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RecG helicase activity at three- and four-strand DNA structures

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ABSTRACT

The RecG helicase of *Escherichia coli* is necessary for efficient recombination and repair of DNA *in vivo* and has been shown to catalyse the unwinding of DNA junctions *in vitro*. Despite these findings, the precise role of RecG remains elusive. However, models have been proposed in which RecG promotes the resolution of linked duplexes by targeting three-strand junctions present at D-loops. One such model postulates that RecG catalyses the formation of four-strand (Holliday) junctions from three-strand junctions. To test this model, the DNA binding and unwinding activities of RecG were analysed using synthetic three- and four-strand junctions. The substrate specificity of RecG was found to depend critically on the concentrations of ATP and $MgCl_2$ and under certain conditions RecG preferentially unwound three-strand junction DNA. This was at least partly due to the larger inhibitory effect of $MgCl_2$ on the binding of four-strand as opposed to three-strand junctions by RecG. Thus RecG may be targeted to three-strand junctions *in vivo* whilst still being able to branch migrate the four-strand junctions formed as a result of the initial helicase reaction. The increase in the dissociation constant of RecG on conversion of a three-strand into a four-strand junction may also facilitate resolution of the four-strand junction by the RuvABC complex.

INTRODUCTION

The important roles of DNA repair and recombination in maintaining both prokaryotic and eukaryotic cell viability are well characterised. However, it is only recently that the intimate linkage between recombination, repair and replication of DNA has been appreciated (1). A key discovery was that the *Escherichia coli* replication protein PriA, known to be required for the assembly of a replication fork at sites other than *oriC*, is important for efficient recombination, repair of double-strand breaks and maintenance of high cell viability (2,3). This finding has led to models of recombination that predict a major role for DNA replication in the formation of mature recombinant chromosomes (1,4). Such models have in common the ability of a DNA end to initiate recombination via the coordinated activities of the RecBCD and RecA proteins (5). The single-stranded DNA generated by RecBCD nuclease/helicase is bound by RecA to form a nucleoprotein filament that promotes pairing and strand exchange with a homologous duplex. The initial product of this reaction is a D-loop that can be targeted by structure-specific DNA-binding proteins to continue the recombination reaction (Fig. 1). If the invading strand has a 3'-end then it is postulated that a replication fork can assemble at the 3'-end in the D-loop by the action of PriA (3,6–8). The coordinated assembly of two replication forks has been postulated to occur during the repair of double-strand breaks and the integration of linear DNA fragments into the *E.coli* chromosome during conjugation and transduction (4,9). A one-ended event has been proposed as a mechanism for the repair of collapsed replication forks during normal chromosomal replication (10). Thus the assembly of replication forks at D-loops may be critical for the maintenance of chromosomal integrity.

DNA replication primed via a D-loop generates two duplexes linked by a three-strand DNA junction. To allow the separation of these duplexes this junction must be removed. How this is achieved is unknown but two very different models have been postulated, both of which rely on the ability of RecG to drive branch migration of the junction along the DNA. In the first model, a single-stranded endonuclease with specificity for D-loops has been suggested to cleave the displaced strand (11,12). RecG can then resolve the structure by driving branch migration of the junction to the nick (Fig. 1A). This model is consistent with the known ability of RecG to target junction structures *in vitro* (13,14). The identification of an *E.coli* endonuclease activity targeted to D-loops has lent further support to this model (15).

In the second model, RecG is also postulated to bind to the three-strand junction. However, instead of moving the junction to a nick in the D-loop, RecG drives migration of the junction in the opposite direction into regions of duplex:duplex DNA pairing (Fig. 1B) (16). The three-strand junction would thus be converted into a four-strand (or Holliday) junction that could be targeted and resolved by the coordinated branch migration and endonuclease activities of the RuvABC complex (17). The ability of RecG to target three- and four-strand junctions, D-loops and R-loops, and to counter strand exchange mediated by RecA support this hypothesis (16,18–21). An interaction between RecG and D-loops was also suggested following the identification of mutations in *priA* that suppress the DNA repair and recombination defects associated with *recG* mutations (6).

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Either of these models could account for the reduced DNA repair and recombination seen in *recG* mutants. Both models depend upon the ability of RecG to specifically recognise three-strand junctions and to drive their branch migration along the DNA. They differ in that the second model assumes RecG will end up bound to a four-strand Holliday junction. In this study we therefore compared the relative levels of DNA binding and unwinding by RecG using synthetic three- and four-strand junctions. We have found that under different concentrations of $MgCl_2$ and ATP, which act both to modify DNA junction structure and to provide the energy required for helicase activity, the relative substrate specificities of RecG can be dramatically altered. This is related to the possible *in vivo* levels of free Mg^{2+} and ATP and their effect on the role(s) of RecG.

MATERIALS AND METHODS

Protein purification

All chromatography, centrifugation and dialysis was performed at 4°C. Protein concentrations were determined with a modified Bradford assay (Bio-Rad) using bovine serum albumin as a standard (22). Protein concentrations were expressed as moles of protein monomer.

