

# DNA structure specificity of Rap endonuclease

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## ABSTRACT

The Rap protein of phage  $\lambda$  is an endonuclease that nicks branched DNA structures. It has been proposed that Rap can nick D-loops formed during phage recombination to generate splice products without the need for the formation of a 4-strand (Holliday) junction. The structure specificity of Rap was investigated using a variety of branched DNA molecules made by annealing partially complementary oligonucleotides. On Holliday junctions, Rap endonuclease shows a requirement for magnesium or manganese ions, with  $Mn^{2+}$  supporting 5-fold more cleavage than  $Mg^{2+}$ . The location of endonuclease incisions was determined on 3'-tailed D-loop, bubble, flayed duplex, 5'-flap and Y junction DNA substrates. In all cases, Rap preferentially cleaves at the branch point of these molecules. With a flayed duplex, incisions are made in the duplex adjacent to the single-strand arms. Comparison of binding and cleavage specificities revealed that Rap is highly structure-specific and exhibits a clear preference for 4- and 3-stranded DNA over Y and flayed duplex DNA. Almost no binding or cleavage was detected with duplex, partial duplex and single-stranded DNA. Thus Rap endonuclease shows a bias for structures that resemble D-loop and Holliday junction recombination intermediates.

## INTRODUCTION

Many bacteriophages, such as  $\lambda$ , can perform homologous recombination reactions that contribute to DNA repair and genomic variability. A critical function of the phage  $\lambda$  Red pathway of recombination is the repair of double-strand DNA breaks that can arise following exposure to ionising radiation, collapse of replication forks, restriction endonuclease incision or cleavage at *cos* by the terminase protein (1–3). Intact  $\lambda$  chromosomes are used as a template for the repair process which entails the exchange of homologous DNA strands. Broken ends are first processed by the Red $\alpha$  exonuclease which specifically degrades DNA with blunt or nearly blunt ends to expose a 3' single-strand tail (4,5). This single-strand is used by the host RecA protein to initiate a search for a homologous chromosome and initiate strand pairing and exchange (6). The resulting 3-stranded intermediate, or D-loop, contains a 3' end which can prime DNA replication (7). Nicking at specific points in the D-loop may generate a splice-type product (8,9),

whereas further strand exchange into the adjacent duplex DNA of the invading molecule creates a 4-stranded structure known as a Holliday junction (7). This Holliday structure can be eliminated by the introduction of symmetrically related incisions (7,10). Formation and resolution of Holliday junctions at each duplex end of the chromosome break with concomitant DNA replication will repair the double-strand break and restore intact chromosomes (3,11).

Recent experiments have revealed that  $\lambda$  carries a structure-specific endonuclease, Rap, that can target recombination intermediates generated by the Red system (12) (T.Tarkowski and F.W.Stahl, personal communication). The *rap* (*ninG*) gene is located in the non-essential *ninR* region of lambda (13) and highly conserved *rap* genes are found in a number of lambdoid phages including P22, H-19B,  $\phi$ 21, 933W and PS34 (GenBank accession nos X78401, AF034975, AJ237660, AF125520 and AJ011580, respectively). Deletion mutants of *rap* (recombination adept with plasmid) show a 100-fold reduction in  $\lambda$ -plasmid recombination catalysed by the host RecBCD-dependent pathway (14,15). Reduction in recombination proficiency and loss of focusing of recombination events are also exhibited by *rap* mutants in the Red pathway (T.Tarkowski and F.W.Stahl, personal communication). Purified Rap protein introduces nicks in substrates that mimic D-loop and Holliday junction recombination intermediates (12), leading to the proposal that Rap could cleave D-loops to generate the splice recombinants predicted by the break-join model (8,12). In an attempt to define which recombination intermediates Rap acts upon, we have characterised the cleavage and structure specificity of the Rap endonuclease on a range of branched DNA structures.

## MATERIALS AND METHODS

### Enzymes and reagents

Rap (29K his-tag) and RusA proteins were purified as described (12,16). Protein concentrations were determined using a modified Bradford Assay (Bio-Rad) with bovine serum albumin as a standard. Amounts of Rap and RusA are expressed as moles of protein monomer. T4 polynucleotide kinase was purchased from BRL, [ $\gamma$ - $^{32}P$ ]ATP from Amersham Pharmacia Biotech and poly(dI:dC) from Sigma. All other reagents were from BDH or Sigma and were of analytical grade.

