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On the Mechanism of RecA-Mediated Repair of Double-Strand Breaks: No Role for Four-Strand DNA Pairing Intermediates

Qun Shan and Michael M. Cox*
Department of Biochemistry
University of Wisconsin, Madison
Madison, Wisconsin 53706

Summary

RecA protein will bind to a gapped duplex DNA molecule and promote a DNA strand exchange with a second homologous linear duplex. A double-strand break in the second duplex is efficiently bypassed in the course of these reactions. We demonstrate that the bypass of double-strand breaks is not explained by a mechanism involving homologous interactions between two duplex DNA molecules, but instead requires the ATP-mediated generation of DNA torsional stress brought about by the action of RecA. The results suggest new pathways for the repair of double-strand breaks and underline the need for new paradigms to explain the alignment of homologous DNAs during genetic recombination.

Introduction

The question of how two DNA molecules interact so as to align homologous DNA sequences is one of the central problems in recombination research. Watson-Crick base pairing is the only documented way that two random sequence DNA strands can stably interact (Frank-Kamenetskii and Mirkin, 1995). Thus, alignment of two duplex DNAs of similar sequence might be preceded by strand separation in each of them. For purposes of genetic recombination, the formation of interwound three-stranded (triplex) or four-stranded (quadruplex) DNA structures as DNA pairing intermediates has been proposed as an alternative mechanism for DNA–DNA alignment. Since homologous recombination must accommodate almost any sequence, a triplex or quadruplex structure formed as an intermediate in this process would represent a novel DNA species.

There are no reports that a quadruplex forms spontaneously in solution, and only one report of a possible recombination triplex (called R-DNA) observed at temperatures below 25°C (Shchyolkina et al., 1994). In the absence of direct observation of these DNA species in solution, published research has concentrated on DNA structures associated with reactions promoted by the RecA protein of *Escherichia coli*. Proposals for a recombination triplex are discussed in detail elsewhere (Stasiak, 1992; Baliga et al., 1995; Frank-Kamenetskii and Mirkin, 1995; Kubista et al., 1996; Podyminogin et al., 1995, 1996; Roca and Cox, 1997; Zhou and Adzuma, 1997). We focus here on evidence for the existence of a recombination quadruplex structure as a mechanism to align two duplex DNAs.

*To whom correspondence should be addressed.

The hypothetical recombination quadruplex is a four-stranded DNA pairing intermediate in which two homologous duplex DNA molecules are interwound and interact via major groove–major groove interaction. Proposals for a quadruplex structure as a recombination intermediate have appeared on numerous occasions over a period spanning nearly three decades (McGavin, 1971; Wilson, 1979; Fishel and Howard-Flanders et al., 1984; Fishel and Rich, 1988). Although little evidence for the existence of the quadruplex has emerged, the idea still plays a prominent role in modern reviews, textbooks, and reports describing recombination in general and RecA protein in particular (West, 1992; Kowalczykowski and Eggleston, 1994; Voet and Voet, 1995; Eggleston et al., 1997).

In vitro, RecA protein promotes efficient DNA strand exchange reactions between homologous DNA molecules. Reactions can involve either a single strand and a duplex (a three-strand reaction), or two duplex DNAs, one of which has a single-strand gap (a four-strand reaction) (Kowalczykowski and Eggleston, 1994; Cox, 1995; Kubista et al., 1996; Roca and Cox, 1997). RecA protein typically forms a filament on a circular single-stranded DNA or a circular gapped duplex. The DNA within the filament is extended and any bound duplex DNA is underwound by nearly 40%. RecA is also a DNA-dependent ATPase. When a duplex DNA is added that is homologous to the RecA-bound DNA, a DNA strand exchange reaction ensues.

For purposes of this discussion, DNA strand exchange can be divided into two phases, one that does not depend on ATP hydrolysis and another that does. In the first phase, the two DNAs are aligned and a significant exchange of strands generating 1–2 kilobase pairs of hybrid DNA can occur within 2 min (Menetski et al., 1990; Rehrauer and Kowalczykowski, 1993; Jain et al., 1994; Kowalczykowski and Krupp, 1995; Shan et al., 1996). This will be referred to as the DNA pairing phase, and is where the triplex or quadruplex species in question have been invoked as intermediates. ATP hydrolysis permits an additional phase in which the exchanged DNA is greatly lengthened. The reaction also becomes unidirectional and acquires the capacity to bypass significant structural barriers in one or both DNA substrates (Kim et al., 1992a, 1992b; Jain et al., 1994; Shan et al., 1996). This will be called the extended exchange phase.

For RecA, there is much evidence that the DNA pairing phase can handle three, but not four DNA strands (Cox, 1995). Physical studies have consistently demonstrated that no more than three DNA strands can be readily accommodated within the interior helical groove of a RecA filament, where the fundamental DNA pairing process occurs (Müller et al., 1990; Takahashi et al., 1991; Wittung et al., 1994; Cox, 1995; Kubista et al., 1996; Roca and Cox, 1997). Although RecA promotes an efficient four-strand exchange reaction, pairing in these reactions is always initiated within the single-strand gap; i.e., four-strand exchanges must be initiated as three-strand reactions (Conley and West, 1990; Lindsley and Cox, 1990; Chow et al., 1992). Finally and significantly,

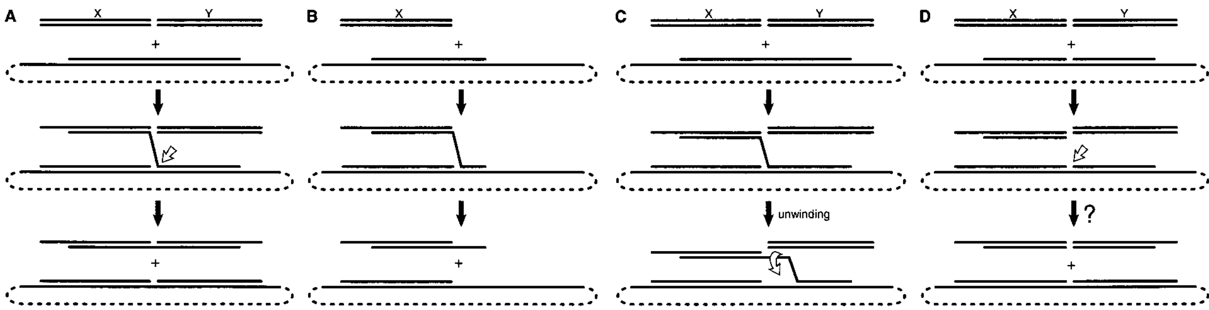

Figure 1. Bypass of Double-Strand Breaks during Four-Strand Exchange Reactions Promoted by RecA Protein

(A) The double-strand break bypass reaction (West and Howard-Flanders, 1984). The gapped duplex substrate is depicted linearly, with its circularity indicated with the dashed line. Once the first linear duplex undergoes exchange, the second is presumed to initiate exchange in a region (open arrow) where it must pair with a duplex DNA segment within the filament.

(B) Unwinding of a distal duplex DNA segment after a four-strand exchange reaction. After the reciprocal exchange in step 1, RecA protein will unwind 100 bp or more in a duplex region attached to the region already exchanged, so as to separate the reaction products (MacFarland et al., 1997).

