Sequence and Functional-Group Specificity for Cleavage of DNA Junctions by RuvC of *Escherichia coli*

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ABSTRACT: RuvC is the DNA junction-resolving enzyme of *Escherichia coli*. While the enzyme binds to DNA junctions independently of base sequence, it exhibits considerable sequence selectivity for the phosphodiester cleavage reaction. We have analyzed the sequence specificity using a panel of DNA junctions, measuring the rate of cleavage of each under single-turnover conditions. We have found that the optimal sequence for cleavage can be described by (A∼T)TT↓(C>G∼A), where ↓ denotes the position of backbone scission. Cleavage is fastest when the cleaved phosphodiester linkage is located at the point of strand exchange. However, cleavage is possible one nucleotide 3′ of this position when directed by the sequence, with a rate that is 1 order of magnitude slower than the optimal. The maximum sequence discrimination occurs at the central TT in the tetranucleotide site, where any alteration of sequence results in a rate reduction of at least 100-fold and cleavage is undetectable for some changes. However, certain sequences in the outer nucleotides are strongly inhibitory to cleavage. Introduction of base analogues around the cleavage site reveals a number of important functional groups and suggests that major-groove contacts in the center of the tetranucleotide are important for the cleavage process. Since RuvC binds to all the variant junctions with very similar affinity, any contacts affecting the rate of cleavage must be primarily important in the transition state. Introduction of the optimal cleavage sequence into a three-way DNA junction led to relatively efficient cleavage by RuvC, at a rate only 3-fold slower than the optimal four-way junction. This is consistent with a protein-induced alteration in the conformation of the DNA.

Homologous genetic recombination is important in DNA rearrangements and in the repair of double-strand breaks in DNA.The pathway is probably best understood in *Escherichia coli* (*1*), where a four-way junction generated by the strand exchange action of RecA undergoes branch migration catalyzed by RuvAB (*2*−*6*) and is ultimately resolved into duplex species by RuvC (*7, 8*).

RuvC is a member of the class of junction-resolving enzymes, reviewed in White et al. (*9*). These enzymes are relatively small proteins (14−41 kDa) that act in dimeric form to generate paired cleavages in four-way DNA junctions. They exhibit strong structural specificity for DNA junctions, and in general complexes of these junction-resolving enzymes with DNA junctions are not displaced by a 1000-fold excess of duplex DNA of the same sequence. By contrast, as a class the junction-resolving enzymes are indifferent to the sequence of the junction in terms of binding affinity. Thus the interaction is fundamentally a structure-selective one, requiring molecular recognition of the DNA conformation.

When it comes to the cleavage reaction, however, some of the junction-resolving enzymes exhibit considerable sequence specificity. For example, while CCE1 of *Saccharomyces cerevisiae* will bind to any four-way DNA junction with equal affinity, it cleaves most efficiently at the sequence 5′ ACT↓A (where ↓ indicates the cleavage site), with the fastest rate of cleavage when the scissile bond is presented at the point of strand exchange (*10*). Similarly, RuvC (*11*) and RusA (*12, 13*) exhibit considerable specificity in terms of the sequences at which cleavage can occur. To dissect the sequence selectivity of CCE1 in some detail we have previously employed a method in which the cleavage rates of DNA junctions are determined under single-turnover conditions while the base sequence and critical functional groups are systematically varied. In the present paper we have applied this approach to analyze the determinants of sequence selectivity for RuvC, from which we have found that the optimal sequence for cleavage can be described by (A∼T)TT↓(C>G∼A). The fastest rate of cleavage occurs when the cleavage site is located at the point of strand exchange, although some cleavage can occur one nucleotide to the 3′ side if directed by the sequence. The optimal cleavage site is also cleaved at a significant rate when incorporated into a three-way DNA junction.

RESULTS

RuvC Cleaves the DNA Backbone Most Efficiently at the Point of Strand Exchange. Preliminary experiments (data not shown) revealed that a junction containing the sequence 5′-TTT↓C (where ↓ indicates the position of cleavage) was cleaved efficiently by RuvC. Junction RC1 was synthesized containing this sequence with the cleavage site located at the point of strand exchange (Figure 1A). The bases of the

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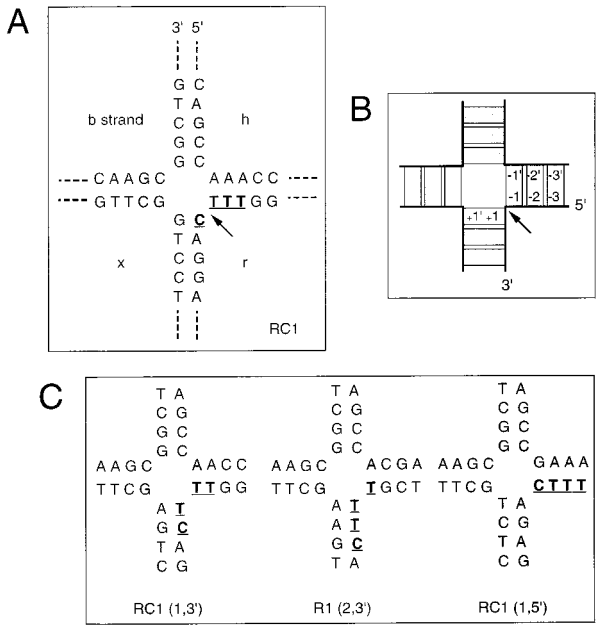

FIGURE 1: Junction RC1, the parent sequence used in these studies. (A) Central sequence of junction RC1. The RuvC target sequence TTTC is shown underscored. The junction is constructed by hybridization of four strands each of 30 nt, called b, h, r, and x as shown. The point of strand exchange lies at the center of each strand, thus creating a junction with four arms each of 15 bp in length. For the RuvC cleavage experiments the r strand is radioactively 5′-^{32}P-labeled. The position of RuvC cleavage is indicated by the arrow. (B) Numbering scheme for the RuvC recognition sequence. (C) Variant four-way junctions in which the TTTC sequence is shifted relative to the point of strand exchange. Only the central sequences are shown.