### Oligonucleotides

Oligonucleotides were synthesised on an Applied Biosystems 380B DNA synthesiser using cyanoethyl chemistry. Each oligonucleotide was deprotected, precipitated in ethanol and

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20 µg purified on a 15% (w/v) polyacrylamide gel containing 7 M urea. The band containing the full-length oligonucleotide was visualised by UV shadowing, excised and extracted by soaking in water overnight. The sequences of the oligonucleotides used were: 1, 5'-GACGCTGCCGAATTCTGGCTTGC-TAGGACATCTTTGCCACGTTGACCC; 2, 5'-ATCGAT-AGTCTCTAGACAGCATGTCCTAGCAAGCCAGAATTC-GGCAGCGT; 3, 5'-TGGGTCAACGTGGGCAAAGATGTC-CTAGCAATGTAATCGTCTATGACGTT; 4, 5'-CAACGT-CATAGACGATTACATTGCTAGGACATGCTGTCTAGA-GACTATCGA; 5, 5'-TGGGTCAACGTGGGCAAAGATGT-CCGGACATGCTGTCTAGAGACTATCGA; 6, 5'-GACGC-TGCCGAATTCTACCAGTGCCTTGCTAGGACATCTTT-GCCACCTGCAGGTTACCC; 7, 5'-GGGTGAACCTGC-AGGTGGGCAAAGATGTCCTAGCAAGGCACTGGTAG-AATTCGGCAGCGTC; 8, 5'-AAAGATGTCCTAGCAAGG-CAC; 9, 5'-TGGGTCAACGTGGGCAAAGATGTCC; 10, 5'-GGGTGAACCTGCAGGTGGGCGGCTGCTCATCGTAGGT-TAGTTGGTAGAATTCGGCAGCGTC; and 11, 5'-TTTCTAC-AGGATCGTTCGTCGATCGACCGGATATCTATGAGA.

### Construction of junction substrates

Small branched DNA structures and relevant control substrates were made by annealing oligonucleotides (see above) varying in length from 21 to 61 nt essentially as described (17). The oligonucleotide combinations used to make each DNA substrate are shown in Table 1. Each branched structure contained a specific oligonucleotide <sup>32</sup>P-labelled at its 5' end. DNA concentrations (moles of junctions) were calculated from the specific activity of the <sup>32</sup>P-labelled oligonucleotide used in their construction. Due to the instability of certain junctions, the substrates used for mapping cleavage sites were annealed and used in assays without further purification. These substrates therefore contain a variety of unlabelled, annealed DNA species. As a result of this, direct comparisons between Rap cleavage activity on these substrates cannot be reliably estimated.

**Table 1.** Composition of branched DNA substrates

Substrate	Oligonucleotide composition	DNA structure
X junction/J12	1+2+3+4	
3-strand junction	1+2+3	
flayed duplex	1+2 or 1+3	
Y junction	1+2+5	
duplex	6+7	
partial duplex	6+8	
single strand	6	
5'-flap	1+2+9	
bubble	6+10	
3'-tailed D-loop	6+10+11	

### DNA binding assays

Bandshift assays (20 µl) were performed in binding buffer (50 mM Tris-HCl pH 8.0, 5 mM EDTA, 1 mM dithiothreitol, 5% glycerol and 100 µg/ml BSA) using 0.25–0.5 nM of

<sup>32</sup>P-labelled DNA substrate. Samples were incubated on ice for 15 min before loading 12 µl on a 4% polyacrylamide gel in 6.7 mM Tris-HCl pH 8.0, 3.3 mM sodium acetate and 2 mM EDTA. Electrophoresis was at 10 V/cm for 1 h 45 min. Gels were dried and visualised by autoradiography.

### DNA cleavage assays

Cleavage of <sup>32</sup>P-labelled DNA (0.25 or 0.5 nM) by Rap was assayed at 37°C in cleavage buffer (50 mM Tris-HCl pH 8.0, 1 mM dithiothreitol and 100 µg/ml BSA) containing 0.5 mM MnCl<sub>2</sub>. Reactions (20 µl) were terminated by adding 5 µl of stop buffer (100 mM Tris-HCl pH 8.0, 2.5% SDS, 100 mM EDTA and 10 mg/ml proteinase K) and incubated for a further 10 min at 37°C. Following addition of 5 µl of loading buffer (0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol and 15% v/v Ficoll type 400), 15 µl was electrophoresed on 10% polyacrylamide gels in 90 mM Tris-borate, 2 mM EDTA at 12 V/cm for 2 h. Gels were dried, autoradiographed and the amount of DNA cleavage quantified using a Molecular Dynamics PhosphorImaging system with ImageQuant software.

