Targeting Holliday Junctions by the RecG Branch Migration Protein of Escherichia coli

(Received for publication, February 11, 1998, and in revised form, May 21, 1998)

Matthew C. Whitby‡‡ and Robert G. Lloyd¶
From the ‡‡Microbiology Unit, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, United Kingdom and the ¶Department of Genetics, University of Nottingham, Queens Medical Center, Nottingham NG7 2UH, United Kingdom

The RecG protein of Escherichia coli is a junction-specific DNA helicase that drives branch migration of Holliday intermediates in genetic recombination and DNA repair. The reaction was investigated using synthetic X-junctions. RecG dissociates X-junctions to flayed duplex products, although DNA unwinding of the heterologous arms is limited to ≤30 base pairs. Junction unwinding requires Mg²⁺ and the hydrolysis of ATP. X-junction DNA stimulates the ATPase activity of RecG. ATPase activity is also stimulated by linear duplex DNA, although to a lesser extent than by X-DNA, but not by linear single-stranded DNA. In situ 1,10-phenanthroline-copper footprinting shows that RecG binds to the strand cross-over point at the center of the X-junction. Substrate recognition by RecG was investigated using DNAs that represented the various component parts of an X-junction. The minimal DNA structure that RecG forms a stable complex with is a flayed duplex, suggesting that this is the critical feature for junction recognition by RecG. Junction binding and unwinding also depend critically on the concentration of free Mg²⁺, excess free cation dramatically inhibiting both processes. These inhibitory effects are not mediated specifically by Mg²⁺; e.g. both Ca²⁺ and hexaminecobalt(III) chloride also inhibit X-junction binding and unwinding by RecG. The relative abilities of these cations to inhibit RecG-junction binding is correlated with their respective abilities to stack X-junction DNA. From this we conclude that RecG is unable to bind or binds very poorly to fully stacked X-junctions.

General genetic recombination is a key process in biology that is required for promoting genetic diversity, repairing damaged DNA, and ensuring that chromosomes are correctly segregated at cell division. A central feature of the reaction is a reciprocal exchange of single strands between two DNA duplexes. Strand exchange creates a heteroduplex joint within reciprocal exchange of single strands between two DNA duplexes. Strand exchange creates a heteroduplex joint within a reaction requiring both strands of each DNA to participate. The DNA helicase must unwind a strand from each of the unbound arms and drive their branch migration (6). The reaction catalyzed by RuvAB is reasonably well understood. A tetramer of RuvA binds to the junction point and holds the DNA in an open configuration that allows assembly of a hexameric ring of RuvB around each of the diametrically opposed homologous arms flanking the RuvA-junction complex (7, 8). The two rings face each other across the junction, and in a reaction requiring hydrolysis of ATP, each ring rotates the bound DNA and draws it through the hollow core of the RuvB assembly. This novel reaction unwinds a strand from each of the unbound arms and winds them together to pass through the RuvB ring. As each arm is rotated, the net effect of this specialized helicase activity is to move the junction point along the DNA.

How RecG interfaces with Holliday junctions and drives their branch migration is less clear. The 76-kDa RecG polypeptide is a DNA-dependent ATPase that binds specifically to Holliday intermediates and, in the presence of Mg²⁺ and ATP, catalyzes branch migration of the junction point (9–11). RecG has a number of structural motifs that are well conserved in DNA and RNA helicases (9). In agreement with this, we have shown that RecG can unwind partial duplex DNAs, although it does so rather inefficiently (12). Helicase activity is stimulated on branched DNA structures and targeted specifically to the junction point (12). From these observations, it was proposed that RecG drives the branch migration of Holliday junctions by targeting and unwinding DNA at or near the junction crossover point. The link between DNA unwinding and branch migration was further supported by a RecG mutant with an Ala to Val substitution in helicase motif III that retains the ability to target junctions but is deficient in DNA unwinding and branch migration (13). Recently, Mahdi et al. (14) have used various truncated and mutant forms of RecG to show that the N-terminal region of the protein is required for junction-specific binding, while the C-terminal region is critical for DNA unwinding. Since both the junction targeting and helicase activities are encoded within a single polypeptide, the mode of action of RecG is likely to differ from that of RuvAB.

To gain more insights into the mechanism of branch migration, we have analyzed further features of RecG’s interaction with X-junctions in vitro. In particular, we concentrate here on how RecG targets X-junctions. Data are presented indicating that RecG binds to the center of the X-junction by recognizing
principally the displacement of two strands of DNA from a common junction arm. We also show that the unwinding of X-junctions by RecG is remarkably sensitive to the level of free Mg$^{2+}$. This sensitivity correlates directly with an inability of RecG to bind to the fully stacked X-junction. The possible significance of these observations is discussed in relation to how RecG might function in vivo.

**MATERIALS AND METHODS**

**Enzymes and Reagents**—RecG, RuvA, and RuvB were purified as described (15, 16). Amounts of protein were estimated by a modified Bradford assay using a Bio-Rad protein assay kit and bovine serum albumin (Amersham Pharmacia Biotech) as the standard. Details of the procedures of Parsons et al. (17) were used. This junction contains a homologous core of 12 bp, within which the junction point is free to branch-migrate, whereas the static X-junction (X-0) made from oligonucleotides 1–4 contains 270 ng of RecG and 16 ng of X-0, labeled in one of its component oligonucleotides (Molecular Dynamics). We have used oligonucleotides—Oligonucleotides were synthesized on an Applied Biosystems 380B DNA synthesizer using cyanoethyl chemistry. Each oligonucleotide was deprotected, precipitated in ethanol, and purified on a 12% (v/v) polyacrylamide gel containing 7 M urea. The bands containing full-length oligonucleotides were cut out and extracted from the gel by soaking in water over night.