Wild-type RecG was purified from a BL21(DE3) *E.coli* strain carrying plasmid pGS772, in which *recG* was expressed from an IPTG-inducible promoter (13). Two batches of 500 ml of cells in LB broth containing 10 g/l NaCl were grown at 37°C in 1 l baffled flasks to an absorbance of 0.65 at 650 nm. IPTG was then added to 0.4 mM and growth continued at 37°C for another 3 h. The cells were pelleted by centrifugation, resuspended in 25 ml of 40 mM Tris–HCl (pH 8), 2 mM EDTA and frozen in liquid nitrogen prior to storage at –80°C. The cell suspension was subsequently thawed at 37°C and DTT added to 1 mM. The cells were sonicated on ice, NaCl was added to 1 M and the suspension was then centrifuged at 19 000 r.p.m. for 15 min using a Sorvall SS-34 rotor. The supernatant was filtered through a 5 μm filter unit (Millipore) and RecG was precipitated from this solution by addition of ammonium sulphate to 40% saturation and centrifugation as above for 20 min. The precipitate was resuspended in 9 ml of buffer A (50 mM Tris–HCl, pH 7.5, 1 mM EDTA, 1 mM DTT) and dialysed against the same buffer. This was then loaded onto a 15 ml heparin–agarose column (Sigma) and bound proteins eluted with a 160 ml 0–0.5 M KCl gradient in buffer A. The RecG, which eluted between 0.3 and 0.4 M KCl, was dialysed into buffer B (50 mM sodium phosphate, pH 7.4, 1 mM EDTA, 1 mM DTT, 0.5 M ammonium sulphate) and loaded onto a 5 ml Phenyl Sepharose 6 Fast Flow column (Pharmacia). Bound proteins were eluted with a stepped gradient of 0.5–0 M ammonium sulphate in buffer B, with RecG eluting at 0 M salt. RecG fractions were dialysed against buffer A plus 0.1 M KCl and then bound to a 3 ml single-stranded DNA–cellulose column (Sigma). Proteins were eluted with a 0–1 M KCl gradient in buffer A, with RecG eluting between 0.4 and 0.6 M KCl. The RecG-containing pool was diluted with buffer A to 0.1 M KCl and then loaded onto a 1 ml Q Sepharose Fast Flow column (Pharmacia). The column was washed with buffer A and the RecG, which did not bind, was then precipitated by addition of ammonium sulphate to 40% and centrifugation as above. The pellet was resuspended in 5 ml of buffer A plus 0.2 M KCl, passed through a 0.45 μm filter (Millipore) and run through a 1.6 × 78 cm Sephacryl S200 HR gel filtration column (Pharmacia) using buffer A plus 0.2 M KCl. The RecG eluted as a peak of apparent M_r 62 000 and was dialysed against buffer A plus 50% glycerol before storage at –80°C.

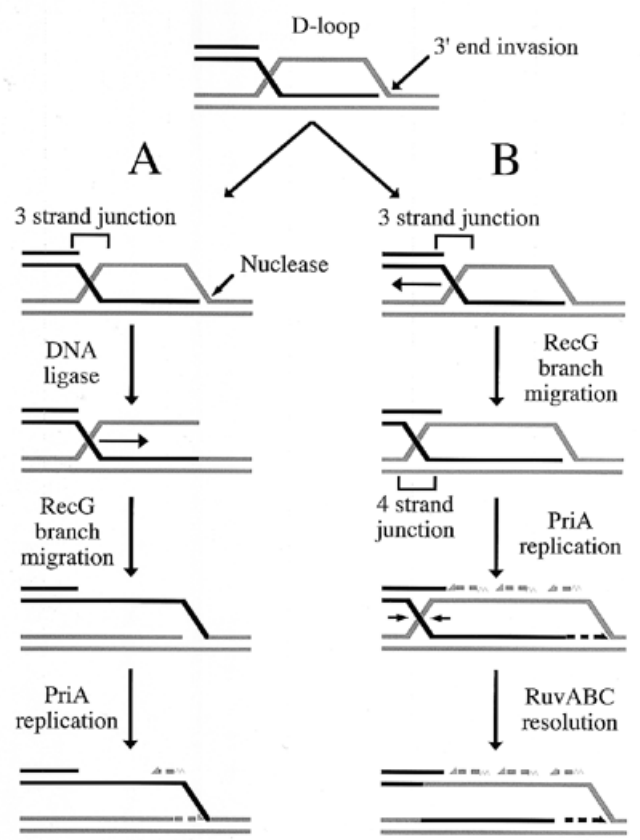

Figure 1. Models of RecG helicase activity at D-loops generated by RecA from single-stranded DNA with a free 3'-end. (**A**) A single-stranded endonuclease cleaves the displaced strand of the D-loop and the 5'-end generated by this cleavage is ligated to the 3'-end of the invading single-stranded DNA. Targeting of the RecG branch migration protein to the three-strand junction at the D-loop then allows this junction to be removed by unwinding of the junction towards the end of the displaced single-stranded DNA, thus resolving the two duplexes behind the future site of replication fork assembly. This assembly is catalysed by the binding of PriA to the DNA structure generated by the action of RecG helicase activity. In the alternative model presented in (**B**) the three-strand junction present at the D-loop is unwound by RecG towards the region of duplex:duplex DNA pairing rather than a nick in the D-loop. The four-strand junction produced by RecG catalysis is then targeted by the RuvABC complex which cleaves two opposing strands of the junction, thus resolving the two duplex DNA molecules. Simultaneously, replication fork assembly is catalysed by the binding of PriA to the 3'-end of the D-loop. Note that only one of the two possible outcomes of RuvC cleavage is shown.

