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Branch migration of three-strand recombination intermediates by RecG, a possible pathway for securing exchanges initiated by 3′-tailed duplex DNA

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RecG protein is required for normal levels of recombination and DNA repair in *Escherichia coli*. This 76 kDa polypeptide is a junction-specific DNA helicase that acts post-synaptically to drive branch migration of Holliday junction intermediates made by RecA during the strand exchange stage of recombination. To gain further insight into the role of RecG, we studied its activity on three-strand intermediates formed by RecA between circular single-stranded and linear duplex DNAs. Once RecA is removed, RecG drives branch migration of these intermediates by a junction-targeted activity that depends on hydrolysis of ATP. RuvAB has a similar activity. However, when RecG is added to a RecA strand exchange reaction it severely reduces the accumulation of joint molecule intermediates by driving branch migration of junctions in the reverse direction to that catalysed by RecA strand exchange. In comparison, RuvAB has little effect on the reaction. We discuss how reverse branch migration by RecG, which acts counter to the 5′→3′ polarity of RecA binding and strand exchange, could serve to promote or abort the early stages of recombination, depending on the orientation of the single DNA strand initiating the exchange relative to the adjacent duplex region.

Key words: DNA repair/RecA/RecG/recombination/RuvAB

Introduction

The exchange of single-strands between two DNA duplexes is an important stage in the initiation of genetic recombination. It provides the means to establish homologous contacts and leads directly to Holliday intermediates that can be resolved to recombinant products. Most of what we know about homologous pairing and strand exchange has come from studies of the *Escherichia coli* RecA protein, though the recent discovery of eukaryotic homologues of RecA suggests a common reaction mechanism may apply in all organisms (Shinohara *et al.*, 1992; Ogawa *et al.*, 1993; Shinohara *et al.*, 1993).

Three clearly defined stages have been identified in the RecA reaction (for recent reviews see West, 1992; Kowalczykowski and Eggleston, 1994). In the pre-synaptic stage, RecA polymerizes on single-stranded DNA in the 5′→3′ direction to form a helical nucleoprotein filament that can extend to adjacent duplex regions. Within the filament the DNA is extended and, if duplex, underwound. Extending the DNA is critical for the next stage of the reaction, synapsis, in which a homologous duplex is sought, brought into homologous alignment within the filament and driven rapidly to exchange strands with the resident molecule. Strand exchange links the two molecules together and creates a short heteroduplex joint. If pairing initiates within a single-stranded region bound by RecA, the exchange leads to a three-stranded junction. However, in duplex–duplex pairings strand exchange is reciprocal and generates a four-stranded Holliday junction. In both cases strand exchange is unidirectional and proceeds with the same 5′→3′ polarity as the polymerization of RecA on the initiating single-strand. In the final stage, called post-synapsis, the region of heteroduplex DNA is extended as naked duplex DNA is spooled in one end of the filament and heteroduplex DNA is spilled out the other (Radding, 1991). Strand exchange continues in the 5′→3′ direction, but unlike the initial synaptic exchange requires hydrolysis of ATP. It also proceeds more slowly.

In *E.coli* two other enzymes, RuvAB and RecG, have evolved to help drive the post-synaptic stage of strand exchange (West, 1994; Whitby *et al.*, 1995). Both act catalytically to drive branch migration of Holliday junctions along the DNA. In the case of RuvAB, a combination of junction-specific DNA binding by RuvA and protein–protein interaction between RuvA and RuvB targets a specialized assembly of RuvB hexamer rings to the duplex DNA adjacent to a Holliday junction (Parsons and West, 1993; Stasiak *et al.*, 1994). Once targeted, RuvB drives the junction along the DNA in a reaction that depends on hydrolysis of ATP (Parsons *et al.*, 1992; Tsaneva *et al.*, 1992b). RuvB has DNA helicase activity and West and colleagues have proposed that it drives branch migration by locally unwinding DNA at the junction (Tsaneva *et al.*, 1993; Tsaneva and West, 1994). Genetic studies indicate that the RuvAB-mediated branch migration reaction is linked intrinsically with the resolution of recombination intermediates by RuvC protein (Mandal *et al.*, 1993). RuvC is an endonuclease that resolves Holliday intermediates into recombinant products by a dual incision activity targeted specifically to junctions which cleaves two strands of the same polarity. Cleavage is favoured at sequences with the consensus 5′-A/T T T ↓ G/C-3′ (Shah *et al.*, 1994). One of the principal functions of RuvAB may be to locate junctions at these sequences (Shah *et al.*, 1994).

RecG behaves in many ways like RuvAB. It is a DNA-dependent ATPase, binds specifically to model Holliday junctions and dissociates these structures in reactions which depend on hydrolysis of ATP. It also drives branch migration of Holliday intermediates made by RecA (Lloyd and Sharples, 1993a,b; Whitby *et al.*, 1993). RecG will also unwind partial duplex substrates. The processivity of unwinding is low compared with RuvAB and proceeds with the opposite (3′→5′) polarity (Whitby *et al.*, 1994). The similar properties of RecG and RuvAB are reflected

in vivo, where both enzymes seem to provide overlapping activities to promote recombination and repair (Lloyd, 1991). However, several observations suggest that RecG and RuvAB are not simple alternatives. First, both *recG* and *ruv* single mutants are sensitive to radiation and somewhat deficient in recombination (Otsuji *et al.*, 1974; Lloyd *et al.*, 1984). Second, RecG cannot substitute for RuvAB to facilitate junction resolution by RuvC (Mandal *et al.*, 1993). Third, there is a functional overlap between RecG and RuvC, which indicates that RecG may function to resolve junctions independently of the RuvABC pathway (Lloyd, 1991). A clue as to how RecG could eliminate junctions has come from model four-strand recombination reactions in which RecA catalyses pairing and strand exchange between gapped circular and linear duplex DNA molecules (Whitby *et al.*, 1993). In these reactions RecG inhibits heteroduplex formation by driving branch migration in the reverse direction to that driven by RecA strand exchange. This reverse branch migration has the potential to remove Holliday junctions *in vivo* by aborting the initial exchange. We have suggested previously that such an activity may have a role in recombinational repair of UV damage and may also help to eliminate unproductive exchanges in genetic crosses (Whitby *et al.*, 1993; Ryder *et al.*, 1994).

