Functional interactions between the Holliday junction resolvase and the branch migration motor of *Escherichia coli*

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Homologous recombination generates genetic diversity and provides an important cellular pathway for the repair of double-stranded DNA breaks. Two key steps in this process are the branch migration of Holliday junctions followed by their resolution into mature recombination products. In *E. coli*, branch migration is catalysed by the RuvB protein, a hexameric DNA helicase that is loaded onto the junction by RuvA, whereas resolution is promoted by the RuvC endonuclease. Here we provide direct evidence for functional interactions between RuvB and RuvC that link these biochemically distinct processes. Using Holliday junctions, RuvB was found to stabilize the binding of RuvC to a junction and to stimulate its resolvase activity. Conversely, RuvC facilitated interactions between RuvB and the junction such that RuvBC complexes catalysed branch migration. The observed synergy between RuvB and RuvC provides new insight into the structure and function of a RuvABC complex that is capable of facilitating branch migration and resolution of Holliday junctions via a concerted enzymatic mechanism.

**Keywords:** DNA helicase/DNA repair/protein–DNA interactions/recombination/resolution

**Introduction**

Recombination is required for the generation of genetic diversity and for the repair of post-replication gaps and double-stranded DNA breaks caused by radiation and chemical damage. Studies of recombination proteins from *Escherichia coli* have helped to define each stage of the recombination process and to elucidate the molecular interactions that occur during DNA pairing, strand exchange and the resolution of recombination intermediates (Kowalczykowski *et al.*, 1994; Lloyd and Low, 1996). Recent studies indicate that the key steps of recombination have been conserved during evolution (Ogawa *et al.*, 1993a,b; Story *et al.*, 1993; Hyde *et al.*, 1994; Sung, 1994; Baumann *et al.*, 1996) and the bacterial proteins now provide a paradigm with which eukaryotic enzymes can be compared.

During the late stages of recombination in *E. coli*, intermediates made by RecA-mediated homologous pairing and strand exchange are processed into mature recombinants by the RuvA, RuvB and RuvC proteins (Shinagawa and Iwasaki, 1996; West, 1997). The motor of branch migration, RuvB, is an asymmetric ring-shaped hexameric helicase that binds DNA with low affinity. However, RuvB is specifically loaded onto Holliday junctions by RuvA, forming a tripartite structure in which RuvA is flanked by two RuvB rings. Biochemical and structural studies show that tetramers of RuvA bind the junction and impose a 4-fold symmetric square-planar structure on the DNA (Parsons *et al.*, 1995; Rafferty *et al.*, 1996). The two hexameric rings of RuvB are oppositely oriented through contacts with RuvA and drive branch migration by passing the DNA through the central cavity of each ring. Holliday junction resolution is mediated by the RuvC endonuclease, a homodimer that introduces symmetrically related nicks in two strands of like polarity (Dundurdaie *et al.*, 1991; Iwasaki *et al.*, 1991; Bennett *et al.*, 1993; Ariyoshi *et al.*, 1994). The nuclease binds Holliday junctions in a structure-specific way, yet it promotes efficient cleavage at the 3'-side of a thymidine residue within the consensus sequence 5'-G/T/T↓G/C-3' (Shah *et al.*, 1994b; Shida *et al.*, 1996). These results indicate that base-specific contacts are required for optimal resolution (Shah *et al.*, 1997).

Genetic and biochemical studies suggest that RuvAB-mediated branch migration and RuvC-mediated Holliday junction resolution may be linked, possibly by formation of a RuvABC complex. Firstly, mutations in *ruvA*, *ruvB* or *ruvC* result in similar genetic phenotypes, i.e. increased UV-sensitivity and a modest defect in recombination (Lloyd *et al.*, 1984; Sharples *et al.*, 1990). In each case, the defect can be corrected by over-expression of RusA, a cryptic Holliday junction resolvase of *E. coli* (Mandal *et al.*, 1993), indicating that all *ruv* mutants are resolution-defective. Secondly, monoclonal antibodies directed against RuvA, RuvB or RuvC inhibit RuvC-mediated Holliday junction resolution in *in vitro* reconstitution experiments (Eggleston *et al.*, 1997). Thirdly, RuvA and RuvC are able to bind simultaneously to a synthetic Holliday junction to form a RuvAC–junction complex (Whitby *et al.*, 1996). Finally, direct interactions between RuvB and RuvC are indicated by protein cross-linking studies carried out in the absence of DNA (Eggleston *et al.*, 1997).

