Activation of RuvC Holliday junction resolvasse in vitro

Rajvee Shah, Richard J.Bennett and Stephen C.West*
Imperial Cancer Research Fund, Clare Hall Laboratories, South Mimms, Herts EN6 3LD, UK

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ABSTRACT

The Escherichia coli RuvC protein is an endonuclease that resolves Holliday junctions. In vitro, the protein shows efficient structure-specific binding of Holliday junctions, yet the rate of junction resolution is remarkably low. We have mapped the sites of cleavage on a synthetic junction through which a crossover can branch migrate through 26 bp and find that ≥ 90% of the junctions were cleaved at one site. This observation of sequence-specific cleavage suggests that inefficient resolution may be due to DNA binding events which occur away from the cleavage site and are therefore non-productive. Holliday junction resolution by RuvC protein can be stimulated by a number of factors including: (i) the presence of Mn2+ (rather than Mg2+) as the divalent metal cofactor, (ii) alkaline pH (≤ 10), and (iii) elevated temperature. These observations may indicate that other proteins are required for efficient RuvC-mediated resolution.

INTRODUCTION

The ruv locus on the E.coli chromosome encodes three genes that are required for genetic recombination and DNA repair [1]. The ruvA and ruvB genes encode the RuvA and RuvB proteins which together promote the branch migration of Holliday junctions [2, 3]. The third gene, ruvC, encodes the 19 kDa RuvC protein, an endonuclease that resolves Holliday junctions [4, 5].

In previous in vitro studies, it was shown that purified RuvC binds specifically to small synthetic Holliday junctions, as measured by band-shift assays. In the presence of the divalent metal ion Mg2+, RuvC promotes junction-cleavage to produce nicked duplex products. The resolution reaction occurs via the introduction of symmetrical nicks into two DNA strands of like polarity, and the nicked DNA products can subsequently be repaired by DNA ligase [4, 6].

Following the development of a simple method of purification for RuvC protein [7], studies of its DNA substrate specificity and mechanism of resolution were initiated. We found that the protein was highly specific for 3- and 4-stranded junctions and was inactive on Y-junctions or duplex DNA containing mismatches or heteroduplex loops [8]. In studies with synthetic Holliday junctions, through which the junction could branch migrate through 12 base pairs (junction X12), we found that cleavage occurred in a sequence-specific manner such that the majority of junctions were resolved by the introduction of nicks at a single site [6]. Thus, cleavage occurred at the 3'-side of a thymine residue within the sequence 5'-TGTCCCT. Further evidence for the requirement for specific DNA sequences at the cleavage site was obtained in two ways: firstly, we modified the junction X12 such that A-T base pairs were replaced by G-C base pairs (to make a ‘T-less’ junction), and secondly, we constructed a junction (X0) that lacked homologous sequences around the junction point. We found that the junctions X0 and ‘T-less’ were bound efficiently by RuvC but that resolution activity was reduced to almost undetectable levels [6].

The demonstration of sequence-specific resolution helps to reconcile data which indicate that RuvC protein binds Holliday junctions efficiently whereas in vitro cleavage is inefficient [6]. In the work described here, we have used a different synthetic Holliday junction, which contains a homologous core of 26 base pairs through which the junction can potentially branch migrate, to confirm that cleavage occurs in a sequence-specific manner. In addition, we have investigated the cleavage reaction in greater detail and have found three factors that stimulate RuvC-mediated resolution in vitro.

MATERIALS AND METHODS

Enzymes
Homogeneous RuvC protein was purified as described [7]. It was stored at −70°C in 20 mM Tris−HCl (pH 8.0), 1 mM EDTA, 1 mM dithiothreitol, and 10% [v/v] glycerol. RuvC was diluted as required in the same buffer containing 50 mM KCl prior to use. T7 endonuclease I was purified as described [9], and T4 endonuclease VII [10] was a gift of Dr J. Kemper (University of Cologne). T4 polynucleotide kinase was purchased from Pharmacia.