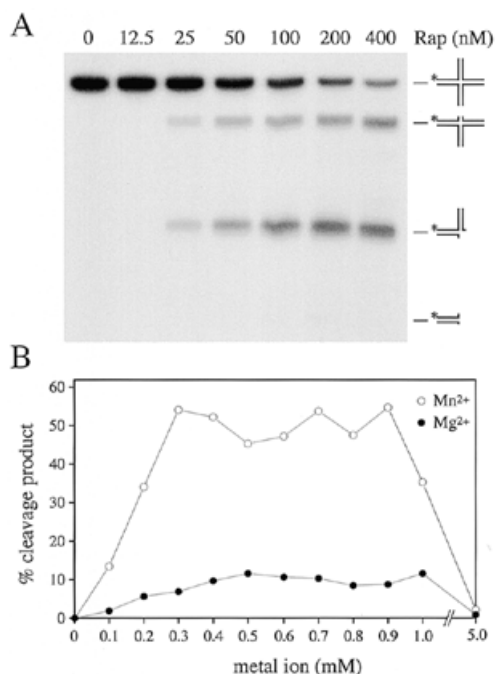
### Mapping cleavage sites

Separate junction preparations were made so that each was 5'-<sup>32</sup>P-labelled in a different strand. These were incubated at 37°C for 1 h with 200 nM Rap in cleavage buffer. Samples were deproteinised in stop buffer and precipitated with 0.4 vol of 10 M ammonium acetate, 0.02 vol 0.1 µg/ml glycogen and 3 vol of ethanol. Dried pellets were resuspended in denaturing loading buffer (0.3% w/v bromophenol blue, 0.3% w/v xylene cyanol, 10 mM EDTA and 97.5% formamide) to give equivalent radioactivity in each lane. Samples of 1–2 µl were boiled for 2 min and separated by gel electrophoresis on 15% polyacrylamide-7 M urea at 4.4 V/cm for 4 h. A+G Maxam-Gilbert sequencing reactions were performed on each oligonucleotide and run on the same gel to provide markers. Cleavage sites were mapped by reference to the sequencing ladder with a 1.5 base allowance made to compensate for the nucleoside eliminated in the sequencing reaction. Gels were dried and analysed by autoradiography and phosphorimaging.

## RESULTS

### Reaction conditions for cleavage of Holliday junctions by Rap

The Rap endonuclease introduces nicks in branched DNA substrates that resemble D-loop and Holliday junction recombination intermediates (12). Cleavage of a synthetic Holliday (X or 4-strand) junction gives products that are consistent with independent incisions at the branch point, yielding three-, two- and one-arm DNA fragments (Fig. 1A) (12). The basic buffer requirements of Rap endonuclease were established in a variety of reaction conditions using a 50 bp Holliday junction containing a homologous core of 12 bp (J12). Rap showed a requirement for divalent metal ions with a clear preference for manganese over magnesium (Fig. 1B; Table 2). Over the optimal range in MnCl<sub>2</sub> (0.3–0.9 mM), Rap cleaved on average 5-fold better in manganese than in magnesium (Fig. 1B). Cleavage was generally reduced at low (<0.3 mM) and high (>1 mM) concentrations of metal ion (Fig. 1B). No significant endonuclease activity was detected in the presence of Ca<sup>2+</sup>, Co<sup>2+</sup>, Ni<sup>2+</sup> or Zn<sup>2+</sup> ions (Table 2). A small amount of junction

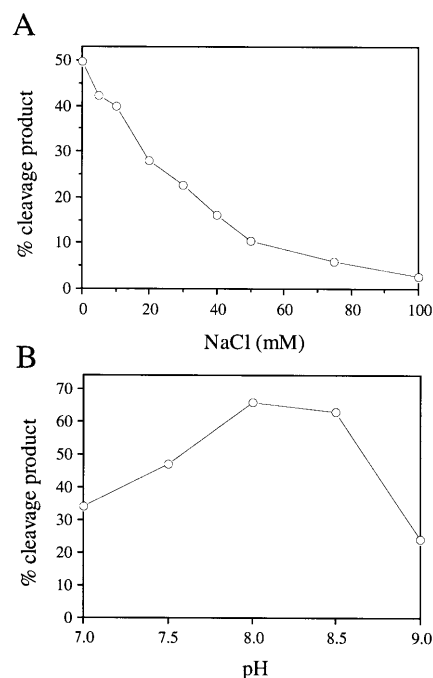


**Figure 1.** Effect of magnesium and manganese ions on cleavage of Holliday junctions by Rap. (A) Cleavage of synthetic Holliday junction J12. Reactions were performed in cleavage buffer (containing 0.5 mM MnCl<sub>2</sub>) with 0.25 nM <sup>32</sup>P-labelled J12 at 37°C for 1 h and electrophoresed on 10% polyacrylamide at 12 V/cm for 90 min. The predicted products of two incisions on different strands are represented on the right. An asterisk indicates the labelled strand. The single arm product can be seen on phosphorimages but is not readily detectable on autoradiographs as it comprises only one-sixth of the total products. (B) Effect of magnesium and manganese ions on cleavage of J12. Reactions were performed in cleavage buffer with 0.25 nM <sup>32</sup>P-labelled J12 and 200 nM Rap at the indicated concentrations of MgCl<sub>2</sub> or MnCl<sub>2</sub>. Values are the mean of two independent experiments.

cleavage was detected with Cu<sup>2+</sup> ions (Table 2). The endonuclease activity on X junction DNA was extremely sensitive to NaCl concentration (Fig. 2A). In Tris buffer with Mn<sup>2+</sup>, maximal junction cleavage was noted between pH 8.0 and 8.5 (Fig. 2B).