**DNA Substrates**—DNA substrates were made by annealing combinations of oligonucleotides 5 (5′-AAAGTAAAGCTCTATGCGCCAT-3′), 6 (5′-TTTCTCATTTT-3′), 7 (5′-CAACGGTATAGACGATTACATTGCTTAACAG-3′), 8 (5′-TGGGTCAACGTGGGCAAAGATGTCCTAGC-3′), 9 (5′-GGTGATGGACATCTTTGCCCACGTTGAC-3′), 10 (5′-ATCGATAGTCTC-3′), 11 (5′-CTACGGCCATAGCTGGTAGAATTCGGC-3′), 12 (5′-CAACGGTATACGATTACATTGCTTAACAG-3′), 13 (5′-CCATGAGATTCGTTTAGAATTCGGC-3′), 14 (5′-CAACGCTATGACGATTACATTGCTTTGCCCACGTTGAC-3′), 15 (5′-AAATGAGCTCTTTCTAACCTTCTTCCGATCATG-3′), 16 (5′-TGGGTCAACGTGGGCAAAGATGTCCTAGC-3′), 17 (5′-CAACGGTATACGATTACATTGCTTAACAG-3′), 18 (5′-ATCGATAGTCTC-3′), 19 (5′-GGTGATGGACATCTTTGCCCACGTTGAC-3′), 20 (5′-GAGCTGCGAAATCGCCATAGCTGGTAGAATTCGGC-3′), 21 (5′-CAACGGTATACGATTACATTGCTTAACAG-3′), 22 (5′-ATCGATAGTCTC-3′), 23 (5′-CAACGGGATTACATTGCTTAACAG-3′), and 24 (5′-CAACGGGATTACATTGCTTAACAG-3′) as indicated following the procedures of Parsons et al. (17). The synthetic Holliday junction consists of a 32-bp duplex region and a 16–20 nucleotide region of single-stranded DNA, whereas the flayed duplex substrates H and I each consist of a 26-bp duplex region and a 24–26-nucleotide region of unpaired single-stranded DNA, whereas the flayed duplex substrates H and I each consist of a 32-bp duplex region and a 16–20 nucleotide region of unpaired single-stranded DNA. Prior to annealing, DNA substrates were labeled at the 5′-end of one of their component oligonucleotides as indicated using [γ-32P]ATP and polynucleotide kinase. Annealed substrates were purified by nondenaturing electrophoresis on 6% polyacrylamide gels followed by electrophoresis. The concentration of DNA substrates was estimated by relating the specific activity of the labeled oligonucleotide to the activity of the purified substrate and is expressed in molar concentrations of DNA substrate.

**ATPase Assay**—Reactions (100 μl) contained 66 mM RecG in 20 mM Tris-HCl, pH 7.5, 2 mM DTT, 5 mM MgCl2, 5 mM ATP (of which a fraction was [α-32P]ATP), and 100 μM BSA. 720 ng of 60-mer X-12, linear duplex (oligonucleotides 18 and 23), or ssDNA (oligonucleotide 19) was included in the reaction mixture as indicated. Reactions were incubated at 37 °C, and at specified intervals 10-μl samples were taken and stopped by the addition of 2 μl of 0.5 M EDTA. The release of [α-32P]ADP from [α-32P]ATP was assayed by thin layer chromatography on polyethyleneimine-cellulose and measured by detection of the labeled products using a Molecular Dynamics PhosphorImager (model 4400i). ImageQuant software (Molecular Dynamics, Inc.) was used to analyze phosphor images.

**Binding Assays**—Reaction mixtures (20 μl) contained labeled substrate DNA in buffer 2 (25 mM Tris-HCl, pH 8.0, 1 mM DTT, 100 μg/ml BSA, 6% glycerol) as indicated. Reactions were started typically by the addition of RecG, held on ice for 10 min, and then loaded immediately onto a 4% native polyacrylamide gel in low ionic strength buffer (6.7 mM Tris-HCl, pH 8.0, 3.3 mM sodium acetate, 2 mM EDTA) with the voltage already applied at 200 V, unless otherwise indicated. Competitor DNA (poly(dIdC)-poly(dIdC)) (Amersham Pharmacia Biotech) added during the course of some reactions was mixed in by four smooth pipetting movements using a P20 Gilson Pipetman. Samples were run into the gel typically for 20 min at 200 V. Following this, the voltage was lowered to 160 V, and electrophoresis was continued for 20 min with buffer recirculation occurring throughout. Both buffer and gel were precooled at 4 °C, but electrophoresis was at room temperature. Gels were dried on 3 MM Whatman paper, quantified using a model SF PhosphorImager and ImageQuant software (Molecular Dynamics), and autoradiographed.

**Junction Unwinding Assays**—Reaction mixtures (20 μl) contained labeled substrate DNA in either buffer 1 (20 mM Tris-HCl pH 7.5, 2 mM DTT, 100 μg/ml BSA) or buffer 2 (25 mM Tris-HCl, pH 8.0, 1 mM DTT, 100 μg/ml BSA, 6% glycerol) and MgCl2, ATP, and protein as indicated. After incubation at 37 °C, typically for 30 min, reactions were terminated by adding one-fifth volume of stop mix (2.5% SDS, 200 μM EDTA, 10 mg/ml proteinase K) and incubating for a further 10 min at 37 °C to denature the mixture. Products were analyzed by electrophoresis through 10% native polyacrylamide gels at 190 V using a standard Tris-borate buffer system. Gels were dried on 3 MM Whatman paper, quantified using a model SF PhosphorImager and ImageQuant software (Molecular Dynamics), and autoradiographed.