(C) Alternative mechanism for double-strand break bypass, based on the observation in (B). After the exchange of the first linear duplex fragment, continued unwinding of the gapped duplex DNA would open a single-stranded region (open arrow) in which the second linear fragment could initiate DNA strand exchange as a three-stranded reaction.

(D) The presence of a nick in the exchanging strand of the gapped duplex should block double-strand break bypass by the mechanism of (C), but should not block bypass if it proceeds via a four-strand DNA pairing interaction at a point indicated by the open arrow.

a four-strand exchange exhibits an absolute requirement for ATP hydrolysis (Kim et al., 1992b; Shan et al., 1996), even though considerable exchange can occur in a three-strand reaction without ATP hydrolysis. The dependence on ATP hydrolysis associates a four-strand exchange reaction uniquely with the extended exchange phase of DNA strand exchange.

In bacteria, the biochemistry is complemented by studies highlighting the importance of single-stranded DNA to initiate recombination in vivo. The early phases of recombination are replete with enzymes (nucleases and helicases) whose function is to convert duplex DNA to single strands for RecA binding and initiation of DNA pairing (Smith, 1989; Kowalczykowski et al., 1994), obviating any need for duplex–duplex interactions.

On the other side of the issue, two types of evidence provide indirect support for the existence of a four-stranded DNA pairing intermediate in RecA-mediated DNA strand exchange reactions involving two duplexes. First, when RecA protein is bound to a gapped duplex DNA, the complex promotes some homology-dependent underwinding of a second circular duplex DNA, even when that homology is limited to the duplex region of the gapped substrate (Conley and West, 1989, 1990; Chiu et al., 1990; Lindsley and Cox, 1990; Chow et al., 1992). However, the signal obtained is very weak and readily explained by a three-strand rather than a four-strand interaction (Cox, 1995). The second piece of evidence for four-stranded DNA pairing intermediates is more compelling. In a four-strand exchange reaction between a gapped duplex DNA (to which RecA is bound), and a linear duplex DNA, a double-strand break in the linear duplex (dividing the duplex into two fragments) can be bypassed (West and Howard-Flanders, 1984). The second linear DNA fragment to be exchanged in these reactions must initiate exchange at a point where the gapped substrate is double-stranded (Figure 1A). Therefore, this result supports the notion that initiation of strand exchange can involve a duplex–duplex DNA pairing process.

Recent work has suggested a potential alternative explanation of the double-strand break bypass phenomenon that does not involve duplex–duplex pairing. RecA protein can simply unwind regions of DNA of 100 bp or more in particular exchange contexts. For example, the DNA within a heterologous insertion in the linear duplex DNA is unwound in the process of bypassing this structural barrier during a three-strand exchange (Jwang and Radding, 1992). During a four-strand exchange, duplex DNA in the gapped and RecA-bound substrate, beyond the region of reciprocal exchange with the second duplex, is unwound to effect separation of the exchange products (Figure 1B) (MacFarland et al., 1997). Both of these processes require ATP hydrolysis. In both cases, the DNA is not directly unwound by RecA protein bound to it, because a nick in or near the DNA being unwound (which should not affect RecA binding) abolishes the unwinding (Jwang and Radding, 1992; MacFarland et al., 1997). Instead, unwinding involves an indirect application of DNA torsional stress. In effect, the DNAs must be rotated to bring about the strand separation. A similar process might bring about the bypass of a double-strand break. Once the first linear DNA fragment has undergone exchange, the RecA-bound duplex could be unwound beyond the region exchanged. This would create single-stranded DNA within which a second linear DNA fragment could initiate exchange as a three-strand reaction (Figure 1C). The present study was initiated to investigate this possibility.

Results

Experimental Design

RecA-mediated four-strand exchange reactions were monitored by agarose gel electrophoresis. DNA substrates are described in Figure 2. A standard four-strand exchange reaction occurs between the gapped duplex, simply called GD, and the linear duplex substrate (LDS). A double-strand break in the linear duplex DNA substrate is created by restriction digestion, dividing the

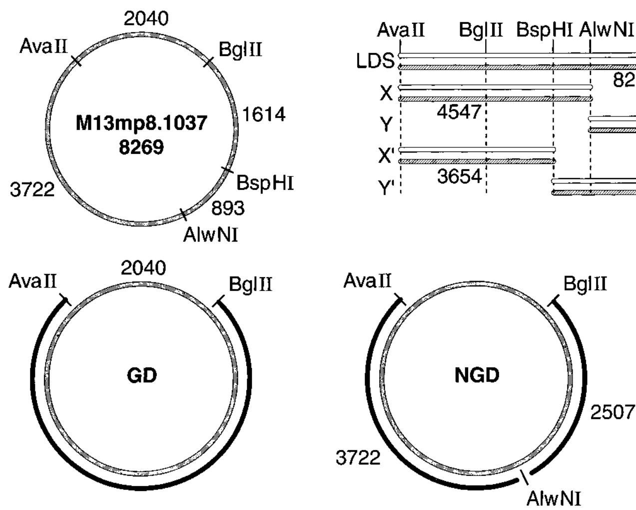

Figure 2. DNA Substrates Used in This Study

All DNA molecules were based on M13mp8.1037 (8269 bp total). Numbers around the periphery in the molecule at top left are distances, in bp, between the restriction sites indicated. The gapped and nicked gapped DNAs are shown at bottom, and the linear duplex substrates are shown at the upper right. Symbols here and in subsequent figures are: (GD), gapped duplex DNA; (NGD), gapped duplex DNA with a nick in the shortened strand at the AlwNI site; (LDS), linear duplex substrate; (X), (Y), (X′), (Y′), fragments of LDS as shown. Strand shading is maintained in all subsequent figures.

linear molecule into two fragments, X and Y. The normal polarity of RecA-mediated DNA strand exchange would lead to fragment X being exchanged before Y, a sequence confirmed below. A nick can be placed in the gapped duplex, coincident with the cleavage at AlwNI that separates X and Y, creating a nicked gapped duplex (NGD). The linear DNA substrate was also processed to generate a double-strand break at the BspHI site, creating linear fragments X′ and Y′ as alternative substrates with a DS break that is not coincident with the nick in NGD.

The experimental premise is straightforward: if double-strand break bypass during these reactions involves four-stranded DNA pairing, a nick in the gapped duplex in the exchanging strand (coincident with the double-strand break, Figure 1D) should not affect bypass. However, if the bypass mechanism involves DNA unwinding as outlined in Figure 1C, the nick in the gapped duplex should prevent bypass of double-strand breaks. Reaction conditions were based on those reported by West and Howard-Flanders (1984).

