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Recognition and manipulation of branched DNA by the RusA Holliday junction resolvase of *Escherichia coli*

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ABSTRACT

Homologous recombination is a fundamental cellular process that shapes and reshapes the genomes of all organisms and promotes repair of damaged DNA. A key step in this process is the resolution of Holliday junctions formed by homologous DNA pairing and strand exchange. In *Escherichia coli*, a Holliday junction is processed into recombinant products by the concerted activities of the RuvA and RuvB proteins, which together drive branch migration, and RuvC endonuclease, which resolves the structure. In the absence of RuvABC, recombination can be promoted by increasing the expression of the RusA endonuclease, a Holliday junction resolvase encoded by a cryptic prophage gene. Here, we describe the DNA binding properties of RusA. We found that RusA was highly selective for branched molecules and formed complexes with these structures even in the presence of a large excess of linear duplex DNA. However, it does bind weakly to linear duplex DNA. Under conditions where there was no detectable binding to duplex DNA, RusA formed a highly structured complex with a synthetic Holliday junction that was remarkably stable and insensitive to divalent metal ions. The duplex arms were found to adopt a specific alignment within this complex that approximated to a tetrahedral conformation of the junction.

INTRODUCTION

The RusA protein of *Escherichia coli* is a DNA endonuclease that resolves Holliday intermediates formed during genetic recombination and DNA repair (1). Holliday junctions made in *E.coli* by RecA-mediated homologous pairing and strand exchange are normally processed into viable recombinants by the RuvA, RuvB and RuvC proteins (2). RuvA and RuvB act together to catalyse branch migration, with RuvA providing the means to recognise the four-way branched structure of the junction and RuvB the helicase motor to drive the point of strand exchange along the DNA (3–5). RuvC is an endonuclease that catalyses junction resolution by introducing symmetrically-related nicks in two strands of like polarity in a sequence-dependent manner (6). Recent studies indicate that the branch migration and resolution reactions are probably coupled via the formation of a RuvABC–junction complex that enables RuvC to monitor the DNA sequence for cleavable sites during the course of branch migration (3,7,8).

Mutations in *ruvA*, *ruvB* or *ruvC* confer sensitivity to UV light, ionising radiation, and mitomycin C, and reduce the efficiency of recombination (9–11). In each case, the defect can be corrected by increasing the expression of the RusA resolvase (1,12). RusA is encoded by a cryptic prophage gene (*rusA*) and is normally expressed very poorly, if at all (12). However, it can be induced to suppress *ruv* mutations by insertion of either IS*2* or IS*10* upstream of the *rusA* coding region, or by cloning *rusA* in a multicopy plasmid (12,13). Suppression depends on RecG (12,13), a junction-specific DNA helicase that drives branch migration of Holliday intermediates and other branched DNA molecules (14–17).

The native RusA protein is a homodimer of 14 kDa subunits (18). In the presence of divalent metal ions, it resolves synthetic Holliday junctions to nicked duplex DNA products by a dual strand incision mechanism similar to that catalysed by RuvC (1,18). It also resolves Holliday intermediates formed by RecA (1). Strand cleavage is targeted to particular DNA sequences located symmetrically at the junction and occurs with the highest efficiency to the 5′ side of a CC dinucleotide (1,18). This sequence-specificity is probably the reason why RusA-mediated suppression of *ruv* mutations depends on RecG. Presumably, junctions have to be located at sites recognised by RusA before they can be resolved. RecG-mediated branch migration may enable this requirement to be satisfied. However, there is no suggestion that RecG and RusA interact with each other to provide a coupled branch migration and resolution reaction as in the RuvABC system. Indeed, genetic evidence indicates that RusA binds and resolves appropriately located junctions without the aid of a specific branch migration protein (12,13), which implies that RusA has to rely on repeated cycles of DNA binding and dissociation until it finds a junction located at a cleavable site. The efficiency of resolution will depend therefore on the affinity of RusA for junction DNA, the rate of dissociation of the complexes formed at non-cleavable sites, and on the rate of branch migration. In this work we focus on the DNA binding properties of RusA.

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MATERIALS AND METHODS

Proteins

RusA (18) and RuvC (19) proteins were purified as described elsewhere. Amounts of RusA and RuvC are expressed as moles of the monomeric protein.