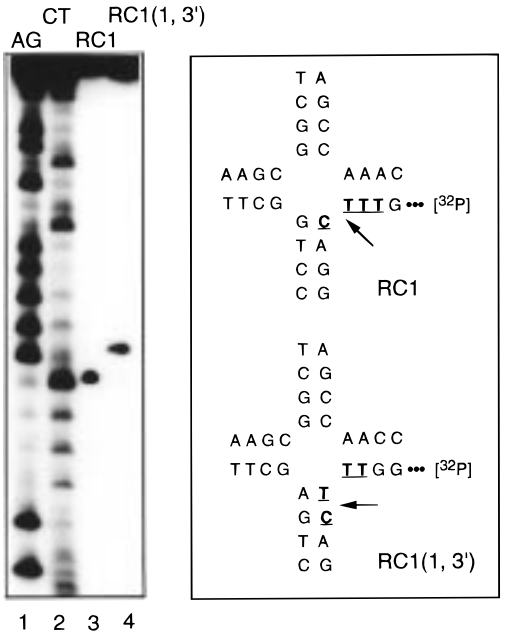

FIGURE 2: Cleavage of four-way DNA junctions by RuvC as a function of the location of the TTTC sequence relative to the point of strand exchange. Junctions RC1 and RC1 (1,3′) radioactively 5′-^{32}P-labeled on the r strands were incubated with RuvC, and the products were separated by electrophoresis on a sequencing gel. The autoradiograph is shown. The deduced positions of RuvC cleavage are indicated by the arrows on the schematics shown on the right. The sequence markers were generated by chemical cleavage of RC1 r strand. Lane 1, A + G sequence marker; lane 2, C>T sequence marker; lane 3, RuvC cleavage of junction RC1; lane 4, RuvC cleavage of junction RC1 (1,3′).

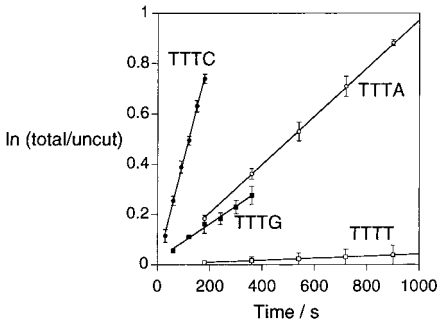

FIGURE 3: Reaction progress plots for RuvC cleavage of junction RC1 and sequence variants in the +1/+1′ position. Single-turnover kinetic analysis was carried out as described under Materials and Methods. Four-way DNA junctions radioactively 5′-^{32}P-labeled on the r strands at a concentration of 80 nM were incubated with 800 nM RuvC in 20 mM Tris (pH 8), 15 mM $MgCl_2$, 20 mM NaCl, 0.2 mM DTT, and 0.1 mg/mL BSA at 37 °C. Samples were removed at different times and the extent of cleavage was determined by sequencing gel electrophoresis and phosphorimaging. The data are plotted as the natural logarithm of the ratio (total junction/uncleaved junction) as a function of time of cleavage. The first-order rate constant for the cleavage reaction was obtained from the slope of the line fitted to the data by linear regression. The data are means of triplicate measurements, and the error bars are the standard deviations. The calculated rate constants are presented in Table 1.

tetranucleotide sequence are designated −3, −2, −1, and +1, indicating their position relative to the point of cleavage, and their base-pairing partners are indicated by primes (Figure 1B). Derivatives of this junction were made in which the TTTC sequence was repositioned in order to shift the cleavage site one nucleotide 5′ [junction RC1(1,5′)], one nucleotide 3′ [junction RC1(1,3′)], or two nucleotides 3′ [junction RC1(2,3′)] to the point of strand exchange (Figure 1C). It was found that RuvC acted efficiently when the cleavage site was positioned at the point of strand exchange or one nucleotide 3′ to it (Figure 2) The cleavage rates determined by kinetic analysis under single-turnover conditions were 416×10^{-5} s^{-1} for RC1, 56.6×10^{-5} s^{-1} for RC1(1,3′), and 0.47×10^{-5} s^{-1} for RC1(2,3′). While the enzyme was tolerant of moving the cleavage site in the 3′ direction, no cleavage was detected for junction RC1(1,5′). Mapping the position of cleavage by analysis of the products by electrophoresis in denaturing sequence gels revealed that RuvC cleaved at the same sequence in all cases except that of junction RC1(2,3′), where cleavage was found to occur at TT↓TC in addition to TTT↓C; however, in both cases the rate of this cleavage was very slow.

Effect of Changes to Consensus Sequence. Having established that a junction containing the TTT↓C sequence (with the cleavage site located at the point of strand exchange) is the most rapidly cleaved by RuvC, we varied this sequence systematically by single changes of base pairs and analyzed the kinetics of cleavage by this enzyme. Junctions RC2−RC13 were derived from the sequence of junction RC1 by alteration of one nucleotide at one of the four positions together with the necessary alterations to the complementary strand to maintain Watson−Crick base pairing. Cleavage rates were determined under single-turnover conditions; examples of the kinetic data are shown in Figure 3, and the results are summarized in Table 1. Differences in activation free energy ($\Delta\Delta G^{\circ\ddagger}$) for the cleavage of the altered sequences

Table 1: First-Order Rate Constants and $\Delta\Delta G^{\circ\ddagger}$ Values for Junction RC1 and Derivatives[a]

position	A	C	T	G
−3	348 (0.11)	4.4 (2.8)	416	3.1 (3.0)
−2	3.6 (2.9)	4.0 (2.9)	416	4.4 (2.8)
−1	2.2 (3.23)	<0.1 (>5)	416	<0.1 (>5)
+1	97.8 (0.89)	416	4.2 (2.83)	78.8 (1.03)

[a] First-order rate constants were determined under single-turnover conditions in triplicate as described and are expressed in reciprocal seconds $\times 10^{-5}$. The numbers in parentheses are calculated differences in activation free energy ($\Delta\Delta G^{\circ\ddagger}$ values in kilocalories per mole) derived by comparison to the fastest cutting sequence (junction RC1). These and all kinetic experiments reported in this paper were performed in triplicate, and standard errors were calculated (e.g., see Figure 3). Experimental error was generally found to be less than 10%.

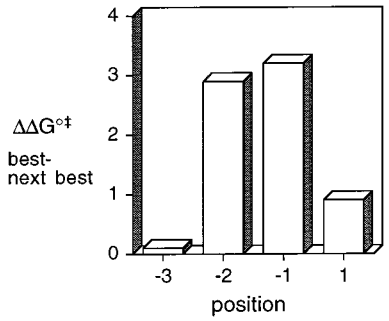

FIGURE 4: Sequence discrimination by RuvC as a function of nucleotide position in the tetranucleotide cleavage sequence. The difference in free energy of activation ($\Delta\Delta G^{\circ\ddagger}$, kilocalories per mole) between the fastest and second fastest cutting sequence variant is plotted as a function of nucleotide position in the cleavage site of junction RC1.