DNA substrates
Synthetic Holliday junctions (X12, X26, and X0) and duplex DNA were prepared by annealing the appropriate oligonucleotides [11]. One strand of each DNA substrate was 5'-32P-labelled prior to annealing, using T4 polynucleotide kinase and [γ-32P]ATP (Amersham Corporation). Unless stated otherwise, junction X12 and its duplex control were labelled in oligonucleotide 2. The oligonucleotides used to make X12 (oligonucleotides 1–4) and its linear duplex control (oligonucleotides 2 and 5) have been described [11]. Junction

*To whom correspondence should be addressed
X26 was made from oligonucleotides A, B, C, and D [12]. The oligonucleotides for X0 are as described [6].

Annealed DNA substrates were purified by gel electrophoresis and their concentrations determined by calculation of specific activities using the DE81 filter binding method [13]. 5'-32P-labelled oligonucleotides were sequenced by the chemical cleavage method [14]. A 1.5 base allowance was made to compensate for the nucleoside eliminated in the sequencing reaction.

The plasmids pDEA-7Z f(+), and pDEA2 were constructed by David Adams. pDEA-7Z f(+). was 3.0 kb in size, contains a unique SphI site and was made by replacing the ScaI–BsaI fragment of pGEM-7Z f(+) (Promega) with the ScaI–BsaI fragment of pBR322. pDEA2 (3.55 kb) was constructed by ligating a 643 nucleotide fragment of pACYC184 (nt. 2057–2700) to the 2.9 kb SphI–NsiI fragment of pDEA-7Z f(+). Circular gapped duplex pDEA-7Z f(+). DNA, containing a defined 175 nucleotide single-stranded gap between the BsaI and PstI restriction sites, was prepared essentially as described [15]. Linear duplex DNA was made by PstI digestion of pDEA2 and 3'-32P-end labelled with (α-32P) ddATP (Amersham) using terminal transferase.

Resolution of Holliday junctions by RuvC

Reaction mixtures (20 μl) containing 5'-32P-labelled DNA were incubated with RuvC protein at 37°C for varying times. Unless stated otherwise, the standard cleavage buffer was 50 mM Tris–HCl (pH 8.0), 10 mM MgCl2, 1 mM dithiothreitol, and 100 μM/ml bovine serum albumin. Products were processed in one of two ways. Firstly, reactions were stopped by the addition of 5 μl loading buffer (50% [v/v] glycerol, 0.1% [w/v] bromphenol blue and 0.1% [w/v] xylene cyanol) and 3 μl 10% [w/v] SDS, and analysed by 6% neutral PAGE, or secondly the DNA products were ethanol precipitated, denatured and analysed by denaturing PAGE. 32P-labelled DNA was detected by autoradiography. The percent resolution was quantitated from dried gels using a Molecular Dynamics Model 425E PhosphorImager with ImageQuant software.

Melting temperature determination

5'-32P-labelled DNA (2 ng) was diluted into 200 μl buffer containing 50 mM Tris–HCl (pH 8.0) or 50 mM borate–NaOH (pH 10), 10–15 mM divalent cation (as indicated), 1 mM dithiothreitol and 100 μM/ml bovine serum albumin. Aliquots (20 μl) were transferred to 5 ml glass tubes which were then sealed with parafilm. Reactions were incubated for 15 min at temperatures ranging from 30°C to 85°C prior to rapid quenching on ice for 2 min. After the addition of 7 μl loading dye (25% [v/v] glycerol, 0.1% [w/v] bromphenol blue and 0.1% [w/v] xylene cyanol), aliquots (15 μl) were analysed by 6% neutral PAGE. Melting of the DNA substrate was determined by PhosphorImager analysis of dried gels. The melting temperature (Tm) was determined to be the temperature at which 50% of the DNA was found to be denatured.

Branch migration of recombination intermediates

Recombination intermediates (α-structures) were prepared from circular gapped duplex pDEA-7Z f(+) DNA and 3'-32P-end labelled linear duplex pDEA2 DNA using RecA protein, essentially as described [15]. The substrates share 1536 bp of homology through which RecA protein drives strand exchange up to a heterologous block (643 bp). Protein-free recombination intermediates were isolated from a Sepharose CL-2B column as described [16], except that the elution buffer contained 2 mM MgCl2 instead of 15 mM MgCl2. Intermediates (5 ng) were then diluted 5-fold into 10 μl buffer containing 50 mM Tris–HCl (pH 8.0) or 50 mM borate–NaOH (pH 10.0), 10 mM divalent cation (MgCl2 or MnCl2), 1 mM dithiothreitol and 100 μg/ml bovine serum albumin. Incubation was at either 37°C or 55°C for up to 7 h, and at various times aliquots were taken and the reactions stopped by chilling on ice. After the addition of 5 μl loading dye (50% [v/v] glycerol, 0.1% [w/v] bromphenol blue and 0.1% [w/v] xylene cyanol), the DNA was analysed by agarose gel electrophoresis. Loss of recombination intermediates by branch migration was quantitated from dried gels by PhosphorImager analysis.