DNA substrates

Oligonucleotides were synthesised on an Applied Biosystems 380B DNA synthesiser and purified by denaturing PAGE. Synthetic Holliday junctions were made by annealing four partially complementary oligonucleotides of 80 (Junction 1) or 49–51 (J0, J3, J4, J12 and J26) nucleotides in length. The point of strand crossover is either fixed centrally within the structure (static junctions, J0 and Junction 1) or free to branch migrate within a central core of homology (mobile junctions J3, J4, J12 and J26). The sequences of the oligonucleotides used for Junction 1 (20), J0 (7), J3 (18), J4 (18), J12 (14) and J26 (21), have been described. A three-way duplex junction (Y-DNA) was made using 50mer oligonucleotides 1, 4 and 6 (22). Linear duplex DNA was made using 50mer oligonucleotides 1 and 5 (14). The J0, J12, Y-DNA and linear duplex substrates shared at least one strand in common. A linear duplex with three contiguous non-complementary base pairs in the middle of the molecule (bubble DNA) was made by annealing oligonucleotide 5 (14) with oligonucleotide 7 (5′-GACGCTGCCGAATTCTGGCTTGCATCGACATCTTTGCCCACGTTGACCC-3′ (the mis-matched bases are underlined). A cruciform structure was made by annealing two oligonucleotides, each containing an inverted repeat capable of forming a hairpin: oligonucleotides 8 (5′-GACGCTGCCGAATTCTGGCTTGCGACTGAGATCAGTCTAGGACATCTTTGCCACGTTGACCC-3′) and 9 (5′-GGGTCAACGTGGGCAAAGATGTCCTACTGACTAGAGTCAGGCAAGCCAGAATTCGGCAGCGTC-3′). The inverted repeat is underlined in each case. Annealing followed the procedures described (23), except for Junction 1 when the mixture of oligonucleotides was allowed to cool slowly to room temperature after the initial heating at 95°C. In all cases, one of the strands was labelled at the 5′ end prior to annealing using [γ-^{32}P]ATP and T4 polynucleotide kinase. Labelled substrates were purified by non-denaturing 10% PAGE (or 8% PAGE for Junction 1) and electroelution. Unlabelled J0 and linear duplex DNAs were made as described above, except that oligonucleotides were annealed in equimolar ratios and the resulting substrates were not purified by PAGE. Unlabelled poly(dI).poly(dC) double-stranded DNA was from Promega.

Preparation of Junction 1 for comparative gel electrophoresis

Each arm of Junction 1 carries a site for restriction by either *Bam*HI, *Eco*RI, *Hin*dIII or *Xba*I (20). Three preparations of the junction, each ^{32}P-labelled in a different strand, were purified, digested with an appropriate pairwise combination of restriction enzymes, loaded directly onto a 10% native polyacrylamide gel, and electrophoresed at 90 V for 16 h. Bands corresponding to the six junction species with unique combinations of two long arms and two short arms were excised from the gel and electroeluted into TBE buffer, dialysed against DNA storage buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 50 mM NaCl), and used directly in binding assays.

Cleavage assays

Cleavage of ^{32}P-labelled substrate DNA by RusA and RuvC was assayed at 37°C in buffer [25 mM Tris–HCl pH 8.0, 1 mM DTT, 100 µg/ml BSA, 6% (v/v) glycerol] containing 10 mM $MgCl_2$. Reactions (20 µl final volume) were terminated after 30 min by adding 5 µl stop mix (2.5% SDS, 200 mM EDTA, 10 mg/ml proteinase K) and deproteinised by incubating for a further 10 min at 37°C. DNA products were analysed by native PAGE, using 10% gels in TBE (90 mM Tris–borate, pH 8.0, 2 mM EDTA). Gels were dried, and labelled products were detected using a PhosphorImager (Molecular Dynamics, model 425) and by autoradiography.

Standard bandshift assays

^{32}P-labelled substrate DNA (0.3 ng) was mixed on ice with RusA or RuvC in binding buffer [50 mM Tris–HCl pH 8.0, 5 mM EDTA, 1 mM DTT, 100 µg/ml BSA, 6% (v/v) glycerol] in a final volume of 20 µl. In competition assays, unlabelled competitor DNA was mixed with labelled DNA before adding protein. After 10–15 min on ice, protein–DNA complexes were resolved by non-denaturing PAGE using 4% gels in low ionic strength buffer (6.7 mM Tris–HCl pH 8.0, 3.3 mM sodium acetate, 2 mM EDTA). Gels were cooled to 4°C before use, but electrophoresis was at room temperature with continuous buffer recirculation. Gels were dried and analysed as described above.