DNA substrates

The synthetic oligonucleotides used to construct the junction structures in this study were: (1) 5'-GACGCTGCCGAATT-CTGGCTTGCTAGGACATCTTTGCCCACGTTGACCC-3';

(2) 5'-TGGGTCAACGTGGGCAAAGATGTCCTAGCAATGTAATCGTCTATGACGTT-3'; (3) 5'-CAACGTCATAGACGATTACATTGCTAGGACATGCTGTCTAGAGACTATCGA-3'; (4) 5'-ATCGATAGTCTCTAGACAGCATGTCCTAGCAAGCCAGAATTCGGCAGCGT-3'; (5) 5'-CCTCGAGAAGCTTCCGGTAGCAGCGAGAGCGGTGGTTGAATTCCTCGAGG-3'; (6) 5'-CCTCGAGGAATTCAACCACCGCTCTTCTCAACTGCAGTCTAGACTCGAGG-3'; (7) 5'-CCTCGAGTCTAGACTGCAGTTGAGAGCTTGCTAGGACGGATCCCTCGAGG-3'; (8) 5'-CCTCGAGGGATCCGTCCTAGCAAGCGCTGCTACCGGAAGCTTCTCGAGG-3'.

Two sets of three- and four-strand junctions were constructed using these oligonucleotides employing the procedure of Parsons *et al.* (23) in which one of the strands in each junction was labelled at the 5'-end with [γ-^{32}P]ATP. The first set had a branch point that was free to migrate within a 12 bp homologous core, flanked by 19–20 bp heterologous arms to prevent spontaneous dissociation (24). The four-strand junction was designated 4J12 and was made using oligonucleotides 1–4. The three-strand version, 3J12, was constructed using oligonucleotides 1, 2 and 4, with strand 2 labelled. The second set of junctions also employed 50mer oligonucleotides (based on junction 1 in Duckett *et al.*; 25) but lacked a homologous core so that the branch point was unable to migrate. This static four-strand junction, 4J0, was made using oligonucleotides 5–8. The three-strand junction, 3J0, was made with oligonucleotides 5, 7 and 8, with strand 5 labelled. A set of three-strand versions of J0 was also made using all four possible three-strand combinations of oligonucleotides 5–8 as illustrated in Figure 6, specifically to test the polarity of unwinding by RecG. The oligonucleotide which provided the free 5'-end of each junction was radiolabelled. The concentrations of all DNA substrates were estimated by monitoring the specific activity of each labelled oligonucleotide after end-labelling and the final activity of the purified substrate. Concentrations were in moles of junction substrate.

DNA binding and unwinding assays

Band shift assays in the absence of $MgCl_2$ were performed on ice and quantified as described using 0.1 nM junction DNA in 50 mM Tris–HCl (pH 8), 5 mM EDTA, 1 mM dithiothreitol, 100 μg/ml bovine serum albumin and 6% (v/v) glycerol (21). Assays in the presence of $MgCl_2$ were performed in an identical manner except that EDTA was replaced by the indicated concentrations of $MgCl_2$ in the reaction buffer and gel running buffer.

The relative rates of dissociation of RecG from junction DNA were measured essentially as described (26). RecG (5 nM) was preincubated on ice for 15 min with 0.05 nM 4J12 or 3J12 junction DNA in a total volume of 72 μl. An aliquot of 10 μl of the binding reaction was then loaded onto a 4% polyacrylamide gel in 6.7 mM Tris–HCl (pH 8), 3.3 mM sodium acetate and 2 mM EDTA running at 160 V. Non-specific competitor DNA [8 μl of poly(dI·dC)] was then added at 5 mg/ml to the reaction on ice. Another 10 μl was immediately removed and loaded onto the gel under current; this time point was taken as 0. Further 10 μl samples were taken at 2, 5, 10 and 30 min and electrophoresis continued for a further 60 min after the final time point was taken. A parallel reaction in which the competitor DNA was present with the junction DNA prior to the addition of RecG provided a measure of the efficiency of competition so as to ensure that any RecG dissociated from the junction DNA was trapped by the competitor DNA. Reactions were performed either with 5 mM EDTA in the binding buffer, as described above, or in the same buffer with the EDTA replaced by 2 mM $MgCl_2$. Control experiments in which water was substituted for the competitor DNA excluded the possibility that shear forces generated by mixing the binding reactions did not by themselves cause disruption of the RecG–DNA interactions as measured by the band shift assay (data not shown).

The assays for junction dissociation by RecG were performed at 37°C in 20 mM Tris–HCl (pH 7.5), 2 mM dithiothreitol, 100 μg/ml bovine serum albumin and the indicated concentrations of RecG, ATP and $MgCl_2$ and used 0.3 nM of junction DNA. $MgCl_2$ and ATP optima were estimated in 20 μl reaction volumes. The reactions were started by the addition of RecG and after 10 min the reactions were stopped by adding 5 μl of 100 mM Tris–HCl (pH 7.5), 2.5% (w/v) SDS, 200 mM EDTA, 10 mg/ml proteinase K and incubating at 37°C for 10 min. Reactions to analyse the polarity of junction unwinding were performed in an identical manner except that 50 nM RecG was used in 5 mM $MgCl_2$, 5 mM ATP at 37°C for 30 min. The rates of junction dissociation by RecG were measured by performing 120 μl reactions. Aliquots of 10 μl were removed at the indicated times after the addition of RecG and deproteinised by addition of 2.5 μl of stop solution as described above. The percentage of junction dissociation in reactions was analysed by electrophoresis through 10% polyacrylamide gels and quantification using a phosphorimager (21).