In this work, we present the first biochemical evidence for functional interactions between the RuvB branch migration motor and RuvC resolvase, indicating a close link between the processes of branch migration and Holliday junction resolution. The studies provide strong support for models that invoke the coupling of these two processes *in vivo* through the formation of a RuvABC Holliday junction processing complex.
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Results

Resolution of junctions containing different lengths of homology

Using chemically modified junctions it was shown previously that RuvC-mediated Holliday junction resolution occurred with greatest efficiency when the incision site (in the sequence 5′-ATTG-3′) was located at, or within, 1 bp of the cross-over position (Bennett and West, 1996). However, since Holliday junctions will often be located away from sites of preferred cleavage, binding by RuvC will frequently result in the formation of non-productive RuvC–junction complexes. We therefore set out to define factors that could influence junction location and thereby stimulate the efficiency of resolution.

To do this, the actions of RuvC on two related synthetic Holliday junctions were investigated. The junctions, designated X4 and X12, contain a RuvC consensus cleavage sequence (5′-ATTG-3′) located within a common strand (strand 1) and differ only in the length of homology (4 or 12 bp) that they contain (Figure 1). Because of the short length of homology in junction X4, the consensus sequence and the cross-over are coincident. This is less likely to be the case with X12.

When incubated with RuvC, junction X4 was cleaved efficiently into nicked duplex DNA products, as detected by neutral PAGE (Figure 2, top panel). In contrast, junction X12 was poorly resolved and two product bands were observed. These arise from cleavage at two alternative resolution sites within the 12 bp homologous core, as revealed by denaturing PAGE (Figure 2, bottom panel). Analysis of the cleavage reactions over a 45 min time course revealed that resolution of X4 was maximal within 5 min. In contrast, cleavage of X12 was still continuing at 45 min (data not shown). The difference in cleavage efficiency between X4 and X12 was not due to different binding efficiencies. Both junctions were bound similarly by RuvC (Figure 3A), with a relatively strong affinity (\(K_d = 1 \times 10^{-10} \text{ M}\)) compared with linear duplex DNA (\(K_d = 5 \times 10^{-8} \text{ M}\)) (Figure 3B). These results indicate that the greater length of homology in X12 affects cleavage efficiency, presumably due to the formation of RuvC–junction complexes at non-cleavable sequences.

RuvB facilitates RuvC-mediated Holliday junction resolution

The need to locate the cross-over to RuvC’s preferred cleavage sequence may underlie the requirement for RuvA and RuvB activity during Holliday junction resolution in vivo (Mandal et al., 1993). We therefore investigated whether branch migration could stimulate the resolution of junction X12 by RuvC.

Elevated temperatures are known to increase the rate of spontaneous branch migration (Panyutin and Hsieh, 1994). When RuvC-mediated resolution reactions were carried out at 55°C, the extent of cleavage of X12 was
but is incapable of binding DNA (Mezard et al., 1996) and presumably also to bind DNA. Indeed, not shown), which corresponds to the minimal amount of ATP required to stimulate junction resolution by RuvC was 250 nM (data not shown). The results obtained with X12 indicate that spontaneous branch migration or the presence of RuvB protein can elevate the efficiency of Holliday junction resolution by RuvC. Presumably, cleavage of X4 is efficient due to the location of the cleavage sequence at the cross-over, thus circumventing the need for branch migration or the stabilization of RuvC–junction complexes by RuvB.

Relationship between cleavage efficiency and cross-over location

The finding that resolution of junction X12 can be stimulated by branch migration (Figure 4A) indicates that the cross-over and cleavage sites may be non-coincident with this junction. To test this hypothesis, the locations of the cross-over in RuvC–X4 and RuvC–X12 complexes were determined using hydroxyl radicals. Although this chemical probe is generally used to randomly cleave DNA for footprinting purposes, it was previously observed that the Fe(II) ion used to generate the radicals is bound by the active site (R.J. Bennett, N.F.P. Hagan, R.G. Lloyd and S.C. West, unpublished observations). The technique therefore provides an indication of the cross-over position in the RuvC–junction complex.