Dissociation of RusA from junction DNA

Binding reactions (140 µl final volume) containing 2.1 ng ^{32}P-labelled J0 DNA and either 16 nM RusA or 32 nM RuvC were mixed in binding buffer and incubated for 15 min on ice to allow formation of protein–DNA complexes. A 20 µl sample was removed from each mixture and loaded on 4% low ionic strength polyacrylamide gel running with 200 V applied. Unlabelled J0 DNA (4 µg) in 2 µl was added quickly to the remainder and the mixture left on ice. Further 20 µl samples were removed at intervals and loaded on the same gel with 200 V applied. Five minutes after each loading, the voltage was reduced to 160 V. Electrophoresis was continued for 100 min after loading the last sample. Gels were processed as described above.

Comparative gel electrophoresis of RusA–Junction 1 complexes

The six unique substrates generated by restriction of Junction 1 were each mixed with RusA (final concentration 100 nM) in 10 µl binding buffer (50 mM Tris–HCl pH 8.0, 1 mM DTT, 6% glycerol, 100 mg/ml BSA) and left on ice for 10 min. For analysis in the absence of cations, the binding reactions also contained EDTA at a final concentration of 2 mM, and the complexes were resolved by native PAGE using 8% gels in TBE buffer. For analysis in the presence of Mg^{2+}, the binding reactions contained 4 mM $MgCl_2$ and complexes were resolved by native PAGE using 8% gels in low ionic strength buffer (6.7 mM Tris–HCl pH 8.0, 3.3 mM sodium acetate) containing 0.2 mM $MgCl_2$. Gels were run for 15–18 h at 130 V with continuous buffer recirculation, dried, and exposed to X-ray film.

RESULTS

The RusA and RuvC endonucleases are small homodimeric proteins that resolve Holliday junctions by introducing symmetrically-related nicks in two strands of like polarity (1,24,25). In the case of RuvC, the protein binds junction DNA with a high degree of selectivity (26,27) to form a complex in which the junction is held in an open conformation that enables each of the RuvC subunits to make base-specific contacts with the DNA at the site of cleavage (28,29). Cleavage is thought to occur when the two target strands are docked into the catalytic centres located at the base of large clefts within each RuvC subunit where four acidic residues coordinate Mg^{2+} for the hydrolysis of the sugar-phosphate chain (30,31). As a first step in the analysis of the resolution reaction catalysed by RusA, we set out to investigate its affinity for junction DNA and its ability to manipulate the conformation of the DNA at the point of strand crossover.

Binding of RusA to junction DNA

To investigate the binding of RusA to junction DNA, we initially used a small synthetic X-junction containing a homologous core of 12 bp (J12). Binding was monitored using a standard electrophoretic mobility-shift assay. RuvC was used as a control since it is known to form a well defined complex with a substrate of this type. The two proteins resolved J12 to nicked duplex DNA products with similar efficiencies (Fig. 1A). However, they showed significant differences in DNA binding (Fig. 1B). At low concentrations of protein, RusA bound the DNA to form a single complex (complex I) with a sharply-defined mobility (lanes b–d). As the amount of protein was increased, complex I was replaced by a series of progressively slower-migrating species (complexes II–V) until finally all the DNA was bound in a single well-retarded complex (complex V, lanes e–g). In contrast, RuvC gave a single retarded species over the range of protein concentrations tested (lanes i–n).

The selectivity of RusA for junction DNA was investigated by monitoring binding in the presence of unlabelled linear duplex, poly(dI).poly(dC), DNA. We used a level of protein that was sufficient in the case of RusA to bind all of the labelled junction in complex V (Fig. 1C, lane b). The addition of linear duplex DNA to the binding reactions before the addition of RusA eliminated the formation of this complex. As the concentration of linear duplex DNA increased, most of the labelled junction migrated instead in the position of complex I, and ultimately as free DNA (Fig. 1C, lanes c–f). Under these same conditions, RuvC bound 100% of the labelled junction at all concentrations of the competitor DNA (lanes i–l). These results show that RusA has a significant affinity for linear duplex DNA, relative to RuvC. However, RusA was able to bind some of the J12 molecules to form complex I even when linear duplex DNA was present in >33 000-fold excess over junction DNA (lane f), which indicates that RusA, like RuvC, has a much greater affinity for branched DNA. We conclude that the ladder of RusA–DNA complexes detected with J12 in the absence of linear duplex competitor arises from a combination of high affinity binding to the junction (complex I) at low concentrations of protein followed by low affinity binding at secondary sites (complexes II–V) as the concentration of protein increases.