All binding and dissociation assays were performed in duplicate or triplicate and means of the data were used for graphical presentation.

RESULTS

Relative levels of RecG helicase activity for three- and four-strand junction structures under different $MgCl_2$ and ATP concentrations

The aims of this work were to test the hypothesis that the preferred *in vivo* substrates of RecG are three-strand rather than four-strand junctions (Fig. 1A and B) and whether the role of RecG is to catalyse the branch migration of three-strand junctions at D-loops into four-strand junctions to allow subsequent resolution of the two linked duplexes (Fig. 1B). To analyse the relative specificity of RecG for unwinding three- and four-strand DNA structures we employed small synthetic DNA junctions constructed as described in Materials and Methods. One set (4J0 and 3J0) lacked a region of homology and thus the branch point had a defined position at the centre of each junction. However, junctions *in vivo* contain large tracts of homology through which the branch point is free to migrate. To try and mimic this feature a second set of junctions (4J12 and 3J12) had a 12 bp region of homology through which the branch point could spontaneously move, although this meant that the branch point was not well defined in these structures and could possibly occupy any position within the 12 bp homologous region.

Earlier studies indicated that the concentrations of Mg^{2+} and ATP were important factors in determining the level of unwinding of four-strand junctions by RecG (26,27). Therefore,

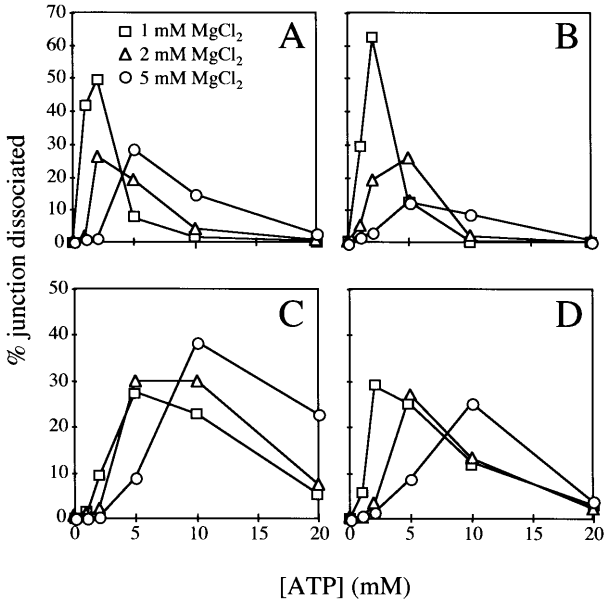

Figure 2. $MgCl_2$ and ATP optima for RecG helicase activity on three- and four-strand DNA junctions. The level of junction unwinding was monitored using 4J0 (**A**), 3J0 (**B**), 4J12 (**C**) and 3J12 (**D**) at the indicated concentrations of ATP and $MgCl_2$. RecG was present at a final concentration of 1 (A and B) or 0.01 nM (C and D).

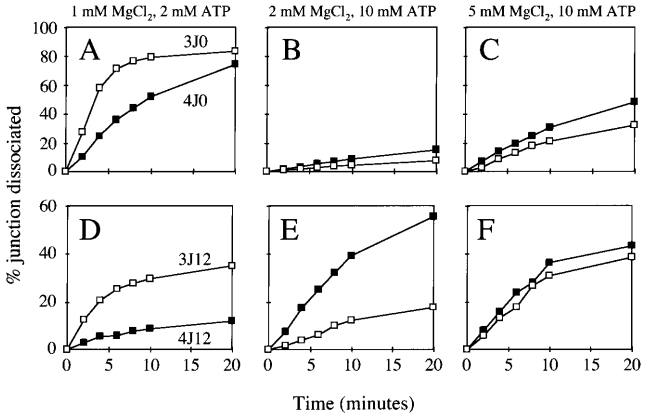

Figure 3. Relative rates of unwinding three- and four-strand junctions by RecG under different $MgCl_2$ and ATP concentrations. The rates of unwinding of 4J0 and 3J0 by 4 nM RecG (**A–C**) and of 4J12 and 3J12 by 0.01 nM RecG (**D–F**) were measured in the presence of: (A and D) 1 mM $MgCl_2$, 2 mM ATP; (B and E) 2 mM $MgCl_2$, 10 mM ATP; (C and F) 5 mM $MgCl_2$, 10 mM ATP. Filled square, four-strand junctions; open square, three-strand junctions.