When bound by RuvC, X4 showed a defined hypersensitivity to hydroxyl radicals between the G and C residues in the 5′-ATTGC-3′ sequence (Figure 5, lane c). In control studies, using a junction with a fixed cross-over point, the site of hydroxyl radical hypersensitivity was mapped 2 bp from the 3′-side of the cross-over (Bennett et al., 1993 and data not shown). The cross-over in X4 is therefore located at the centre of the 4 bp 5′-ATTG-3′ homologous core, as shown in Figure 1. In contrast, when the cross-over position was mapped in the X12–RuvC complex (Figure 5, lane h), we observed that it was located 3 or 4 bp away from the 5′-ATTG-3′ sequence. The major position is indicated in Figure 1.

Enhanced resolution activity may be explained partly by the observation that RuvB stabilised the association of RuvC with junction X12, as determined by band-shift analysis (Figure 4D, lanes g–i). Stabilization required the DNA binding properties of RuvB, since RuvB<sup>D113N</sup> did not elicit a similar effect (lane j). However, RuvB also stabilised the binding of RuvC to X4 (Figure 4D, lanes b–d) without stimulating the resolution of this junction (Figure 4C, lanes a–c), even at low RuvC concentrations (data not shown). The results obtained with X12 indicate that spontaneous branch migration or the presence of RuvB protein can elevate the efficiency of Holliday junction resolution by RuvC. Presumably, cleavage of X4 is efficient due to the location of the cleavage sequence at the cross-over, thus circumventing the need for branch migration or the stabilization of RuvC–junction complexes by RuvB.

A RuvBC complex can promote branch migration

Since RuvB stabilizes the binding of Holliday junctions by RuvC and stimulates resolution of the junction, we investigated whether interactions between RuvB and RuvC could also lead to branch migration. Surprisingly, a large
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Fig. 4. Stimulation of RuvC-mediated junction resolution by spontaneous branch migration or RuvB. (A) Effect of increased reaction temperature on junction cleavage by RuvC. 5′-32P-labelled junctions X4 and X12 were incubated in the presence or absence of RuvC at the indicated temperatures for 30 min in cleavage buffer. Resolution products were deproteinised and analysed by 8% neutral PAGE followed by autoradiography.

(B) Resolution of junctions X4 and X12 by RuvC at 37°C and at 55°C. Junction cleavage was analysed as in (A) and quantified. □: X4, 37°C; ■: X4, 55°C; ○: X12, 37°C; ●: X12, 55°C. (C) Effect of RuvB on RuvC-mediated Holliday junction resolution. 5′-32P-labelled junctions X4 and X12 were preincubated for 5 min at 37°C with the indicated amounts of RuvB or RuvB\(^{D113N}\), followed by the addition of RuvC and further incubation for 30 min at 37°C. DNA products were deproteinised and analysed by 8% neutral PAGE. In this experiment Mg\(^{2+}\) and ATP\(\gamma S\) were present at 15 mM and 1 mM, respectively. In lanes d and i, ATP\(\gamma S\) was omitted. (D) Effect of RuvB on RuvC–junction complex formation. RuvC was incubated with 5′-32P-labelled junctions X4 or X12 after preincubation of the DNA with the indicated amounts of RuvB or RuvB\(^{D113N}\). Protein–DNA complexes were analysed by 6% neutral PAGE followed by autoradiography.

increase in the formation of branch migration products was observed when RuvC was added to reactions containing RuvB (Figure 6). Similar results were obtained with X4 (lanes a–e) and X12 (lanes f–j). Reactions were carried out in the presence of Ca\(^{2+}\) to block resolution (Shah et al., 1994a) and a mixture of ATP and ATP\(\gamma S\) (4 ATP : 1 ATP\(\gamma S\)). With ATP alone, RuvBC-mediated branch migration occurred at a reduced level (lane l), whereas in the presence of ATP\(\gamma S\) few branch migration products could be seen (lane k). In the absence of RuvB, RuvC was unable to promote branch migration (data not shown). These results indicate that RuvC, like RuvA, can target RuvB rings to a Holliday junction. However, RuvBC-mediated branch migration was found to occur with a much lower efficiency than that observed with RuvAB. Indeed, ~100-fold more RuvC protein than RuvA was required to effect a similar level of RuvB-mediated branch migration (data not shown). Moreover, the presence of RuvA reduces the stoichiometric requirement for RuvB ~50-fold (Müller et al., 1993; Mitchell and West, 1996), whereas RuvC does not (data not shown).