A Single Nick in Gapped Duplex DNA Abolishes RecA-Mediated Bypass of Double-Strand Breaks

As shown in Figure 3 (reaction 2), the bypass of a double-strand break during a four-strand exchange reaction occurs efficiently, consistent with published results (West and Howard-Flanders, 1984). The reaction is compared with a normal four-strand exchange without a double-strand break (reaction 1). The formation of nicked circular duplex (P_1) and linear (P_2) products followed similar kinetics, and about 60% of the substrate DNA was converted to products in both reactions. Under these reaction conditions, the presence of a double-strand break in the linear duplex DNA substrate has no detectable effect on the overall efficiency of the reaction. We note that the relatively high concentration of Mg^{2+} and the SSB were critical to the efficiency of the bypass reaction (data not shown). A similar efficiency of double-strand break bypass was achieved in six separate trials carried out under these conditions.

The key experiment is presented in Figure 4. The double-strand break bypass (reaction 2) is compared with the same reaction using the nicked gapped duplex (reaction 3) in (A). The linear product of the latter reaction is divided into two fragments that roughly comigrate with the substrate fragments X and Y, and no P_2 band is generated. However, a band corresponding to P_1 is generated in reaction 3, appearing at substantially earlier times than in reaction 2. As shown in the schematic drawing, the exchange of fragment X only will result in the generation of a reaction intermediate called P_1', which is indistinguishable on the gel from P_1. Since a shorter length of DNA must be exchanged to generate P_1', the kinetics of the reaction are consistent with the generation of this species. A double-strand break bypass would require the exchange of fragment Y as well as fragment X, and the fluoro-image of the ethidium-stained gel in (A) does not allow us to determine if this has occurred.

To monitor the fate of fragment Y in reaction 3, the linear dsDNA substrate was 5′ end-labeled with ^{32}P prior to cleavage with AlwNI. This procedure labels only one of the two strands in fragments X and Y. In fragment X, the labeled strand is the one that ends up in product P_3, so an exchange involving only fragment X does not introduce label into P_1'. The opposite strand is labeled in fragment Y, so that exchange with fragment Y leading to double-strand break bypass would introduce label into P_1. The same gel in (A) was therefore visualized with a phosphor-imager, as shown in Figure 4B. In reaction 2, the double-strand break bypass generates labeled products P_1 and P_2. In reaction 3, where only P_1' can be expected, no label is introduced into the P_1 band. Some label is seen transiently at a position expected for branched reaction intermediates, appearing with kinetics consistent with the exchange involving fragment X alone. Figure 4B demonstrates that a nick in the gapped duplex DNA substrate abolishes the double-strand bypass reaction.

The nick in the gapped duplex is not sufficient by itself to halt DNA strand exchange. A four-strand exchange reaction using the NGD substrate and a full-length linear

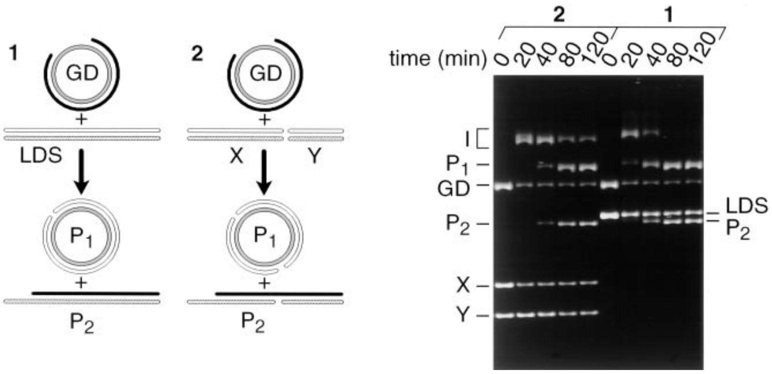

Figure 3. RecA-Mediated Bypass of a Double-Strand Break during a Four-Strand Exchange Reaction

The individual reactions in the gel are identified by reaction numbers to match the schematic diagram at left. Reaction 1 (at right in the gel) is a normal four-strand exchange. Reaction 2 is the DS break bypass. The bypass is indicated by the formation of both products P_1 and P_2. Strand exchange reactions in this and subsequent figures were carried out at 37°C and contained 20 mM Tris–HCl (pH 7.5), 25 mM Mg chloride, 2 mM DTT, 2 mM ATP, 0.1 mg/ml BSA, 5% (w/v) glycerol, an ATP regeneration system (8 mM phosphocreatine, 8 units ml^{-1} phosphocreatine kinase), gapped duplex DNA (9.7 μM), linear duplex (12 μM total), RecA protein (5 μM), and SSB protein (0.15 μM). Symbols are as defined in Figure 2, except that (I) denotes intermediates.

duplex DNA proceeded as well as the standard four-strand exchange in reaction 1 (data not shown). A reaction like reaction 3, but substituting substrates X′ and Y′ for X and Y (see Figure 2), also proceeded as efficiently as reactions 1 and 2 (data not shown), showing that bypass is not affected if the double-strand break of the linear duplex is offset from the nick in the gapped DNA (in this case by 907 bp).

The Bypass of Double-Strand Breaks Involves a Sequential Exchange of Fragments X and Y

If a four-stranded DNA pairing intermediate existed and represented the fundamental pairing mechanism by which RecA promotes a four-strand exchange, one might expect fragments X and Y to be exchanged or at least paired concurrently. A concurrent exchange of multiple nonoverlapping DNA fragments is readily observed in three-strand exchange reactions (Bedale and Cox, 1996), providing one avenue for the rapid repair of gaps and double-strand breaks in vivo. In contrast, the results presented in Figure 5 demonstrate that the exchange of multiple fragments (double-strand break bypass) occurs sequentially in a four-strand reaction.

Reactions 2 and 3 were again followed, but fragments X and Y were added at different times. One of the fragments was preincubated with the gapped or nicked gapped duplex and allowed to react for 60 min before addition of the other fragment. In this experiment, only fragment Y was labeled (in both strands), and the reactions were again monitored both with a fluoro-imager (A) and a phosphor-imager (B). For reaction 2 (no nick in the gapped substrate), addition of fragment X first leads to the production of a branched intermediate evident in (A) after 60 min, which is rapidly chased into products upon addition of fragment Y as seen in both panels. When fragment Y is added first, no reaction is seen in either panel for the first 60 min, not even a weak formation of intermediates that might suggest a pairing interaction. After fragment X is added, products are generated, but with slow kinetics, indicating that X must be exchanged before Y can react. For reaction 3 (using

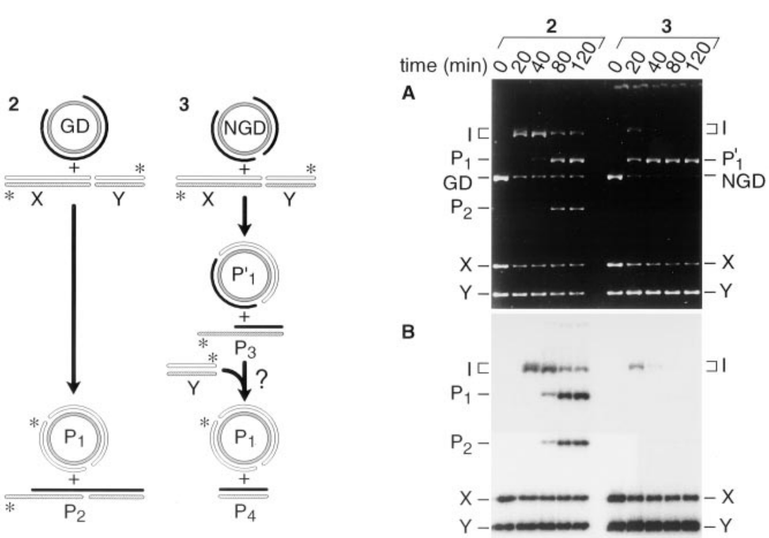

Figure 4. The Bypass of Double-Strand Breaks Is Abolished by a Nick in the Gapped Duplex DNA Substrate