Hydroxyl radical footprinting

Binding reactions containing 5'-32P-labelled junction DNA (~5 ng) and RuvC protein were incubated in 50 mM Tris –HCl (pH 8.0). The reaction mixtures were dried 5-fold into 10 μl buffer containing 50 mM Tris–HCl (pH 8.0) or 50 mM borate–NaOH (pH 10.0), 10 mM dithiothreitol and 100 μg/ml bovine serum albumin. Incubation was at either 37°C or 55°C for up to 7 h, and at various times aliquots were taken and the reactions stopped by chilling on ice. After the addition of 5 μl loading dye (50% [v/v] glycerol, 0.1% [w/v] bromphenol blue and 0.1% [w/v] xylene cyanol), the DNA was analysed by agarose gel electrophoresis. Loss of recombination intermediates by branch migration was quantitated from dried gels by PhosphorImager analysis.

Figure 1. Resolution of a Holliday junction (X26) containing a 26 bp region of homology. (A) Four uniquely 5'-32P-labelled junctions (1 ng; labelled in strands 1, 2, 3, or 4 as indicated) were incubated with RuvC (100 ng) in cleavage buffer at 37°C. The DNA products were analysed by 12% denaturing PAGE (lanes marked RuvC), flanked by G+A and T+C sequence ladders. The reactions containing 32P label are indicated. (B) Summary of the cleavage sites. The dotted lines indicate the central 26 bp of homology. The major and minor sites of cleavage are marked by large and small arrows. Asterisks indicate the major sites of cleavage in the junction X12 [6].
of a junction become hypersensitive to hydroxyl radicals, at sites located 1–2 nucleotides immediately 3’ to the crossover point [6]. Figure 2A shows the hydroxyl radical pattern on all four strands of X26 in the absence and presence of RuvC. In the absence of protein (Figure 2A, lanes b, g, l, and q) hydroxyl radicals attacked the DNA to give a uniform ladder of bands. In the presence of RuvC (Figure 2A, lanes c, d, h, i, m, n, r, and s), two regions of DNA (in strands 1 and 3) were hypersensitive to radical attack. The sites of hypersensitivity were mapped using marker DNA fragments and are summarised in Figure 2B. Remarkably, the patterns of hydroxyl radical attack were identical to those found previously with X12 [6], and indicate that there are two major populations of junction, with the crossovers positioned as indicated in Figure 2B.

To summarise the data of Figure 1 and 2 and link it to our previous work, we have analysed two junctions, X12 and X26, and find that the crossover position in each RuvC–junction complex maps to the same site(s). However, the sites of cleavage of X12 and X26 were found to differ, even though X26 contains the region of homology found in X12. Thus, RuvC binding occurs at sites that are unsuitable for cleavage (due to the protein’s sequence specificity), and suggests that resolution requires

Figure 2. Hypersensitivity of RuvC–Holliday junction complexes to hydroxyl radicals. (A) Four 5'-32P-labelled junctions (X26; labelled in strands 1, 2, 3, or 4 as indicated) were incubated with the indicated amounts of RuvC. Reaction mixtures were then treated with hydroxyl radicals as described in Materials and Methods and the products were analysed by denaturing PAGE. (B) Summary of hydroxyl radical sites of hypersensitivity on the RuvC–junction complex. The sites of hypersensitivity, resolution and the predicted crossover sites are indicated.
movement of the crossover position (branch migration) to place it close to RuvC’s recognition sequence. In addition, we note that some minor cleavage sites in X26 (shown in Figure 1B), are located 9 bp away from the predicted strand crossover positions (Figure 2B). These results support earlier observations suggesting that RuvC binding and cleavage are distinct events [6].