(rate constant k_m) relative to that of the TTT↓C sequence (rate constant k_s) are calculated from

$$\Delta\Delta G^{\circ\ddagger} = -RT \ln (k_m/k_s) \quad (1)$$

The analysis confirms that the preferred sequence for RuvC cleavage is TTT↓C; all changes to this sequence lead to lower rates of cleavage. However, the discrimination between the best and next-best sequences is not the same at all positions, as shown in Figure 4. From this point of view the greatest discrimination occurs at the −2 and −1 positions, with $\Delta\Delta G^{\circ\ddagger}$ (best/next-best) of 2.8 and 3.2 kcal mol^{-1}, respectively. The difference in cleavage rate at the −3 position between TTT↓C and ATT↓C is small, corresponding to a $\Delta\Delta G^{\circ\ddagger}$ of only 0.11 kcal mol^{-1}. The discrimination at the +1 position is also relatively weak, except against T, since the effect of replacing C with either A or G corresponds to $\Delta\Delta G^{\circ\ddagger} \leq 1$ kcal mol^{-1}. The consensus cleavage sequence can therefore be written (A∼T)TT↓(C>G∼A).Thus C and G ($\Delta\Delta G^{\circ\ddagger}$ = 2.8 and 3.0 kcal mol^{-1}, respectively) are unfavorable at −3 and T is unfavorable at +1 ($\Delta\Delta G^{\circ\ddagger}$ = 2.8 kcal mol^{-1}). The most severely discriminatory position remains −1, where substitution by a G·C base pair in either orientation results in undetectable rates of cleavage ($\Delta\Delta G^{\circ\ddagger}$ > 5 kcal mol^{-1}). In terms of discriminating the best and worst sequence at each position, −1 is clearly the most stringent, while there is little to choose between the other three positions.

Double Substitutions in the Sequence Have Approximately Additive Effects. To investigate whether the interactions between the enzyme and the DNA at the different nucleotide positions are independent of each other, two junctions (RC37 and RC38) were synthesized with alterations at two of the positions in the sequence. In junction RC37 the double change to TGT↓G resulted in a $\Delta\Delta G^{\circ\ddagger}$ of 4.2 kcal mol^{-1}, which is reasonably close to the sum of the $\Delta\Delta G^{\circ\ddagger}$ values of the two equivalent single changes (TTT↓G = 1.0 kcal mol^{-1}, TGT↓C = 2.8 kcal mol^{-1}) of 3.8 kcal mol^{-1}. This was also true for junction RC38, where a double change to CTT↓A resulted in $\Delta\Delta G^{\circ\ddagger}$ of 4.6 kcal mol^{-1} (approximately the sum of the components, i.e., CTT↓C = 2.8 kcal mol^{-1}, TTT↓A = 0.9 kcal mol^{-1}, sum = 3.7 kcal mol^{-1}). These results suggest that the interactions at each position are to a first approximation independent of one another, with changes at each position having additive changes in activation free energy.

Table 2: First-Order Rate Constants and $\Delta\Delta G^{\circ\ddagger}$ Values for Derivatives of Junction RC1 with Mismatches and Base Analogues[a]

position	junction	sequence h	sequence r	k_c ($\times 10^{-5}$ s^{-1})	$\Delta\Delta G^{\circ\ddagger}$ (kcal mol^{-1})
−3	RC14	G	T	5.5	2.7
	RC15	T	T	272	0.3
	RC16	^{7C}A	T	101	0.9
	RC17	2AP	T	3.0	3.0
	RC26	A	C	13	2.1
	RC27	A	A	5.5	2.7
	RC28	A	U	164	0.6
	RC35	T	G	1.2	3.6
	RC36	C	A	114	0.8
−2	RC18	G	T	210	0.4
	RC19	T	T	111	0.8
	RC20	^{7C}A	T	64	1.2
	RC21	2AP	T	29	1.6
	RC29	A	C	143	0.7
	RC30	A	A	25	1.7
	RC31	A	U	30	1.6
−1	RC22	G	T	43	1.4
	RC23	T	T	311	0.2
	RC24	^{7C}A	T	30	1.6
	RC25	2AP	T	44	1.4
	RC32	A	C	329	0.1
	RC33	A	A	79	1.0
	RC34	A	U	236	0.4

[a] ^{7C}A = 7-deazaadenine and 2AP = 2-aminopurine.

Introduction of Base-Pair Mismatches and Base Analogues. In an effort to gain a further insight into the recognition of the cleavage sequence, a series of four-way junctions were synthesized where single nucleotides at positions −1, −2, and −3 were changed without changing the base-pairing partner (i.e., in the nucleotides at the positions denoted by primes). This resulted in the generation of non-Watson−Crick base mismatches. All of these junctions appeared to assembled correctly insofar as having normal electrophoretic mobility during purification in polyacrylamide, and they were bound with similar affinities as junction RC1 by RuvC (refer ahead to Table 3). We also constructed junctions in which one of the nucleotides was substituted by a base analogue in order to probe the role of selected functional groups. Cleavage rates were measured under single-turnover conditions and are summarized in Table 2. We note that single nucleotide changes at positions −1 and −2 had smaller effects than when both base-pairing partners were changed simultaneously.

Variation at Position −3, −3′. From the data presented in Table 1, RuvC appears to cleave most efficiently with an A·T base pair in either orientation at position −3. Thus TTT↓C (junction RC1) and ATT↓C (junction RC3) are cleaved at similar rates, while changes to CTT↓C or GTT↓C both result in $\Delta\Delta G^{\circ\ddagger}$ values of about 3 kcal mol^{-1}. To try to dissect this further, base mismatches were introduced. The biggest effects came when a guanine was introduced on either of the strands or when the adenine (−3′) was replaced by 2-aminopurine. These changes resulted in 100-fold lower cleavage rates, corresponding to $\Delta\Delta G^{\circ\ddagger} \sim 3$ kcal mol^{-1}. This could be due to the altered geometry of the wobble base pairs (*14*) or perhaps to the intrusion of the 2-NH_2 groups into the minor groove. An A·A mismatch at −3 also severely reduced the cleavage rate, while a T·T mismatch had a very small effect. Replacement of the T (at −3) by dU resulted in only a 3-fold reduction in cleavage rate, suggesting that contact with the 5-methyl group in the major groove is not very important. Replacement of adenine (−3′) by 7-deazaadenine resulted in a 4-fold reduction in cleavage rate ($\Delta\Delta G^{\circ\ddagger} = 0.9$ kcal mol^{-1}). This is a relatively small effect on RuvC cleavage, much smaller than the A to G transition, for example, and suggests that the adenine N7 does not make a critical contact with the protein in the transition state.