To gain further insight into the activities of RecG and RuvAB we investigated their effect on three-strand intermediates formed by RecA. Intermediates of this type are likely to feature early in recombination, as the initiating event most probably involves an exchange between duplex DNA and a RecA-coated single-strand (Lindsley and Cox, 1990). RecA requires single-stranded regions to load on DNA. This requirement is satisfied *in vivo* by helicases and exonucleases that act pre-synaptically to unwind and selectively degrade one strand of a duplex DNA molecule (Kowalczykowski *et al.*, 1994). Our results show that both RecG and RuvAB catalyse branch migration of three-strand intermediates provided they are free of RecA. In reactions where RecA is present to drive strand exchange, RecG strongly inhibits the formation of heteroduplex intermediates, whereas RuvAB has little or no effect. The implications of these findings are discussed in terms of the events needed to promote homologous strand exchanges during recombination and DNA repair.

Results

Branch migration of three-stranded junctions by RecG

The well-documented strand exchange reaction catalysed by RecA between circular single-stranded ϕX174 (+) strand DNA and ^{32}P-labelled linear duplex ϕX174 DNA (three-strand reaction) provides an *in vitro* model for the early stages of synapsis (Figure 1; West, 1992). In the presence of Mg^{2+}, ATP and, for optimal efficiency, SSB, RecA polymerizes on the single-strand and then catalyses homologous pairing and strand exchange with the linear duplex to form intermediates where the two DNA molecules are joined by a point of crossover. Although the initial contacts can occur at any point along the DNA, strand separation requires a free single-strand end. Because of the 5′→3′ polarity of RecA, exchanges are favoured at one end of the duplex and once initiated proceed unidirectionally to give nicked circular and linear single-strand products. We used this model system to investigate whether RecG can branch migrate three-strand junctions. The ^{32}P-labelled intermediates from a three-strand reaction were deproteinized, purified by gel filtration and then incubated with RecG. Figure 2A shows that the intermediates form a broad smear of slowly migrating DNA in agarose gels, within which more defined bands can be distinguished. The data show that some of this material disappears on incubation with RecG. With 1–5 nM RecG, dissociation of this subsection of the intermediates was achieved within 30 min (lanes e and f). Quantitation of the gel analysis showed that these intermediates were converted to complete strand exchange products (data not shown). Since we observed no nuclease activity in the reaction, we conclude that RecG must drive branch migration of the junction point to the DNA ends.

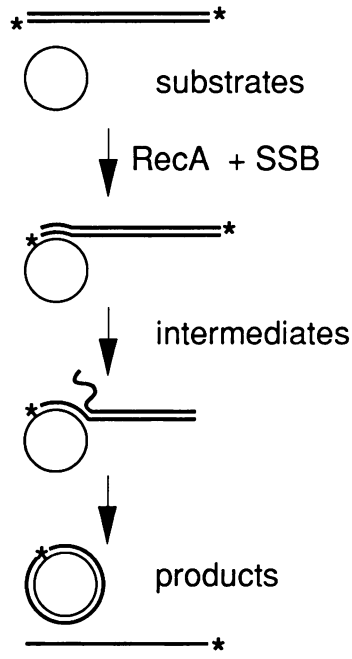

Fig. 1. Schematic representation of the RecA-mediated three-strand reaction. The reaction between the ^{32}P-labelled (asterisk) linear duplex DNA and circular single-stranded DNA is described in the text.

We noticed that a considerable subfraction of the faster migrating intermediates was refractory to dissociation by RecG. A similar level of partial dissociation was observed using intermediates from a three-strand reaction with M13mp18 (+) strand DNA and ^{32}P-labelled linear duplex M13mp18 DNA (data not shown), from which we conclude that the resistance to dissociation is not some feature peculiar to ϕX174 DNA. To investigate this phenomenon we first studied the kinetics of intermediate formation by RecA. The intermediates formed during the first 2 min of the reaction were found to have the same migration pattern in agarose gels as those resistant to dissociation by RecG (data not shown). Previous studies revealed that most of the net strand exchange occurs in the first 2 min of the reaction, in what is called the burst phase (Kahn and Radding, 1984; Jain *et al.*, 1994). This strand exchange extends through 1–2 kb of DNA and is independent of ATP hydrolysis. We therefore analysed reactions where the ATP was replaced with the poorly hydrolysable ATPγS.

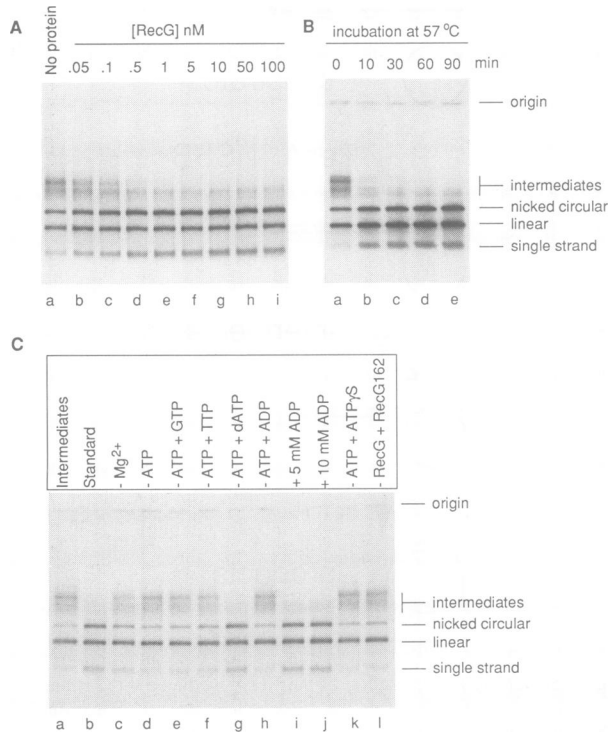

Fig. 2. Dissociation of three-strand junctions made by RecA. (**A**) Branch migration of junctions by RecG. Deproteinized intermediates were incubated with RecG at 37°C for 30 min before stopping the reaction and analysing the products by agarose gel electrophoresis and autoradiography. Reactions contained 5.7 μM DNA, 5 mM $MgCl_2$, and 5 mM ATP. (**B**) Thermal dissociation of intermediates. Deproteinized intermediates were incubated at 57°C for the time indicated before chilling on ice and analysis as in (A). (**C**) Reaction requirements. Deproteinized intermediates were incubated in the presence (lanes b–k) or absence (lanes a and l) of 100 nM RecG. The standard reaction (lane b) contained 20 mM Tris–HCl, pH 7.5, 5 mM ATP, 5 mM $MgCl_2$, 2 mM DTT, 100 μg/ml BSA, 2.2 μM DNA in a final volume of 20 μl. The reaction was varied as indicated in lanes c–l. For lanes e–h and lane k ATP was substituted with 5 mM of the indicated nucleotide. For lane l RecG was substituted with 100 nM RecG162 in an otherwise standard reaction mixture. Reactions were incubated for 30 min at 37°C before stopping and analysis by gel electrophoresis.