the amounts of dissociation of 4J0 and 3J0 and of 4J12 and 3J12 were measured in the presence of a range of $MgCl_2$ and ATP concentrations. For the static junctions 4J0 and 3J0 two main points arose from the activity profiles (Fig. 2A and B). Firstly, the maximal RecG activity on both substrates occurred at 1 mM $MgCl_2$, 2 mM ATP. At higher $MgCl_2$ concentrations RecG catalytic activity was substantially reduced, irrespective of the ATP concentration. Secondly, RecG activity was inhibited whenever the $MgCl_2$:ATP ratio was greater than 1:1 or less than 1:4. The mobile junctions 4J12 and 3J12 gave a different pattern of activities as compared with 4J0 and 3J0 (Fig. 2C and D). The levels of RecG activity with the J12 junctions were substantially reduced when $MgCl_2$:ATP was greater than 1:2. However, the maximal levels of unwinding of 4J12 and 3J12 generally occurred over a wider range of and at higher $MgCl_2$ and ATP concentrations. Therefore, the levels of unwinding of the J12 junctions were less sensitive to the $MgCl_2$:ATP ratio as compared with the J0 junctions as long as the ATP concentration exceeded that of $MgCl_2$. Very similar results were obtained with other three-strand versions of J0 and J12 employing different combinations of oligonucleotides 5–8 and 1–4, respectively, which eliminated the possibility that the three-strand results were specific for 3J0 or 3J12 (data not shown). The activity pattern obtained for J12 (Fig. 2C) was also observed for other four-strand junctions constructed with 50mer oligonucleotides but containing 11 and 3 bp regions of homology (data not shown). This indicated that the relative activities obtained under different conditions with 4J12 were applicable to other junctions which possessed regions of homology through which the junction crossover point could migrate. It should also be noted that higher RecG concentrations were required to unwind both 4J0 and 3J0 as compared with junctions containing a homologous core (Fig. 2 and data not shown). This has been attributed to the inhibitory effect of larger heterologous arms on the ability of RecG to unwind junctions (26).

Rates of three- and four-strand junction unwinding by RecG

Three sets of conditions used in Figure 2 were employed to determine the actual rates of three- and four-strand junction unwinding so as to obtain an accurate measure of the relative substrate specificity of RecG. Concentrations of $MgCl_2$ and ATP were chosen that gave high levels of dissociation of either three- or four-strand junctions as measured in Figure 2. The rates of unwinding for 4J0 and 3J0 were maximal at 1 mM $MgCl_2$, 2 mM ATP, with the rate of 3J0 dissociation exceeding that of 4J0 (Fig. 3A). Rates of dissociation under other conditions were lower for both 4J0 and 3J0 but 4J0 was unwound at a slightly higher rate than 3J0 (Fig. 3B and C). Thus, under reaction conditions which gave maximal unwinding of both 4J0 and 3J0, 3J0 was the preferred substrate of RecG.

The same three sets of reaction conditions were used for 4J12 and 3J12. At 1 mM $MgCl_2$, 2 mM ATP the rate of unwinding of 3J12 exceeded that of 4J12 (Fig. 3D). However, with 2 mM $MgCl_2$, 10 mM ATP the rate of dissociation of 4J12 was higher than 3J12 (Fig. 3E). Thus the relative substrate specificities of RecG for the three- and four-strand junctions depended critically upon the $MgCl_2$ and ATP concentrations. Indeed, reaction conditions could be obtained which gave approximately equal rates of dissociation of both junctions (Fig. 3F).

The data in Figure 3 also support the observations in Figure 2, since the rates of dissociation of the J12 junctions were higher than those for the J0 junctions over a broader range of $MgCl_2$ and ATP concentrations.

Effect of $MgCl_2$ upon the binding affinities of RecG for three- and four-strand junctions

The levels of free Mg^{2+} ions within an *E.coli* cell are estimated to be only 1–2 mM, despite a total intracellular Mg^{2+} concentration of ~100 mM (28,29). This discrepancy arises because the majority of Mg^{2+} within the cell is bound to polyanions such as nucleic acids. Therefore, DNA *in vivo* is likely to be complexed with Mg^{2+}. However, a previous study demonstrated that the *in vitro* unwinding of four-strand junction DNA by RecG was inhibited by Mg^{2+} ions (26). This inhibition was attributed to Mg^{2+} ions promoting folding of the 4J12 junction DNA used in the assays from an open, square planar conformation into a stacked conformation. This suggested that the switching of substrate specificities of RecG for the three- and four-strand junctions observed in Figure 3 may have been due to the differential effects of Mg^{2+} on such structures. This was investigated using band shift assays to monitor binding of the J12 and J0 junctions in the presence of Mg^{2+}. In the absence of $MgCl_2$, RecG bound 4J12 with a higher affinity than 3J12 (Fig 4A and Table 1). Increasing levels of $MgCl_2$ (1–5 mM) raised the relative dissociation constants of RecG for both junctions (Fig. 4B–D and Table 1). However, this increase was much less marked for 3J12 than for 4J12 and resulted in the affinity of RecG for 3J12 being higher than for 4J12 in the presence of $MgCl_2$.

Table 1. Relative dissociation constants of RecG binding to three- and four-strand junctions

[$MgCl_2$] (mM)	Relative dissociation constant (nM)			
	4J12	3J12	4J0	3J0
0	0.8	6.4	0.2	20
0.5	ND[a]	ND	13	50
1	>100	20	50	100
2	>100	50	>100	>100
5	>100	>100	ND	ND

Values were determined from the band shift data presented in Figure 4.
[a]ND, not determined.

The pattern of relative affinities of RecG for 4J0 and 3J0 was similar to that obtained for the J12 junctions. In the absence of $MgCl_2$, RecG had a lower relative dissociation constant for 4J0 than for 3J0 (Fig. 4E and Table 1). The presence of $MgCl_2$ (0.5–2 mM) also increased the relative dissociation constants of RecG for both 4J0 and 3J0, but in contrast to the J12 junctions this did not lead to a higher binding affinity of RecG for 3J0 compared with 4J0 (Fig. 4F–H and Table 1). However, the relative dissociation constant for 3J0 was 100-fold higher than for 4J0 in the absence of $MgCl_2$ but only 2-fold higher in 1 mM $MgCl_2$ (Table 1). Thus the inhibitory effect of $MgCl_2$ was greater for 4J0 than for 3J0, which reflected the pattern seen with the J12 junctions.

