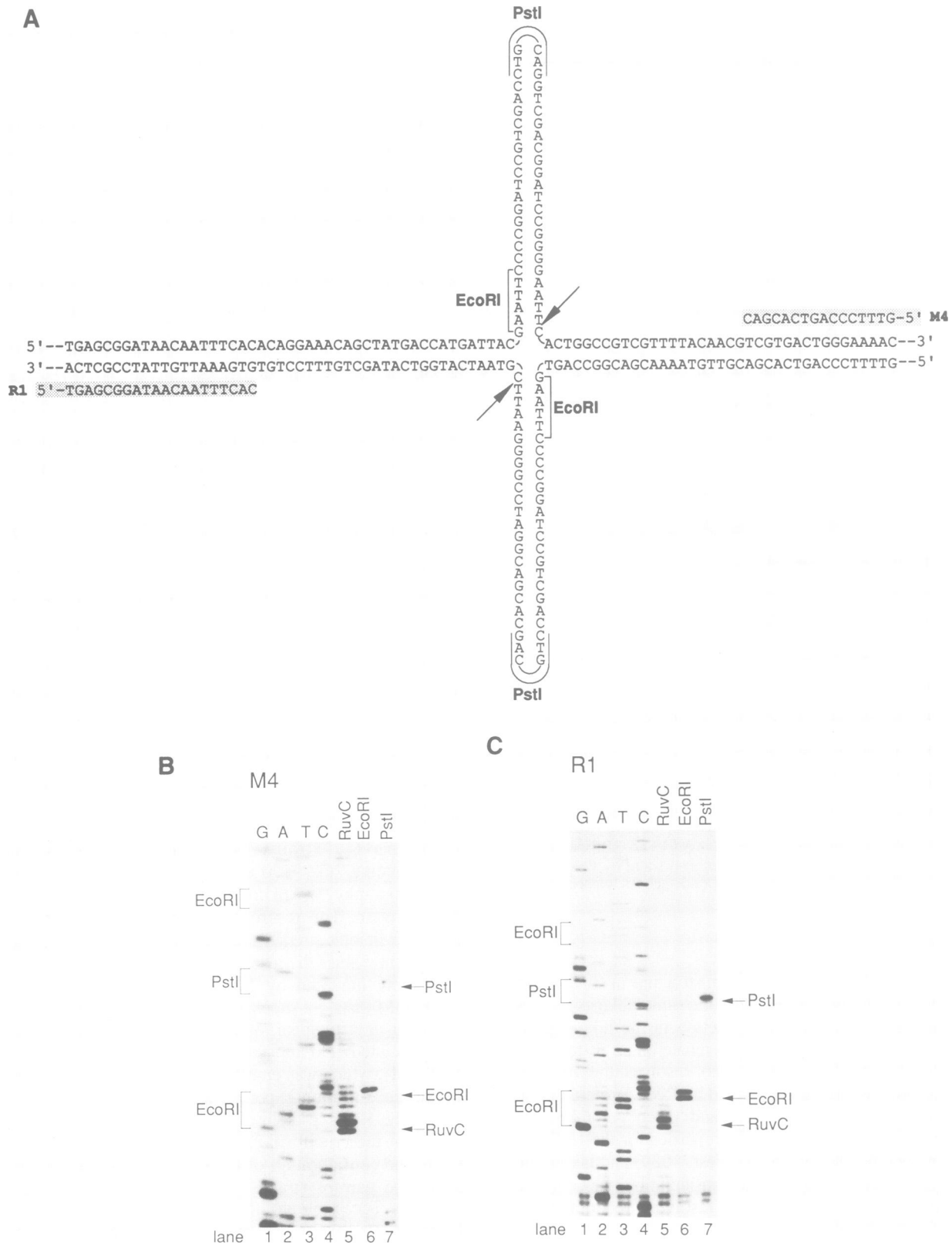

Fig. 6. Determination of DNA sequences of the RuvC cleavage sites in the cruciform of pUC4 DNA. (**A**) The DNA sequence around the inverted repeat of pUC4 DNA and the sequences of the R1 and M4 primers are shown. The arrows indicate the major cleavage sites by RuvC protein. (**B**) and (**C**) Primer extension experiments with the M4 (B) and R1 (C) primers. In both panels; lanes 1–4, the sequencing ladders, lanes 5, 6 and 7, the products of primer extension reactions with pUC4 DNA cleaved by RuvC, *Eco*RI and *Pst*I, respectively. The arrows in the right indicate the positions of the major products which are compensated for the extra insertion by *Taq* DNA polymerase. In the left, the bracketed areas indicate the positions of the ladders for the recognition sequences of the *Eco*RI or *Pst*I restriction enzyme.

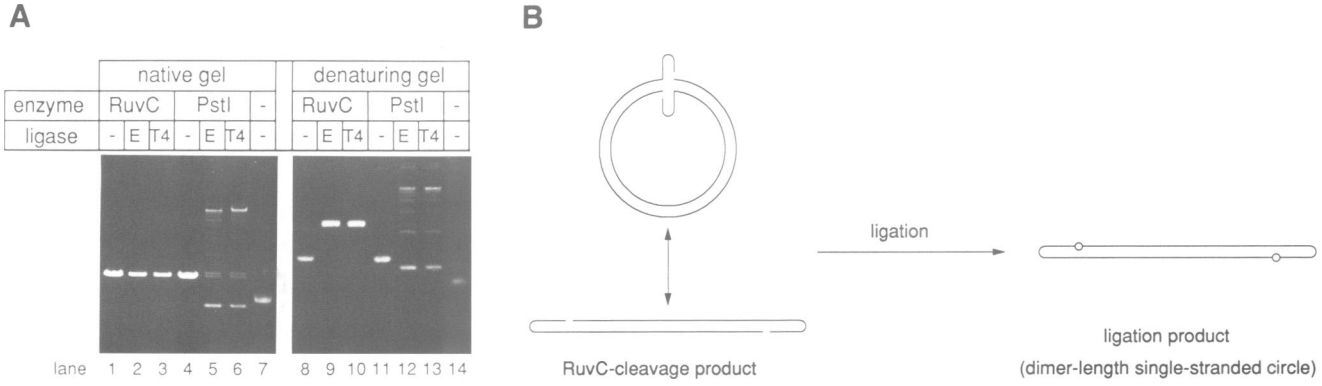

Fig. 7. Ligation of RuvC-cleaved pUC4 DNA. Pre-heated pUC4 DNA was cleaved with RuvC protein, deproteinized with phenol, and ligated with *E.coli* DNA ligase in the presence of NAD or with T4 DNA ligase in the presence of ATP. For the control, *Pst*I-linearized pUC4 