The intermediates formed migrated to the same position in agarose gels as those produced in the first 2 min of the standard reaction (data not shown). From these data we suspected that at least some of the intermediates resistant to dissociation by RecG might be those formed during the burst phase of strand exchange. Models for the initial stages of synapsis envisage that two DNA molecules come together in the RecA filament and align with each other by non-Watson–Crick bonding (Howard-Flanders *et al.*, 1984). In reactions between single-stranded and double-stranded DNA such pairing leads to the formation of so-called triplex DNA (Hsieh *et al.*, 1990; Menetski *et al.*, 1990; Umlauf *et al.*, 1990; Rao *et al.*, 1991). Triplex DNA formed during three-strand reactions is remarkably thermostable following removal of RecA (Rao *et al.*, 1991). We used this characteristic to see if some of the intermediates resistant to dissociation by RecG might be composed of triplex DNA. Deproteinized intermediates were incubated at 57°C, sampled at intervals and analysed on agarose gels (Figure 2B). The data show that some of the intermediates were dissociated more rapidly than others during the incubation at 57°C. These appear to correspond to the slower migrating intermediates dissociated by RecG (Figure 2A). The faster migrating intermediates are clearly more stable. However, the pattern of migration is not entirely in agreement with these being the same intermediates resistant to RecG. The exact nature of the refractory intermediates therefore remains to be elucidated. Further possibilities are considered in a subsequent section (see below).

Reaction requirements

The standard reaction which supported dissociation of RecA-free intermediates by RecG contained 5 mM $MgCl_2$ and 5 mM ATP (Figure 2C, lane b). Omission of either $MgCl_2$ (lane c) or ATP (lane d) abolished dissociation. ATP could be replaced by dATP (lane g), but not by GTP (lane e), TTP (lane f) or ADP (lane h). ATP could not be substituted by the poorly hydrolysable analogue ATPγS (lane k), implying a dependence on the hydrolysis of ATP. The addition of either 5 mM ADP (lane i) or 10 mM ADP (lane j) to the standard reaction had no observable effect. However, higher concentrations of ADP or shorter reaction times revealed that ADP can block dissociation (data not shown), which suggests that ADP accumulation inhibits RecG. Dissociation requires RecG protein with a functional helicase motif III. RecG162, which contains an Ala→Val substitution in this motif, is inactive (lane l), although it retains junction binding and ATPase activities (Sharples *et al.*, 1994). These data show that the dissociation of three-strand junctions proceeds under the same conditions and with the same requirements as those needed for branch migration of Holliday junctions (Lloyd and Sharples, 1993a,b; Whitby *et al.*, 1993). They support the view that RecG dissociates three-strand intermediates by branch migration of the junction point.

Rate and directionality

We monitored the dissociation of RecA-free recombination intermediates at different times after the addition of RecG (Figure 3A, lanes a–g). Sixty per cent or more of the susceptible intermediates were dissociated to product DNAs within 2 min at 37°C. Quantitation of the gel shown in Figure 3A shows that significant amounts of product were detected after only 30 s (Figure 3B). Given that φX174 DNA is 5386 bases long and assuming the junction is located about halfway along the molecule, we calculate the rate of branch migration to be ~80 bp/s. This is somewhat faster than the RecG rate of branch migration of Holliday intermediates made in a four-strand reaction (Whitby *et al.*, 1993). However, the rate would be much lower if the junction was located on average towards one end of the molecule, a possibility we cannot rule out, as only a fraction of three-strand intermediates are dissociated by RecG.

The data in Figure 3B also show that RecG has a strong directional bias. About 90% of the intermediates dissociated were converted to nicked circular and single-strand product DNAs. This bias was observed in several independent experiments. A similar bias was observed using intermediates based on M13mp18 DNA (data not shown). We reported previously that RecG also dissociates Y-shaped junctions with a strong preference for one of the three possible directions (Whitby *et al.*, 1994). This

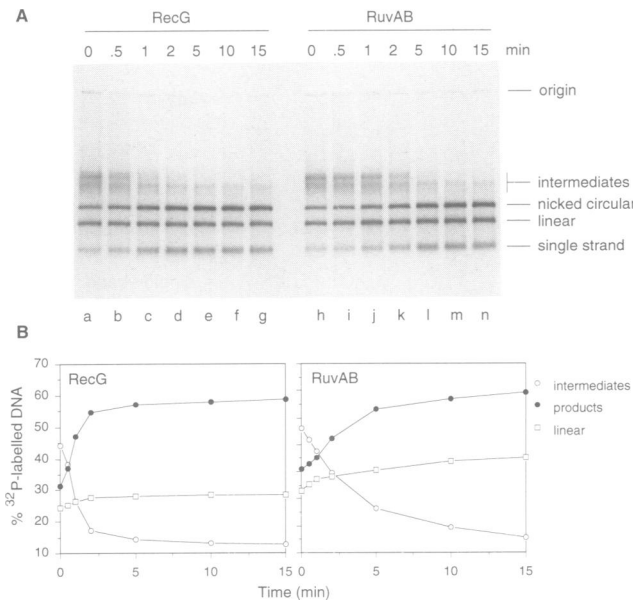

Fig. 3. Rate and directionality of branch migration by RecG and RuvAB. (**A**) Gel analysis of reaction products. Deproteinized recombination intermediates were incubated with 100 nM RecG (lanes a–g) or 272 nM RuvA plus 560 nM RuvB (lanes h–n). Reaction mixtures (100 µl) contained 5.7 µM DNA in reaction buffer containing 10 mM $MgCl_2$ and 3 mM ATP. Reactions were incubated at 37°C and aliquots (10 µl) were taken at intervals and processed as described in Materials and methods. (**B**) Quantitation of the gels shown in (A).

was attributed in part to a sequence-dictated folding of the junction that influences how it is seen by RecG. Three-strand junctions formed by RecA may also fold in different ways according to the local DNA sequence or its location within the intermediate. If RecG acts preferentially on junctions folded in a particular way, it could explain both the directionality of RecG and its inability to act on a substantial fraction of the available intermediates.