Thus $MgCl_2$ preferentially reduced the affinity of RecG for four-strand as opposed to three-strand junctions and in the case

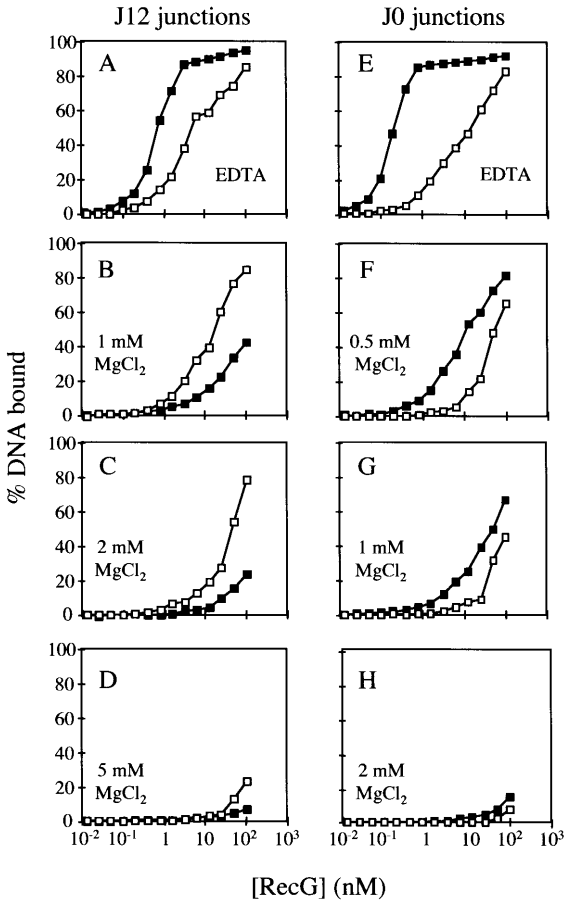

Figure 4. Effect of $MgCl_2$ on three- and four-strand junction binding by RecG. Levels of RecG binding to 4J12 and 3J12 (**A–D**) and to 4J0 and 3J0 (**E–H**) were monitored using band shift assays. Binding reactions and electrophoresis were performed in the presence of EDTA or $MgCl_2$ as indicated. Filled square, four-strand junctions; open square, three-strand junctions.

of the J12 junctions actually reversed the relative substrate binding specificity of RecG. This differential effect of Mg^{2+} may therefore explain, at least in part, the different rates of three- and four-strand junction unwinding measured under various $MgCl_2$ and ATP concentrations (Fig. 3). These data also suggest that binding of junction DNA may be a rate-limiting step of branch migration catalysed by RecG.

Relative rates of dissociation of RecG from 4J12 and 3J12

Mg^{2+} has been shown to increase the dissociation rate of RecG from 4J12 (26). Therefore, it was possible that the switch in binding specificity of RecG from 4J12 to 3J12 in the presence of $MgCl_2$ could have been due to a preferential increase in the dissocation rate of RecG from 4J12 as opposed to 3J12. An estimate of the relative levels of dissociation of RecG from 4J12 and 3J12 was obtained by preincubating RecG with labelled junction DNA and then adding non-specific competitor DNA to act as a sink for any RecG that dissociated from the junction DNA prior to electrophoresis. In the absence of

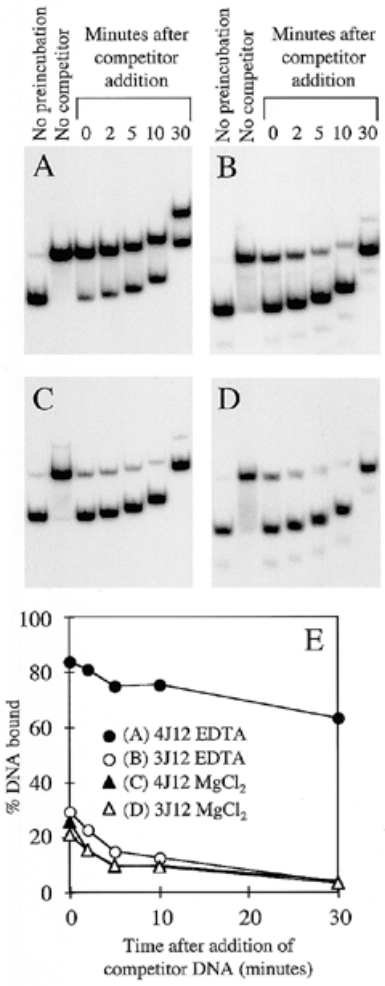

Figure 5. Relative rates of dissociation of RecG from 4J12 and 3J12 junction DNA. The rates at which RecG dissociated from 4J12 (**A** and **C**) and from 3J12 DNA (**B** and **D**) were estimated from band shift gels as described in Materials and Methods. RecG was preincubated with 4J12 or 3J12 and a sample was then electrophoresed into a gel containing EDTA (No competitor). Poly(dI·dC) was then added to act as a sink for any RecG which dissociated from the junction DNA and samples were run into the gel immediately after (0 min) and 2, 5, 10 and 30 min after competitor addition. A control reaction in which RecG was added to a mixture of both the labelled junction DNA and the competitor DNA (No preincubation) was also performed to ensure that the competitor inhibited any dissociated RecG from rebinding to labelled junction DNA. Binding reactions contained EDTA (A and B) or 2 mM $MgCl_2$ (C and D). (**E**) Quantification of the mean levels of binding in (A)–(D) and in a second set of reactions.