**In Situ 1,10-Phenanthroline-Copper Footprinting**—Binding reactions (50 μl) containing 270 ng of RecG and 16 ng of X-0, labeled in one of its component oligonucleotides, were set up in buffer containing 2 mM EDTA in parallel with control reactions containing no RecG as indicated. Following a 10-min incubation at 4 °C, binding reactions were run on a 6% non-denaturing polyacrylamide gel in low ionic strength buffer (6.7 mM Tris-HCl pH 8.0, 3.3 mM sodium acetate, 2 mM EDTA). Electrophoresis was at 4 °C for 2 h at 160 V with continuous circulation of buffer. X-0 and RecG-X-0 complexes were detected by autoradiography at 4 °C, and the appropriate bands were excised from the gel, chopped into evenly sized small pieces, and immersed in 100 μl of 50 mM Tris-HCl (pH 8.0). 10 μl of OP-Cu mix (2 mM 1,10-phenanthroline and 0.45 mM CuSO4) and 10 μl of 58 mM mercaptopyrrolic acid were then added, and the mixture was incubated at room temperature for 10 min. The reaction was stopped by the addition of 20 μl of 28 μM 2,9-dimethyl-1,10-phenanthroline (18). 270 μl of 0.5 M ammonium acetate, 1 mM EDTA was then added, and the DNA was loaded in the gel by incubation overnight at 37 °C with gentle agitation in a thermomixer (Eppendorf). The DNA was then precipitated with ethanol, washed twice with 70% ethanol, resuspended in loading dye (98% formamide, 1 mM EDTA), then loaded onto a 15% polyacrylamide sequencing gel containing 7 M urea. Gels were dried and analyzed using a model SF PhosphorImager and ImageQuant software (Molecular Dynamics) and by autoradiography.

**RESULTS**

**The Binding and Unwinding of X-junctions by RecG**—We and others have used synthetic X-junctions as substrates to study the in vitro behavior of enzymes that process Holliday junctions during genetic recombination and DNA repair. For many of the studies on RecG described below, an X-junction made from four oligonucleotides of 48–51 nucleotides each has been used. This junction contains a homologous core of 12 bp, 1

1The abbreviations used are: bp, base pair(s); DTT, dithiothreitol; ssDNA, single-stranded DNA; BSA, bovine serum albumin; ATPγS, adenosine 5′-O-(thiotriphosphate).
within which the junction point is free to branch-migrate and therefore provides a close mimic of a natural Holliday intermediate. The heterologous arms flanking the core limit spontaneous unwinding of the substrate by branch migration of the junction point to the DNA ends. This junction will be referred to as X-12. To begin to characterize the mechanism of RecG branch migration, we have studied some general features of RecG’s interaction with X-12 in vitro.

Two basic qualities of a Holliday junction processing enzyme are that it binds to the junction with a high degree of specificity and, under appropriate reaction conditions, catalyzes either its branch migration or resolution by endonucleolytic cleavage. Both binding and branch migration of X-12 by RecG can be analyzed by simple gel-based assays (Fig. 1). In Fig. 1A, RecG junction binding is analyzed by a band shift assay. Two distinct RecG-X-12 complexes are detected. Complex 1 forms at low concentrations of RecG (lanes b–i), but as the concentration of RecG increases and essentially all of the junction DNA has been bound, it is replaced by the slower migrating complex 2 (lanes j–m). Based on assays like this, we estimate that complex 1 is formed by the binding of a single monomer or a dimer of RecG per junction, which agrees with previous estimates (10) and is consistent with gel filtration data indicating that RecG is a monomer in solution. Based on its relative mobility, complex 2 is unlikely to involve more than two dimers or a single tetramer of RecG. RecG’s specificity for X-12 under these conditions can be demonstrated by its lack of binding to any of the individual oligonucleotides used to make X-12 or to a linear duplex DNA of related nucleotide sequence (Fig. 1A, lanes l–m). It is evident from the lack of complex 2 formation at concentrations of RecG that are saturating for the formation of complex 1 that RecG’s affinity for binding X-12 to form complex 1 is considerably higher than it is for binding X-12 to form complex 2.

RecG’s ability to convert X-12 to flayed duplex products is shown in Fig. 1B. It dissociates the substrate by branch-migrating the junction point followed by unwinding one pair of heterologous arms (Fig. 1C). The junction is a symmetrical structure, and as such RecG can unwind two different pairs of arms to catalyze branch migration in either of the two possible directions as evidenced by the production of two different flayed duplexes (Fig. 1B, lane d). This reaction is dependent on Mg\(^{2+}\) and a hydrolyzable form of ATP (lanes b–d).

Hydrolysis of ATP—RecG is a DNA-dependent ATPase that needs to hydrolyze ATP to branch-migrate and unwind X-junctions. X-junction DNA is bound by RecG with higher specificity and affinity than other DNAs; it should therefore provide a better cofactor for the hydrolysis of ATP by RecG than other DNAs. To test this possibility, we analyzed the hydrolysis of ATP in the presence of a range of different but sequence-related DNAs (Fig. 2). Little or no hydrolysis was observed in the absence of DNA or in the presence of a ssDNA oligonucleotide. Some hydrolysis was detected in the presence of linear duplex DNA; however, hydrolysis was most stimulated in the presence of X-junction DNA, with the rate being nearly four times greater than with the linear duplex DNA. The same total quantity of DNA was used in each of these reactions, but similar results were obtained also when equimolar amounts of DNA were used (data not shown). The lack of ATP hydrolysis in the presence of ssDNA appears to contradict the observation of Lloyd and Sharples (10) that X174 ssDNA can promote ATP hydrolysis by RecG. This apparent anomaly probably reflects a size difference and the likely ability of X174 ssDNA to form secondary structures that could mimic junctions.