Three-strand intermediates are also dissociated by RuvAB

The RuvA and RuvB proteins together have been shown to drive branch migration of four-strand intermediates made by RecA (Tsaneva *et al.*, 1992b). We found that they also act on three-strand intermediates (Figure 3A, lanes h–n). Like RecG, RuvAB dissociated a specific subfraction of the intermediates, but the rate of dissociation was a little slower, at least under our assay conditions. With intermediates based on ϕX174 we also repeatedly observed a bias towards dissociating these intermediates to nicked circular and single-strand product DNAs. The bias was less marked than with RecG. In the reaction shown, 30% of the intermediates released the linear duplex substrate DNA. However, with intermediates based on M13mp18 this bias was not evident and in some experiments dissociation favoured the production of the linear duplex substrate (data not shown). The reason for the differences between RecG and RuvAB is not clear. We assume the two enzymes have different requirements for junction recognition and are therefore affected differently by the available junction conformations.

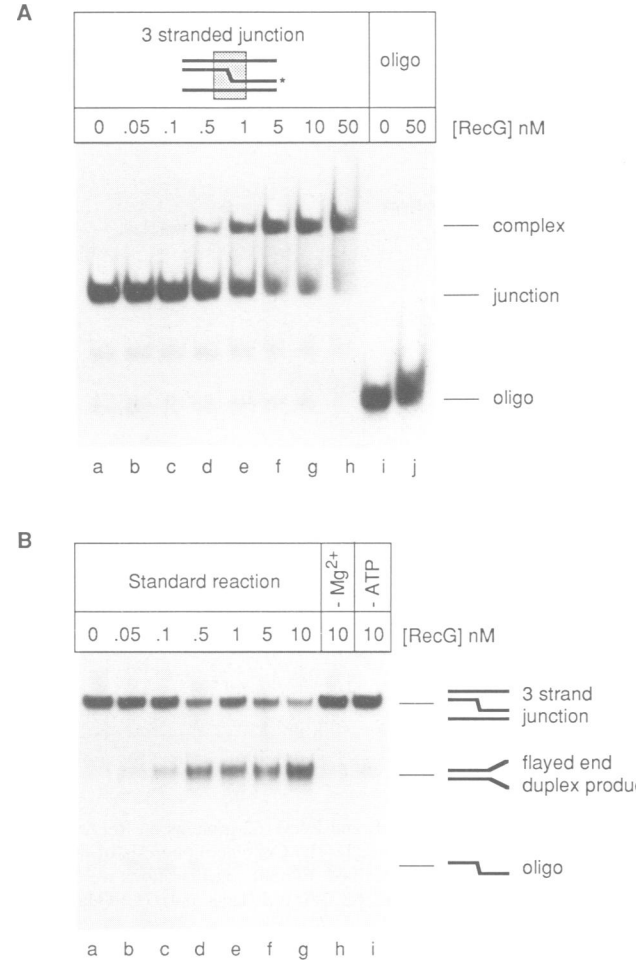

Fig. 4. Interaction of RecG with model three-stranded junctions. (**A**) Band-shift assay showing binding of RecG to the three-stranded junction. (**B**) Dissociation of the three-stranded junction by RecG.

RecG is targeted to three-strand junctions

Although RecG is a junction-specific DNA helicase, it can unwind partial duplex DNA molecules to some extent, with a 3′→5′ polarity with respect to flanking ssDNA (Whitby *et al.*, 1994). This activity has the same reaction requirements as the branch migration of Holliday junctions. It is possible that RecG dissociates the three-strand intermediates made by RecA using this non-targeted activity. We therefore examined whether RecG could be targeted to a three-strand junction. We used a synthetic junction for this purpose, made by annealing short oligonucleotides (62–64 nt). Two of the oligonucleotides share no significant regions of homology, but the third is partially homologous to the other two. Upon annealing, these form a three-stranded junction as shown in Figure 4A, with a junction point that is free to branch migrate within a central region of shared homology (depicted in Figure 4A by the shaded region). When this junction was mixed with increasing amounts of RecG and the mixture electrophoresed on a low ionic strength polyacrylamide gel, a well-defined protein–DNA complex was observed (Figure 4A, lanes b–h). No binding to equivalent linear ssDNA (lane j) or linear double-stranded DNA molecules (data not shown) was observed under these conditions. RecG was also able to dissociate the junction to yield flayed-end duplex