DNA was ligated similarly. The ligation products were analyzed by gel electrophoresis. (**A**) Lanes 1–7, analysis by 0.8% standard agarose gel electrophoresis. Lanes 8–14, analysis by 0.8% alkaline denaturing agarose gel electrophoresis. Lanes 1 and 8, RuvC-linearized pUC4 DNA. Lanes 2, 3, 9 and 10, RuvC-linearized pUC4 DNA was treated with *E.coli* DNA ligase (lanes 2 and 9) or with T4 DNA ligase (lanes 3 and 10). Lanes 4 and 11, *Pst*I-linearized pUC4 DNA. Lanes 5, 6, 12 and 13, *Pst*I-linearized pUC4 DNA was treated with *E.coli* DNA ligase (lanes 5 and 12) or with T4 DNA ligase (lanes 6 and 13). Lanes 7 and 14, intact closed circular pUC4 DNA. E and T4 indicate *E.coli* DNA ligase with NAD and T4 DNA ligase with ATP, respectively. In lanes 5, 6, 12 and 13, the upper bands are oligomers produced by ligation. (**B**) Schematic diagram of the ligation product of RuvC-cleaved pUC4 DNA.

corresponding cruciform structures which reflect the linking numbers of the superhelicity of individual plasmids. Therefore, DNA topology rather than specific sequence appears to be a major determinant of specific interaction of RuvC protein in the endonuclease reaction.