Structure-specificity of DNA binding

The crossover in J12 is located in a homologous core of 12 bp within which it can move by branch migration. To see if this homology and the associated potential for variation in the length of the duplex arms affects RusA binding, we used junctions in which the homologous core was either eliminated (J0), extended to 26 bp (J26), or restricted to the sequence 5′-GTCC-3′ (J4) or 5′-TCC-3′ (J3). In each case, we detected a ladder of five reasonably well defined complexes (Fig. 2, panels i and ii, and data not shown). The pattern was essentially the same as that observed with J12 over the range of protein concentrations tested (Fig. 1B). It is clear that a homologous core is not essential for DNA binding and that the ability to branch migrate does not affect the pattern of complexes formed. It is also evident that the location of a CC dinucleotide target for strand cleavage at or near the crossover in J3 and J4 does not increase the affinity of RusA for the DNA. This result implies that RusA binds junctions independently of the sequence at the crossover and that sequence-specificity is exhibited at the cleavage step of the resolution reaction.

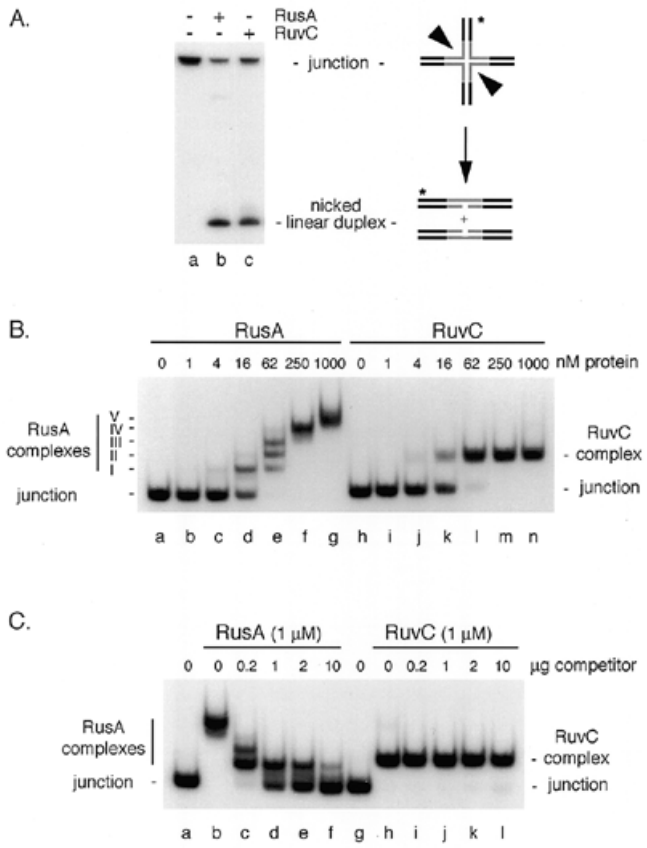

Figure 1. Holliday junction binding and resolution by RusA and RuvC. (**A**) Cleavage assay showing resolution of a junction J12 to nicked duplex DNA products. Reactions contained 0.15 ng ^{32}P-labelled J12 DNA and 20 nM RusA or RuvC as indicated. Junction and nicked duplex DNAs are shown schematically on the right. The central core of homology is shown in lighter shading. Arrowheads indicate the strand cleavages needed to achieve resolution. The ^{32}P-labelled strand is indicated by an asterisk. (**B**) Band-shift assay showing formation of protein–DNA complexes. Binding reactions contained 0.3 ng ^{32}P-labelled J12 DNA and protein as indicated. (**C**) Competition assay showing the effect of poly(dI).poly(dC) competitor DNA on the binding of junction DNA. Reactions contained 0.3 ng ^{32}P-labelled J12 DNA, 1 µM RusA or RuvC, and unlabelled poly(dI).poly(dC) as indicated.