Discussion

By analysing the ability of RuvC to resolve two synthetic Holliday junctions, both containing a RuvC consensus sequence (5′-ATT↓G-3′), we observed that the cleavage efficiency was low when the sequence was located several base pairs away from the cross-over point. In contrast, when the consensus cleavage sequence was coincident with the cross-over, efficient Holliday junction resolution was observed. These results have important biological implications for recombination since they indicate that Holliday junction resolution in vivo will be inefficient unless cellular factors co-operate to bring the cross-over to sites where RuvC-mediated resolution can occur.

Stimulation of RuvC-mediated Holliday junction resolution by RuvB

RuvC binds Holliday junctions irrespective of the DNA sequence (Bennett et al., 1993, and Figure 3), resulting in a local disruption of base pairing in the region of the cross-over (Bennett and West, 1995). Using synthetic Holliday junctions containing different homologous cores, we found that sequence effects contribute to favour the formation of RuvC–junction complexes in which the cross-over lies at preferred sites (Figure 5). Rather surprisingly, these sites can lie away from the sequences at which RuvC effects cleavage.

The experiments described in this work show that the branch migration motor RuvB stimulates junction resolution by RuvC protein. One possibility is that RuvB helps localize the cross-over to the cleavage site by promoting limited branch migration. Alternatively, the interaction of RuvB with the RuvC–junction complex leads to its stabilization and indirectly facilitates cleavage.
Fig. 5. Determination of the cross-over and cleavage sites in junctions X4 and X12. To determine the cross-over position, preformed RuvC–junction complexes were treated with hydroxyl radicals (•OH) as described in Materials and methods. Reaction products were analysed by denaturing PAGE (lanes b, c, g and h), alongside junction cleavage products (lanes d and i). A + G and C > T sequence ladders permit a comparison of cross-over position relative to the sites of RuvC cleavage. The amounts of label in each lane were adjusted to yield bands of equal intensity. Owing to the nature of the DNA modification, it was necessary to make a 1 nt allowance when comparing DNA fragments produced by hydrogen radical treatment and chemical cleavage. The 4 and 12 bp regions of homology are indicated by a dashed line.

Although we cannot distinguish between these possibilities, denaturing PAGE of the X12 cleavage products, obtained after incubation with RuvB and RuvC, indicated the presence of novel cleavage sites outside of the region of homology (data not shown). Since spontaneous branch migration is unable to go through mismatches or short regions of heterology (Panyutin and Hsieh, 1993) and elevated temperature only increased cleavage within the homologous core (data not shown), these sites are indicative of resolution occurring as RuvB promotes movement of the cross-over. In support of this notion, inclusion of RuvA led to a further increase in branch migration and cleavage in the heterologous arms (data not shown).

Stimulation of RuvB-mediated branch migration by RuvC

In previous work, it was shown that high concentrations of RuvB alone can branch migrate recombination intermediates made by RecA-mediated strand exchange (Tsaneva et al., 1992b). However, branch migration of synthetic Holliday junctions by RuvB was not observed previously (Parsons et al., 1992). These reactions require the presence of RuvA, which targets RuvB to the junction (Parsons and West, 1993) and induces structural changes to the DNA (Parsons et al., 1995; Rafferty et al., 1996). The observation that RuvC can act in a similar way, to facilitate the interaction of RuvB with a synthetic junction and promote branch migration, was unexpected and most likely results from the stabilization of RuvB on the junction through additional contacts with RuvC (Eggleston et al., 1997). RuvBC-mediated branch migration was, however, less efficient than comparable RuvAB-catalysed reactions, leading us to propose that RuvA is the primary protein for loading RuvB onto the Holliday junction. Nonetheless, the results indicate that interactions between RuvB, RuvC and the Holliday junction can result in the formation of an active RuvBC–junction complex capable of limited branch migration and resolution.