Reaction 2 is the same bypass reaction shown in Figure 3. Reaction 3 makes use of a gapped duplex with a nick in the exchanging strand coincident with the double-strand break separating X and Y. Both panels show the same gel, with (A) showing a fluoro-image of the gel stained with ethidium bromide, and (B) the same gel monitored with a phosphor-imager. P_1' is the product generated by exchange of the nicked gapped duplex (NGD) with fragment X alone. In reaction 3, P_1 (or P_1') is the only new detectable product generated, since P3 and P4 comigrate with the substrates X and Y. A band corresponding to P_1 is formed quite early in reaction 3 (A). A ^{32}P label placed on the ends of one strand in each linear substrate (*) allows a discrimination between P_1 and P_1', since a label will appear in the P_1 band only if fragment Y undergoes exchange.

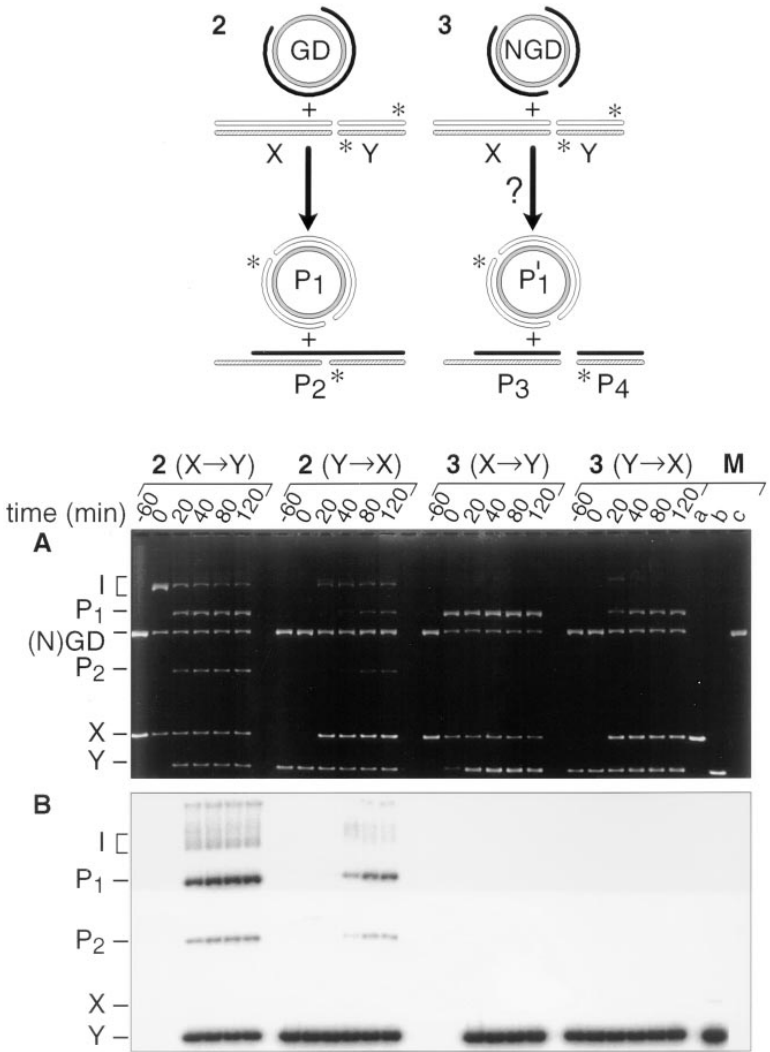

Figure 5. The Fragments X and Y Are Exchanged Sequentially Rather Than Concurrently in a Four-Strand Exchange Reaction

The reactions shown here are the same as in Figure 4, except that the fragments X and Y are added at different times. There are four experiments; from left to right in the panels, the first two are reaction 2 and the others are reaction 3, each with six time points. Both panels show the same gel, with (A) and (B) representing a fluoro-image and a phosphorimage, respectively. Both strands of fragment Y are labeled in all cases as indicated for reaction 2. In the first reaction, at left, fragment X is added alone for 60 min. Immediately after the 0 time point, fragment Y is added. In the second reaction, fragment Y is added alone at −60 min. Reaction intermediates and products are generated slowly and only after the addition of X immediately after the 0 time point. In the third and fourth reactions, involving the nicked gapped duplex, a similar reaction protocol is used adding X or Y first, respectively. As seen in (B), there is no incorporation of label into products indicating a reaction with Y to generate either intermediates or products in the third or fourth reactions. Symbols are defined in the legends to Figures 2–4.

the nicked gapped duplex), no reaction is seen with fragment Y regardless of the order of addition, confirming that the nick blocks double-strand break bypass. The results indicate that fragment Y interacts only with the branched DNA intermediate formed after the complete exchange of fragment X. If a nick is present in the gapped DNA, or if fragment X is not provided, no interaction of fragment Y with the RecA-bound gapped DNA is detected.

The order of fragment exchange in these reactions is consistent with the documented polarity of RecA exchange reactions involving three strands, proceeding 5′ to 3′ with respect to the single-stranded DNA in the gap of the gapped duplex. A sequential exchange of DNA fragments was also evident in the original work on double-strand break bypass in a four-strand context (West and Howard-Flanders, 1984), although the reason why fragment X must be exchanged before an interaction with fragment Y can be seen was not addressed.

ATP Hydrolysis Is Required for RecA-Mediated Bypass of dsDNA Breaks during a Four-Strand Exchange Reaction

All four-strand exchange reactions promoted by RecA protein exhibit a requirement for ATP hydrolysis, so that examining this parameter for the bypass reaction is not straightforward. We elected to make use of the RecA K72R mutant, which binds but does not hydrolyze ATP (Rehrauer and Kowalczykowski, 1993). This mutant also forms mixed filaments with wild-type RecA protein (Shan et al., 1996). The presence of the K72R mutant partially poisons the ATPase activity of the entire filament, but the mixed filaments are still capable of promoting significant DNA strand exchange in three-strand reactions (Shan and Cox, 1996, 1997; Shan et al., 1996). When RecA is bound to dsDNA, the mutant protein is readily exchanged into a wild-type filament, displacing some of the wild-type protein and creating mixed filaments (Shan and Cox, 1996).