$MgCl_2$ RecG–4J12 complexes were stable, with only a small proportion of free DNA appearing over the time course (Fig. 5A and E). However, the presence of 2 mM $MgCl_2$ destabilised the RecG–4J12 complexes, which supported an increase in the dissociation rate of RecG from 4J12 in the presence of Mg^{2+} (Fig. 5C and E; 26). In contrast, the stability of RecG–3J12 complexes was low whether $MgCl_2$ was present or not and were equivalent to the result obtained with 4J12 in $MgCl_2$ (Fig. 5B, D and E). This indicated that in the absence of $MgCl_2$ the rate of dissociation of RecG from 4J12 was lower

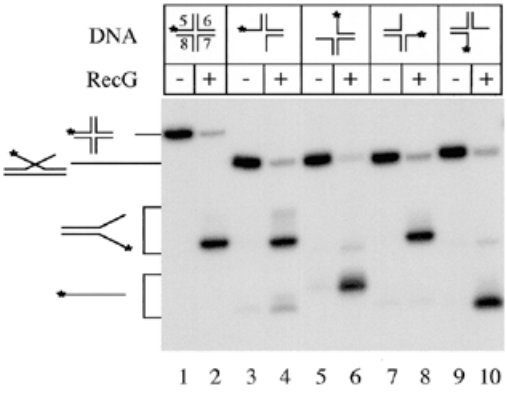

Figure 6. Polarity of unwinding of three-strand junctions by RecG. Unwinding assay gel of 4J0 (lanes 1 and 2) and all four possible three-strand versions of J0 (lanes 3–10). The five junctions, using oligonucleotides 5–8 as described in Materials and Methods, were constructed so that the free 5'-end of each three-strand junction was radiolabelled as indicated with an asterisk. The positions of intact four- and three-strand junctions are marked, as are the flayed duplex and single-stranded oligonucleotide products. The polarity of RecG action was deduced from the production of labelled flayed duplex or single-stranded oligonucleotide which indicated unwinding of the oligonucleotide arm with a free 3'-end or a free 5'-end, respectively.

than from 3J12 and may have explained the lower relative dissociation constants of RecG with four-strand junctions as compared with three-strand junctions (Table 1). However, the presence of $MgCl_2$ caused an increase in the rate of RecG dissociation from 4J12 to approximately the level seen with 3J12. This may be one reason why the substrate binding affinity of RecG shifted from four-strand to three-strand junctions in the presence of $MgCl_2$ (Fig. 4 and Table 1).

Polarity of unwinding of three-strand junctions by RecG

The relative substrate specificities analysed above indicate that RecG preferentially targets three-strand junctions under certain conditions. A three-strand junction has polarity since one single-stranded arm has a 5'-end whereas the other single-stranded arm ends 3'. The model proposed in Figure 1A predicts that RecG preferentially unwinds the arm that has a free 5'-end, thus driving the crossover point towards the D-loop. In contrast, Figure 1B suggests that RecG preferentially unwinds the arm that has a free 3'-end, thus moving the crossover away from the D-loop towards the region of duplex:duplex DNA pairing. To investigate whether either of these predictions is accurate, and by inference which model of RecG catalysis is correct, all four possible three-strand versions of J0 were constructed and the free 5'-end of each junction was radiolabelled (Fig. 6). If RecG unwound three-strand junctions as predicted by Figure 1A then each of the three-strand versions of J0 in Figure 6 would be preferentially unwound to give labelled single-stranded oligonucleotides. If the second model is correct (Fig. 1B) then each three-strand version of J0 would be unwound to give labelled flayed duplexes. The use of J0 rather than 4J12 excluded the possibility that any observed polarity would be due to the crossover point being preferentially

located towards one end of the junction. Two of the three-strand junctions were unwound to give primarily labelled flayed duplexes (Fig. 6, lanes 4 and 8) whereas the other two three-strand junctions were unwound with the reverse polarity to give mainly labelled single-stranded oligonucleotides (Fig. 6, lanes 6 and 10). RecG also failed to display a preference for unwinding either the free 3'- or 5'-end of three-strand junctions containing 2, 4 and 12 bp homologous cores and this lack of polarity was unaffected by the concentrations of $MgCl_2$ and ATP (data not shown). There was also no correlation between the G+C content of the duplex arms of each junction and the direction of unwinding (data not shown). Thus RecG did not preferentially unwind the free 3'- or 5'-ends of three-strand junctions *in vitro*.

DISCUSSION

The models presented in Figure 1 predict that RecG specifically targets three-strand junctions and, in the second model (Fig. 1B), that RecG catalyses the unwinding of a three-strand junction at a D-loop to generate a four-strand structure. These predictions have been tested by comparing the rates of unwinding of, and binding affinities for, model three- and four-strand junctions by RecG *in vitro*. We have shown that the relative rates of unwinding depend critically on the concentrations of $MgCl_2$ and ATP. This dependence can be attributed, at least in part, to the differential effects of $MgCl_2$ on the relative dissociation constants of RecG for three- as compared with four-strand junction binding (Figs 4 and 5 and Table 1). The complex nature of this effect is most likely due to the requirement of Mg^{2+} for ATP hydrolysis by RecG whilst at the same time it inhibits binding of RecG to four-strand junctions (13,26). The inhibition of DNA binding is due to the ability of Mg^{2+} to promote the folding of a four-strand junction from an open, square planar comformation, which is bound with high affinity by RecG, to a stacked structure, which is bound less well (26,30). Therefore, the optimal level of Mg^{2+} for the unwinding of four-strand junctions by RecG is a delicate balance between these opposing effects.