Variation at Position −2, −2′. RuvC appears to recognize thymine specifically at position −2, with a change to any other Watson−Crick base pair resulting in a 100-fold reduction in cleavage rate (Table 1). However, individually changing bases led to relatively small reductions in rate (2−4-fold) except for changing the thymine (−2) to adenine, which resulted in a 16-fold rate reduction. These effects are evidently nonadditive. For example, changing T·A at this position to T·G or C·A gave rate changes corresponding to $\Delta\Delta G^{\circ\ddagger} = 0.4$ and 0.7 kcal mol^{-1} respectively, while changing both bases to give the base pair C·G led to a $\Delta\Delta G^{\circ\ddagger} = 2.9$ kcal mol^{-1}. Although replacing the adenine (−2′) with guanine (to form a T·G mismatch) had a very small effect on cleavage rate (2-fold only), substitution by 2-aminopurine had a much larger effect (14-fold reduction). Substitution of the T·A base pair to generate the two possible homobase mispairs gave 4-fold and 17-fold rate reductions for T·T and A·A mismatches, respectively. Removal of the thymine (−2) 5-methyl group (dU·A pair) led to a 14-fold reduction in cleavage rate, while replacement of the adenine (−2′) with 7-deazaadenine gave a 6.5-fold rate reduction.

Variation at Position −1, −1′. As we have noted above, the −1 position exhibits the tightest discrimination of any in the tetranucleotide recognition element. Any base other than thymine at this position results in a reduction in cleavage rates of 200-fold or more when normal base pairing is maintained ($\Delta\Delta G^{\circ\ddagger} \geq 3.2$ kcal mol^{-1}). When a G·C base pair was introduced in either orientation, no cleavage could be detected whatsoever. Therefore the $\Delta\Delta G^{\circ\ddagger}$ value for this change is at least 5 kcal mol^{-1}, based on an estimation of a lower limit of rate measurement of 10^{-6} s^{-1} for the assay. However, introducing base mismatches had much smaller effects, with the largest resulting from replacement of T·A by T·G with a rate reduced by a factor of 10-fold ($\Delta\Delta G^{\circ\ddagger} = 1.4$ kcal mol^{-1}). In general, the thymine (−1) could be replaced with other pyrimidines with only small effects on cleavage rate ($\Delta\Delta G^{\circ\ddagger} \leq 0.35$ kcal mol^{-1}), and substitution by deoxyuracil led to a rate reduction of only 1.8-fold, indicating that a contact with the 5-methyl group is not important. Replacement of adenine (−1′) with 7-deazaadenine (^{7C}A)[1] gave a 14-fold slower rate of cleavage. This suggests that the N7 of adenine (−1′) is important for transition state contacts by RuvC, although the base can be replaced by thymine (giving a T·T mismatch) with very little effect on cleavage rate. In general, substitution of the adenine (−1′) tended to give larger effects on cleavage rate compared to thymine (−1) substitutions.

Variation at Position +1, +1′. Attempts were made to dissect sequence recognition at this position. However, introducing base mismatches led to the site of cleavage shifting to one nucleotide 3′ of the junction in many cases, thus preventing systematic analysis.

Influence of Junction Sequence on RuvC Binding Affinity. Electrophoretic retardation analysis was used to investigate the binding affinity of RuvC (as described under Materials and Methods). In common with other junction-resolving enzymes (*15, 16*), RuvC forms two retarded complexes with different mobilities (Figure 5A). Complex I appears at lower concentrations, while a further complex that is retained in the wells appears at higher protein concentrations. The binding data were quantified and fitted to a simple model in which dimeric RuvC binds to the junction (Figure 5B), from which a dissociation constant could be calculated (see Materials and Methods). The experimental data exhibited binding over a narrower range of protein concentration compared to the theoretical model; this could indicate a component arising from monomer−dimer equilibrium for the protein. This analysis was repeated for a number of variant four-way junctions. It was found that RuvC bound with similar affinity (all within 1.1 kcal mol^{-1}) to a number of different junctions used in this study, as summarized in Table 3. No correlation was seen between binding affinity and cleavage rate. The apparent dissociation constant was close to 2 nM over a range of DNA junctions, comparable to the affinity of CCE1 for four-way DNA junctions (*17*).

Binding and Cleavage of Three-Way DNA Junctions by RuvC. West and co-workers observed some cleavage of incomplete four-way junctions assembled from three strands, but no cleavage was found in perfect three-way junctions (*18*). By contrast, some activity of RuvC on three-way DNA junctions was found by Shinagawa and co-workers (*19*). In the latter study a requirement for homology was claimed; however, homology has no meaning in a three-way junction, and this is more likely to reflect a stringent sequence requirement for cleavage. To resolve this issue we have examined the cleavage of three-way DNA junctions by RuvC, using a variety of junctions containing the consensus sequence optimized for the four-way junction. A perfect three-way junction [i.e., a 3H junction (*20*)] was generated by hybridizing a new single strand (h′r′) to the h and r strands of junction RC1, thereby leaving the r and x arms containing the TTT↓C consensus sequence (Figure 6). The resulting junction Y0 was cleaved by RuvC at a rate that was 2.7-fold slower than that for four-way junction RC1 (Table 4). This corresponds to a $\Delta\Delta G^{\circ\ddagger}$ of only 0.6 kcal mol^{-1} and

[1] Abbreviations: K_D, dissociation constant; K_A, association constant; PCR, polymerase chain reaction; BSA, bovine serum albumin; ^{7C}A, 7-deazaadenine; 2AP, 2-aminopurine.