Reaction 2 was carried out in two separate tubes, and the fragments X and Y were added sequentially. ATP was replaced by dATP in this experiment, to meet the requirements of DNA pairing reactions promoted by the mutant protein (Rehrauer and Kowalczykowski, 1993; Shan et al., 1996). After 60 min of reaction with fragment X, the RecA K72R mutant was added to one of the tubes, and fragment Y was then added to both tubes. Based on previous work (Shan and Cox, 1996), the amount of RecA K72R added should bring the overall mutant protein content in the resulting mixed filaments to about

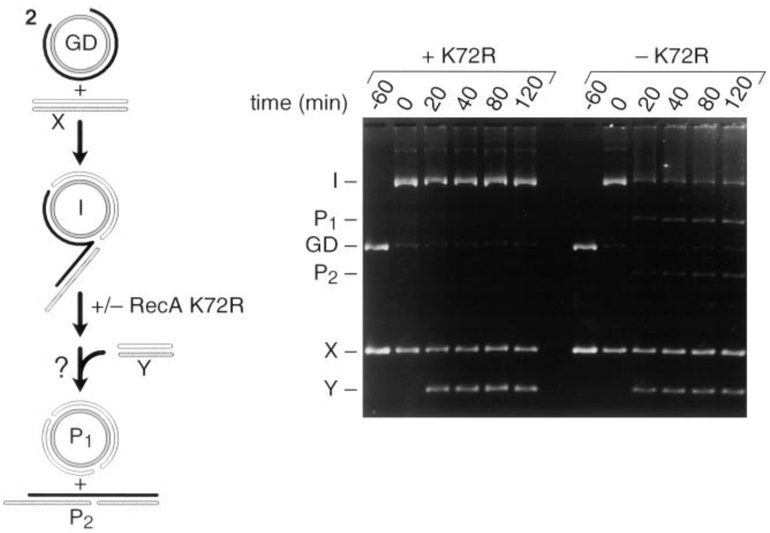

Figure 6. The Bypass of DS Breaks Requires ATP Hydrolysis

Reactions were carried out as described in the Experimental Procedures and the legend to Figure 3, with dATP replacing ATP. The protocol is similar to the first reaction in Figure 5. Reactions are preincubated for 60 min with fragment X, generating branched strand exchange intermediates. There was then a second short incubation (10 min) in the presence or absence of the RecA mutant K72R (5 μM) protein. Fragment Y is added immediately after the 0 time point in both reactions. Symbols are defined in the legends to Figures 2–4.

50% in the incubation period before fragment Y is added. As can be seen in Figure 6, product formation indicating an exchange of fragment Y proceeded in the tube without the mutant protein, but not at all in the tube in which the mutant protein was added. RecA K72R mutant protein, which retains the fundamental DNA pairing activity of RecA protein, blocks the bypass of double-strand breaks just as it blocks four-strand exchange reactions in general (Shan et al., 1996).

Discussion

The reactions described in this report are often complicated to describe, but the conclusion derived from them is not. RecA protein can efficiently promote the bypass of double-strand breaks during a four-strand exchange reaction in vitro. However, the bypass mechanism does not involve the formation of a four-stranded DNA pairing intermediate. The bypass is abolished by placing a nick in the gapped duplex DNA substrate (to which RecA is initially bound), positioned coincident with the double-strand break in the linear duplex DNA substrate. This condition should not affect bypass if the second DNA fragment initiates exchange by forming a four-stranded DNA pairing intermediate.

A hypothesis is useful only to the extent that reasonable predictions arising from it are borne out experimentally. There are a range of predictions elicited by the hypothesis that RecA protein aligns two duplex DNAs by formation of an interwound quadruplex DNA pairing intermediate. The most obvious prediction is that alignment of two homologous duplex DNAs within the RecA filament should be experimentally demonstrable. However, nearly two decades of work on RecA protein in dozens of laboratories has failed to provide compelling evidence that four DNA strands can be bound in the interior of a RecA filament under any set of conditions. This failure does not reflect a lack of effort (Müller et al., 1990; Takahashi et al., 1991; Wittung et al., 1994; Cox, 1995; Kubista et al., 1996; Roca and Cox, 1997). The introduction to the original work on double-strand break bypass (West and Howard-Flanders, 1984) alluded to the difficulties encountered in detecting duplex–duplex interactions in numerous studies. That difficulty continues (Cox, 1995). In contrast, RecA-mediated DNA pairing involving three DNA strands elicits a strong signal in a wide range of assays (Cox, 1995; Roca and Cox, 1997).

The present work is premised on a related prediction of the quadruplex hypothesis (West and Howard-Flanders, 1984). If RecA can align two duplex DNA molecules within the filament groove, such alignment between fragment Y and the gapped duplex DNA substrate might facilitate the double-strand break bypass seen in reaction 2 (Figures 3–5). There is no reason the introduction of a nick in the gapped duplex should affect the hypothetical interaction of the two duplexes. In fact, the positioning of a nick coincident with the double-strand break in the other DNA substrate potentially eliminates a structural barrier that could prevent productive initiation of strand exchange by fragment Y, such that a nick might allow concurrent exchange of fragments X and Y. Instead, double-strand break bypass is abolished and no reaction of fragment Y is observed.

The four-strand exchange reactions provide an important test of models for RecA protein–mediated DNA strand exchange. A wide range of published models (Howard-Flanders et al., 1984; Burnett et al., 1994; Morel et al., 1994; Kowalczykowski and Krupp, 1995) either explicitly or implicitly require the binding of all four DNA strands within a RecA filament to effect a four-strand exchange reaction. None of these models attempts to explain why exchanges involving four strands (but not three strands) should be completely dependent on ATP hydrolysis.

How can RecA protein promote a four-strand exchange with a filament that can accommodate only three DNA strands in its interior? The experiment in Figure 4 provides a clue. If a double-strand break in the linear duplex is to be bypassed, the exchanging strand of the gapped DNA substrate must provide a structural connection between the hybrid DNA in the segments already exchanged and the unexchanged DNA beyond the double-strand break. Bypass would be readily explained if the unexchanged DNA was simply unwound downstream of the hybrid DNA created by the exchange of fragment X. This would create a region of single-strand DNA in which fragment Y could initiate exchange as a three-stranded reaction. A recent study using substrates like those depicted in Figure 1B (MacFarland et al., 1997) demonstrates that DNA unwinding will occur under these conditions, at this location, and with a DNA species like that present after exchange of fragment X, as long as the exchanging strand of the gapped duplex remains intact. The observed unwinding of the gapped duplex substrate is most easily explained if one DNA in the exchanged region is rotated about the other (or the two DNA substrates are each rotated about their longitudinal axes [Honigberg and Radding, 1988]), and the torsional stress thus generated is translated into an unwinding of connected DNA beyond the exchanged region (MacFarland et al., 1997). Facilitated DNA rotation, coupled to ATP hydrolysis, has been proposed as a mechanism to augment the fundamental DNA pairing process in three-strand exchanges, permitting the extended exchange phase of the reaction (Cox, 1994; Roca and Cox, 1997). A similar process may be uniformly required for four-strand exchanges, providing at least one mechanism for exchange without introducing all four DNA strands into the RecA filament interior as well as an explanation for the requirement for ATP hydrolysis.