In contrast to the extensive studies conducted on the structure of four-strand junctions, nothing is known about the conformations adopted by three-strand junctions and what the effect of Mg^{2+} might be on these structures. However, the observation that the optimal $MgCl_2$ and ATP concentrations for unwinding of three-strand junctions by RecG differed from those for four-strand junctions indicates that Mg^{2+} has different effects on the structures adopted by three- and four-strand junctions. The finding that $MgCl_2$ preferentially increased the relative dissociation constants of RecG for four- as opposed to three-strand junctions supports this conclusion (Figs 4 and 5) and indicates that the structures adopted by three-strand junctions in the presence of Mg^{2+} were less inhibitory to binding by RecG than the stacked-X conformations observed for four-strand junctions. Previous studies have indicated that a flayed duplex is the critical feature of junctions recognised by RecG (21,26). Mg^{2+} may reduce the accessibility of this feature in four-strand junctions to a greater extent than in three-strand junctions.

The binding and unwinding data obtained in this study may also be relevant to the ability of RecG to unwind R-loops formed by the invasion of duplex DNA by a homologous RNA (19,20). R-loops share many of the features present in D-loops and it is possible that the conditions which favour unwinding of three-strand junctions by RecG may also enhance the dissociation of R-loops.

What relevance do these data have to the identification of the target(s) of RecG *in vivo*? The importance of $MgCl_2$ and ATP concentrations in determining the substrate targeting of RecG indicate that the *in vivo* levels of these factors are crucial to understanding the specificity of RecG. The concentration of ATP in *E.coli* and *Salmonella typhimurium* has been estimated to be ~3 mM (31,32). However, ATP concentration in *E.coli* varies according to growth conditions and has also been reported to transiently increase 2- to 3-fold after DNA damage or inhibition of DNA synthesis (33–35). Therefore, intracellular ATP concentration may lie somewhere between 1.5 and 9 mM depending on the state of the cell. In contrast, the total concentration of Mg^{2+} in *E.coli* is estimated to be ~100 mM (29). However, most of this is bound to polyanions within the cell such as nucleic acids and the concentration of Mg^{2+} in solution, and not associated with polyanions, has been estimated to be only 1–2 mM (28). It is also unclear whether this estimate of free Mg^{2+} includes that bound to nucleotides such as ATP or whether the concentration of free Mg^{2+} varies according to the state of the cell. Thus, the *in vivo* concentrations of ATP and Mg^{2+} are not sufficiently well characterised to unequivocally determine the substrate specificity of RecG *in vivo* using the data presented here. However, the measured *in vivo* ranges of ATP and free Mg^{2+} suggest it is possible that intracellular conditions may favour the unwinding of three- as opposed to four-strand junctions by RecG. The variation in the levels of ATP seen under different growth conditions and during the SOS response also raises the intriguing possibility that the substrate specificity of RecG could alter in response to the environment. A mechanism whereby RecA catalysis is regulated by changes in Mg^{2+} and ATP concentrations has been proposed previously and the action of RecBCD enzyme has also been shown to be altered by changes in the concentration of these factors *in vitro* (36,37).

The models presented in Figure 1 also predict that RecG has a distinct polarity of unwinding three-strand junctions, although this polarity is different for the two models. However, there was no preferential unwinding of either the free 3'- or 5'-end of junctions (Fig. 6) and so the two models of RecG catalysis could not be distinguished by these data alone. Other factors may dictate the polarity of RecG action at such junctions *in vivo*. Previous studies have demonstrated that the presence of RecA in an *in vitro* strand exchange reaction has a marked effect upon the direction of branch migration catalysed by RecG (16,18). Therefore, it is possible that the polarity of RecG action at D-loops may be determined by RecA bound to the D-loop rather than the asymmetry of the three-strand junction. It should also be noted that the lack of RecG specificity for free 3'- or 5'-ends *in vitro* is consistent with RecG acting at D-loops formed by invading single-stranded DNA 5'-ends as well as those formed by 3'-ends (38). It has been postulated that RecG may actually act at both putative types of D-loop by countering RecA-mediated strand exchange and that this may have opposite effects upon the promotion of recombination from D-loops, leading to the formation of four-strand junctions from 3'-end invasion events but the abortion of 5'-end invasion events (6,16).

The data presented in this study indicate that RecG may be preferentially targeted to three-strand junctions. However, only small changes in ATP and Mg^{2+} levels are required to alter the relative specificity of RecG for three- as opposed to four-strand junctions. This may be a consequence of the ability of RecG to branch migrate three-strand into four-strand junctions, since to do so RecG must be able to act as a helicase upon both structures. Data presented here indicate that the dissociation constant of RecG for a junction increases upon conversion of the junction from a three-strand into a four-strand structure in the presence of Mg^{2+}. This would facilitate the targeting of such junctions by the RuvABC complex, leading to their resolution and separation of the two duplexes, which supports the model presented in Figure 1B. We are currently developing *in vitro* DNA substrates that may accurately reproduce a strand invasion event so as to dissect such post-synaptic catalytic processes.

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