The *ruvC* mutants as well as the *ruvA* and *ruvB* mutants are sensitive to DNA damaging agents and are recombination deficient in *recBC sbcA* or *recBC sbcBC* backgrounds, suggesting that RuvA, RuvB and RuvC proteins are involved in the RecE and RecF recombination pathways (Lloyd *et al.*, 1984, 1987). The phenotypes of these different *ruv* mutants are indistinguishable, indicating functional cooperativity among them (Lloyd *et al.*, 1984, 1987; Iwasaki *et al.*, 1989b; Sharples *et al.*, 1990). The *ruvB* mutants are defective in processing the RecA protein-mediated recombination intermediates formed between F′ factor and the recipient chromosome (Benson *et al.*, 1991), and the RuvA–RuvB protein complex renatures the cruciform structure in pUC4 DNA, which is analogous to branch migration in recombination (Shiba *et al.*, 1991). Therefore, we infer that the RuvA–RuvB protein complex facilitates migration of the Holliday structure formed by RecA protein, and RuvC protein resolves the Holliday structure *in vivo*. Müller *et al.* (1990) demonstrated that RecA protein alone mediates formation of the Holliday structure between a gapped and linear duplex DNA and completes the strand exchange reaction by itself *in vitro*. However, the RuvA–RuvB complex might also interact specifically with the Holliday junction and facilitate the migration of the junction. Although the protein complex might transiently compete with RuvC protein for interaction with the Holliday junction, the RuvA–RuvB complex might participate in the resolution reaction by changing the topology of the Holliday junction to be more accessible to the RuvC endonuclease. The *ruv* mutants are almost normal in conjugative recombination in wild type backgrounds, but they are sensitive to DNA damaging agents. In wild type cells, genetic recombination might be efficiently accomplished by the RecBCD pathway, but for efficient recombination repair,

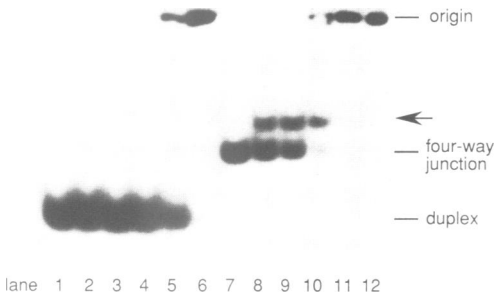

Fig. 8. Binding of a synthetic four-way junction by RuvC protein. Various concentrations of RuvC protein were incubated with ^{32}P-labeled synthetic four-way junction (0.2 μM, lanes 1–6) or ^{32}P-labeled duplex DNA (0.1 μM, lanes 7–12). Complexes between DNAs and RuvC protein were analyzed by polyacrylamide gel electrophoresis and radiolabeled DNA was detected by autoradiography. Lanes 1 and 7, no protein. Lanes 2 and 8, 0.07 μM of RuvC protein. Lanes 3 and 9, 0.14 μM of RuvC protein. Lanes 4 and 10, 0.35 μM of RuvC protein. Lanes 5 and 11, 0.7 μM of RuvC protein. Lanes 6 and 12, 1.4 μM of RuvC protein. The arrow indicates the retarded four-way junction due to the formation of complex with RuvC protein.

the function of RuvABC proteins would be required. DNA damaging treatments may produce recombinogenic DNA structures such as gaps in DNA, which may be processed by RecA protein into interwound DNA molecules. The RuvA–RuvB protein complex might be required particularly for processing these repair intermediates by facilitating branch migration along the damaged DNA, and RuvC protein might complete repair process by resolving the interwound molecules.

Materials and methods

Reagents

S-Sepharose Fast Flow, denatured DNA–cellulose and Superdex 75 16/60 were from Pharmacia LKB; P11–phosphocellulose was from Whatman; hydroxylapatite columns (KB-0012) from Koken, Tokyo; and Bradford reagent from Bio-Rad. [γ-^{32}P]ATP was from Amersham.