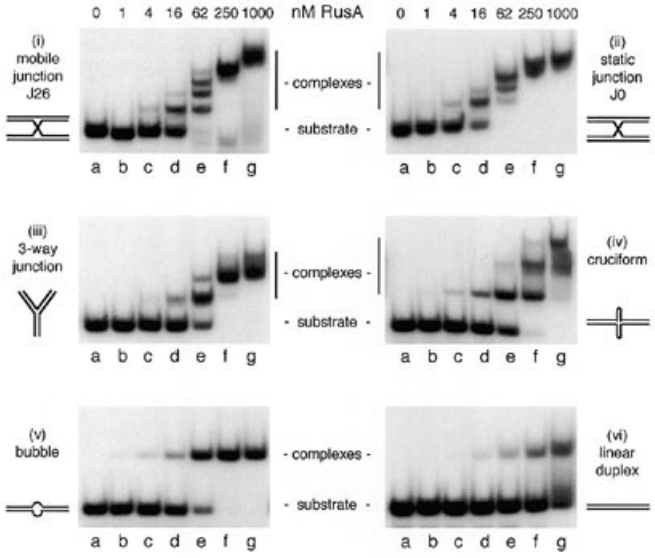

Figure 2. Structure-specificity of DNA binding by RusA. Panels i–vi are gel assays showing binding of RusA to the structures depicted. Binding reactions contained 0.3 ng of the ^{32}P-labelled substrate and RusA as indicated.

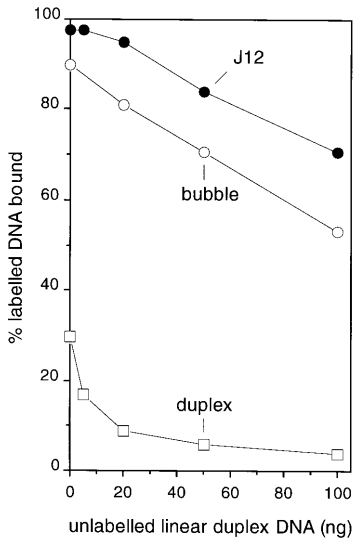

Figure 3. Competition assays showing the affinity of RusA for different DNA substrates. Binding reactions containing 0.3 ng of the indicated ^{32}P-labelled DNA, various concentrations of unlabelled linear duplex DNA, and 62.5 nM RusA, were mixed on ice and analysed by native PAGE as described in Materials and Methods.

We next investigated whether DNA binding is affected by the number and length of the duplex DNA arms extending from the branch point. RusA bound to a three-way junction related in sequence to J0 (Fig. 2, panel iii). The pattern of retarded bands indicated that RusA formed at least three different complexes with this substrate, possibly four. A cruciform structure with two long arms of 23 and 25 bp, respectively, and two short hairpin arms, each of 6 bp, was used to investigate whether the pattern of binding was affected by the length of the duplex arms. In this case, three well separated complexes were detected (panel iv).

These results show that the number of complexes formed upon binding of RusA to junction DNA is determined by the number and length of the duplex arms extending from the branch point. It is therefore tempting to conclude that the ladder of five complexes observed with a Holliday junction is the result of RusA binding initially to the branch point and then to each duplex arm in turn. The fact that only three complexes were detected with the cruciform structure indicates that the two 6 bp hairpin arms are not long enough to provide stable binding sites following binding of RusA to the four-way branch point.

We also monitored binding to a 50mer linear duplex molecule containing three mismatched base pairs located centrally (bubble DNA), and to a related molecule base-paired along its entire length. A single complex was detected in both cases (panels v and vi). However, RusA appeared to have a higher affinity for the bubble DNA and the complex formed was more sharply defined. The binding of RusA to these substrates was analysed in more detail using the unlabelled 50mer linear duplex as a competitor (Fig. 3). The results confirmed that RusA has a high affinity for the bubble DNA, almost as high as for J12 DNA used as a control. More than 50% of the bubble molecules were bound by RusA even when linear duplex competitor was present in >300-fold excess. We assume the distortion of the DNA by the mismatches provides RusA with a stable binding site. This may involve interactions with single-stranded DNA. Band shift assays revealed that RusA has a greater affinity for single-stranded DNA than for linear duplex DNA. For instance, in reactions containing 62 nM RusA and 0.3 ng of either 50mer oligonucleotide or linear duplex DNA, we found that 59% and 24%, respectively, of the labelled DNA was bound by RusA.

Global conformation of a Holliday junction bound by RusA

Previous studies have shown that RuvC protein and other resolving enzymes such as CCE1 from *Saccharomyces cerevisiae* manipulate the structure of a Holliday junction to form a complex in which the crossover is held in an open conformation (28,32). To determine the conformation of a junction bound by RusA, we compared the relative mobilities of six derivatives of a four-way junction each carrying two long (40 bp) and two short arms (15 bp). The method used was developed initially to investigate the structure of a Holliday junction (20,33), but has since been extended to study the conformation of junction DNA bound by protein (28,32,34,35). It relies on the fact that the relative mobilities of the six junction species is determined largely by the angle subtended by the two long arms.