The proposed mechanism for double-strand break bypass is presented in more detail in Figure 7. The RecA filament binds to the gapped duplex DNA. Fragment X initiates a DNA strand exchange reaction within the gap by a standard three-stranded pairing mechanism within the groove that does not require ATP hydrolysis (not shown). Some part of fragment X remains outside the filament, and facilitated rotation of this segment of X about the gapped duplex moves the exchange into the four-stranded region and creates a Holliday intermediate (B), which in turn migrates slowly down the filament in a reaction coupled to ATP hydrolysis. When the crossover migrates to the end of fragment X (C), the rotation continues so as to unwind a region of DNA in the gapped duplex beyond the end of the already exchanged DNA by means of the resulting applied torsional stress (inset). This creates a region of single-stranded DNA in which the second linear duplex fragment (Y) initiates exchange as a three-stranded reaction (D). Exchange of fragment Y continues by the same facilitated rotation mechanism until complete. With a nick in the gapped duplex coincident with the double-strand break (see Figure 1D), DNA torsional stress generated by the rotation of the two hybrid DNA segments could not be transmitted into the continued unwinding of the gapped DNA molecule. By permitting fragment Y to initiate exchange as a three-strand reaction, the bypass of double-strand breaks is thereby brought into concert with all other DNA strand exchange reactions promoted by RecA protein, all of which must be initiated by a pairing process involving no more than three DNA strands (Conley and West, 1990; Lindsley and Cox, 1990; Cox, 1995).

The quadruplex hypothesis faces additional problems. As a mechanism for homologous recognition, a four-stranded DNA pairing intermediate with duplexes interacting in their major grooves is difficult to reconcile with new information about DNA pairing in a three-stranded reaction. Four laboratories have provided evidence that a RecA-bound single strand is approached via the minor groove of the duplex DNA substrate (Kumar and Muniyappa, 1992; Baliga et al., 1995; Podyminogin et al., 1995, 1996; Zhou and Adzuma, 1997). This work strongly suggests that non-Watson-Crick base interactions in the major groove do not play a role in homologous alignment. Instead, DNA–DNA alignment could involve a rapid sampling mechanism, with bases rotating and interacting via transient Watson-Crick interactions.

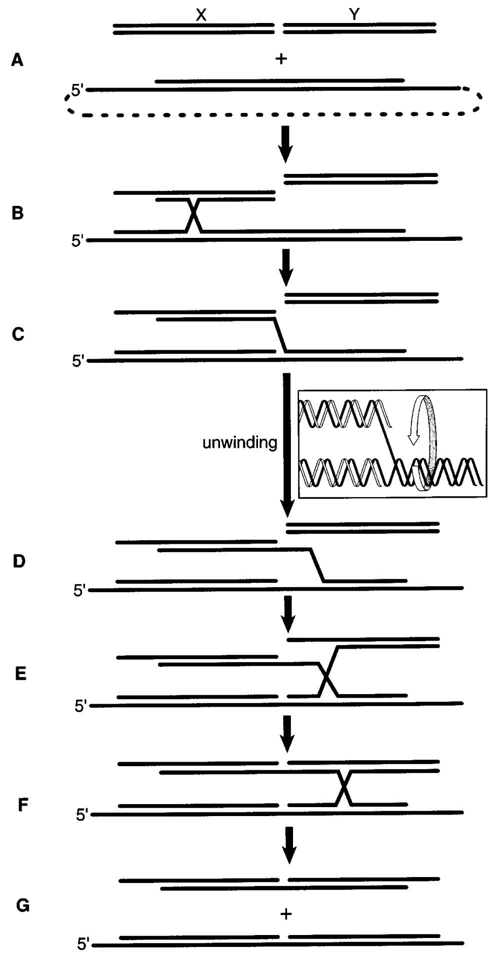

Figure 7. Scheme for DS Break Bypass Mediated by RecA Protein in the Course of a Four-Strand Exchange Reaction

The gapped duplex is depicted linearly (with the dashed lines in [A] there to indicate that the two ends are actually joined). Fragment X is exchanged first, proceeding to completion (C). At this point, the remaining duplex DNA in the original gapped molecule must be partially unwound (D). This is brought about by the indirect application of DNA torsional stress, since a nick at this position in the exchanging strand blocks the bypass. This could occur by rotating the two hybrid DNA segments, as described previously (MacFarland et al., 1997) and in the inset. The unwinding creates a short single-stranded region where fragment Y can initiate exchange as a three-strand reaction (E). Completion of exchange generates the final products (G).

When combined with earlier results, RecA is shown to provide two potential pathways for the repair of double-strand breaks. If RecA protein is bound to single-stranded DNA, complementary and nonoverlapping duplex DNA fragments can be paired and exchanged efficiently and concurrently in a process that may often require no ATP hydrolysis (Bedale and Cox, 1996). If RecA protein is bound to dsDNA, the bypass of double-strand breaks is an ordered process requiring the aid of an ATP-dependent motor.

The bypass of double-strand breaks during four-strand exchanges has been the only evidence put forward in support of a four-stranded DNA pairing intermediate that, prior to the present study, did not have an obvious alternative explanation. We now find it reasonable to question the existence of four-stranded DNA pairing intermediates. While it is virtually impossible to completely disprove the existence of recombination intermediates in which two duplexes are paired and interwound, these hypothetical structures have clearly lost their predictive value. At a minimum, neither physical studies on DNA structure or experience with DNA pairing promoted by RecA protein recommends a recombination quadruplex as a useful hypothesis for homologous alignment, and there is nothing on which to base its continued prominence in the recombination literature. There should also be nothing in the way of a more aggressive consideration and exploration of alternative mechanisms for the four-strand DNA exchange reactions promoted by RecA protein.

Experimental Procedures

Enzymes and Biochemicals

Escherichia coli RecA protein (wtRecA) and RecA K72R mutant were purified by a procedure developed for the RecA K72R mutant protein (Shan et al., 1996). RecA protein was stored in R buffer (20 mM TrisOAc 80%+ [pH 7.5], 1 mM DTT, 0.1 mM EDTA, and 10% [w/v] glycerol). All RecA protein preparations were more than 95% pure and free of detectable nuclease activities. The concentration of RecA protein preparations were determined by absorbance at 280 nm using an extinction coefficient of $\epsilon_{280} = 0.59\ A_{280}\ mg^{-1}$ ml (Craig and Roberts, 1981). *E. coli* single-stranded DNA binding protein (SSB) was purified as described (Lohman et al., 1986) with the minor modification that a DEAE-Sepharose column was added to ensure removal of single-stranded exonucleases. The concentration of SSB protein was determined by absorbance at 280 nm using an extinction coefficient of $\epsilon_{280} = 1.5\ A_{280}\ mg^{-1}$ ml (Lohman and Overman, 1985). Tris buffer was from Fisher Scientific. Restriction endonucleases and T4 polynucleotide kinase were purchased from New England BioLabs. Proteinase K, phosphocreatine, and phosphocreatine kinase were purchased from Sigma. Ultrapure dATP, ATP, and DEAE-Sepharose resin were from Pharmacia Biotech Inc. Hydroxylapatite resin was from Bio-Rad. [γ-^{32}P]ATP was purchased from Amersham.