Construction of a plasmid for RuvC protein overproduction

To utilize the powerful $\phi 10$ promoter and the efficient ribosome binding site of a T7 system in pET8-C vector (Studier *et al.*, 1991) for *ruvC* expression, the 6 base sequence, TGATGG, around the initiation codon of *ruvC* (Takahagi *et al.*, 1991) carried on M13hs271 was converted to the *Nco*I cleavage sequence, CCATGG, by oligonucleotide-directed mutagenesis using an Amersham mutagenesis kit. This gave M13hs641. The 1-kb *Nco*I–*Eco*RI fragment (in which the *Eco*RI end was converted to a blunt end) of M13hs641, which contains the *ruvC* coding region, was inserted between the *Nco*I site and the *Bam*HI-converted blunt site of pET8-C, giving pHS641. In pHS641, the *ruvC* coding region was placed downstream of the $\phi 10$ promoter and the ribosome binding site of pET8-C at an optimal position for expression.

Purification of RuvC protein

E.coli BL21(DE3) carrying pLysE (Studier *et al.*, 1991) was employed as a host for the RuvC overproducing plasmid pHS641. The strain was grown in 3 l of LB medium (plus chloramphenicol at 10 μg/ml and ampicillin at 50 μg/ml) to an OD_{600} of 0.5 and overproduction of RuvC protein was induced by adding IPTG to the culture to a final concentration of 1 mM. After incubation for a further 4 h, the cells were harvested by centrifugation.

All subsequent purification steps were carried out at 4°C. The cells were suspended in 40 ml of buffer R [50 mM Tris–HCl (pH 7.5), 1 mM EDTA, 7 mM 2-mercaptoethanol, 10% glycerol] and were disrupted by sonication. The clear supernatant (fraction 1) obtained by centrifugation at 20 000 r.p.m. for 45 min in a Beckman JA20 rotor was directly applied onto an S-Sepharose Fast Flow column (bed volume 40 ml) which had been equilibrated in buffer R. The column was washed with three column volumes of buffer R and developed with 500 ml of a linear gradient of 0–500 mM NaCl in buffer R. The fractions eluting at about 300 mM NaCl contained RuvC protein; these fractions were pooled and dialyzed against buffer R containing 100 mM NaCl. The dialyzed solution (fraction 2) was applied onto a phosphocellulose column (bed volume 20 ml). This column was washed with two column volume of buffer R containing 100 mM NaCl and developed with 200 ml of a linear gradient of 100–600 mM NaCl in buffer R. The fractions eluting at ~400 mM NaCl contained RuvC protein (fraction 3); these fractions were pooled and dialyzed against phosphate buffer containing 50 mM potassium phosphate (pH 7.4), 200 mM NaCl, 7 mM 2-mercaptoethanol and 10% glycerol. The dialyzed solution was diluted by addition of the same volume of 10 % glycerol solution containing 7 mM 2-mercaptoethanol and adjusted to pH 6.8 with phosphoric acid. The solution was applied onto a hydroxylapatite column. This column was washed with one column volume of buffer P [100 mM potassium phosphate (pH 6.8), 7 mM 2-mercaptoethanol, 100 mM NaCl, 10% glycerol], and developed with seven column volumes of a linear gradient of 100–300 mM $KHPO_4$ in buffer P. RuvC protein was eluted at ~260 mM $KHPO_4$. The fractions were immediately dialyzed against buffer R containing 200 mM NaCl (fraction 4). The dialyzed solution was diluted with the same volume of buffer R containing 20 mM $MgCl_2$ and applied onto a single-stranded DNA cellulose column pre-equilibrated with buffer R containing 100 mM NaCl and 10 mM $MgCl_2$, and developed with six column volume of a linear gradient of 100–500 mM NaCl in buffer R containing 10 mM $MgCl_2$. RuvC protein was eluted at about 300 mM NaCl, pooled and dialyzed against storage buffer [20 mM Tris–HCl (pH7.5), 200 mM KCl, 0.1 mM EDTA, 2 mM 2-mercaptoethanol, 50% glycerol]. The protein was stored at −20°C.