The Limit of Unwinding DNA at Holliday Junctions—RecG can unwind partial duplex DNAs of 26 bp but fails to unwind ones of 52 bp (12). To see if DNA unwinding at Holliday junctions is similarly restricted, we examined RecG’s activity on a number of different X-junctions that varied the number of base pairs needed to be unwound before the substrate could be dissociated to flayed duplex products. We first examined an X-junction with the same homologous core as X-12 but made with oligonucleotides of approximately 60 nucleotides in length (60-mer X-12) so as to give heterologous arms of 24–25 bp instead of the 18–19 bp that X-12 has. RecG readily unwinds this junction (Fig. 3A, lane b), as does RuvAB (lane c). However, when we used an equivalent sized junction without a homologous core (60-mer X-0) so that 30 bp had to be unwound, we observed very little unwinding by RecG (lane e), whereas RuvAB seemed to have no trouble (lane f). To test whether the
poor unwinding of 60-mer X-0 by RecG was due to the increased number of base pairs to be unwound or the absence of a homologous core, we tested RecG's activity on a smaller static X-junction. This smaller junction, made from oligonucleotides approximately 50 nucleotides in length, is unwound much more readily than the 60-mer X-0 junction (Fig. 3B). The activity is similar to that seen with the 60-mer X-12 junction, which is perhaps not too surprising, since the two junctions have homologous arms of the same length (24–25 bp). We also examined the 50-mer X-12 junction in the same series of experiments. This junction, which has homologous arms of only 18–19 bp, is unwound with noticeably greater efficiency than any of the other junctions. These data show that there is a direct correlation between the ability of RecG to unwind a junction and the number of base pairs in the homologous arms flanking the junction point. RecG's limit for DNA unwinding at X-junctions is ≤30 bp, which lies within the range estimated for the limit of unwinding of partial duplex DNA substrates.

RecG binds to X-junctions at the Point of Strand Crossover—The binding specificity of RecG for X-junctions presumably reflects its ability to recognize some structural feature of this type of DNA. To gain a better understanding of what this might be, we sought to locate where RecG was binding on X-junction DNA using in situ 1,10-phenanthroline-copper footprinting. For these studies, we used an X-junction without a mobile core (X-0) so that the position of the junction crossover point would be fixed and therefore known precisely. X-0 shares one common oligonucleotide with X-12 and is bound by RecG to form complex 1 with similar efficiency as X-12 (data not shown). RecG-X-0 junction complexes were excised from band shift gels containing 2 mM EDTA and reacted with the 1,10-phenanthroline-copper reagent as described under “Materials and Methods.” Four reactions were performed in parallel, each containing X-0 labeled in a different strand. The products of these reactions were analyzed on a 15% polyacrylamide gel containing 7 M urea (Fig. 4A). Regions of protection from attack by the 1,10-phenanthroline-copper reagent were detected in all four strands of X-0, which was bound by RecG to form complex 1. These were located at and flanking the point of strand crossover in X-0 and exhibited approximately 2-fold symmetry (Fig. 4B). Essentially the same results were obtained when complex 2 was footprinted (data not shown). In the absence of metal ions, X-junctions adopt a structure in which the four arms of the junction extend toward the corners of a square and as such are regarded to have 4-fold symmetry. However, it is clear from the data in Fig. 4 that RecG does not bind to X-0 to give a 4-fold symmetrical pattern of protection from 1,10-phenanthroline-copper. This suggests that X-0, in the absence of metal ions, may not be wholly 4-fold symmetrical and as such may present a favored binding interface to RecG. Alternatively, particular nucleotide sequences at the junction point could influence the binding preferences of RecG.

RecG binds to flayed duplex DNA—Genetic and biochemical data have provided evidence for an involvement of RecG in processing early recombination intermediates (D-loops) and R-loops, in addition to its role in branch-migrating Holliday junctions (15, 19–22). For example, RecG binds to a range of different branched DNAs in vitro, including D-loops, three-strand junctions, and Y-junctions. To determine what the minimal DNA structure is for specific binding by RecG, we constructed a range of DNA substrates of decreasing complexity.
Targeting Holliday Junctions by RecG

Fig. 4. In situ 1,10-phenanthroline-copper footprinting of complex 1 of RecG with X-0 isolated from a 6% polyacrylamide binding gel. A, 15% sequencing gel of footprinting reactions (see "Materials and Methods"). Bars alongside +RecG lanes indicate regions of protection as determined by PhosphorImager analysis. B, schematic of the central region of X-0 showing regions protected by RecG (shaded).

Constant ($K_D$) for RecG binding to X-12 is approximately 0.5–1.5 nM, whereas for three-strand junctions and Y-junctions (substrates A and B) it is approximately 5 nM, and for part Y-junctions (substrates C and D) and flayed duplex DNA (substrate E) it is in the range of 10–50 nM (data not shown). These data indicate that RecG’s affinity for X-junctions is 5–10-fold higher than for three-strand junctions and Y-junctions, and 10–100-fold higher than for part Y-junctions and flayed duplex molecules.