**Activation of RuvC-mediated resolution by Mn^{2+}**

To further understand the binding/cleavage paradox, we investigated how the reaction conditions can be altered to favour junction-resolution by RuvC. Previous studies showed that Mn^{2+} ions are required as a cofactor for RuvC-mediated cleavage of Holliday junctions [6, 7]. To assess the suitability of other divalent cations, resolution reactions were performed on a synthetic Holliday junction (X12) in the presence of MgCl\(_2\), CaCl\(_2\), ZnCl\(_2\), CoCl\(_2\), MnCl\(_2\) and CuCl\(_2\) (Figure 3). We observed that cleavage of the junction occurred in the presence of 1 and 10 mM MgCl\(_2\) (lanes b and c), whereas Ca\(^{2+}\), Zn\(^{2+}\), Co\(^{2+}\) and Cu\(^{2+}\) ions were unable to support resolution at these concentrations. Significantly, more cleavage occurred in the presence of Mn\(^{2+}\) ions (lanes j and k).

The experiment presented in Figure 4 compares the ability of Mn\(^{2+}\) and Mg\(^{2+}\) to act as divalent metal cofactor for RuvC-mediated resolution over the range 0–40 mM. In both cases, optimal resolution reactions were observed at 10 mM and the initial rates of cleavage in Mn\(^{2+}\) and Mg\(^{2+}\) were 0.680 and 0.098 fmol junction/min respectively (Figure 5A), a rate difference of seven-fold. Denaturing PAGE studies showed that the sites of RuvC-mediated cleavage in the presence of either Mn\(^{2+}\) or Mg\(^{2+}\) were identical, indicating that Mn\(^{2+}\) does not significantly alter sequence specificity of RuvC on this junction (data not shown). The presence of Mn\(^{2+}\) also stimulated resolution which occurred in the presence of Mg\(^{2+}\) (Figure 5B).

With Mg\(^{2+}\) at 10 mM, stimulation was optimal when 4 mM Mn\(^{2+}\) was added. No stimulation was seen when either Mg\(^{2+}\) or Ca\(^{2+}\) was used as the supplementing cation.

The effect of Mn\(^{2+}\) on the activity of other Mg\(^{2+}\)-dependent resolvases (T4 endonuclease VII and T7 endonuclease I) was also studied. We found that Mn\(^{2+}\) substituted as an efficient cofactor for cleavage by these enzymes, but did not detect stimulation of resolution activity (data not shown).

**Resolution in Mg\(^{2+}\) is stimulated by alkaline pH and high temperatures**

To study the effects of varying pH on RuvC activity, resolution reactions were performed in a variety of buffers, in the presence of either 10 mM Mg\(^{2+}\) or 10 mM Mn\(^{2+}\) (Figure 6). In Mg\(^{2+}\)-dependent reactions, resolution was greatest above physiological pH, with the most efficient reactions taking place at our highest assay point, pH 10.0. In contrast, in the presence of Mn\(^{2+}\), resolution activity was optimal at physiological pH (~8.0).

At elevated temperatures, we also observed a striking enhancement of RuvC-mediated Holliday junction resolution (Figure 7). The initial rate of reaction (V\(_i\)) increased dramatically with increasing temperature and a 27-fold increase in rate was observed between 37°C and 65°C. Over an extended time course, the optimum temperature for resolution was observed to be 55°C, with 70% of the substrate resolved within 15 min (data not shown). At 37°C, less than 5% resolution was observed after 15 min, rising to a maximum level of resolution of 35% after 3 h. Interestingly, related experiments showed that enzymatic activity was abolished following incubation of RuvC (in the presence of substrate) at 55°C for 5 min. This indicates that at temperatures which gave rise to a greatly increased efficiency of resolution, the protein was being inactivated.

As observed with Mn\(^{2+}\)-dependent stimulation, activation by high pH and temperature did not affect the specificity of junction cleavage (data not shown). Significantly, we observed that the levels of activation seen at high temperature could be further enhanced by Mn\(^{2+}\) (data not shown). In addition, stimulation of resolution by high temperature was specific for reactions catalysed

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**Figure 3.** Effect of divalent cations on RuvC-mediated Holliday junction resolution. 5'-\(^{32}\)P-labelled junction (X12; 1 ng) was incubated with RuvC (100 ng) at 37°C for 60 min in cleavage buffer containing either 1 mM or 10 mM divalent cation as indicated in the heading. Reactions were analysed by neutral PAGE, and \(^{32}\)P-labelled DNA was detected by autoradiography as described in Materials and Methods.