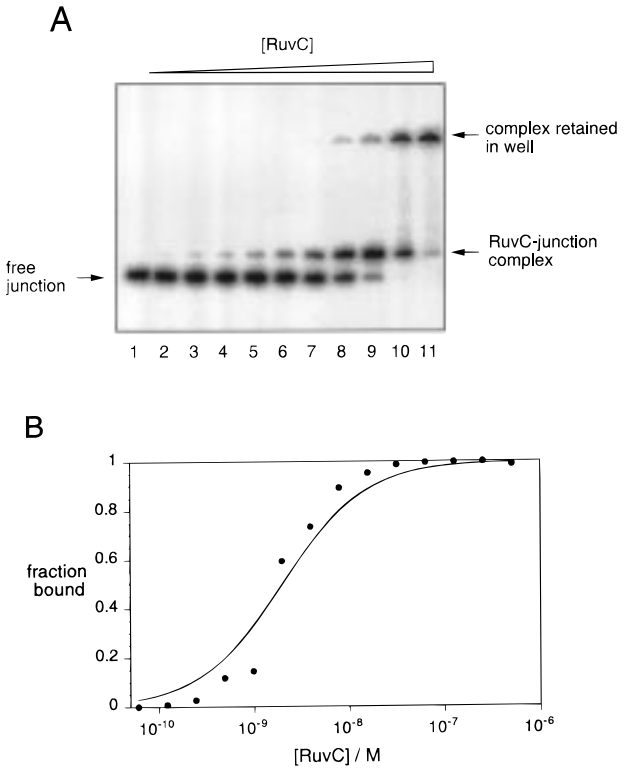

FIGURE 5: Binding of RuvC to the four-way DNA junction RC1. (A) Binding of RuvC to junction RC1 observed by electrophoretic retardation analysis. Radioactive junction RC1 (0.25 nM) was incubated with a series of RuvC concentrations as described under Materials and Methods. Free junction and complexes were separated by electrophoresis in 5% polyacrylamide under nondenaturing conditions. The dried gel was exposed to a storage phosphor screen, and the phosphorimage is presented. The following RuvC concentrations were used in the incubations: lane 1, 61 pM; lane 2, 0.12 nM; lane 3, 0.24 nM; lane 4, 0.49 nM; lane 5, 0.98 nM; lane 6, 2 nM; lane 7, 3.9 nM; lane 8, 7.8 nM; lane 9, 16 nM; lane 10, 31 nM; lane 11, 63 nM. (B) Isotherm for RuvC binding to junction RC1. Plot of the fraction of RuvC-bound junction as a function of RuvC concentration in the incubation. The experimental data are shown by the points (•), and the line was obtained by fitting the binding to eq 2 by nonlinear regression. The experimental binding is clearly a sharper function of RuvC concentration than that of the simple binding model, indicating an element of cooperativity in the interaction. Apparent dissociation constants calculated from these and related data are presented in Table 3.

Table 3: Binding Affinity of RuvC to a Selection of Four-Way Junctions[a]

junction	description	K_D[a] (nM)	$\Delta\Delta G^\circ_{bind}$ (kcal mol^{-1})	$\Delta\Delta G^{\circ\ddagger}$ (kcal mol^{-1})
RC1	reference	1.9		
RC1 (1,3′)	TTTC moved 1 nt 3′	0.9	−0.46	1.2
RC1 (2,3′)	TTTC moved 2 nt 3′	2.5	0.17	4.2
RC17	2AP at −2	0.3	−1.1	3.0
RC29	C·A mismatch at −2	1.0	−0.40	0.7
RC32	C·A mismatch at −1	0.7	−0.62	0.1

[a] K_D represents an apparent dissociation constant obtained by fitting to a simple binding model.

represents a significant rate of cleavage of the three-way junction. The affinity of binding for a perfect three-way junction was approximately 10-fold lower than that for the four-way junction that contains the same sequence. If we define a specificity parameter as k_c/K_D we obtain values of 2.2×10^6 and 6.9×10^4 M^{-1} s^{-1}, respectively, for the four- and three-way junctions, i.e., there is a 32-fold reduction in specificity for the three-way junction.

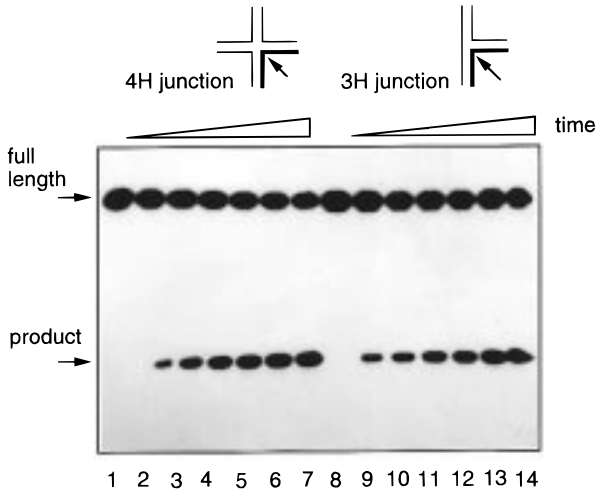

FIGURE 6: Comparison of RuvC cleavage of three- and four-way DNA junctions. Radioactively labeled 3H and 4H junctions were incubated with RuvC as described under Materials and Methods, and aliquots were removed at 30 s intervals. The products were analyzed by electrophoresis in a 12% polyacrylamide gel under denaturing conditions, and the autoradiograph is shown. Lanes 1−7, cleavage of the four-way junction RC1; lanes 8−14, cleavage of the three-way junction Y0. Lanes 1 and 8, junction incubated with RuvC in the presence of EDTA; lanes 2−7 and 9−14, junction incubated with RuvC in the presence of 15 mM $MgCl_2$ for 30, 60, 90, 120, 150, and 180 s, respectively.

Table 4: Binding Affinities and Cleavage Rates of Three-Way Junctions by RuvC[a]

junction	K_D[a] (nM)	k_c ($\times 10^{-5}$ s^{-1})	$\Delta\Delta G^\circ_{bind}$ (kcal mol^{-1})	$\Delta\Delta G^{\circ\ddagger}$ (kcal mol^{-1})
Y0	22	154	1.5	0.61
YA1	20	217	1.4	0.40
YA2	20	127	1.4	0.73
YA3	10	165	1.0	0.57
YA4	14	160	1.2	0.59

[a] K_D represents an apparent dissociation constant obtained by fitting to a simple binding model.

The structure of three-way DNA junctions is profoundly affected by the presence of additional unpaired bases located at the point of strand exchange (*21*−*23*), whereby the additional conformational flexibility allows pairwise helical stacking between arms. We therefore added oligoadenine insertions (A_n, where $n = 1$, 2, 3, or 4) at the center of the h′r′ strand (Figure 6), thus generating $3HS_n$ ($n = 1$−4) junctions. This resulted in small increases in binding affinity that are probably due to the increased flexibility of these junctions. However, we observed very little alteration in the cleavage rate (Table 4), consistent with an opening of the structure by the enzyme. When binding to three-way junctions, RuvC adopts complex I only over a narrow range of protein concentration. The appearance of complex II occurs at a similar protein concentration as that for four-way junctions, indicating that the formation of complex II is considerably less structure-specific than that of complex I. Fitting to the simple binding model (Figure 7) showed a greater deviation between theory and experiment, possibly indicating a greater degree of cooperativity in binding for the three-way junction.