### Structure specificity of binding

In previous studies, Rap was found to bind preferentially to Holliday junctions rather than linear duplex DNA (12). The structure selectivity of Rap was further examined by assessing its ability to recognise and cleave a simple range of DNA substrates (Fig. 3). Each substrate was labelled with <sup>32</sup>P in a single strand and used at equimolar concentrations. In gel retardation assays, Rap formed a single complex with the Holliday, 3-strand and Y junctions (Fig. 3A, lanes a–f and j–l). A second complex was detectable with Holliday and 3-strand junctions at higher Rap concentrations (200 and 400 nM; data not shown). Smearing of the junction with the flayed duplex, and to a lesser degree with the 3-strand and Y, suggested a less stable DNA–protein interaction (Fig. 3A, lanes f, i and l). No binding was detected with duplex, partial duplex and single-stranded DNA in keeping with the structure selectivity of Rap (Fig. 3A, lanes m–v). A range of Rap concentrations from 1.56 to 400 nM was used to more accurately assess binding to X, 3-strand, Y and



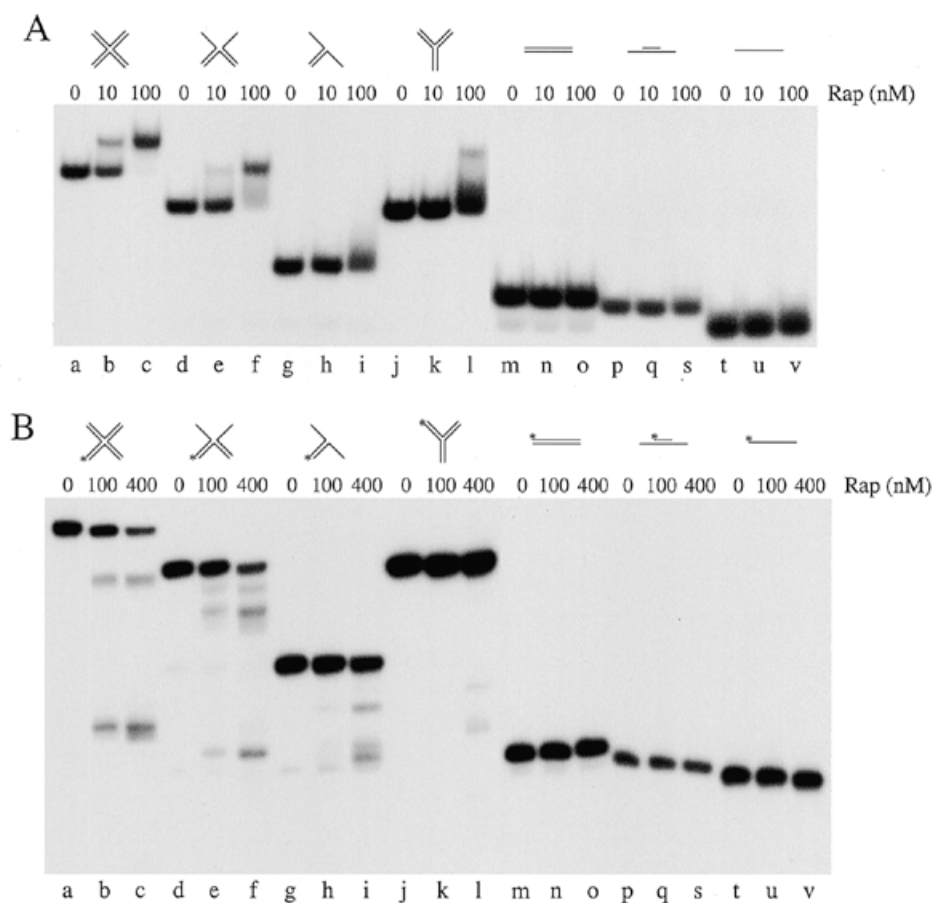
**Figure 2.** Effect of (A) NaCl and (B) pH on cleavage of Holliday junction DNA by Rap. Reactions contained 200 nM Rap and 0.25 nM <sup>32</sup>P-labelled J12 in cleavage buffer (containing 0.5 mM MnCl<sub>2</sub>) and were incubated at 37°C for 1 h. Values are the mean of two independent experiments.

flayed duplex structures. Rap showed a preference for binding in the order X, 3-strand, Y and flayed duplex (Fig. 4A).