We constructed an immobile junction (Junction 1) with unique restriction sites in each arm (Fig. 4A) and made the six required species by cleaving the DNA with appropriate pairs of restriction enzymes. Junction 1 does not have CC dinucleotides located symmetrically at the crossover and is not cleaved by RusA. This enabled us to conduct the analysis both in the presence and absence of Mg^{2+}. Without RusA, the six junction species migrated in EDTA gels (Fig. 4B) with the expected four slow, two fast pattern characteristic of an open, square conformation, whereas in the presence of Mg^{2+} (Fig. 4C) they migrated with a two slow, two intermediate, two fast pattern, reflecting the antiparallel stacked X-structure arising from stacking of the duplex arms, with arm B stacked on H, and arm R stacked on X (20).

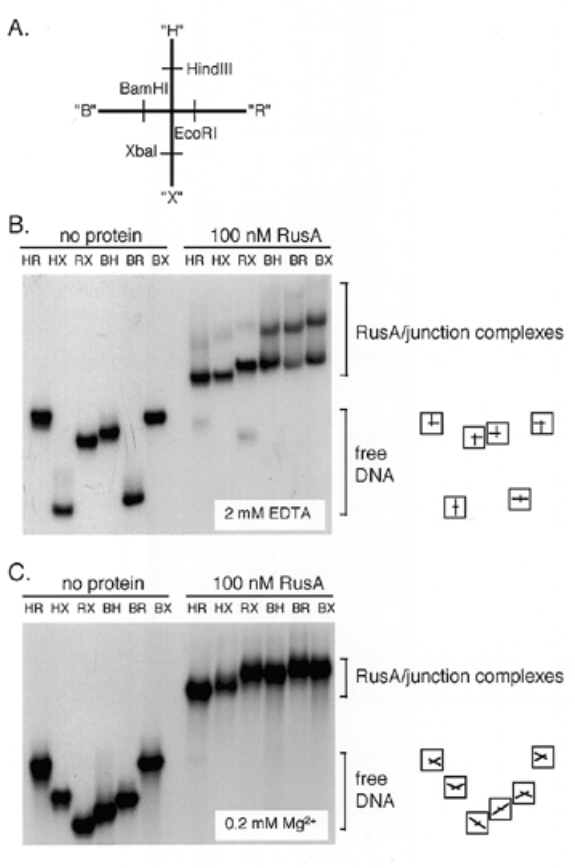

Figure 4. Comparative gel electrophoresis of RusA–junction complexes. Six species of Junction 1 were prepared, each with a unique combination of two long and two short arms. The relative electrophoretic mobilities of each species with and without protein was analysed in the presence or absence of Mg^{2+}, using native PAGE as described in Materials and Methods. (**A**) Arrangement of restriction sites in the duplex arms of Junction 1 and nomenclature for labelling each intact arm. (**B**) Gel assay showing the electrophoretic mobility pattern of the six junction species in the absence of Mg^{2+}, with and without RusA. The conformation of the junction arms deduced from the relative mobilities of the six protein-free junction species is shown in the boxes on the right. (**C**) Same as (B) except that the analysis was conducted in the presence of Mg^{2+}.

When RusA was added, a new pattern of retarded bands was observed. However, in this case the pattern was essentially the same whether Mg^{2+} was present or not (Fig. 4B and C). The only significant difference was that while one complex was detected with each species in Mg^{2+}, three complexes were seen in the presence of EDTA. These three complexes were especially clear with the BH, BR and BX species (Fig. 4B), but were also formed with the other three junction species and are clearly visible on longer exposures of the original autoradiograph. We repeated the assay in EDTA using 25 ng of poly(dI).poly(dC) DNA in the binding reactions. The two complexes with slower mobilities were no longer detected, but otherwise the results were identical (data not shown). The mobility of the one remaining complex was the same in each case as that of the fastest migrating complex seen in the absence of competitor. From these results we conclude that the slower migrating complexes arise from the binding of RusA to one or both of the two long arms of a junction already bound by RusA at the crossover. The fact that we did not see the five complexes detected with X-junctions with four duplex arms of 25 bp (Figs 1 and 2) indicates that the two short (15 bp) arms of the Junction 1 species used do not provide stable binding sites. The results also indicate that the binding of RusA to the duplex arms is reduced in the presence of Mg^{2+}.

The similar mobility of the stable complexes formed with each of the six species of Junction 1 both in Mg^{2+} and in EDTA indicated that all the possible pairs of junction arms subtend angles that are approximately the same. This is consistent with a tetrahedral structure. However, given the small differences in the mobility of the six complexes, we cannot exclude alternative conformations. Nevertheless, it is clear that RusA manipulates the junction and imposes a conformation that resists coaxial stacking of the duplex arms.