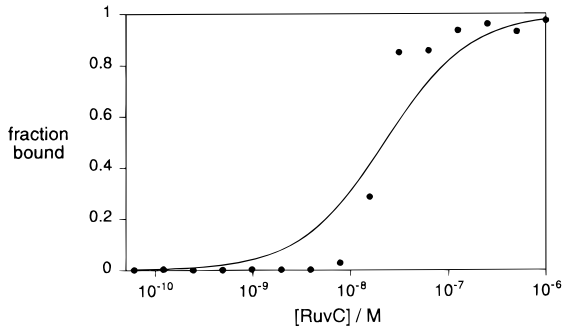

FIGURE 7: Isotherm for RuvC binding to a three-way DNA junction Y0. The extent of binding for different RuvC concentrations was analyzed by electrophoretic retardation analysis and plotted as a function of RuvC concentration. The experimental data are shown by the points (•), and the line was obtained by fitting the binding to eq 2 by nonlinear regression. As with the four-way junction, the experimental binding is clearly a sharper function of RuvC concentration than that of the simple binding model, indicating considerable cooperativity in the interaction, probably due to monomer−dimer equilibria in RuvC. Apparent dissociation constants calculated from these and related data are presented in Table 4.

DISCUSSION

DNA Sequence Specificity for Cleavage of Junctions by RuvC. Our results confirm that RuvC exhibits strong sequence specificity for the resolution cleavage of DNA four-way junctions, despite binding with equal affinity to junctions of any sequence. Using a single-turnover kinetic assay, we have determined the optimal cleavage site to be (A∼T)TT↓(C>G∼A). This is in reasonable agreement with the conclusions of West and colleagues (*11*) by analysis of cleavage within a junction that could sample many sequences by branch migration, where a consensus sequence of (A∼T)TT↓(G∼C) was deduced. Using our kinetic analysis, we found that variants of the site other than those defined by the optimal sequence were cleaved at least 95-fold more slowly, corresponding to $\Delta\Delta G^{\circ\ddagger}$ values of around 3 kcal mol^{-1}. This is of the order of the energy of a hydrogen bond and indicates specific interaction between the protein and the DNA in the transition state.

The crystal structure of RuvC has been solved (*24*). In this structure two highly conserved lysine residues (K107 and K118) are among a number of basic residues in close proximity to the acidic residues thought to be involved in coordinating Mg^{2+} within the active site (*25*). These two residues are on the same side of the putative DNA binding cleft and are in a position to make specific hydrogen bonds with the bases close to the cleavage site. It is therefore conceivable that they are involved in the sequence recognition. Hagan et al. (*26*) have shown that changes in glycine (G114) and alanine (A116) residues that would be expected to alter the relative positioning of these residues led to changed sequence selectivity. It has also been found that mutation of lysine 118 led to loss of RuvC activity [unpublished data reported in Ariyoshi et al. (*24*)].

The major candidates for making such interactions in the DNA are the −1/−1′ and −2/−2′ positions, where sequence discrimination is greatest. A detailed analysis of the effects of all the sequence variants is not possible, and we cannot explain all the data at present. In particular, the effects of mismatches are difficult to rationalize in a number of cases, although we recognize that these can be complicated by the changes in local DNA structure that they impose (reviewed in refs *27* and *28*). However we note that in the critical −1 and −2 positions the effect of introducing mismatches is often to relax the discrimination. For example, the effect of reversing the T•A base pairs at −1/−1′ and −2/−2′ positions to A•T is a reduction in cleavage rate of 190- and 116-fold, respectively. Yet placing an A•A mismatch at these positions gives rate reductions of only 5- and 17-fold, respectively, while the corresponding T•T mismatches at these positions lead to even smaller rate reductions of 1.3- and 3.7-fold, respectively. Perhaps the clearest data come from functional group changes, especially those not expected to alter the basic DNA structure. The methyl group at the C5 of thymine (−2) appears to interact with the protein since its removal by deoxyuracil replacement leads to a 14-fold reduction in rate ($\Delta\Delta G^{\circ\ddagger}$ = 1.6 kcal mol^{-1}). This suggests a hydrophobic contact in the transition state. Substitution of the complementary adenine (−2′) by 7-deazaadenine led to a 7-fold slower cleavage rate, which could indicate an interaction with adenine N7. We have noted that sequence discrimination is greatest at the −1 position. The N7 of the complementary adenine (−1′) appears to be important in the transition state since the 7-deazaadenine-substituted variant at this position is cleaved 14-fold more slowly than the optimal sequence. Taken together, these effects suggest that major-groove recognition could be important at the −2 and −1 positions.

Variation of Cleavage Position and Conformational Flexibility of the RuvC−Junction Complex. We have found that RuvC has some flexibility in terms of its site of cleavage relative to the point of strand exchange. By changing the position of the optimal sequence relative to the junction center it was found that RuvC is able to cleave at three different positions. Cleavage was most efficient when it occurred immediately at the point of strand exchange. However, the measured cleavage rate one nucleotide 3′ to the center (i.e., within a formally double-stranded region) was only reduced by a factor of 7 ($\Delta\Delta G^{\circ\ddagger}$ = 1.23 kcal mol^{-1}), which is actually rather less than that resulting from many changes in sequence. Exceptionally, RuvC can also cleave two nucleotides removed from the junction center in the 3′ direction; however, this is energetically unfavorable ($\Delta\Delta G^{\circ\ddagger}$ = 4.18 kcal mol^{-1}). It is possible that there is sufficient flexibility in RuvC to allow reorientation of the active site on the DNA, although this does not seem probable. Distortion of the DNA seems a more likely explanation for these data. In fact, it has been observed that all the junction-resolving enzymes studied to date substantially distort the global structure of DNA junctions on binding (*13, 17, 26, 29−32*). Comparative gel electrophoresis experiments suggest that RuvC changes the structure of the junction into an open form of an antiparallel X-shape, and chemical reactivity to permanganate indicates a loss of base stacking at the center (*30*). Such opening of the center could facilitate the repositioning of the junction sequence within the active site of the enzyme.

This flexibility of RuvC action is further underlined by its ability to bind and cleave three-way DNA junctions, which

are characterized by a very different DNA structure in the absence of proteins (*22, 23, 33*−*36*). Three-way junctions containing the optimal RuvC sequence are cleaved at rates within a factor of 2−4 of the four-way junction and are bound with an affinity about 10-fold lower (a $\Delta\Delta G^{\circ}_{bind}$ of only $\leq$1.5 kcal mol^{-1}). Once again it seems that the protein can impose the structure required for cleavage on the DNA. The binding and cleavage of the perfectly paired 3H junction is particularly revealing, since the free DNA is unstacked under all conditions (*22, 23, 33*). The relatively efficient cleavage of this junction indicates that an open conformation is an acceptable substrate for RuvC.