**Figure 4.** Determination of optimal divalent cation concentrations for cleavage. 5'-\(^{32}\)P-labelled junction (X12; 1 ng) was incubated with 5 ng RuvC at 37°C for 30 min in cleavage buffer containing the indicated concentrations of either MgCl\(_2\) or MnCl\(_2\). Reactions were stopped and analysed by neutral PAGE as described. The percentage of junction resolved was determined using a PhosphorImager.
by RuvC, since the activity of T7 endonuclease I was inhibited at temperatures over 45°C, and in the case of T4 endonuclease VII, was completely abolished at 55°C (data not shown).

Factors that stimulate resolution decrease junction stability

To examine whether Mn²⁺, alkaline pH and elevated temperature stimulate resolution by affecting DNA structure, we determined the melting temperature of the synthetic Holliday junction X12 under a variety of reaction conditions. As shown in Figure 8A, the junction was most stable in 10 mM MgCl₂, pH 8.0, at which a melting temperature (Tₘ) of 72°C was observed. When Mg²⁺ was replaced with Mn²⁺, the junction was less stable and the Tₘ was lowered by 10°C to 62°C. Similarly, high pH reduced the melting temperature of the DNA. In the absence of divalent cations the junction melted at the lowest temperature (58°C). In control experiments, the stability of duplex DNA of the same length (60 bp) was measured and melting temperatures were found to be similar to those observed for the junction (data not shown).

Interestingly, the presence of Mn²⁺ ions was found to reduce the melting temperature of the junction, even in the presence of 10 mM Mg²⁺ (Figure 8B). These conditions are similar to those at which cleavage activity is greatly activated, as shown in Figure 5B. These effects appear to be restricted to Mn²⁺, since addition of Ca²⁺ had no effect on the melting temperature (Figure 8B), nor on the activation of RuvC (Figure 5B).

Figure 5. Activation of RuvC cleavage of Holliday junctions by Mn²⁺. (A) Time courses of Mg²⁺ and Mn²⁺-dependent resolution. Reactions containing 5'-3²P-labelled junction (X12; 1ng) were incubated at 37°C in cleavage buffer containing either 10 mM MgCl₂ or 10 mM MnCl₂ for 2 min prior to the addition of 10 ng RuvC. Reactions were stopped at the times indicated and the products analysed as described in Figure 4 legend. (B) Addition of Mn²⁺ stimulates Mg²⁺-dependent resolution by RuvC. Cleavage reactions containing 5'-3²P-labelled junction (X12; 1ng) and RuvC protein (10 ng) were incubated at 37°C for 5 min. The cleavage buffer contained 10 mM MgCl₂ and was supplemented with the indicated concentrations of MnCl₂, CaCl₂ or MgCl₂. Reaction were stopped and the products analysed as described in Figure 4 legend.

Figure 6. pH profile of RuvC-mediated Holliday junction resolution. Reactions contained 5'-3²P-labelled junction (X12; 1 ng) and RuvC protein (5 ng) in a series of buffers containing either 10 mM MgCl₂ or 10 mM MnCl₂. Resolution activity was assayed over a pH range by replacing the Tris—HCl, pH 8.0 in the standard cleavage buffer (Materials and Methods) with the following buffers (at 50 mM): sodium acetate, pH 5.5; 2-(N-Morpholino)ethanesulphonic acid (MES), pH 6.0 and 6.5; 3-(N-Morpholino)propanesulphonic acid (MOPS), pH 6.5, 7.0 and 7.5; Tris—HCl, pH 7.5 and 8.0; borate—NaOH, pH 8.0, 8.5, 9.0, 9.5 and 10; glycine pH 9.5 and 10.0. Reactions were stopped after 30 min at 37°C and were analysed by neutral PAGE. The percent resolution was determined as described in Figure 4 legend.