Stability of RusA–junction complexes

RusA catalyses resolution of synthetic Holliday junctions by targeting cleavage to the 5′ side of CC dinucleotides (18). The selectivity of RusA for branched molecules implies that it should have no difficulty finding junctions. However, since there is no evidence that RusA can promote branch migration, either alone or in conjunction with other proteins, its ability to promote resolution must rely on repeated cycles of DNA binding and dissociation of the complex until it finds a junction located at a cleavable site. The efficiency of resolution will depend therefore on the rate of dissociation of complexes formed at non-cleavable sites and on the rate of branch migration of the RusA-free junction.

We measured the rate of dissociation of RusA from ^{32}P-labelled junction DNA by adding a large excess (>2000-fold) of unlabelled junction to preformed complexes and then measuring the amount of labelled DNA that remained bound over a period of time. J0 was used for this experiment as it lacks a target site for resolution. To reduce any possible complications arising from the binding of RusA to the junction arms, the amount of RusA used was limited to that needed to form complex I (Fig. 5A, lane a). The gel assay used to detect DNA binding revealed that a significant amount of labelled complex I was present 60 min after the addition of excess cold junction (Fig. 5A, lanes b–f). Quantification of the data showed that ~25% of the complexes dissociated immediately (Fig. 5B). This loss most probably reflects the duplex DNA binding activity of RusA and the dissociation of those complexes in which RusA was bound in a non-structure specific manner to one or more arms of the junction. The remaining 75% showed no signs of dissociation and probably reflect stable binding of RusA at the crossover.

As a control, we measured the rate of dissociation of a RuvC–junction complex. In this case, most of the complexes dissociated within 5 min (Fig. 5A, lanes g–l, and B). We repeated the analysis using a mobile junction (J11) that is cleaved efficiently by RusA (18). J0 was used as the unlabelled competitor. The results were essentially the same. In this case, 80% of the RusA–J11 complexes remained 60 min after addition of the competitor DNA (data not shown). We conclude that RusA binds a Holliday junction at the crossover to form a complex that is very stable. This property is likely to have implications for the resolution of Holliday junctions *in vivo*.

DISCUSSION

In previous studies we showed that the RusA endonuclease can provide an efficient system for the resolution of Holliday junctions in the absence of RuvC (1,12,13). We have shown here

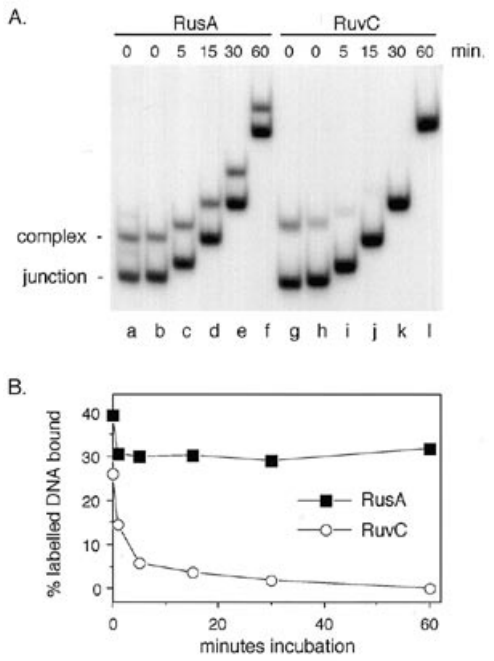

Figure 5. Dissociation of RusA– and RuvC–junction complexes. (**A**) Gel assay showing dissociation of RusA and RuvC from junction J0. Binding reactions containing ^{32}P-labelled J0 DNA and either RusA or RuvC were mixed as described in Materials and Methods and incubated on ice to allow formation of protein–DNA complexes. A sample was removed from each mixture (lanes a and g) before adding a >2000-fold excess (over labelled DNA) of unlabelled J0 DNA. Incubation was continued on ice and samples were removed for analysis at the times indicated (lanes b–f and h–l). Complexes were resolved by non-denaturing PAGE, using 4% gels in a low ionic strength buffer. All samples were loaded immediately on the gel with voltage applied. Gels were dried and labelled DNA quantified by analysis of phosphorimages. (**B**) Quantification of the per cent of labelled J0 DNA bound in (A).