Relationship between Sequence Dependence and Structural Dependence. The first junction-resolving enzymes to be discovered were the bacteriophage proteins T4 endonuclease VII (*37*) and T7 endonuclease I (*38, 39*), and these were found to have a relatively weak sequence preference for cleavage. However, enzymes isolated subsequently have generally been found to have significant sequence selectivity in terms of their cleavage reactions while remaining fundamentally structure selective in terms of binding. CCE1 of *S. cerevisiae* exhibits strong sequence selectivity in the cleavage of DNA junctions, with an optimal sequence of ACT↓A (*10*), and RuvC has a comparable level of sequence discrimination. We note that the enzymes exhibiting sequence-selective junction cleavage in general bind with higher affinity than the less selective phage enzymes; RuvC, CCE1 (*17*), YDC2 (*16*), and RusA (*13*) all bind with affinities in the range K_D = 1.6−5 nM, while T4 endonuclease VII is significantly weaker at K_D = 40 nM (*40*). However, this difference could be coincidental.

When RuvC was first isolated, it was reported that the enzyme was selective for DNA junctions containing significant stretches of internal homology (*18, 19, 41*), in contrast with the previously studied phage enzymes. However, we see that this is really not a fundamental distinction of this enzyme. Relatively good rates of cleavage can be obtained on junctions that cannot undergo a single step of branch migration and even on three-way DNA junctions. Nevertheless, the effect of a strong sequence preference tends to provide a de facto selection for homologous four-way junctions. Only these junctions can undergo branch migration and thus display a series of sequences in different orientations relative to the point of strand exchange for the enzyme to sample. Only six out of 256 sequences in a random tetranucleotide match the RuvC optimal sequence, and this selectivity therefore precludes the cleavage of the majority of structures other than homologous four-way junctions on a simple statistical basis. Enzymes such as RuvC and CCE1 are therefore directed to branched DNA by their strong structural selectivity, but their cleavage activity is subject to a powerful filter imposed by the sequence selectivity. Thus these enzymes will be effectively restricted to acting upon the kinds of DNA intermediates important in homologous recombination. By contrast the lower sequence filtering of the phage enzymes results in a wider range of substrates, consistent with their more general role in the processing of branch points in replicated DNA. This wider activity could be deleterious in a cellular enzyme, and this is clearly demonstrated by the strong toxicity of T4 endonuclease VII and T7 endonuclease I when overexpressed in *E. coli*. However, sequence selectivity is not the sole preserve of the cellular enzymes, since RusA [which is of lambdoid phage origin (*42*)] exhibits a significant sequence preference for cleavage at ↓CC (*12, 13*).

In summary, we find that the cleavage sequence specificity of RuvC is very pronounced, comparable to that of CCE1 of *S. cerevisiae*. The discrimination is strongest at the central two base pairs of the tetranucleotide recognition sequence, where major-groove contacts appear to be important in the transition state. The result of this strong sequence selectivity is effectively to restrict cleavage to four-way DNA junctions containing homology, although this is not intrinsic to the mechanism of action of the enzyme.

MATERIALS AND METHODS

Expression and Purification of RuvC. A *ruvC* gene was amplified from *E. coli* JM101 DNA by use of the polymerase chain reaction (PCR). RuvC protein was overexpressed by inserting the *ruvC* gene into pET-19b (Novagen), which was then transformed into BL21(DE3) pLysS. Cells were grown at 37 °C to an absorbance A_{660} = 0.5 and induced by addition of IPTG to a final concentration of 0.05 M for 2 h. Cells were harvested by centrifugation and lysed by sonication. The lysate was centrifuged and the supernatant was dialyzed against 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 10% glycerol, and 1 mM DTT (R buffer) supplemented with 0.1 M KCl. The precipitate was resuspended in R buffer supplemented with 0.5 M KCl and applied to a gel-filtration column. Fractions were analyzed by polyacrylamide gel electrophoresis in SDS. Those containing RuvC were applied to a Poros HS 20/100 ion-exchange column installed on a Bio-Cad Sprint HPLC apparatus (PerSeptive Biosystems) and eluted with a 400 mM−2 M NaCl gradient in 10 mM MES (pH 6), 1 mM EDTA, and 0.2 mM DTT. RuvC in peak fractions was essentially pure as judged by polyacrylamide gel electrophoresis in SDS-containing buffer, where only a single band was visible by Coomassie blue staining.

Oligonucleotide Synthesis. Oligonucleotides were synthesized by β-cyanoethyl phosphoramidite chemistry (*43, 44*) on a 394 DNA/RNA synthesizer (Applied Biosystems). 2-Aminopurine and 7-deazaadenine deoxyribonucleoside phosphoramidites were purchased from Glen Research. Following deprotection, oligonucleotides were purified by electrophoresis in 12% polyacrylamide containing 7 M urea, and DNA was recovered from gel fragments by electroelution and ethanol precipitation.

Construction of DNA Junctions. DNA junctions were constructed from four (or three, for three-way junctions) oligonucleotides each of 30 nt, to create junctions with four (or three) arms each of 15 bp in length. The r strand of each junction was radioactively 5′-^{32}P-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. Stoichiometric quantities of this strand and the three (or two) unlabeled strands were annealed by incubation in 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM DTT, 0.1 M spermine, and 0.1 mM EDTA for 3 min at 85 °C, followed by slow cooling overnight. Junctions were purified by electrophoresis in 5% polyacrylamide. Bands were excised and the DNA was recovered by electroelution and ethanol precipitation.

(A) Junctions Used To Examine Cleavage with Respect to the Point of Strand Exchange. Junctions based on the RC1

sequence contain the consensus TTTC sequence shown in boldface type, with the point of strand exchange indicated by the | symbol. All sequences are written 5′ to 3′.