From these data, we conclude that the minimal branched DNA structure bound by RecG to yield a specific complex that is detectable by band shift analysis is a flayed duplex molecule. Indeed, the flayed duplex may represent the basic structural element that RecG recognizes in all of the junctions that it binds to. If this is true, then it also provides an explanation for why RecG binds readily to X-12 to form complex 2 but does not readily form a second complex with any of the other substrates used in this investigation. Basically, X-12 can be considered to contain two distinct “flayed duplex” components, whereas each of the other substrates contains only one. To test this idea, we constructed a further substrate consisting of two 18-bp duplex regions flanking a heterologous loop region of 19 bases. We reasoned that RecG should be able to target both flayed duplexes formed at the junctions between double-stranded and single-stranded DNA at either end of the loop and therefore would readily form both RecG-junction complex 1 and complex 2. The loop substrate was incubated with different concentrations of RecG, and binding was analyzed as previously. As predicted, two specific RecG-DNA complexes were observed (Fig. 5C, lanes f–h). The faster complex migrated in the gel to approximately the same position as complex 1 formed with X-12, whereas the slower complex migrated to approximately the same position as complex 2 formed with X-12. Furthermore, it is evident that RecG forms complex 2 far more readily with the loop substrate than with X-12 (Fig. 5C and data not shown). Complex 2 formation with X-12 may be impeded by steric interference between the two molecules of RecG attempting to dock on the same junction. Such conflict may not arise with the loop substrate because its flayed duplex components are sufficiently spaced to avoid such clashes. These data are consistent with RecG binding readily to both flayed duplex junctions at either end of the loop.

To see if RecG’s ability to bind a particular DNA substrate correlates with its ability to unwind that substrate, we analyzed the unwinding of substrates A–G by RecG (Fig. 4D). Very low levels (<1%) of unwinding were observed with the two flayed duplex DNA molecules (substrates E and F, lanes j and l) and the partial duplex molecule (substrate G, lane n). We also detected very little unwinding of the other two flayed duplex molecules (substrates H and I) and the loop DNA (data not shown). In contrast, efficient unwinding of substrates A, B, C, and D was observed (lanes b, d, f, and h). The three-strand junction (substrate A) was dissociated by the removal of either oligonucleotide 2 or 3 predominantly. Small amounts of free oligonucleotide 1 were also observed that could have come directly from dissociation of the three-strand junction or from a secondary reaction on the flayed duplex molecules produced following oligonucleotide 2 or 3 removal. The Y-junction (substrate B) was dissociated in similar fashion by removal of either oligonucleotide 2 or 4 predominantly. Dissociation of substrates C and D was predominantly by removal of the short oligonucleotide in each case. These data show that the ability of RecG to bind to a DNA substrate does not always correlate with its ability to unwind that substrate. Furthermore, it is clear that, although RecG will readily bind to a three-way junction with only one duplex arm (i.e. a flayed duplex), efficient DNA un-
winding by RecG requires at least two of the arms to be double-stranded.

The Mg$^{2+}$/ATP Ratio Affects the Unwinding of X-12 by RecG—As shown in Fig. 1, RecG depends on both ATP and Mg$^{2+}$ to unwind X-12. Previous studies have indicated that branch migration catalyzed by RecG favors high concentrations of ATP (10, 16) and is inhibited by high concentrations of MgCl$_2$ (11). To investigate further RecG’s dependence on ATP and Mg$^{2+}$ for unwinding X-12, we analyzed the rate of the reaction at four concentrations of MgCl$_2$ and a fixed concentration of ATP. The rate was reduced dramatically with Mg$^{2+}$ in molar excess over ATP. A 10-fold reduction was observed when the molar ratio was increased from 1:2 to 2:1 (Fig. 6A). The negative effect of excess Mg$^{2+}$ was reversed by reestablishing a more favorable ratio during the reaction (Fig. 6B). These data show that the unwinding of X-12 by RecG depends critically on the Mg$^{2+}$/ATP ratio, with the optimal ratio being 1:1 and junction unwinding being significantly inhibited when there is excess free Mg$^{2+}$.

Mg$^{2+}$ Reduces RecG Junction Binding—The inhibition of junction unwinding by excess Mg$^{2+}$ could be explained by an inhibition of RecG junction binding. We investigated this possibility by comparing junction binding in the presence of 2 mM EDTA, 200 $\mu$M MgCl$_2$, and 5 mM MgCl$_2$. X-12 was incubated

Fig. 5. Binding and unwinding DNA substrates that mimic the component parts of an X-junction. A and B, band shift assay showing RecG binding to a range of DNA substrates. Reactions (20 $\mu$L) contained 0.7 nM substrate DNA in reaction buffer 2 containing 2 mM EDTA and RecG as indicated. Reactions were incubated on ice for 10 min before loading onto a 4% polyacrylamide binding gel as described under “Materials and Methods.” A schematic diagram of each DNA substrate is shown at the top. Each substrate is made from the oligonucleotides indicated by number on each respective schematic. All substrates are $^{32}$P-labeled at the 5’-end of one of their component oligonucleotides as indicated by the asterisk. C, same as above except for the concentrations of X-12 and the loop substrate DNA, which were 1.5 and 0.5 nM, respectively. D, unwinding of substrates A–G by RecG. Reactions (20 $\mu$L) contained 0.7 nM substrate DNA in 20 mM Tris-HCl, pH 8.0, 2 mM DTT, 2 mM MgCl$_2$, 5 mM ATP, 100 $\mu$g/ml BSA, and 100 nM RecG as indicated.
Targeting Holliday Junctions by RecG

Inhibition of Junction Binding and Unwinding by Other Cations—The inhibitory effect of MgCl₂ on junction binding could be due to its interaction with RecG and/or DNA. In order to distinguish between these possibilities, we sought to determine whether the inhibitory effects of MgCl₂ were specific to Mg²⁺ or could be manifested by other cations. We first examined the effect of CaCl₂. Ca²⁺ was unable to substitute for Mg²⁺ in the unwinding of X-12 by RecG (data not shown). However, both junction binding and unwinding in the presence of Mg²⁺ were inhibited by CaCl₂ to a similar extent as by MgCl₂ (data not shown). These data indicate that inhibition is not mediated specifically by Mg²⁺. Furthermore, they suggest that the inhibitory effects of Mg²⁺ may be mediated largely by its interaction with the X-junction DNA.