DNA

Duplex DNA and circular single-stranded DNA were derived from bacteriophage M13mp8. Bacteriophage M13mp8.1037 (8269 bases) is bacteriophage M13mp8 with a 1040 bp EcoRV–EcoRV fragment from the *E. coli* galT gene inserted into the SmaI site (Lindsley and Cox, 1990). The concentration of dsDNA and ssDNA stock solutions were determined by absorbance at 260 nm, using 50 and 36 μg $ml^{-1} A_{260}^{-1}$, respectively, as conversion factors. DNA concentrations are expressed in terms of total nucleotides. Full-length linear duplex DNA substrates were generated by complete digestion of supercoiled M13mp8.1037 DNA by appropriate restriction endonucleases under conditions recommended by the supplier. The protein was removed by extraction with phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1). The DNA was finally concentrated by ethanol precipitation. Shorter linear dsDNA fragments were generated by cutting supercoiled M13mp8.1037 with appropriate restriction endonucleases followed by gel purification (Maniatis et al., 1982). In some cases, dsDNA fragments were ^{32}P-radiolabeled at 5′ ends by T4 polynucleotide kinase using conditions suggested by the manufacturer's instructions.

Preparation of Gapped Duplex DNA

Gapped duplex DNA molecules were prepared by large-scale RecA-mediated strand exchange reactions between circular ssDNA and linear duplex DNA molecules (Shan et al., 1996). The standard circular duplex DNA with a defined gap used in this study, GD_{2040}, was prepared from circular M13mp8.1037 ssDNA and an AvaII–BglII-cut duplex DNA fragment of M13mp8.1037 (6229 bp). Another version of GD_{2040} with a precise nick at AlwNI restriction sit, made use of two linear dsDNA fragments (BglII–AlwNI, 2507 bp and AlwNI–AvaII, 3722 bp). All linear duplex DNAs used in preparation of gapped duplex DNA were from M13mp8.1037 and gel-purified.

Strand Exchange Reaction Conditions

Unless otherwise indicated, DNA strand exchange reactions (50 μl) were performed at 37°C in a strand exchange buffer containing 20 mM Tris–HCl (80% cation), 25 mM Mg chloride, 2 mM DTT, 0.1 mg/ml BSA, 5% (w/v) glycerol, an ATP regeneration system (8 mM phosphocreatine, 8 units ml^{-1} phosphocreatine kinase) (West and Howard-Flanders, 1984). Gapped duplex DNA (9.7 μM) and linear duplex (12 μM) were added, and a 7.5 μl aliquot was taken at a 0 time point. RecA (5 μM) was added and preincubation carried out for 5 min at 37°C before ATP or dATP (2 mM) and SSB protein (0.15 μM) were added to initiate DNA strand exchange. The pH of this reaction mixture after the addition of all reaction components (with storage buffer replacing proteins and DNAs) was 7.4.

Monitoring DNA Strand Exchange with Agarose Gel Electrophoresis

Aliquots (7.5 μl) of the DNA strand exchange reactions described above were removed at 20, 40, 80, and 120 min, and the reactions stopped by addition of 1/4 volume of stopping buffer (60 mM EDTA, 5% SDS, 25% [w/v] glycerol, 0.2% bromophenol blue). Samples were electrophoresed overnight in a 0.8% agarose gel at 2 V cm^{-1}. The gel was stained with ethidium bromide (1 mg/ml) for at least 30 min and destained for at least 2 hr. The gel was then photographed over an ultraviolet transilluminator and/or FluoroImaginer SI (Molecular Dynamics). When radiolabeled DNA substrates were employed, the same gel was also dried and exposed to a PhosphorImager screen. The intensities of DNA bands were quantified with ImageQuant software (version 4.2). To correct for variability in sample loading onto the agarose gel, bands corresponding to full-length circular hybrid duplex product and linear dsDNA substrate were quantified as the fraction of the total fluorescing DNA or radioactivity in a given gel lane.

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References

Baliga, R., Singleton, J.W., and Dervan, P.B. (1995). RecA oligonucleotide filaments bind in the minor groove of double-stranded DNA. Proc. Natl. Acad. Sci. USA *92*, 10393–10397.

Bedale, W.A., and Cox, M. (1996). Evidence for the coupling of ATP hydrolysis to the final (extension) phase of RecA protein–mediated DNA strand exchange. J. Biol. Chem. *271*, 5725–5732.

Burnett, B., Rao, B.J., Jwang, B., Reddy, G., and Radding, C.M. (1994). Resolution of the three-stranded recombination intermediate made by RecA protein. An essential role of ATP hydrolysis. J. Mol. Biol. *238*, 540–554.

Chiu, S.K., Wong, B.C., and Chow, S.A. (1990). Homologous pairing in duplex DNA regions and the formation of 4-stranded paranemic joints promoted by RecA protein—effects of gap length and negative superhelicity. J. Biol. Chem. *265*, 21262–21268.

Chow, S.A., Chiu, S.K., and Wong, B.C. (1992). RecA protein–promoted homologous pairing and strand exchange between intact and partially single-stranded duplex DNA. J. Mol. Biol. *223*, 79–93.

Conley, E.C., and West, S.C. (1989). Homologous pairing and the formation of nascent synaptic intermediates between regions of duplex DNA by RecA protein. Cell *56*, 987–995.

Conley, E.C., and West, S.C. (1990). Underwinding of DNA associated with duplex–duplex pairing by RecA protein. J. Biol. Chem. *265*, 10156–10163.

Cox, M.M. (1994). Why does RecA protein hydrolyze ATP? Trends Biochem. Sci. *19*, 217–222.

Cox, M.M. (1995). Alignment of three (but not four) DNA strands in a RecA protein filament. J. Biol. Chem. *270*, 26021–26024.

Craig, N.L., and Roberts, J.W. (1981). Function of nucleoside triphosphate and polynucleotide in Escherichia coli recA protein–directed cleavage of phage lambda repressor. J. Biol. Chem. *256*, 8039–8044.

Eggleston, A.K., Mitchell, A.H., and West, S.C. (1997). In vitro reconstitution of the late steps of genetic recombination in E. coli. Cell *89*, 607–617.

Fishel, R.A., and Rich, A. (1988). The role of left-handed Z-DNA in general genetic recombination. In Mechanisms and Consequences of DNA Damage Processing, E.C. Friedberg and P.C. Hanawalt, eds. (New York: Alan R. Liss), pp. 23–32.

Frank-Kamenetskii, M.D., and Mirkin, S.M. (1995). Triplex DNA structures. Annu. Rev. Biochem. *64*, 65–95.

Honigberg, S.M., and Radding, C.M. (1988). The mechanics of winding and unwinding helices in recombination: torsional stress associated with strand transfer promoted by RecA protein. Cell *54*, 525–532.

Howard-Flanders, P., West, S.C., and Stasiak, A. (1984). Role of RecA protein spiral filaments in genetic recombination. Nature (London) *309*, 215–219.

Jain, S.K., Cox, M.M., and Inman, R.B. (1994). On the role of ATP hydrolysis in RecA protein–mediated DNA strand exchange III. Unidirectional branch migration and extensive hybrid DNA formation. J. Biol. Chem. *269*, 20653–20661.

Jwang, B., and Radding, C.M. (1992). Torsional stress generated by RecA protein during DNA strand exchange separates strands of a heterologous insert. Proc. Natl. Acad. Sci. USA *89*, 7596–7600.