Pairwise associations of RuvAB, RuvBC and RuvAC indicate the formation of a RuvABC branch migration–resolution complex

In the present work, we describe functional interactions between RuvB and RuvC on a synthetic Holliday junction, leading to branch migration and enhanced resolution efficiency. These studies confirm and extend previous observations of physical interactions between the two proteins in solution (Eggleston et al., 1997). Based on the structure of RuvC (Ariyoshi et al., 1994) and properties of the RuvC–junction complex (Bennett and West, 1995), we suggest that hexameric rings of RuvB interact with RuvC to form a RuvBC complex (Figure 7B) reminiscent of that formed by RuvAB (Figure 7A).

Using band-shift assays, Lloyd and co-workers recently demonstrated the formation of a RuvA–RuvC–junction complex (Whitby et al., 1996). The mobility of this complex is consistent with a structure in which a RuvC dimer binds a junction that lies on the surface of a RuvA tetramer (Figure 7C). Since both RuvA and RuvC interact with RuvB hexamers, these studies reinforce the proposal that Holliday junction branch migration and resolution may be co-ordinated via formation of a RuvABC complex (Whitby et al., 1996; Eggleston et al., 1997). The complex may adopt a structure similar to that shown in Figure 7C, with a RuvA tetramer and RuvC dimer binding to opposite faces of the Holliday junction and with RuvB rings binding to two arms. RuvAB-mediated branch migration would drive DNA through the RuvABC complex, providing a mechanism by which RuvC can scan the DNA for preferred cleavage sequences. Such a proposal is consistent with genetic data indicating that ruvA, ruvB and ruvC mutants...
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Fig. 7. Model indicating the interactions between the RuvA, RuvB and RuvC proteins and the Holliday junction. (A) Model of the RuvAB branch migration complex, in which RuvA binds the junction and holds it in an unfolded square planar configuration. RuvB hexameric rings, which are oppositely oriented and lie diametrically opposed on two DNA arms, drive branch migration using their DNA helicase activities. Although physical studies indicate that the junction is bound by two tetramers (Yu et al., 1997), one on either face of the junction, in this diagram only one RuvA tetramer is shown for purposes of clarity. Drawing adapted from Parsons et al. (1995) and Rafferty et al. (1996). The arrows indicate the direction of DNA movement during branch migration. (B) Proposed structure of a RuvBC complex capable of limited branch migration and elevated junction-resolution efficiency. In this diagram, a RuvC dimer is shown binding the junction and interacting with two RuvB hexamers. (C) Two views of a hypothetical RuvABC complex in which RuvA and RuvC bind opposite faces of the Holliday junction and stabilise the binding of two RuvB rings. It is proposed that the RuvC dimer may displace a RuvA tetramer, as indicated. In this model, RuvAB promote branch migration, as shown in (A), driving DNA into and through the complex. The RuvC dimer ‘scans’ DNA sequences as they pass through the complex, resulting in efficient resolution at preferred sites. The complex is shown from above and in side view.

exhibit a resolution-defective phenotype (Mandal et al., 1993), and with observations showing that monoclonal antibodies directed against RuvA, RuvB or RuvC inhibit Holliday junction resolution by RuvC in in vitro reconstitution experiments (Eggleston et al., 1997).

In agreement with the studies presented here, the resolution of plasmid-sized recombination intermediates by RuvC was found to be stimulated by the presence of the RuvA and RuvB proteins (D.Zerbib, C.Mézard, H.George and S.C.West, in preparation). Moreover, the physical association of RuvABC with synthetic Holliday junctions has been detected by immunoprecipitation (A.A.Davies and S.C.West, in preparation). The detailed structure of the RuvABC complex remains to be elucidated, as does the mechanism by which RuvC, as part of this complex, recognises cleavable sequences during branch migration.

Materials and methods

Enzymes and DNA
RuvB (Tsaneva et al., 1992a), RuvBD113N (Mézard et al., 1997) and RuvC (Dundordale et al., 1994) were purified as described. Immediately before use, enzymes were diluted in 20 mM Tris–HCl (pH 8.0), 0.5 mM EDTA, 0.5 mM dithiothreitol, 0.5 mg/ml bovine serum albumin and 10% glycerol. Protein concentrations are expressed in moles of protein monomers.