**Fig. 6. The effect of MgCl₂ on the rate of unwinding of X-12 by RecG.** A, reactions (20 μl) contained varying concentrations of RecG and 2.75 nM X-12 in reaction buffer 2 plus 2 mM EDTA or 200 μM MgCl₂ as indicated. Reaction mixtures were incubated on ice for 10 min before loading onto a 4% polyacrylamide binding gel containing 2 mM EDTA or 200 μM MgCl₂ as indicated. The data was quantitated by PhosphorImager analysis, and the percentage of X-12 bound for each concentration of RecG was plotted against the logarithm of the protein molarity.

**Fig. 7. The effect of MgCl₂ on the binding of X-12 by RecG.** Reaction mixtures (20 μl) contained varying concentrations of RecG with 1.6 nM 32P-labeled X-12 in reaction buffer 2 plus 2 mM EDTA or 200 μM MgCl₂ as indicated. Reactions were incubated on ice for 10 min before loading onto a 4% polyacrylamide binding gel containing 2 mM EDTA or 200 μM MgCl₂ as indicated. The data was quantitated by PhosphorImager analysis, and the percentage of X-12 bound for each concentration of RecG was plotted against the logarithm of the protein molarity.
FIG. 8. Dissociation of RecG from X-12. A–D, band-shift analysis of dissociation of RecG from X-12. Reactions (60 μl) contained 5 nM RecG and 1.4 nM X-12 in buffer 2 plus 2 mM EDTA (A), 5 mM MgCl₂ (B), or 5 mM MgCl₂ plus 5 mM ATP-γ-S (C), or 5 mM MgCl₂ plus 5 mM ADP (D). Reactions were incubated on ice for 10 min either with or without 12 μg of poly(dI-dC)-poly(dI-dC) competitor DNA as indicated. Following incubation, 10-μl samples were loaded from each reaction mixture onto a standard 4% polyacrylamide gel containing 2 mM EDTA as described under "Materials and Methods." 12 μg of poly(dI-dC)-poly(dI-dC) was then added to each of the reactions not containing competitor DNA, and further 10-μl samples from these were loaded onto the gel at timed intervals. E, quantitated data from A–D.

There are at least two ways in which the interaction of Mg²⁺ with X-junction DNA could affect RecG binding: 1) By providing a general screening of the phosphodiester backbone (23, 24) or 2) By affecting the conformation of the X-junction DNA. In the absence of cations the DNA arms of the X-junction adopt a 4-fold symmetrical pattern. However, in the presence of sufficient cations (approximately 100 μM in the case of Mg²⁺) the negative charges of phosphates on the DNA backbone are neutralized, allowing coaxial stacking of junction arms (5). Complete folding appears to require the site binding of a cation at an electronegative cleft created at the center of the stacked X-junction (25). To test both of these possibilities, we compared the effects of different concentrations of MgCl₂, hexamminecobalt(III) chloride, and NaCl on the binding of X-12 by RecG. Hexamminecobalt(III) chloride stacks X-junctions far more efficiently than Mg²⁺, with <2 μM required for complete stacking (5), whereas, Na⁺ can provide a general screening of the phosphodiester backbone but will fully stack an X-junction only at very high concentrations (>0.5 M), probably due to an inability to site-bind at the center of the stacked X-junction (25). Therefore, if general screening of the phosphodiester backbone is responsible for the inhibition of junction binding, then equivalent ionic strengths of Na⁺ and Mg²⁺ should equally affect binding of RecG to X-12, whereas if the junction conformation is important for RecG's ability to bind to X-12, then hexamminecobalt(III) chloride should be a better inhibitor of binding than Mg²⁺.

To compare the effects of these different cations on junction binding, we utilized the dissociation assay shown in Fig. 8 and analyzed binding over a range of concentrations of cations. However, instead of monitoring binding over time following the addition of the competitor DNA, we analyzed the level of binding only immediately following its addition. As mentioned before, this level of binding reflects both ongoing dissociation of RecG from X-12 and the equilibrium between free RecG and RecG bound to X-12. Increasing the concentrations of any of the cations inhibits binding of X-12 by RecG (Fig. 9A). Hexamminecobalt(III) chloride is the best inhibitor, requiring only 10–20 μM to elicit a 50% reduction in the amount of X-12 bound. In comparison, approximately 700–800 μM MgCl₂ or 100 mM NaCl is required to achieve the same reduction in binding. These data do not support the idea that general screening of the phosphodiester backbone is the primary way in which relatively low concentrations of Mg²⁺ inhibit junction binding, because equal ionic strengths of Na⁺ and Mg²⁺ do not elicit the same reduction in junction binding. For example, at 15 mM NaCl, which has an ionic strength equivalent to 5 mM MgCl₂, no reduction in junction binding is observed, whereas at 5 mM MgCl₂ binding is greatly reduced. However, these results do support the idea that junction stacking is responsible for the inhibition of RecG-junction binding observed at relatively low concentrations of Mg²⁺, because the concentrations of MgCl₂ and hexamminecobalt(III) chloride required to inhibit binding correlate well with those required to stack X-junctions (see above).