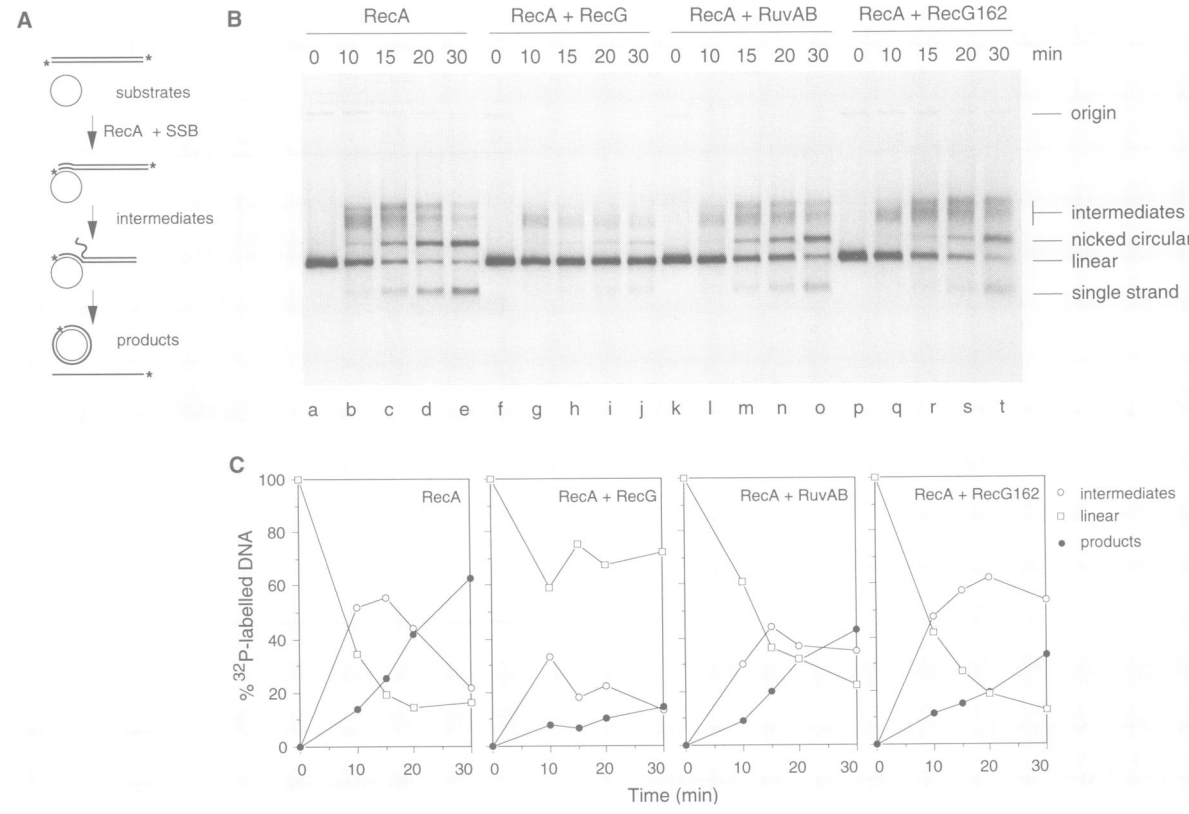

Fig. 5. Effect of RecG, RuvAB and RecG162 proteins on RecA-mediated three-strand exchange. (**A**) Schematic representation of the RecA-mediated three-strand reaction (see Figure 1). (**B**) Gel electrophoresis of reaction products. Reactions were incubated at 37°C and aliquots (9 µl) were taken at intervals and processed as described. Protein concentrations were as follows: RecG, 270 nM (lanes f–j); RuvA and RuvB, 500 nM and 1.1 µM respectively (lanes k–o); RecG162, 380 nM (lanes p–t). (**C**) Quantitation of the gel shown in (A).

products (Figure 4B, lanes b–g). No dissociation was observed in the absence of Mg^{2+} (lane h) or ATP (lane i). These properties are similar to those observed previously for synthetic X- and Y-junction DNAs. They support the view that RecG dissociates the three-strand intermediates made by RecA by a junction-targeted mechanism.

RecG inhibits heteroduplex formation by RecA in the three-strand reaction

Having established that RecG can branch migrate three-strand intermediates, we proceeded to examine the effect of this activity during reactions catalysed by RecA. Three-strand reactions (Figure 5A) were set up in parallel containing RecA, either alone or with RecG, RuvAB or RecG162. The reactions were sampled at intervals and the products analysed by agarose gel electrophoresis. In the reaction containing RecA alone a typical pattern of intermediate accumulation followed by product formation was observed (Figure 5B, lane a–e). Quantitation of the gel shown confirmed the unidirectional progression from linear substrate through intermediates to heteroduplex products and revealed that ~85% of this substrate was utilized in the reaction (Figure 5C). The addition of RecG had a marked effect (Figure 5B, lanes f–j, and C). Less than half the linear duplex substrate was utilized, with the result that substantially fewer intermediates were accumulated and fewer products formed. The inhibitory effect of RecG is remarkable, given its preference for driving RecA-free intermediates to heteroduplex products. By comparison with RecG, the addition of RuvAB had little effect. The reaction was a little slower than with RecA alone, but nearly 80% of the linear duplex substrate was used (Figure 5B, lanes k–o, and C).

RecG is clearly inhibiting the RecA reaction. Examination of the quantified data provides a clue as to how RecG achieves this effect. The intermediates accumulated in the first 10 min slowly disappear over the next 20 min. However, in contrast to the reaction containing RecA alone, much of this material is converted back to substrate DNAs, rather than being processed through to heteroduplex products. This dissociation of intermediates back to substrate DNA was observed in several independent experiments. It suggests that RecG inhibits the reaction by driving branch migration of intermediates in the opposite direction to RecA strand exchange (reverse branch migration). Further support for this idea comes from a comparison of the RecG and RecG162 reactions. As mentioned above, RecG162 cannot drive branch migration, despite retaining both DNA binding and ATPase activities. The reaction containing RecG162 gave a unidirectional progression from linear substrate, to intermediates, to product DNAs, in much the same way as the reaction with RecA alone (Figure 5B, lanes p–t, and C). However, as with RuvAB, the reaction proceeds a little more slowly. We assume the slow progress is due to interference caused by binding of these proteins to the DNA. Both RecG162 and RuvA bind well to the synthetic three-strand junction described in Figure 4 (data not shown). Presumably, by binding to junction DNA RecG162 (and RuvA) hinders

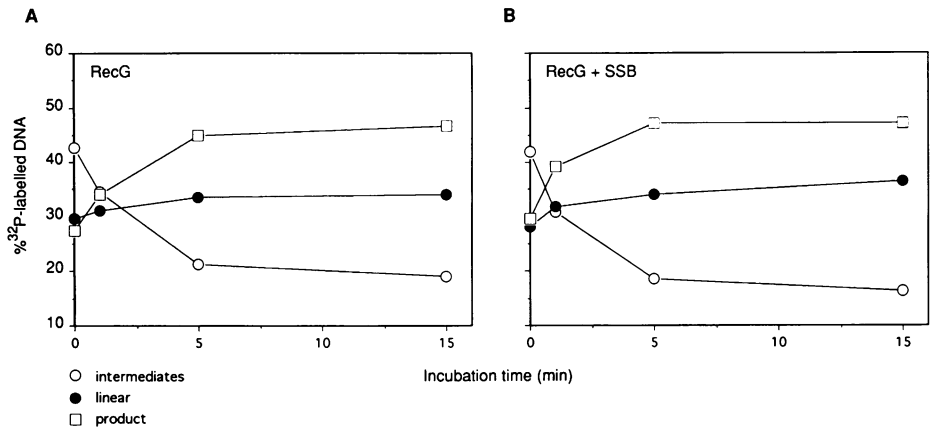

Fig. 6. Effect of SSB on branch migration of three-strand intermediates by RecG. Reactions (60 µl) contained 3.5 µM DNA, 10 mM $MgCl_2$, 3 mM ATP and (**A**) 100 nM RecG or (**B**) 100 nM RecG plus 460 nM SSB. Reactions were incubated at 37°C and aliquots (10 µl) were taken at intervals, processed as described and the products separated by agarose gel electrophoresis before quantification.

the strand exchange catalysed by RecA, thus slowing the processing of intermediates through to products.