The selectivity of Rap for Holliday junctions was determined by monitoring binding in the presence of unlabelled, non-specific competitor DNA. A concentration of Rap was chosen that was sufficient to bind almost all of the junction DNA (Fig. 5, lane b). Increasing concentrations of non-specific competitor DNA were included in the binding mixture containing <sup>32</sup>P-labelled J12 to which the protein was subsequently added. The amount of free junction DNA observed increased relative to the concentration of competitor DNA (Fig. 5, lanes c–g). Nonetheless, a significant proportion of the junction remained bound by Rap in the presence of a 20 000-fold excess of competitor DNA (Fig. 5, lane g). These results confirm the high specificity of Rap for branched DNA molecules.

### Structure specificity of cleavage

The cleavage specificity of Rap was analysed on the same range of substrates in cleavage buffer containing 0.5 mM MnCl<sub>2</sub>. A similar pattern to the binding results emerged, with Rap cutting DNA substrates with a branched composition (Fig. 3B). The 4- (X) and 3-strand junctions were the favoured substrates for cleavage (Figs 3B and 4B). In contrast to the binding data, Rap appeared to show a preference for cleaving the flayed duplex rather than the Y junction (Fig. 4B). However, only one strand needs to be cleaved to give an observable product from the flayed duplex, while two within the same duplex arm are required with a Y junction, which may explain, at least in part, the apparently higher cleavage obtained with a flayed duplex. Some nicking of the partial



**Figure 3.** Binding and cleavage specificity of Rap. Binding (A) and cleavage (B) of Rap on X (lanes a–c), 3-strand (lanes d–f), flayed duplex (lanes g–i), Y (lanes j–l), duplex (lanes m–o), partial duplex (lanes p–s) and single-stranded DNA (lanes t–v). Reactions contained 0.5 nM of  $^{32}\text{P}$ -labelled DNA and Rap protein as indicated. Cleavage assays were performed in cleavage buffer (containing 0.5 mM  $\text{MnCl}_2$ ) at 37°C for 1 h. The labelled strand in each substrate is marked with an asterisk in (B).

**Table 2.** Effect of metal ions on X junction cleavage by Rap

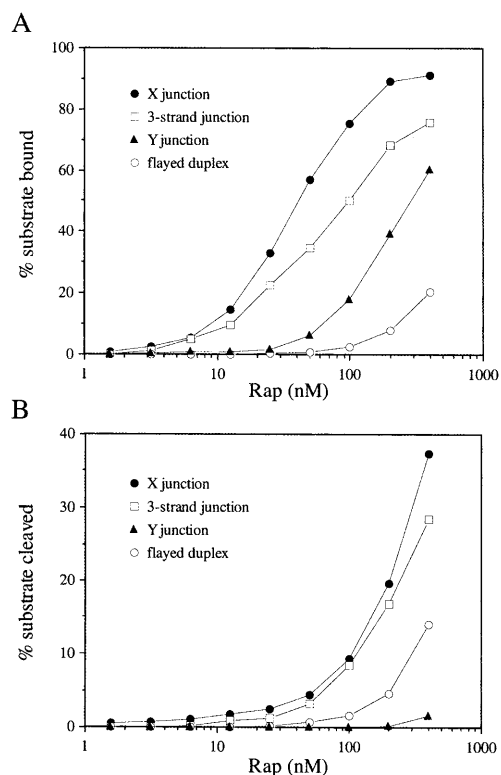
Metal ion	% J12 cleavage
$\text{CaCl}_2$	0.03
$\text{CoCl}_2$	1.66
$\text{CuSO}_4$	3.00
$\text{MgCl}_2$	10.10
$\text{MnCl}_2$	48.94
$\text{NiSO}_4$	1.20
$\text{ZnCl}_2$	0

Reactions contained 200 nM Rap, 0.25 nM J12 and 0.5 mM of the appropriate metal ion in cleavage buffer. Samples were incubated at 37°C for 30 min. Values are the mean of two independent experiments.

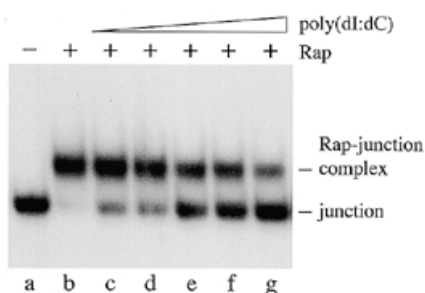
duplex (5.2%), and to a lesser degree single-stranded DNA (2.4%), could be detected with 400 nM Rap, probably because these molecules can occasionally adopt a branched conformation or secondary structure (data not shown). Less than 0.5% cleavage was observed on linear duplex DNA under the same

conditions (data not shown), which emphasises the specificity of Rap for branched DNA.