Hexamminecobalt(III) chloride cannot substitute for Mg²⁺ in the unwinding of X-12 by RecG (data not shown). However, to see if its effect on junction binding also affected junction unwinding, we compared the rates of unwinding X-12 with and without 200 μM hexamminecobalt(III) chloride for two different concentrations of RecG in reactions that also contained an excess of ATP (10 mM) over MgCl₂ (1 mM) to limit any effects of free Mg²⁺ (Fig. 9B). At both concentrations of RecG, the addition of 200 μM hexamminecobalt(III) chloride to the standard reaction reduced the rate of unwinding approximately 5-fold. This amount of inhibition correlates reasonably well with the reduction in junction binding in Fig. 9A, indicating that, as expected, inhibiting junction binding by RecG also perturbs its unwinding.
DISCUSSION

Previous studies have exposed two key features of the mechanism by which RecG catalyzes branch migration. First, RecG binds with a high degree of specificity to Holliday junction DNA; second, RecG is a DNA-dependent ATPase that can unwind short stretches (≤25 bp) of duplex DNA in a reaction that, like branch migration, is dependent on Mg\(^{2+}\) and the hydrolysis of ATP (10, 12). In the present study, we have added to this basic characterization by showing that, as expected for a protein that targets Holliday junctions, the rate of ATP hydrolysis catalyzed by RecG is much more strongly stimulated by X-junction DNA than by linear duplex DNA. Furthermore, we have shown that DNA unwinding at Holliday junctions is limited to ≤30 bp/junction arm. This confirms that DNA unwinding at Holliday junctions and partial linear duplex substrates is similarly restricted. These data are consistent with RecG catalyzing the branch migration of Holliday junctions and other recombination intermediates by unwinding DNA at or near to the junction crossover point. Presumably, limited DNA unwinding is sufficient to promote extensive branch migration of Holliday junctions, three-strand junctions, and R-loops through regions of homology, because for every base pair that is broken a new base pair is formed, thereby preventing the newly unwound strands from snapping back together (11, 15, 26).

The main focus of this paper is the study of Holliday junction binding by RecG. A variety of enzymes have been discovered that bind with some degree of specificity to X-junction DNA. These include not only the branch-migrating proteins RuvAB and RecG, and the resolvases RuvC, RusA, CCE1, SpCCE1, T4 endonuclease VII, and T7 endonuclease I, but also binding proteins without detectable catalytic activity such as HMG1, HU, and CBP from HeLa cells (10, 27–36). Using a variety of enzyme and chemical probes to analyze protein-DNA interactions, it is clear that these proteins, at least where tested, bind to DNA at and around the junction crossover point (36–40). The 1,10-phenanthroline-copper footprinting of the RecG-junction complex presented here shows that RecG too binds at the junction crossover point.

What precisely is RecG recognizing when it binds to X-junction DNA? Many possibilities exist, since the X-junction structure offers a whole array of potential sites for interaction that could singly or in combination provide a potent signature for its identification. These include a high affinity binding site for certain intercalators (41–44), local widening of groove widths (45), and a range of angles subtended within or between DNA helices. In the latter case, the angles presented by a junction are dependent on presence or absence of metal ions. In the absence of metal ions, electrostatic repulsion between the four arms of the junction forces them into a 4-fold symmetrical arrangement in which each arm subtends an angle of 90° with its neighbor. In the presence of metal ions, charges are neutralized, enabling the junction to fold into a more compact structure in which the helical arms stack pairwise upon each other to form an X-shaped structure with 2-fold symmetry (5, 46). This stacked X-structure presents a large angle of approximately 120° and a small angle of approximately 60° between DNA helices.

T4 endonuclease VII has the ability to bind and cleave a range of DNA structures, including X-junctions, that have in common two mutually inclined helices (47). This presents a strong argument for it “measuring” the angle of helical inclination for binding and catalytic activation. RecG’s ability to bind and unwind a range of DNA structures in addition to X-junctions suggests that it too may recognize angles inclined between DNA helices. However, it has now been found that most, if not all, X-junction-binding proteins dramatically alter the conformation of the junction upon binding (7, 39, 48–50). The same appears to be true for RecG. From this it can be concluded that it is not necessarily the initial conformation of the DNA that is critical for recognition but rather an intrinsic ability of that DNA to be molded into the binding site(s) of the protein in question. By analyzing RecG’s ability to bind to various substrates made from the component parts of an X-junction, we conclude that the X-junction complex presented here shows that RecG too binds at the junction crossover point.
Targeting Holliday Junctions by RecG

...it is clear that the minimal structure that it will bind to is a flayed duplex molecule. Therefore, the critical feature for specific protein-DNA interaction is the displacement of two single strands from one end of a common duplex strand. Presumably, RecG contacts each of the three arms emanating from the common junction point in a flayed duplex, manipulating each arm relative to the others so that they are fitted into its binding site(s). If RecG does use this feature to recognize branched DNAs, then multiple binding sites should be available to it, the exact number depending on the DNA species; e.g., X-junctions should present four binding sites, three-strand junctions and Y-junctions should present three binding sites, and flayed duplexes should present only one binding site. The observation from band shift data that only two RecG-X-junction complexes are formed and that only one complex is formed efficiently with Y-junction and three-strand junction DNA indicates that not all binding sites can be bound simultaneously by RecG. Presumably, this is because binding at one site interferes with binding at the other sites either by direct physical interaction between the protein molecules or indirectly by RecG altering and fixing the conformation of the other sites such that they cannot be bound. If the binding sites are separated by an intervening region of DNA, then both sites can be efficiently bound within a single DNA molecule as is the case with the loop substrate (Fig. 5C). The intervening DNA provides both physical separation of protein molecules and a degree of flexibility that could enable both binding sites to be conformationally altered to fit the RecG binding site(s).