Kim, J.I., Cox, M.M., and Inman, R.B. (1992a). On the role of ATP hydrolysis in RecA protein–mediated DNA strand exchange. I. Bypassing a short heterologous insert in one DNA substrate. J. Biol. Chem. *267*, 16438–16443.

Kim, J.I., Cox, M.M., and Inman, R.B. (1992b). On the role of ATP hydrolysis in RecA protein–mediated DNA strand exchange. II. Four-strand exchanges. J. Biol. Chem. *267*, 16444–16449.

Kowalczykowski, S.C., and Eggleston, A.K. (1994). Homologous pairing and DNA strand-exchange proteins. Annu. Rev. Biochem. *63*, 991–1043.

Kowalczykowski, S.C., and Krupp, R.A. (1995). DNA-strand exchange promoted by RecA protein in the absence of ATP: implications for the mechanism of energy transduction in protein-promoted nucleic acid transactions. Proc. Natl. Acad. Sci. USA *92*, 3478–3482.

Kowalczykowski, S.C., Dixon, D.A., Eggleston, A.K., Lauder, S.D., and Rehrauer, W.M. (1994). Biochemistry of homologous recombination in Escherichia coli. Microbiol. Rev. *58*, 401–465.

Kubista, M., Simonson, T., Sjöback, R., Widlund, H., and Johansson, A. (1996). Towards an understanding of the mechanism of DNA strand exchange promoted by RecA protein. In Biological Structure and Function: Proceedings of the Ninth Conversation, The State University of New York, R.H. Sarma and M.H. Sarma, eds. (New York: Adenine Press), pp. 49–59.

Kumar, K.A., and Muniyappa, K. (1992). Use of structure-directed DNA ligands to probe the binding of recA protein to narrow and wide grooves of DNA and on its ability to promote homologous pairing. J. Biol. Chem. *267*, 24824–24832.

Lindsley, J.E., and Cox, M.M. (1990). On RecA protein–mediated homologous alignment of 2 DNA molecules—3 strands *versus* 4 strands. J. Biol. Chem. *265*, 10164–10171.

Lohman, T.M., and Overman, L.B. (1985). Two binding modes in *Escherichia coli* single strand binding protein–single stranded DNA complexes. Modulation by NaCl concentration. J. Biol. Chem. *260*, 3594–3603.

Lohman, T.M., Green, J.M., and Beyer, R.S. (1986). Large-scale overproduction and rapid purification of the *Escherichia coli ssb* gene product. Expression of the *ssb* gene under λ P_L control. Biochemistry *25*, 21–25.

MacFarland, K.J., Shan, Q., Inman, R.B., and Cox, M.M. (1997). RecA as a motor protein: testing models for the role of ATP hydrolysis in DNA strand exchange. J. Biol. Chem. *272*, 17675–17685.

Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982). Molecular Cloning: A Laboratory Manual (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory).

McGavin, S. (1971). Models of specifically paired like (homologous) nucleic acid structures. J. Mol. Biol. *55*, 293–298.

Menetski, J.P., Bear, D.G., and Kowalczykowski, S.C. (1990). Stable DNA heteroduplex formation catalyzed by the *Escherichia coli* RecA protein in the absence of ATP hydrolysis. Proc. Natl. Acad. Sci. USA *87*, 21–25.

Morel, P., Stasiak, A., Ehrlich, S.D., and Cassuto, E. (1994). Effect of length and location of heterologous sequences on RecA-mediated strand exchange. J. Biol. Chem. *269*, 19830–19835.

Müller, B., Koller, T., and Stasiak, A. (1990). Characterization of the DNA binding activity of stable RecA–DNA complexes: interaction between the two DNA binding sites within RecA helical filaments. J. Mol. Biol. *212*, 97–112.

Podyminogin, M.A., Meyer, R.B., and Gamper, H.B. (1995). Sequence-specific covalent modification of DNA by cross-linking oligonucleotides. Catalysis by RecA and implication for the mechanism of synaptic joint formation. Biochemistry *34*, 13098–13108.

Podyminogin, M.A., Meyer, R.B., and Gamper, H.B. (1996). RecA-catalyzed, sequence-specific alkylation of DNA by crosslinking oligonucleotides. Effects of length and nonhomologous base substitution. Biochemistry *35*, 7267–7274.

Rehrauer, W.M., and Kowalczykowski, S.C. (1993). Alteration of the nucleoside triphosphate (NTP) catalytic domain within Escherichia coli RecA protein attenuates NTP hydrolysis but not joint molecule formation. J. Biol. Chem. *268*, 1292–1297.

Roca, A.I., and Cox, M.M. (1997). RecA protein: structure, function, and role in recombinational DNA repair. Prog. Nucleic Acids Res. Mol. Biol. *56*, 129–223.

Shan, Q., and Cox, M.M. (1996). RecA protein dynamics in the interior of RecA nucleoprotein filaments. J. Mol. Biol. *257*, 756–774.

Shan, Q., and Cox, M.M. (1997). RecA filament dynamics during DNA strand exchange reactions. J. Biol. Chem. *272*, 11063–11073.

Shan, Q., Cox, M.M., and Inman, R.B. (1996). DNA strand exchange promoted by RecA K72R. Two reaction phases with different Mg^{2+} requirements. J. Biol. Chem. *271*, 5712–5724.

Shchyolkina, A.K., Timofeev, E.N., Borisova, O.F., Il'icheva, I.A., Minyat, E.E., Khomyakova, E.B., and Florentiev, V.L. (1994). The R-form of DNA does exist. FEBS Lett. *339*, 113–118.

Smith, G.R. (1989). Homologous recombination in E. coli: multiple pathways for multiple reasons. Cell *58*, 807–809.

Stasiak, A. (1992). Three-stranded DNA structure; is this the secret of DNA homologous recognition? Mol. Microbiol. *6*, 3267–3276.

Takahashi, M., Kubista, M., and Nordén, B. (1991). Co-ordination of multiple DNA molecules in RecA fiber evidenced by linear dichroism spectroscopy. Biochimie *73*, 219–226.

Voet, D., and Voet, J.G. (1995). Biochemistry, 2nd Edition (New York, NY: John Wiley and Sons).

West, S.C. (1992). Enzymes and molecular mechanisms of genetic recombination. Annu. Rev. Biochem. *61*, 603–640.

West, S.C., and Howard-Flanders, P. (1984). Duplex–duplex interactions catalyzed by RecA protein allow strand exchanges to pass double-strand breaks in DNA. Cell *37*, 683–691.

Wilson, J.H. (1979). Nick-free formation of reciprocal heteroduplexes: a simple solution to the topological problem. Proc. Natl. Acad. Sci. USA *76*, 3641–3645.

Wittung, P., Nordén, B., Kim, S.K., and Takahashi, M. (1994). Interactions between DNA molecules bound to RecA filament. Effects of base complementarity. J. Biol. Chem. *269*, 5799–5803.

Zhou, X., and Adzuma, K. (1997). DNA strand exchange mediated by the Escherichia coli RecA protein initiates in the minor groove of double-stranded DNA. Biochemistry *36*, 4650–4661.