The products of the cleavage reaction analysed by neutral PAGE can be interpreted based on the known preference of Rap for cleaving the branch points of such molecules (12) (Figs 6 and 7). Because of the different location of cut sites and the range of products that can be generated it is not always easy to predict what the products will be or how they will migrate on polyacrylamide gels. However, some conclusions can be made concerning the major products of these reactions. Two or more independent incisions by Rap close to the centre of an X junction should produce 3-, 2- and 1-arm DNA fragments (Fig. 1A) (12). The migration of the products observed are consistent with this view (Fig. 3B, lanes b and c). The 3-arm product migrates close to the position of the 3-strand and Y junctions (Fig. 3B, lanes c, d and j). The 2-arm product migrates near the position of duplex DNA although always slightly slower (Fig. 3B, compare lanes c and m). This would be expected if incisions were not symmetrical across the point of crossover, yielding a nicked duplex with short single-strand extensions in the middle. The single arm product is not readily detected as only one-sixth of the overall products generated will contain a labelled strand. With the 3-strand junction the products fit with removal of either the 5' or 3' single-strand arms, which migrate



**Figure 4.** Binding and cleavage specificity of Rap on X, 3-strand, flayed duplex and Y junctions. (A) Binding reactions contained 0.25 nM  $^{32}$ P-labelled DNA and Rap protein. (B) Cleavage reactions contained 0.25 nM  $^{32}$ P-labelled DNA and Rap protein in cleavage buffer (containing 0.5 mM MnCl<sub>2</sub>) incubated at 37°C for 1 h. Rap protein was used at 1.56, 3.15, 6.25, 12.5, 25, 50, 100, 200 and 400 nM. Data are the mean of two independent experiments.



**Figure 5.** Competition assay showing the specificity of Rap for Holliday junctions. Reactions (20  $\mu$ l) were performed in binding buffer containing 0.1 ng (0.08 nM)  $^{32}$ P-labelled J12 and poly(dI:dC) at 62.5, 125, 250, 500, 1000 and 2000 ng. Rap protein (200 nM) was added last and samples incubated on ice for 15 min prior to gel electrophoresis.

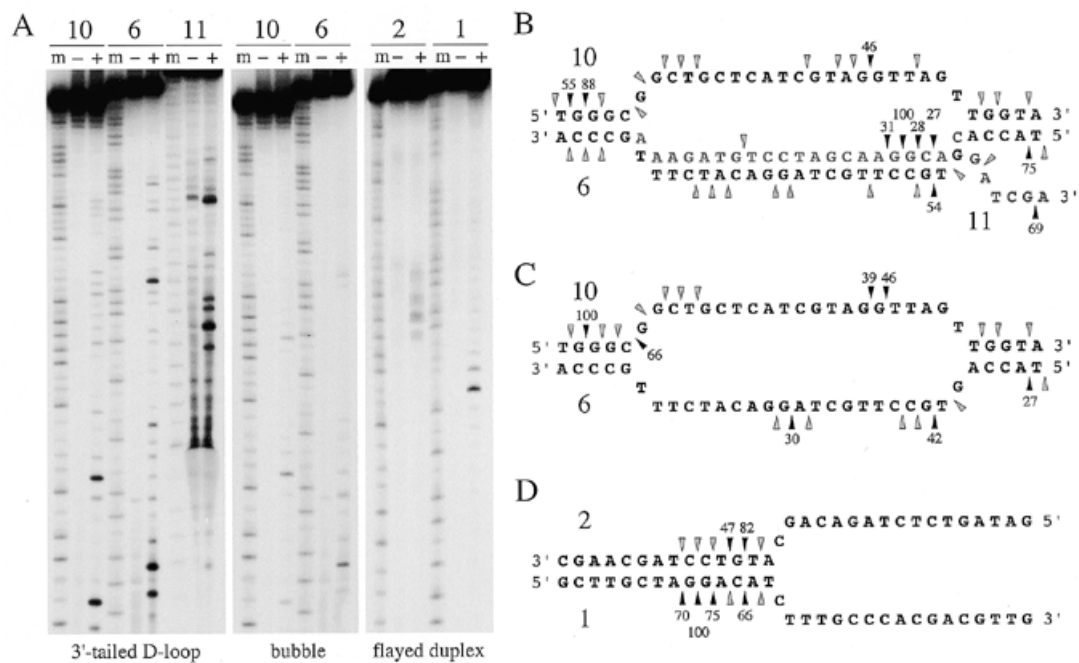
differently, and a product with both single-strand arms removed that resembles a nicked duplex (Fig. 3B, lanes e and f). A similar pattern is observed with the flayed duplex with removal of either, or possibly both, of the single-strand arms (Fig. 6D), although the precise nature of the three major products is unclear (Fig. 3B, lanes h and i). Cleavage of the Y junction gives two major products and probably results from the partial or complete loss of a duplex arm (Figs 3B, lane l and 7B).