SSB does not affect the direction of branch migration by RecG

SSB is normally added to the three-strand reaction to improve the efficiency of joint molecule formation (Kowalczykowski and Eggleston, 1994). We therefore considered the possibility that SSB could be influencing the direction of branch migration by RecG. Although RecG drives RecA-free intermediates predominantly to heteroduplex products, its directionality in the RecA reaction could possibly be altered by SSB. To test this possibility, purified three-strand intermediates were incubated with RecG in the presence or absence of SSB. In the absence of SSB, RecG readily dissociated some 50% of the intermediates, with a bias towards nicked circular and single-strand product DNAs, as described before (Figure 6A). With SSB present the intermediates were dissociated with similar efficiency and much the same bias, though there was some evidence of a greater increase in the linear substrate species (Figure 6B). From these data we conclude that SSB cannot be responsible, at least on its own, for causing RecG to inhibit strand exchange in the RecA reaction. Therefore, by a process of elimination, we suggest that the RecA nucleoprotein filament dictates the binding of RecG to the junction point in such a way that RecG drives branch migration in the reverse direction to that driven by RecA strand exchange. How this happens remains to be determined.

Discussion

In previous studies we demonstrated that RecG catalyses branch migration of Holliday junctions (Lloyd and Sharples, 1993a,b; Whitby *et al.*, 1993; Whitby *et al.*, 1994). Here we have shown that the branch migration activity can also be directed to three-strand intermediates made in reactions catalysed by RecA. The activity in both cases is intimately associated with the ability of RecG to unwind DNA. RecG is a junction-specific DNA helicase, but it can also unwind partial duplexes, with a clear 3′→5′ polarity with respect to the flanking single-strand (Whitby *et al.*, 1994). The latter activity is weak, but could be enough to account for the dissociation of three-stranded intermediates in our assays. Such a possibility is not without precedent, as both UvrD and T4 Dda helicases have been shown to dissociate three-stranded intermediates (Kodadek, 1991; Morel *et al.*, 1993). However, we found that RecG binds specifically to model three-stranded junction DNA and conclude that it dissociates three-stranded intermediates via a junction-targeted mechanism. This is supported by the observation that amounts of SSB which inhibit the non-targeted helicase activity of RecG fail to prevent it from dissociating three-strand intermediates (unpublished work). We found that RuvAB could also dissociate these intermediates. Like RecG, RuvA binds specifically to the model three-strand junctions used in this study (unpublished data). It is likely therefore that RuvAB also dissociates three-strand intermediates via a junction-targeted helicase activity.

Is there any biological significance to this ability of RecG and RuvAB to branch migrate three-strand junctions or does it simply reflect the ability to drive Holliday junctions? While Holliday junctions are key intermediates in recombination, they are not the first products of the strand exchange reaction catalysed by RecA. These are likely to contain a three-strand junction, as all exchanges probably initiate from single-stranded regions of DNA (Lindsley and Cox, 1990). The single-stranded regions available to RecA are likely to be quite extensive. In the RecBCD pathway of recombination, RecBCD enzyme binds to the ends of duplex DNA and, following an encounter with a χ sequence, proceeds to unwind and selectively degrade the 5′-ending strand of the DNA, leaving the 3′-ending strand intact (Kowalczykowski *et al.*, 1994). Unwinding is highly processive, at least *in vitro*, and can generate tracts of single-stranded DNA of ~30 kb/binding event (Roman *et al.*, 1992). During conjugation with Hfr donors a single-strand of Hfr DNA with a 5′ leading end is transferred to the recipient, where it provides a template for lagging-strand synthesis (Willetts and Wilkins, 1984). Discontinuities in the lagging strand provide single-stranded regions for the initiation of recombination that could be quite extensive. Likewise, discontinuities of 1000 bp or more arise during replication

of UV-damaged templates. In yeast, double-strand breaks are processed to expose long 3′ single-strand tails that initiate subsequent exchanges with an intact homologue (Sun *et al.*, 1991). One possible role for RecG and RuvAB in *E.coli*, or their hypothetical equivalents in other organisms, would be to help RecA drive the initial three-strand exchange. After the synaptic stage, RecA drives strand exchange at a rate of 3–10 bp/s (Cox and Lehman, 1981; Kahn *et al.*, 1981; West *et al.*, 1981). Our results indicate that both RecG and RuvAB can improve on this rate when driving (RecA-free) three-strand intermediates. This improvement might be particularly valuable when strand exchange has to be driven through mismatched base pairs in the DNA or through more substantial barriers, such as DNA lesions or heterologous inserts. In the three-strand reaction RecA can bypass mismatches and short heterologous inserts up to 100 bp in the duplex DNA substrate with reasonable efficiency. However, larger inserts provide an increasingly greater block to strand exchange (Bianchi *et al.*, 1983). RuvAB has been shown to facilitate the bypass of heterologous inserts of up to 1 kb in the three-strand reaction (Iype *et al.*, 1994). It is able to do this presumably through its ability to unwind DNA. RecG can also unwind DNA, but its low processivity makes it a poor candidate to help overcome heterology (Whitby *et al.*, 1994).

A role in promoting strand exchange is, however, at odds with our finding that neither RecG nor RuvAB improved the rate of the reaction in the presence of RecA. Indeed, in the case of RecG we observed a very strong inhibition of strand exchange. Moreover, many of the intermediates observed to be formed were driven back to starting material. This reverse branch migration was not detected with RuvAB. The reaction was less efficient, but the effect was rather small and could be explained in terms of RuvA binding to the substrate DNA and inhibiting the progress of RecA (Iype *et al.*, 1994). The reverse branch migration seen with RecG is remarkable, given its propensity to drive intermediates to complete strand exchange products in the absence of RecA.

The ability of RecG to block strand exchange by RecA and to drive the junction point in the intermediates already formed in the reverse direction to that driven by RecA was first observed in the four-strand reaction (Whitby *et al.*, 1993). We hypothesized that reverse branch migration enables RecG to 'resolve' intermediates in recombination and DNA repair by driving strand exchange back to the DNA ends from where strand separation was initiated. It provides a possible explanation for the functional overlap between RecG and RuvC observed *in vivo*. Models of conjugational recombination and daughter strand gap repair of UV-induced lesions encompassing this activity assume the polarity of branch migration catalysed by RecG is in some way dictated by the nature of the RecA nucleoprotein filament (Whitby *et al.*, 1993; Ryder *et al.*, 1994).