Figure 7. Effect of temperature on RuvC-mediated Holliday junction resolution. 5'-3²P-labelled junction (X12; 1 ng) was preincubated in cleavage buffer (at the temperatures indicated) for 2 min prior to the addition of RuvC (5 ng). Reactions were continued for 5, 10 and 15 min, except for temperatures over 50°C where 1, 2.5 and 5 min time points were taken. Reactions were stopped and products analysed by neutral PAGE. The percentage cleavage at each time point was quantitated using a PhosphorImager and the initial rate of reaction (V₁) was determined.
Figure 8. Effect of reaction conditions on the stability of a Holliday junction. (A) Determination of junction melting temperature. 5'-32P-labelled junction DNA (X12; 0.2 ng) was incubated at a range of temperatures in buffers modified from the standard RuvC cleavage buffer, as indicated. (B) Mn2+ lowers the melting temperature in the presence of Mg2+. 5'-32P-labelled junction DNA (X12; 0.2 ng) was incubated at a range of temperatures in standard cleavage buffer containing 10mM MgCl2 supplemented with 4 mM of either MnCl2, MnCl2 or CaCl2, as indicated. In (A) and (B), incubation was stopped after 15 min and the DNA products analysed by neutral PAGE as described in Materials and Methods. The percentage of DNA molecules thermally denatured was determined by PhosphorImager analysis.

Figure 9. Effect of reaction conditions on the dissociation of recombination intermediates by branch migration. Intermediates (5 ng) were incubated in buffers modified from the standard RuvC cleavage buffer, as indicated in Materials and Methods. Reactions were incubated at 37°C or 55°C for up to 7 hours. Reactions were stopped at 1 hour intervals by chilling on ice, and DNA was analysed by agarose gel electrophoresis. Loss of recombination intermediates by branch migration was quantified by PhosphorImager analysis.

Activation is not due to a net increase in the rate of spontaneous branch migration

One plausible mechanism for the stimulation of RuvC activity by Mn2+, pH and temperature is that by lowering the stability of duplex DNA, these factors stimulate branch migration of the Holliday junction to DNA sequences at which cleavage is preferred. However, two experiments argued against this as the sole cause of activation. Firstly, we prepared recombination intermediates (α-structures) using RecA protein and measured their rates of dissociation (due to branch migration) under various reaction conditions (Figure 9). Although we observed an elevated rate of branch migration at 55°C compared with 37°C [16], we failed to observe any significant changes to the overall rate in the presence of Mn2+ or at pH 10.0. In a second series of experiments, we measured cleavage of the synthetic Holliday junction X0. This junction lacks homology and is cleaved by RuvC at very low frequency compared to X12 (Figure 10) or X26 [6, 17]. As shown in Figure 10, the low level of cleavage observed with X0 was stimulated by Mn2+, elevated temperature and alkaline pH. Since this junction cannot branch migrate due to its lack of homology, these results indicate that branch migration alone cannot account for the stimulation of resolution activity.

Figure 10. Stimulation of RuvC-mediated cleavage of a non-branch migratable junction. 5'-32P-labelled junctions (X12 or X10; 1 ng) were incubated with RuvC (20 ng) in buffers modified from the standard RuvC cleavage buffer, as indicated. Incubation was performed at 37°C unless otherwise indicated. Reactions were stopped after 30 minutes and the DNA products analysed by neutral PAGE as described in Materials and Methods. The percentage cleavage was quantitated by PhosphorImager analysis.
In previous experiments we demonstrated that, in addition to recognising Holliday junction structure, RuvC exhibits a sequence-specificity, cleaving at the 3' side of thymine residues [6]. These results were extended here in studies with a synthetic Holliday junction containing 26 bp of homology (X26). Once again, all the cleavage sites mapped to the 3' side of thymine residues. Although there was no obvious consensus sequence shared between the major cleavage sites (5'-TGT1CCCT-3' in junction X12 and 5'-ATT1GCT-3' in junction X26), more extensive studies show that recombination intermediates made by RecA protein are cleaved at preferred sites which share some sequence similarities (R. S. and S.C.W. unpublished data).

Using hydroxyl radicals, we have been able to determine the position of the strand crossover(s) in a synthetic junction by footprinting the RuvC—Holliday junction complex. RuvC causes the DNA immediately 3' to the crossover (in two of the four strands) to become hypersensitive to hydroxyl radical attack [6]. Using the junction X26, we found that in spite of the potential of the crossover to branch migrate through 26 bp, only two populations of the X26 junction existed, and that the positions of the crossover were identical to those found in the junction X12 (Figure 2B and ref [6]). However, we were surprised to find that the sites of cleavage of these two junctions differed markedly, even though X26 contains the 12 bp of homology present in junction X12. These results indicate that efficient RuvC cleavage requires some branch migration of the Holliday crossover until it reaches a site that best satisfies the sequence requirements of the protein. Cleavage of X26 at the sequence 5'-ATT1GCT-3', rather than at 5'-TGT1CCCT-3', indicates that this site provides a better recognition sequence for resolution.