that RusA has a strong selectivity for the four-way structure of a Holliday junction, forming a stable complex (complex I) with the DNA even in the presence of a large excess of linear duplex DNA. As with RuvC, binding is independent of the DNA sequence and does not require homologous sequences at the crossover. However, RusA differs from RuvC in that it will bind with high affinity to a variety of other branched DNA molecules, including a three-way junction and a linear duplex with mismatched base pairs. We have found that it also binds a three-strand junction with two duplex arms and two single strands extending from the branch point, and to a duplex molecule containing a hairpin loop in one strand (results not shown). However, we have detected no significant strand cleavage activity with any of these structure (results not shown). Although RusA is probably of bacteriophage origin (12), the limitation of its strand cleavage activity to four-way junctions with CC dinucleotides located symmetrically within a mobile core of homology distinguishes this enzyme from known bacteriophage resolvases such as T4 endonuclease VII and T7 endonuclease I. Like RusA, these phage enzymes bind a broad spectrum of DNA substrates, but they also cleave all of these structures and are less sequence-selective in their cleavage of four-way junctions (21,36–40).

RusA also differs from RuvC in that it has a much higher affinity for linear duplex DNA. In band-shift assays with branched DNA molecules, this property results in the formation of a characteristic ladder of discrete complexes with increasingly slower electrophoretic mobility as the concentration of RusA used is increased. The number of complexes detected varied with the number and length of the duplex arms extending from the branch point. Five distinct complexes were detected with a four-way junction, of which only the fastest-migrating (complex I) could be detected in the presence of a large excess of linear duplex DNA (Fig. 1C). Complex 1 migrated only marginally faster than the single complex detected with RuvC (Fig. 1B). Since RuvC binds junction DNA as a dimer of 19 kDa subunits (29), and the 14 kDa RusA protein is a dimer in solution (18), we suggest that complex I is formed when a dimer of RusA binds in a structure-specific manner to the branch point, and that the four other complexes detected are formed as an additional dimer binds to each duplex arm in turn.

There was some indication from our studies that the formation of complex I facilitates the binding of RusA to the duplex arms. Slower-migrating complexes were quite prevalent at concentrations of RusA that managed to shift only a small fraction of linear duplex DNA. We also found that RusA had a particularly high affinity for a 50mer duplex molecule with mismatched bases that probably distort the linear structure (Fig. 2, panel v, and Fig. 3). The angled structure of a four-way junction bound by RusA may similarly favour stable binding of additional molecules of RusA to the duplex arms. However, given the affinity of RusA for linear duplex DNA, there is unlikely to be enough free protein *in vivo* to bind next to a junction that is already bound.

Holliday junctions are folded in a stacked X-structure in the presence of Mg^{2+}, but adopt an unfolded square planar structure in the absence of added metal ions (20). We found that in common with other resolvases (35), RusA binds a four-way junction and manipulates the molecule to impose an extended, unstacked structure in a manner that was no longer affected by the presence or absence of Mg^{2+}. The conformation of the junction deduced by comparative gel electrophoresis of RusA complex I approximates to a tetrahedral arrangement of the junction arms, although we could not exclude alternative arrangements. Each of the three other resolvases analysed to date (RuvC, CCE1 and T4 endonuclease VII) also imposes a unique conformation on a four-way junction (35). The significance of the tetrahedral conformation imposed by RusA is unclear at present, especially as it does not fit with the two-fold symmetry one might expect to be associated with a dual strand incision mechanism for junction resolution.

The complex formed by RusA is very stable, at least at 4°C. We found that once RusA had bound to a junction, it could not be removed even when a >2000-fold excess of free junction was added (Fig. 5). Under the same conditions, a RuvC–junction complex dissociated very rapidly. Tight binding of RusA to a Holliday junction could be a limiting factor for resolution *in vivo*. This possibility is supported by previous studies in which we showed that the overexpression of RusA reduces the efficiency of DNA repair, even in strains with a functional RuvABC system (12). It serves to highlight the elegance of the resolvasome model proposed for the RuvABC system (7), whereby DNA can be driven through the RuvABC–junction complex by the powerful RuvB motor. However, there are many species of bacteria that have homologues of RuvAB, but not of RuvC. Most of these species do have a homologue of RusA (G.J.Sharples, personal communication). It remains to be seen whether RuvAB can help to support resolution by RusA in these cases, for instance by displacing RusA from non-cleavable sites or by forming an

alternative resolvasome complex with RusA. This does not appear to be the case in *E.coli* since the expression of RusA fails to confer resistance to UV in a *ruvC recG* strain, despite the presence of RuvAB (12). However, it is also possible to explain this failure by assuming that RecG may help to form Holliday junctions in the first instance (17,41).

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