If RecG does have the ability to drive branch migration in the reverse direction to that driven by RecA, it is patently obvious that it cannot abort all exchanges initiated by RecA. We assume the three-strand intermediates formed early in recombination during the burst phase of synapsis provide the means of ensuring exchanges always get off the ground. If we take it that the directionality of branch

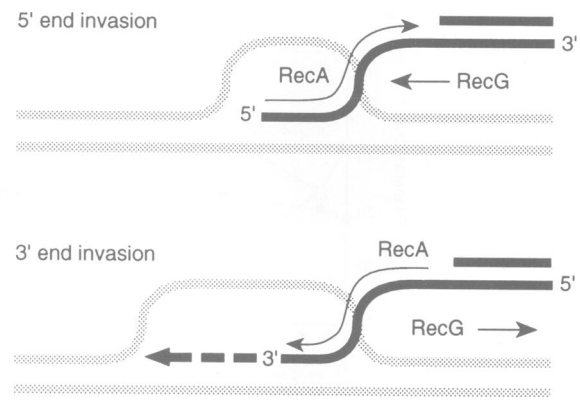

Fig. 7. Possible effect of RecG on RecA-mediated recombination initiated by tailed duplex DNA with either 5′ or 3′ single-strand overhangs. In both cases the direction of RecA filament assembly and strand exchange is indicated by the curved arrow, while the direction of RecG branch migration is shown by a shaded arrow. The 3′ invading strand can prime DNA synthesis, as indicated by the dashed arrow.

migration by RecG is determined by the RecA filament, we can envisage ways in which RecG could both abort and promote exchanges mediated by RecA. These opposite outcomes are illustrated in Figure 7. They are possible because of the unidirectional nature of RecA strand exchange. RecA both polymerizes on single-stranded DNA and catalyses strand exchange with a 5′→3′ polarity. Therefore, if the single-strand is a 5′-tail on a duplex molecule, then RecA will readily catalyse an exchange from the three-stranded region into the duplex:duplex region of the two homologous DNA molecules. It is this class of exchange that RecG would abort by driving the junction back to the 5′ single-stranded end. However, if the tail ends 3′, then the initial exchange is likely to be constrained to the three-stranded region of the two homologous DNA molecules, as the RecA filament extends very poorly in this direction and tends to be discontinuous (Shaner *et al.*, 1987; Shaner and Radding, 1987). In this case reverse branch migration catalysed by RecG would help extend the exchange into the duplex:duplex region.

The need to set up a Holliday junction by moving the three-strand junction into duplex:duplex regions against the polarity of RecA is highlighted in recent models for double-strand break repair (DSBR) based on recombination-dependent priming of DNA replication forks (Figure 8; Asai *et al.*, 1994). Similar models have been proposed for restoration of collapsed replication forks (Kuzminov *et al.*, 1994) and for recombinant formation in conjugational crosses (Smith, 1991). These models have in common a three-strand intermediate generated by 3′-tailed duplex invasion of an intact homologue, which primes the initial replication, that in turn extends the D-loop to allow additional replication proteins to be recruited. This intermediate has to be removed for completion of the repair process. However, RuvC, which removes Holliday junctions by cleaving them, appears to be unable to cleave equivalent three-stranded junctions in the presence of RecA. Furthermore, RuvAB, which readily promotes strand exchange in the four-strand reaction, has relatively little effect on the three-strand reaction. Therefore, RecG may be required to propel the exchange into duplex:duplex regions to generate a Holliday junction which can then be

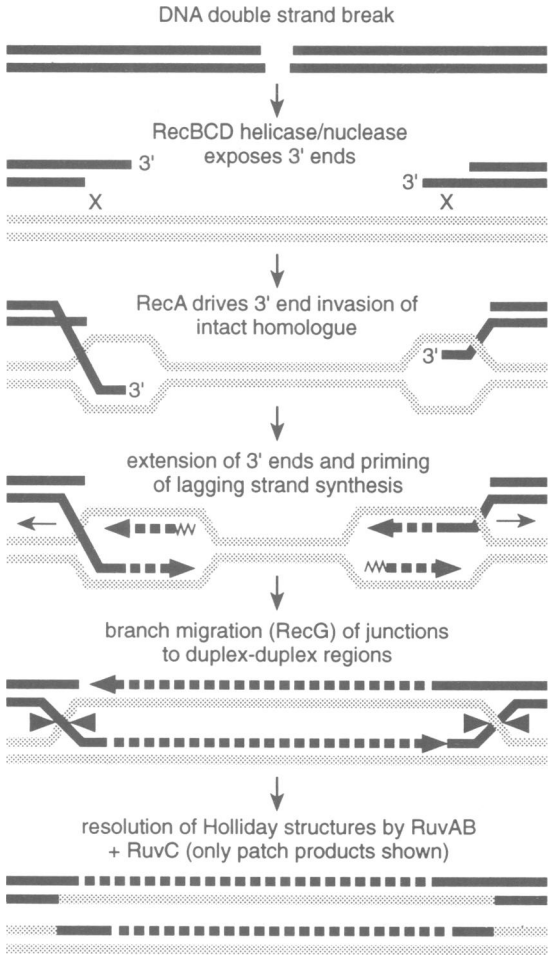

Fig. 8. Model for repair of double-strand breaks by recombination-dependent DNA replication primed by 3′-end invasion (modified from Asai *et al.*, 1994). The model assumes branch migration of the three-strand junctions into duplex regions to form symmetrical Holliday junctions which can then be resolved.

resolved by RuvABC. It is perhaps significant therefore that *recG* mutants are noticeably more sensitive to ionizing radiation than they are to UV light (Lloyd and Buckman, 1991, unpublished results). They also show altered levels of recombination-dependent DNA replication (Asai *et al.*, 1993; Asai and Kogoma, 1994).