Junction RC1

b strand:
TCCGTCCTAGCAAGC GGCTGCTACCGGAAG

h strand:
CTTCCGGTAGCAGCC AAACCGGTGGTTGAA

r strand:
TTCAACCACCGG**TTT** | **C**AGGAACTGCAGTCT

x strand:
AGACTGCAGTTCCTG GCTTGCTAGGACGGA

Junction RC1(1,5′)

b strand:
TCCGTCCTAGCAAGG CGCTGCTACCGGAAG

h strand:
CTTCCGGTAGCAGCG GAAACGGTGGTTGAA

r strand:
TTCAACCACCG**TTTC** | AGAGAACTGCAGTCT

x strand:
AGACTGCAGTTCTCT CCTTGCTAGGACGGA

Junction RC1(1,3′)

b strand:
TCCGTCCTAGCAAGC CGCTGCTACCGGAAG

h strand:
CTTCCGGTAGCAGCG AACCGAGTGGTTGAA

r strand:
TTCAACCACTCGG**TT** | **TC**AGAACTGCAGTCT

x strand:
AGACTGCAGTTCTGA GCTTGCTAGGACGGA

Junction RC1(2,3′)

b strand:
TCCGTCCTAGCAAGC CGCTGCTACCGGAAG

h strand:
CTTCCGGTAGCAGCG ACGACGGTGGTTGAA

r strand:
TTCAACCACCGTCG**T** | **TTC**AGACTGCAGTCT

x strand:
AGACTGCAGTCTGAA GCTTGCTAGGACGGA

(B) Junctions Used in the Study of Sequence Dependence of Cleavage. Junctions RC2− RC13 were created by synthesizing oligonucleotides with one of the bases in the consensus sequence changed, together with the necessary changes in the h or x strands in order to maintain base complementarity. Flanking sequences remained unchanged from the basic junction RC1 sequence.

junction	−3	−2	−1	+1
RC1	*T*	*T*	*T*	*C*
RC2	G	T	T	C
RC3	A	T	T	C
RC4	C	T	T	C
RC5	T	G	T	C
RC6	T	A	T	C
RC7	T	C	T	C
RC8	T	T	G	C
RC9	T	T	A	C
RC10	T	T	C	C
RC11	T	T	T	G
RC12	T	T	T	T
RC13	T	T	T	A

Junctions RC14−RC25 contain single base changes in the h strand of junction RC1 (without changes in the r strand), incorporating a mismatch or base analogue (^{7C}A, 7-deaza-adenine; 2AP, 2-aminopurine).

junction	−3′	−2′	−1′
RC1	*A*	*A*	*A*
RC14	G	A	A
RC15	T	A	A
RC16	^{7C}A	A	A
RC17	2AP	A	A
RC18	A	G	A
RC19	A	T	A
RC20	A	^{7C}A	A
RC21	A	2AP	A
RC22	A	A	G
RC23	A	A	T
RC24	A	A	^{7C}A
RC25	A	A	2AP

Junctions RC26−RC34 contain single base changes in the r strand (without changes in the h strand), incorporating a mismatch or base analogue.

junction	−3	−2	−1	+1
RC1	*T*	*T*	*T*	*C*
RC26	C	T	T	C
RC27	A	T	T	C
RC28	dU	T	T	C
RC29	T	C	T	C
RC30	T	A	T	C
RC31	T	dU	T	C
RC32	T	T	C	C
RC33	T	T	A	C
RC34	T	T	dU	C

Junction RC35 contains a single base change from the h strand of junction RC3, incorporating a base mismatch.

junction	−3′	−2′	−1′
RC3	*T*	*A*	*A*
RC35	G	A	A

Junction RC36 contains a single base change from the r strand of junction RC3, incorporating a base mismatch.

junction	−3	−2	−1
RC3	*A*	*T*	*T*
RC36	C	T	T

Junctions RC37 and RC38 contain two bases changed from the r strand of junction RC1, together with the necessary changes to the h and x strands.

junction	−3	−2	−1	+1
RC1	*T*	*T*	*T*	*C*
RC37	T	G	T	G
RC38	C	T	T	A

(C) Three-Way Junctions. Junction Y0 is a 3H three-way junction derived from junction RC1 by annealing the h and r strands of RC1 to the following oligonucleotide:

h′r′ strand:
AGACTGCAGTTCCTG | GGCTGCTACCGGAAG

The point of strand exchange in this junction is located at the position indicated by |. Junctions YA1, YA2, YA3, and YA4 are derived from junction Y0 by insertion of a one-, two-, three-, or four-adenine sequence, respectively, into the h′r′ strand sequence at the point of strand exchange.

Measurement of Binding Affinity of RuvC for DNA Junctions. A range of concentrations of purified RuvC protein was incubated with 0.25 nM radioactively labeled four-way DNA junction for 15 min in 20 mM Tris (pH 8), 20 mM NaCl, 0.2 mM DTT, and 0.1 mg/mL bovine serum albumin (BSA) (binding buffer) for 15 min at 20 °C in a total volume of 10 μL. One-sixth volume of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, and 15% Ficoll type 40) was added, and the samples were loaded onto 5% polyacrylamide gels. After electrophoresis at 120 V in 1× TBE buffer for approximately 2 h, the gels were dried on Whatmann 3MM paper and exposed to storage phosphor screens for quantification on a Fuji BAS-1500 phosphorimager with MacBAS software (Fuji). Data were analyzed as fraction DNA bound (f_b) versus protein concentration and fitted by nonlinear regression to

$$f_b = \{(1 + K_A P_T + K_A D_T) - [(1 + K_A P_T + K_A D_T)^2 - (4 D_T K_A^2 P_T)]^{1/2}\}/2 D_T K_A \quad (2)$$

where P_T is the total protein concentration, D_T is the total DNA concentration, and K_A is the association constant. The dissociation constant (K_D) is the reciprocal of K_A. At higher concentrations where a discrete further-retarded complex was visible, it was counted as bound DNA. For the calculation RuvC was considered to be a dimer in solution, although the shape of the experimental binding isotherms suggests that this is probably an oversimplification in the case of RuvC and that a monomer−dimer equilibrium is likely to exist.

Kinetic Analysis of DNA Cleavage by RuvC. Rates of DNA junction cleavage were measured with 80 nM radioactively 5′-^{32}P-labeled junction with 800 nM RuvC dimer in binding buffer in a total volume of 30 μL. Under these conditions all junction will be bound by RuvC. Samples were preequilibrated at 37 °C and the reactions were initiated by addition of $MgCl_2$ to a final concentration of 15 mM. At set time points aliquots were removed and the reaction was stopped by addition of an equal volume of 95% (v/v) formamide, 50 mM EDTA (pH 8.0), 0.1% bromophenol blue, and 0.1% xylene cyanol (formamide loading buffer) and heating at 80 °C for 4 min, followed by storage on ice. Reaction products were analyzed by denaturing gel electrophoresis on 0.4 mm thick 12% polyacrylamide gels containing 7 M urea run at 50 °C in TBE buffer. Gels were exposed to storage phosphor screens for quantification on a Fuji BAS-1500 phosphorimager. First-order rate constants were obtained by plotting ln (total/uncut) against time, generating straight lines passing through the origin, of slope k, where k is the first-order rate constant for the reaction. All rate measurements were performed in triplicate, from which mean rate constants and standard errors were calculated. Sequence markers were generated by chemical cleavage reactions (*45*).

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