The protein concentration was determined by the Bradford method with a Bio-Rad protein assay kit.

Other proteins

Restriction endonucleases and DNA modification enzymes were obtained from Takara Shuzo, Kyoto. RecA protein was generously given by Dr T.Horii of this institute.

Cruciform cleavage assay

The cruciform structure in pUC4 DNA was induced in buffer containing 10 mM Tris–HCl (pH 8.0), 1 mM EDTA and 200 mM NaCl by heating at 60°C for 2 h. Standard reaction mixtures (20 μl) contained pre-heated pUC DNA (300 pmol) and RuvC protein (2 pmol) in a buffer containing 20 mM HEPES-KOH (pH 7.6), 50 mM K-glutamate, 8 mM Mg-acetate, 2 mM dithiothreitol (DTT) and 10% glycerol. The mixtures were incubated at 37°C for 20 min and the reactions were terminated by the addition of 0.25 vol stop solution containing 20 mM Tris–HCl (pH 7.5), 20 mM EDTA, 1% SDS, 0.25% bromophenol blue, 0.25% xylene cyanol and 30% glycerol. Samples were analyzed by electrophoresis in a 0.8% agarose gel in a buffer containing 40 mM Tris–acetate (pH 8.0) and 1 mM EDTA. The DNA in the gel was stained with ethidium bromide, illuminated with UV light, and photographed.

Determination of cleavage sites by DNA sequencing

The cleavage sites of pUC4 DNA by RuvC protein were analyzed by the primer extension method using two synthetic primers; M4 primer for 5′-GTTTTCCCAGTCACGAC and R1 primer for 5′-TGAGCGGATAACAATTTCAC (Figure 6A). The 5′ ends of the primers were labeled with [γ-^{32}P]ATP by T4 phage DNA kinase and each labeled primer was extended by incubation with *Taq* DNA polymerase in the presence of four dNTPs (100 μM each) at 60°C for 10 min. The products of the reactions were analyzed by standard DNA sequencing gels (Sambrook *et al.*, 1989). For comparison, the cleavage sites of pUC4 DNA digested with either *Eco*RI or *Pst*I were analyzed by the same method in parallel with the RuvC-cleaved DNA. The sequences of both strands of pUC4 DNA were also analyzed by the dideoxy chain termination method with *Taq* DNA polymerase using the same primers. These reaction products were applied to the same sequencing gels as markers.

Preparation of synthetic four-way junction DNA

Four-way junction DNA was made by annealing four 49mer oligodeoxynucleotides (oligos 1–4) whose sequences have been described by Parsons *et al.* (1990). Duplex DNA was made by annealing oligo 1 with oligo 5 described by Parsons *et al.* (1990). All oligodeoxynucleotides were synthesized by the ABI 380B DNA synthesizer and purified by HPLC. Synthetic four-way junction and duplex DNA were prepared, 5′-labeled with [γ-^{32}P]ATP and T4 kinase, and purified by electrophoresis as described by Parsons *et al.* (1990).

Protein-DNA binding assay

Reaction mixtures (15 μl) containing various amounts of RuvC protein and ^{32}P-labeled four-way junction DNA (0.2 μM) or duplex DNA (0.1 μM) in binding buffer [20 mM HEPES-KOH, (pH 7.6), 50 mM K-glutamate, 2 mM DTT and 10% glycerol] were incubated at 30°C for 15 min. To each sample, 5 μl of loading buffer [20 mM Tris–HCl (pH 7.5), 20% glycerol and 0.1% bromophenol blue] was added and the mixtures were immediately applied onto a 4% polyacrylamide gel. Electrophoresis was carried out at room temperature at 100 V for 1 h in a buffer containing 40 mM Tris–acetate (pH 8.0) and 1 mM EDTA. Gels were dried and DNA was visualized by autoradiography.

Other procedures

Alkaline denaturing gel electrophoresis and standard recombinant DNA techniques were as described by Sambrook *et al.* (1989).