In terms of DSBR, the invasion of a 5′-tail into a homologous duplex, being unable to prime repair DNA synthesis, would serve little purpose unless the 3′-end of the complementary strand was brought into play. Such exchanges would naturally proceed into duplex:duplex regions, because of the polarity of RecA strand exchange, and therefore would be processed readily by RuvABC. However, reverse branch migration of three-strand intermediates catalysed by RecG would ensure that such unproductive exchanges are aborted at an early stage and without any risk of an unnecessary crossover of DNA. In other situations where invasion with a 5′-end could lead to a productive exchange, the same reaction might serve to limit growth of the RecA filament in the 5′→3′ direction, thereby avoiding the risk of sequestering the available RecA during the exchange. Such an activity is likely to be important when two or more exchanges are needed, as with DSBR. It could also be a critical factor in conjugation, where the incoming Hfr single-strand is presented with 5′→3′ polarity.

To conclude, the biochemical evidence presented in this paper provides support for the idea that RecG drives branch migration of junction intermediates in recombination in the reverse direction to that catalysed by RecA-mediated strand exchange. It also reveals further distinctions between the activities of RuvAB and RecG. As we have discussed, these may have biological significance and as such help us to understand why *E.coli* has more than one activity for driving branch migration.

Materials and methods

Proteins

Escherichia coli RecA and SSB were purchased from USB and Pharmacia LKB respectively. RuvA, RuvB, RecG and RecG162 proteins were purified from strains carrying overexpression plasmid constructs as described (Tsaneva *et al.*, 1992a; Sharples *et al.*, 1994; Whitby *et al.*, 1994). Protein concentrations were estimated by a modified Bradford method using a BioRad protein assay kit and bovine serum albumin as standard.

DNA

φX174 (+) strand and φX174 (RF) DNA was purchased from BRL and M13mp18 (+) strand and M13mp18 RF1 DNA from Pharmacia Biotech. φX174 (RF) DNA was linearized by restriction with *Pst*I and was labelled at the 3′-end using terminal transferase and [α-^{32}P]ddATP. M13mp18 RF1 was linearized with *Hin*dIII and labelled at the 5′ end using the Klenow polymerase and [α-^{32}P]ddATP. Model three-stranded junction DNA was made by annealing the following three oligonucleotides: 1, 5′-GACGCTGCCGAATTCTACCAGTGCCTTGCTAGGACATCTTTGCCCACCTGCAGGTTCACCC-3′; 2, 5′-TGGGTGAACCTGCAGGTGGGCAAAGATGTCCTAGCAATGTAATCGTCAAGCTTTATGCCGTT-3′; 3, 5′-CAACGGCATAAAGCTTGACGATTACATTGCTAGGACATGCTGTCTAGAGGATCCGACTATCGA-3′. Oligonucleotide 2 was labelled at the 5′-end prior to annealing using [γ-^{32}P]ATP and polynucleotide kinase. Junction DNA was purified by non-denaturing 6% PAGE followed by electroelution and the concentration measured using DNA DipSticks (Invitrogen, San Diego, CA). DNA concentrations are in moles of nucleotide residues.

RecA-mediated strand exchange reactions

Reactions (50 μl) were mixed on ice and contained 3.6 μM φX174 (+) strand DNA and 3.6 μM ^{32}P-labelled linear duplex DNA in strand exchange buffer [20 mM Tris–HCl, pH 7.5, 2 mM dithiothreitol (DTT), 20 mM phosphocreatine, 12.5 U/ml creatine phosphokinase, 10 mM $MgCl_2$, 3 mM ATP, 100 μg/ml bovine serum albumin (BSA)]. SSB was then added to 0.43 μM and the mixture incubated at 37°C for 2 min before adding 5.8 μM RecA to catalyse strand exchange. Other proteins were added at the same time as RecA, as indicated. Reactions were terminated by adding a one-fifth volume of stop mix (2.5% SDS, 200 mM EDTA, 10 mg/ml proteinase K) and incubating for 10 min at 37°C to deproteinize the mixture.

Preparation of recombination intermediates

A strand exchange reaction (100 μl) was set up on ice with φX174 (+) strand DNA (19.5 μM) and ^{32}P-labelled linear duplex DNA (22.5 μM) in strand exchange buffer with 2 mM ATP. After pre-incubation for 2 min at 37°C with 2.4 mM SSB the reaction was started by the addition of RecA (9.5 μM). After 10 min incubation at 37°C the reaction was terminated by deproteinizing as above. Product DNA was purified by gel filtration chromatography through Sepharose CL-2B as described (Müller *et al.*, 1992). DNA concentrations were determined by the quantitation of radioactivity.

Dissociation of recombination intermediates

Deproteinized intermediates were incubated with or without protein as required in 20 mM Tris–HCl, pH 7.5, 2 mM DTT, 100 μg/ml BSA, with $MgCl_2$ and ATP as indicated. Reactions were incubated at 37°C, unless stated otherwise, and terminated if necessary by deproteinizing as above.

Agarose gel electrophoresis

The products of strand exchange reactions were analysed on 0.8% agarose gels using a 40 mM Tris–HCl, pH 8.0, 1 mM EDTA buffer system. Gels were electrophoresed with buffer recirculation at 6 V/cm for 2.5 h at room temperature. Gels were dried and autoradiographed on Kodak XAR and Amersham β-max film to visualize the DNA. Gels were quantified by phosphorimaging analysis (Molecular Dynamics).

Band-shift assay

Reaction mixtures (20 μl) contained ^{32}P-labelled junction (0.1 μM) or oligonucleotide 2 (0.03 μM) in binding buffer (25 mM Tris–HCl, pH 8.0, 1 mM DTT, 5 mM EDTA, 100 μg/ml BSA, 6% glycerol) and protein as indicated. Reactions were kept for 15 min on ice and then loaded immediately onto 4% polyacrylamide gels in low ionic strength buffer (6.7 mM Tris–HCl, pH 8.0, 3.3 mM sodium acetate, 2 mM EDTA). Electrophoresis was at room temperature for 1 h 45 min at 160 V with continuous circulation of buffer. Gels were dried and autoradiographed.

Dissociation of model three-stranded junction DNA

Reaction mixtures (20 μl) contained junction DNA (24 nM) in 20 mM Tris–HCl, pH 7.5, 2 mM DTT, 5 mM $MgCl_2$, 5 mM ATP, 100 μg/ml BSA and protein as indicated. After incubation at 37°C for 30 min reactions were terminated by deproteinizing as above. Products were analysed by electrophoresis through 10% native polyacrylamide gels at 190 V using a continuously circulated Tris–borate buffer system. Gels were dried on 3MM Whatman paper and autoradiographed.

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