### Location of Rap cleavage sites on branched DNA substrates

Rap introduces nicks in DNA close to the branch points of a synthetic X junction containing a 3 bp homologous core (J3) and a D-loop with a 5' single-strand tail (12). To further characterise the structure specificity of the Rap endonuclease, the cleavage sites on a wider range of branched DNA structures were defined. The substrates studied were a D-loop with a 3' single-strand tail, bubble, flayed duplex, Y junction and 5'-flap (Table 1). Cleavage sites were identified by tagging each strand of a substrate with  $^{32}$ P and analysing the products of the cleavage reaction on denaturing polyacrylamide gels. The results are presented in Figures 6 and 7. In agreement with the Holliday junction and 5'-tailed D-loop data (12), nicking of the DNA occurs predominantly at the branch point of these structures. This is most evident with the flayed duplex (Fig. 6D), Y junction (Fig. 7B) and 5'-flap (Fig. 7C) substrates. In the simplest substrate, the flayed duplex, Rap cleavage is favoured in the duplex region immediately adjoining the single-strand arms (Fig. 6D). Similar results were obtained with a flayed duplex containing a different sequence at the branch point (data not shown). The D-loop and bubble substrates show additional nicks in the central region of the molecules (Fig. 6B and C). This may result from the complex nature of these structures and their propensity to fold in a variety of ways. The 3'-tailed D-loop, bubble and 5'-tailed D-loop (12) substrates have oligonucleotides 6 and 10 in common. Incisions in these strands are frequently located at the same positions (Fig. 6B and C) (12), indicating that the configuration adopted by these three molecules is similar. With all five junction substrates, there was no obvious bias in relation to strand polarity or preference for cleavage at particular sequences. Thus any hotspots for cleavage probably reflect their accessibility within the folded DNA structures.

### DISCUSSION

In this study we have used synthetic DNA substrates to further define the structure specificity of the Rap endonuclease. Rap was found to bind preferentially to DNA molecules containing a branched component and cleave the phosphodiester backbone close to the point of strand crossover or bend. Duplex, partial duplex and single-strand substrates were not generally targeted. With a flayed duplex substrate cleavage was directed to the duplex region adjacent to the branch rather than to the single-stranded DNA arms. A preference for cutting at the border of duplex:single-strand regions fits with a role in nicking D-loops to release a splice recombinant product (8,12). However, a comparison of the binding and cleavage activities of Rap on X and 3-strand [which mimic Holliday junction and D-loop intermediates (18), respectively], suggested a slight preference for X over 3-strand DNA (Fig. 4). Unfortunately this bias was too small to predict whether Rap could target Holliday junctions rather than D-loops *in vivo*. This issue is difficult to address using *in vitro* Holliday junction and D-loop substrates due to the inherent instability of model D-loop substrates. However, the reduced endonuclease activity of Rap on Y and flayed duplex structures supports the notion of Holliday junctions or D-loops being the favoured Rap substrate(s) *in vivo*.



**Figure 6.** Location of Rap cleavage sites on 3'-tailed D-loop (B), bubble (C) and flayed duplex (D) substrates. (A) Junction substrates, each 5'-<sup>32</sup>P-labelled in a different oligonucleotide, were incubated in cleavage buffer without protein (-) or with 200 nM Rap (+) at 37°C for 1 h. A+G sequencing ladders (m) on <sup>32</sup>P-labelled oligonucleotides were used as markers. Reaction products were analysed on 15% denaturing PAGE. The labelled strands are numbered according to the list in Materials and Methods. (B-D) The location of Rap cleavage sites are indicated by arrowheads (black for major sites, shaded for minor sites). Major sites of strand cleavage are represented as a percentage of the most frequently cut site for that substrate. Only the relevant central regions of the substrates are shown. Additional minor cut sites visible by phosphorimaging are omitted for clarity. These sites were located around the core of the D-loop and bubble structures. The invading strand of the D-loop is shaded in (B).