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References

Benson,F. E., Illing,G. T., Sharples,G. J. and Lloyd,R. G. (1988) *Nucleic Acids Res.*, **16**, 1541–1549.

Benson,F., Collier,S. and Lloyd,R. G. (1991) *Mol. Gen. Genet.*, **225**, 266–272.

Connolly,B. and West,S. C. (1990) *Proc. Natl. Acad. Sci. USA.*, **87**, 8476–8480.

Connolly,B., Parsons,C. A., Benson,F. E., Dunderdale,H. J., Sharples,G. J., Lloyd,R. G. and West,S. C. (1991) *Proc. Natl. Acad. Sci. USA.*, **88**, 6063–6067.

de Massy,B., Weisberg,R. A. and Studier,F. W. (1987) *J. Mol. Biol.*, **193**, 359–376.

Dickie,P., McFadden,G. and Morgan,A. R. (1987) *J. Biol. Chem.*, **262**, 14826–14836.

Gellert,M., O'Dea,M. H. and Mizuuchi,K. (1983) *Proc. Natl. Acad. Sci USA.*, **80**, 5545–5549.

Holliday,R. (1964) *Genet. Res.*, **5**, 282–304.

Iwasaki,H., Shiba,T., Makino,K., Nakata,A. and Shinagawa,H. (1989a) *J. Bacteriol.*, **171**, 5276–5280.

Iwasaki,H., Shiba,T., Nakata,A. and Shinagawa,H. (1989b) *Mol. Gen. Genet.*, **219**, 328–331.

Lloyd,R. G., Benson,F. E. and Shurvinton,C. E. (1984) *Mol. Gen. Genet.*, **194**, 303–309.

Lloyd,R. G., Buckman,C. and Benson,F. E. (1987) *J. Gen. Microbiol.*, **133**, 2531–2538.
Mizuuchi,K., Kemper,B., Hays,J. and Weisberg,R. A.(1982) *Cell*, **29**, 357–365.
Müller,B., Jones,C., Kemper,B. and West,S. C. (1990) *Cell*, **60**, 329–336.
Otsuji,N., Iyehara,H. and Hideshima,Y. (1974) *J. Bacteriol.*, **117**, 337–344.
Parsons,C. A., Kemper,B. and West,S. C. (1990) *J. Biol. Chem.*, **265**, 9285–9289.
Radding,C. M. (1988) In Kucherlapati,R. and Smith,G. R. (eds), *Genetic Recombination*. American Society for Microbiology, Washington DC, pp. 193–229.
Sambrook,J., Fritsch,E. F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. Second Edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Sharples,G. J.,Benson,F. E., Illing,G. T. and Lloyd,R. G. (1990) *Mol. Gen. Genet.*, **221**, 219–226.
Shiba,T., Iwasaki,H., Nakata,A. and Shinagawa,H. (1991) *Proc. Natl. Acad. Sci. USA.*, **88**, 8445–8449.
Shinagawa,H., Makino,K., Amemura,M., Kimura,S., Iwasaki,H. and Nakata,A. (1988) *J. Bacteriol.*, **170**, 4322–4329.
Shinagawa,H., Shiba,T., Iwasaki,H., Makino,K., Takahagi,M. and Nakata,A. (1991) *Biochimie*, **73**, 505–507.
Shurvinton,C. E. and Lloyd,R. G. (1982) *Mol. Gen. Genet.*, **185**, 352–355.
Smith,G. R. (1988) *Microbiol. Rev.*, **52**, 1–28.
Studier,F. W., Rosenberg,A. H., Dunn,J. J. and Dubendorff,J. W. (1991) *Methods Enzymol.*, **185**, 60–89.
Takahagi,M., Iwasaki,H., Nakata,A. and Shinagawa,H. (1991) *J. Bacteriol.*, **173**, 5747–5753.
Vieira,J., and Messing,J. (1982) *Gene*, **19**, 259–268.
West,S. C. (1989) In Eckstein,F. and Lilley,D. M. J. (eds), *Nucleic Acids and Molecular Biology*. Springer-Verlag, Berlin, vol. 3, pp.45–55.

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