We have shown that three factors stimulate RuvC-mediated cleavage of synthetic Holliday junctions. An increase in pH, temperature, or the presence of Mn2+ cations, all resulted in a marked increase in the rate of resolution. The effects were not mutually exclusive, so a combination of factors (e.g. elevated temperature and Mn2+) can act to further increase the activity of RuvC. The observed stimulation was specific to RuvC-catalysed cleavage of the Holliday structure, since the two phage resolvases (T4 endonuclease VII and T7 endonuclease I) acted with a reduced or unaltered efficiency under these conditions.

Since all three factors lowered the stability of the synthetic Holliday junction and duplex DNA (data not shown), we used recombination intermediates made by RecA to test whether they increased the rate of spontaneous branch migration. While raising the temperature clearly promoted dissociation of the intermediates by increasing the rate of branch migration, neither alkaline pH nor the presence of Mn2+ cations had any observable effect on dissociation of the intermediates. This result indicates that activation of RuvC under these conditions may not be due simply to an increase in branch migration. This conclusion was supported by experiments performed on a junction that lacked homology (the crossover point is fixed and so incapable of branch migration). This junction is cleaved extremely poorly by RuvC, presumably because of the protein's sequence requirements [6]. However, the low level cleavage of the 'immobile' junction was also stimulated by Mn2+, high temperature and alkaline pH (Figure 10).

Although these factors do not appear to be stimulating resolution via the branch migration of Holliday junctions, it is possible that localised destabilisation or unwinding of the DNA is important for RuvC activity, thus leading to elevated levels of cleavage. In addition, a direct effect of these factors on the RuvC protein is also possible. Stimulation of resolution by increased temperature is likely to be due to an increase in the rate of catalysis of the enzyme in addition to any observed effect upon DNA destabilisation.

Many Mg2+-dependent proteins are able to utilise Mn2+ cations. However, a common characteristic of the effect of Mn2+ is a loss of stringency in the reactions they catalyse. A well-known example is the loss of fidelity by DNA polymerases, caused by direct binding of the Mn2+ to bases on the DNA template, resulting in altered hydrogen bonding properties [18]. Another example is the EcoRV protein which cleaves its recognition sequence in the presence of Mn2+ ions, albeit more slowly than with Mg2+. However, the recognition of suboptimal sequences is stimulated by the substitution of Mg2+ with Mn2+ [19]. Since the consensus sequence for RuvC cleavage is as yet unknown, a similar model cannot be ruled out for RuvC, i.e. that the stringency of RuvC is relaxed by Mn2+ helping it to cleave sub-optimal sites. This may also explain why Mn2+ stimulation of resolvase activity was not observed with T4 endonuclease VII and T7 endonuclease I — enzymes that recognise Holliday junctions but lack sequence-specificity.

A different type of Mn2+-dependent stimulation has been observed during studies of in vitro DNA transposition, where Mn2+ acts as an allosteric effector. For example, whereas efficient transposition of bacteriophage Mu normally requires the MuA and MuB proteins, the requirement for MuB protein can be circumvented by inclusion of Mn2+ cations [20]. It is possible that a similar situation exists with RuvC protein since genetic data indicates that Holliday junction resolution by RuvC requires RuvA and RuvB [21]. We suggest that RuvC interacts with RuvAB (or RuvB alone) to form a branch migration/resolution complex in which RuvAB-mediated branch migration promotes the translocation of the junction until specific resolution sequences are encountered that can be cleaved by RuvC [22]. Alternatively, helix destabilisation may play a direct role in RuvC-mediated resolution since it is known that the RuvA and RuvB proteins exhibit DNA helicase activity in vitro [23]. The presence of Mn2+, alkaline pH or elevated temperature could in part replace a requirement for RuvAB. Further experiments will focus on the protein—protein interactions that occur between RuvA, RuvB and RuvC in order to elucidate the specific requirements needed for efficient Holliday junction processing.

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