Synthetic Holliday junctions X4 and X12 were each composed of four 50-mer oligonucleotides. DNA annealing and purification was essentially as described previously (Parsons et al., 1990). Unless indicated otherwise, strands were 5'-32P-end-labelled in strand 1 prior to annealing. Strand 1 contains a RuvC consensus sequence (5'-ATTG-3') at its centre and is common to junctions X4 and X12. Junction X4 contained: strand 1 (5'-CGCTGGCAATTCTACCTGAGCAATCGTTTATGCC), strand 2 (5'-GGTGAGCTCAGATCTTTATTCTACTAC), and strand 3 (5'-GGCCTCATCAGTATTCTTGAGGATCCGATCA). For DNA-binding analyses, a duplex DNA was made by annealing 32P-labelled strand 1 with a complementary 50-mer oligonucleotide. DNA concentrations are indicated in moles of junctions.

DNA-binding assays
RuvC was incubated with 32P-labelled Holliday junction DNA (1 nM) for 30 min at 37°C in binding buffer [50 mM Tris-acetate (pH 8.0), 0.5 mM Mg(OAc)2, 1 mM DTT, 50 μg/ml bovine serum albumin; total volume 20 μl]. When included, RuvB or RuvBD113N was preincubated with junction DNA in modified binding buffer [containing 1 mM ATPγS and 15 mM CaCl2 instead of 0.5 mM Mg(OAc)2] for 5 min at 37°C.
prior to addition of RuvC. Neutral loading buffer [6 μl of 50% glycerol, 0.1% bromophenol blue] was then added and the products analysed by electrophoresis on a 6% polyacrylamide gel using TBE buffer (2 h at 160 V).

To determine the $K_d$ of RuvC binding, reactions were carried out at low DNA concentrations (0.01 nM) such that variations to the amount of DNA (10-fold) had no significant effect on the percentage of DNA bound. Under such conditions, the $K_d$ is represented by the concentration of protein required to give 50% binding (Rhodes and Fairall, 1997). Junction or duplex DNA was incubated with RuvC in binding buffer (20 μl) on ice for 30 min, followed by the addition of sample buffer [6 μl of 40 mM Tris–acetate (pH 7.5), 4 mM EDTA, 0.4 mg/ml bovine serum albumin, 25% glycerol and 0.1% bromophenol blue] and electrophoresis on 4% low-ionic strength polyacrylamide gels at 160 V for 2.5 h at 4°C (Parsons et al., 1992). The buffer and gel contained 6.7 μl Tris–acetate (pH 8.1), 3.3 mM sodium acetate and 5 mM EDTA, and the reservoir buffer was recirculated. Gels were dried and the intensity of bands was quantified using a PhosphorImager (Molecular Dynamics).

**Junction resolution assays**

$5^{32}$P-labelled junctions (1 nM) were incubated with RuvC in cleavage buffer [50 mM Tris–acetate (pH 8.0), 10 mM Mg(OAc)$_2$, 1 mM DTT and 50 μg/ml bovine serum albumin; total volume 20 μl] for 30 min at 37°C. When required, RuvB was preincubated with the DNA in modified cleavage buffer [containing 15 mM Mg(OAc)$_2$ and 1 mM ATP] for 5 min at 37°C prior to the addition of RuvC. For analysis on neutral gels, reaction mixtures were deproteinised using proteinase K and SDS (to 1 mg/ml and 0.25%, respectively). Neutral loading buffer was added, and samples were analysed on 8% polyacrylamide gels. Electrophoresis was carried out at 160 V for 2 h. To determine the sites of cleavage, samples were analysed on denaturing gels. The DNA was ethanol precipitated and resuspended in 80% formamide, 0.1% bromophenol blue, 0.1% xylene cyanol. Aliquots were loaded onto 12% polyacrylamide gels containing 7 M urea. Following electrophoresis at 65 W for 1 h, gels were fixed for 20 min in 10% acetic acid, 10% methanol. Cleavage gels were deproteinised and analysed on 8% neutral polyacrylamide gels as described above.

**Branch migration assays**

$5^{32}$P-labelled synthetic Holliday junctions (1 nM) were preincubated with RuvB (600 nM) in 50 mM Tris–acetate (pH 8.0), 15 mM CaCl$_2$, 1 mM DTT, 50 μg/ml bovine serum albumin, 1 mM ATP and 0.25 mM ATPyS for 5 min at 37°C (total volume 20 μl). Varying amounts of RuvC were added and incubation was continued for 30 min. Samples were deproteinised and analysed on 8% neutral polyacrylamide gels as described above.

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**References**


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