It is clear that RecG binds to flayed duplex DNA; however, it is also evident from the data presented here and elsewhere that additional substrate complexity is required to stimulate efficient DNA unwinding (see Fig. 5D and Ref. 20). The minimal requirement for efficient DNA unwinding appears to be a junction with at least two duplex arms like the part Y-junctions (substrates C and D, Fig. 5). Interestingly, it is the short oligonucleotide that is unwound from both substrates C and D (Fig. 5D, lanes f and h). Unwinding of both oligonucleotides (oligonucleotides 5 and 6) occurs with approximately the same efficiency despite them having opposite polarities with respect to the junction center. This is unlike RecG’s action on conventional partial duplex Matson style helicase substrates, where unwinding proceeds with a clear 3'-5' polarity with respect to the single strand that is bound (12). These observations together with the binding data suggest that RecG binds to a flayed duplex component of a junction in an orientation dependent fashion. It then proceeds to unwind DNA along both arms of the “flay.” “Unwinding” may be promoted by RecG attempting to anneal two strands displaced from a common junction point (15). This “reverse” helicase action would be efficient for the branch migration of recombination intermediates, because homology between the strands would clearly aid them being brought together.

Observations made here and elsewhere indicate that although a particular DNA species may appear on paper to present the necessary features for specific recognition by RecG, in practice it can be a very poor substrate for binding. For example, RecG’s affinity for flayed duplex molecules and loop DNAs that differ in size and/or nucleotide sequence can vary dramatically (see Fig. 5 and Ref. 20). Furthermore, RecG may prefer only a subset of possible junction binding sites. This appears to be the case when RecG binds to X-0 in the absence of cations, because instead of the predicted 4-fold symmetrical pattern of protection from 1,10-phenanthroline-copper, it yields a 2-fold symmetrical pattern, suggesting that only two of the four possible binding sites on X-0 are efficiently bound by RecG. Presumably, these variations in binding affinity and binding site preference are due to differences in nucleotide sequence. Nucleotide sequence affects junction conformation, which in turn could affect how RecG “sees” the junction. In the case of the flayed duplex and loop substrates, conformational differences may be simply due to limited inter- and intrastrand base pairing of the single-stranded regions of these DNA molecules, whereas in the case of X-junctions the nucleotide sequence is known to affect the crossover isomer bias (51) and presumably also influences the unfolded junction conformation. Alternatively, the nucleotide sequence could influence the malleability of a junction, thereby affecting its ability to be fitted into the RecG binding site(s). This would be analogous to the effect of sequence-dependent DNA bending on CAP protein-DNA binding affinity (52).

RecG depends on Mg\(^{2+}\) for branch migration and DNA unwinding. However, quite low concentrations of free Mg\(^{2+}\) inhibit X-junction unwinding. In the current study, we have shown that the inhibition of X-junction unwinding directly correlates with a reduction in junction binding by RecG. These inhibitory effects are not specifically mediated by Mg\(^{2+}\); e.g., both Ca\(^{2+}\) and hexamminecobalt(III) chloride also inhibit junction binding and unwinding by RecG. The relative abilities of these cations to mediate inhibition closely correlates with their relative abilities to stack X-junctions (5, 25). Furthermore, the respective concentrations of cation at which inhibition is mediated are similar to those required for complete stacking of the X-junction. From this we conclude that the stacking of the X-junction mediated by cations inhibits RecG binding to the junction.

RecG’s apparent inability to bind to the stacked X-junction is in marked contrast to other Holliday junction binding proteins like RuvA, RuvC, and CCE1, whose binding is relatively unaffected by stacking concentrations of Mg\(^{2+}\) (7, 50, 53). This may be significant for RecG’s functioning in vivo. The intracellular level of free Mg\(^{2+}\) is estimated to be on the order of 1 mM (54). If this is true, then naked Holliday junctions would be fully stacked in vivo and RecG’s ability to branch-migrate them would be severely impaired. If this situation occurs in vivo, how might RecG function? One possibility is that RecG activity could be modulated by changes in the level of intracellular free Mg\(^{2+}\). Alternatively, DNA-binding proteins, e.g., RecA, could hold the junction in an unstacked conformation and thereby enable RecG to bind. There may even be no need for RecG to bind to Holliday junctions at all, since branch migration could, presumably, be catalyzed adequately by RuvAB.

Recently, it has become clear that RecG’s function is not restricted to branch-migrating Holliday junctions. For example, RecG can branch-migrate DNA junctions at D-loops (20). This activity appears to be important for ensuring efficient recombination in the presence of the PriA helicase (19). Furthermore, RecG can unwind R-loops, which helps to limit their accumulation in vivo (15, 21, 22). Clearly, these activities do not require RecG’s interaction with a Holliday junction and therefore may not be as sensitive to the concentration of free Mg\(^{2+}\). However, this has yet to be tested. Still, it remains an intriguing possibility that, in vivo, D-loops and R-loops present more attractive targets than Holliday junctions to RecG.

Acknowledgments—We are grateful to David Sherratt for advice and support, Julie Dixon for excellent technical support, and Peter McGlynn for critical reading of the manuscript.

REFERENCES
1. West, S. C. (1992) Annu. Rev. Biochem. 61, 603–640
2. Johnson, R. D., and Symington, L. S. (1993) J. Mol. Biol. 229, 812–820
3. Panyutin, I. G., and Hsieh, P. (1984) Proc. Natl. Acad. Sci. U. S. A. 91, 2921–2925
4. Panyutin, I. G., Biewa, I., and Hsieh, P. (1995) EMBO J. 14, 1819–1826
5. Duckett, D. R., Murchie, A. I., and Lilley, D. M. J. (1990) EMBO J. 9, 583–590
6. West, S. C. (1996) J. Bacteriol. 178, 1237–1241