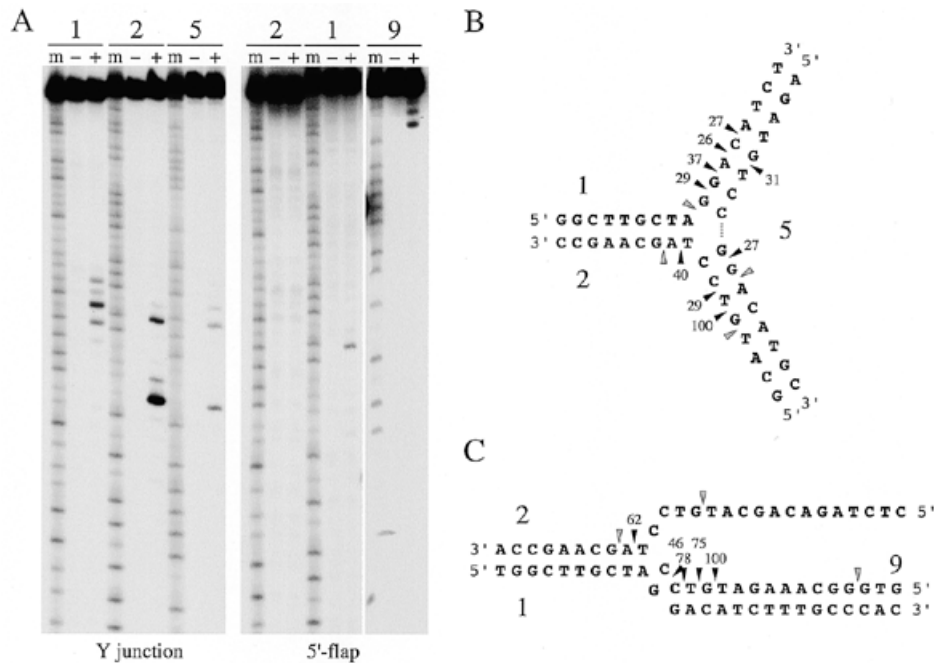
The activities associated with Rap most closely resemble the Holliday junction resolvases encoded by phages T4 and T7 (10,19). T4 endonuclease VII and T7 endonuclease I perform important functions in phage recombination, replication and degradation of the host chromosome. Phages defective in the genes encoding these resolvases accumulate branched DNA molecules which cannot be packaged into phage heads (19). Both endonucleases introduce nicks in a range of branched DNA molecules. For instance, T4 endonuclease VII cleaves Holliday junctions and other branched DNA substrates (20-22), heteroduplex loops (23), mismatches (24), abasic sites (25) and bulky adducts (26). However, Rap differs significantly from the T4 and T7 endonucleases in its apparent inability to introduce symmetrical, paired incisions in 4-way junctions, a characteristic of genuine Holliday junction resolvases (10,19).

Rap also differs from the structure-specific flap endonuclease family of proteins that includes T5 5'-exonuclease and FEN-1 (27-29). The eukaryotic FEN-1 proteins are 5'-3' exonucleases with an associated endonuclease activity which specifically releases the single strand in 5'-flap structures (29). Such flaps occur at Okazaki fragments during DNA replication and following recombination and certain types of DNA repair (29-32). Unlike FEN-1, Rap predominantly introduces nicks in the duplex regions of a 5'-flap structure at either side of the single-strand flap (Fig. 7C) and is clearly not restricted to such structures.

Structure specific cleavage by Rap required either magnesium or manganese ions as a co-factor. Cleavage of an X junction

with a 12 bp homologous core was substantially increased when Mg<sup>2+</sup> was replaced with Mn<sup>2+</sup> (Fig. 1B). Such stimulation of junction cleavage by manganese has been observed previously with the sequence-specific Holliday junction resolvases RuvC and RusA (16,33). This effect was attributed to a combination of localised destabilisation of the DNA with increased branch migration of the mobile core to locate preferred sequences for resolution (16,33). Cleavage by RuvC and RusA was also enhanced by alkaline pH in an apparently similar manner (16,33). In contrast, Rap cleavage is reduced beyond the optimum of pH 8-8.5 (Fig. 2B). Moreover, an 8-fold enhancement of Rap cleavage with manganese over magnesium was also observed using a junction constrained to a 3 bp mobile core (G.J.Sharples, unpublished results). These findings suggest that the reason for the disparity in Rap cleavage activity with Mg<sup>2+</sup> and Mn<sup>2+</sup> is not due to factors that promote cleavage at particular DNA sequences. The possibility that Mn<sup>2+</sup> masks a requirement for additional ( $\lambda$ -encoded) proteins to enhance Rap activity has not been excluded. For example, the requirement for MuB protein in stimulating MuA-mediated transposition can be circumvented by the inclusion of manganese ions (34).

The data presented here indicate that branched structures with four arms are the preferred substrates of Rap. This suggests that Holliday junctions and D-loops are the targets of the Rap endonuclease *in vivo*. However, any preference of Rap for single-stranded regions within such four-way branched structures is not clearly defined by our results. Further analysis



**Figure 7.** Location of Rap cleavage sites on Y junction (B) and 5'-flap (C) substrates. (A) Rap cleavage sites were mapped and analysed as described in the legend to Figure 6. (B and C) The location of Rap cleavage sites are indicated by arrowheads (black for major sites, shaded for minor sites). Major sites of strand cleavage are represented as a percentage of the most frequently cut site for that substrate.

should help to answer this question and determine whether Rap fulfils a dual function in processing both D-loop and Holliday junction intermediates during phage recombination.

## ACKNOWLEDGEMENTS

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