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Effect of Length and Location of Heterologous Sequences on RecA-mediated Strand Exchange*

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We systematically investigated the effect of heterology on RecA-mediated strand exchange between double-stranded linear and single-stranded circular DNA. Strand exchange took place through heterologies of up to 150–200 base pairs when the insertion was at the proximal (initiating) end of the duplex DNA but was completely blocked by an insert of only 22 base pairs placed at the distal end of the duplex. In the case of medial heterology created by insertion either in the duplex or the single-stranded DNA, the ability of RecA to exchange strands decreased as the heterology was shifted toward the distal end of the duplex. These results suggest that two different strand exchange mechanisms operate in the proximal and distal portions of the duplex substrate.

In vitro, RecA protein from *Escherichia coli* has been shown to promote homologous pairing and to exchange strands between single-stranded DNA (ssDNA)[1] and double-stranded (duplex) DNA (dsDNA) (Das Gupta *et al.*, 1980; Cox and Lehman, 1981; West *et al.*, 1981a) or between duplex DNA molecules, provided that one of them carries a single-stranded region (West *et al.*, 1981b, 1982a). RecA first polymerizes on one of the DNA partners (ssDNA or duplex DNA containing single-stranded regions) to form the so-called presynaptic filament, which then participates in the search for homology. Upon homologous recognition, the protein-free duplex partner is enveloped into the presynaptic filament, forming the synaptic filament where the two partners are wound coaxially and where the actual strand switching takes place (for review, see West (1992)).

On the basis of previous results (West *et al.*, 1981a; Kahn *et al.*, 1981; Jwang and Radding, 1992), it is generally believed that the ability of RecA to bypass heterologies is associated with the presence of homologous sequences upstream and downstream of the insert. Therefore, heterology at the proximal (initiating) end of duplex is thought to constitute a complete block for the strand exchange. In addition, RecA has been reported to span heterologous inserts located on ssDNA with much greater efficiency than those on duplex DNA (Bianchi and Radding, 1983), the rationale being that in this situation the insert can fold out of the way.

We have undertaken a systematic study of the effect of heterologous sequences on RecA-promoted strand exchange, with circular ssDNA reacting with linear dsDNA. Inserts ranging from 8 to approximately 300 nucleotides or base pairs (bp) were placed in the ssDNA or at various distances from the ends in the dsDNA. In contrast with previous reports, we found that the ability of RecA to promote strand exchange through inserts of a given size decreased gradually as the inserts were moved from the proximal to the distal end of the duplex via several medial locations. Moreover, strand exchange proceeded with comparable efficiency when identical inserts were located on the single-stranded substrate. To accommodate these results, we propose that directional formation of the synaptic filament from the point of initial homologous contact to the proximal end is coupled with strand separation, immediately followed by strand exchange when the DNA partners are completely homologous. Traversal of stretches of heterology results from the ability of RecA to maintain limited portions of duplex DNA open for a short time without concomitant formation of a heteroduplex product.

EXPERIMENTAL PROCEDURES

Proteins—RecA protein was purified as described (West *et al.*, 1982b). It was at least 99% pure as determined by silver-stained and Coomassie Blue-stained SDS-polyacrylamide gels. Concentration was determined using an extinction coefficient of $\epsilon_{280} = 0.58\ A_{280}\ mg^{-1}$ ml (Craig and Roberts, 1981). RecA protein and SSB (U. S. Biochemical Corp.) assayed separately or in combination, were free of detectable endonuclease and exonuclease activities on dsDNA and ssDNA in the appropriate conformation (linear or circular). Restriction nucleases were from New England Biolabs or Boehringer Mannheim. *E. coli* DNA polymerase I large fragment (Klenow), T4 DNA polymerase, T4 ligase, T4 polynucleotide phosphorylase, and calf intestine alkaline phosphatase were from Boehringer Mannheim, Sequenase was from U.S. Biochemical Corp., and phosphocreatine kinase (type I) was from Sigma.

DNA—Viral and form I DNA from M13mp19 and its derivatives were prepared according to classical procedures (Sambrook *et al.*, 1989). Their identity and purity were verified by restriction mapping and dideoxy sequencing (Sanger *et al.*, 1977).

Construction of DNA Substrates—All phages were derivatives of M13mp19. DNA 1 contained a 308-bp sequence resulting from digestion of pBR322 with *Alu*I and cloned between the *Hin*dIII and *Hin*cII sites. DNA 2 carried a 707-bp segment of the thymidine kinase (*tk*) gene of HSV-1 (Wagner *et al.*, 1981) obtained by restriction of the pAL2/1 plasmid (Waldman and Liskay, 1987) and inserted between *Hin*dIII and *Sma*I. DNA 3 and 4 each contained a 546-bp thymidine kinase segment cloned, respectively, between *Hin*dIII and *Eco*RI and between *Hin*dIII and *Pvu*I. DNA 5 to DNA 7 carried pBR322 *Alu*I segments (135, 106, and 46 bp) inserted by shotgun cloning into the *Hin*cII site. After introduction of an *Nco*I linker into the *Pst*I site, DNA 7 was used as described by Henikoff (1984) to generate nested deletions (DNA 8 to DNA 13). DNA 14 to DNA 16 were deletions of M13mp19 prepared by cutting M13mp19 form I DNA with *Hin*dIII and another restriction enzyme (*Hin*cII for DNA 14, *Sma*I for DNA 15, and *Eco*RI for DNA 16). In all cases the ends were filled with Klenow and ligated with T4 DNA ligase.

The extent of heterology between the substrates used in this study was determined as described in the legend of Fig. 1A.

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[1] The abbreviations used are: ssDNA, single-stranded DNA; dsDNA, double-stranded (duplex) DNA; bp, base pair(s); SSB, ssDNA-binding protein.

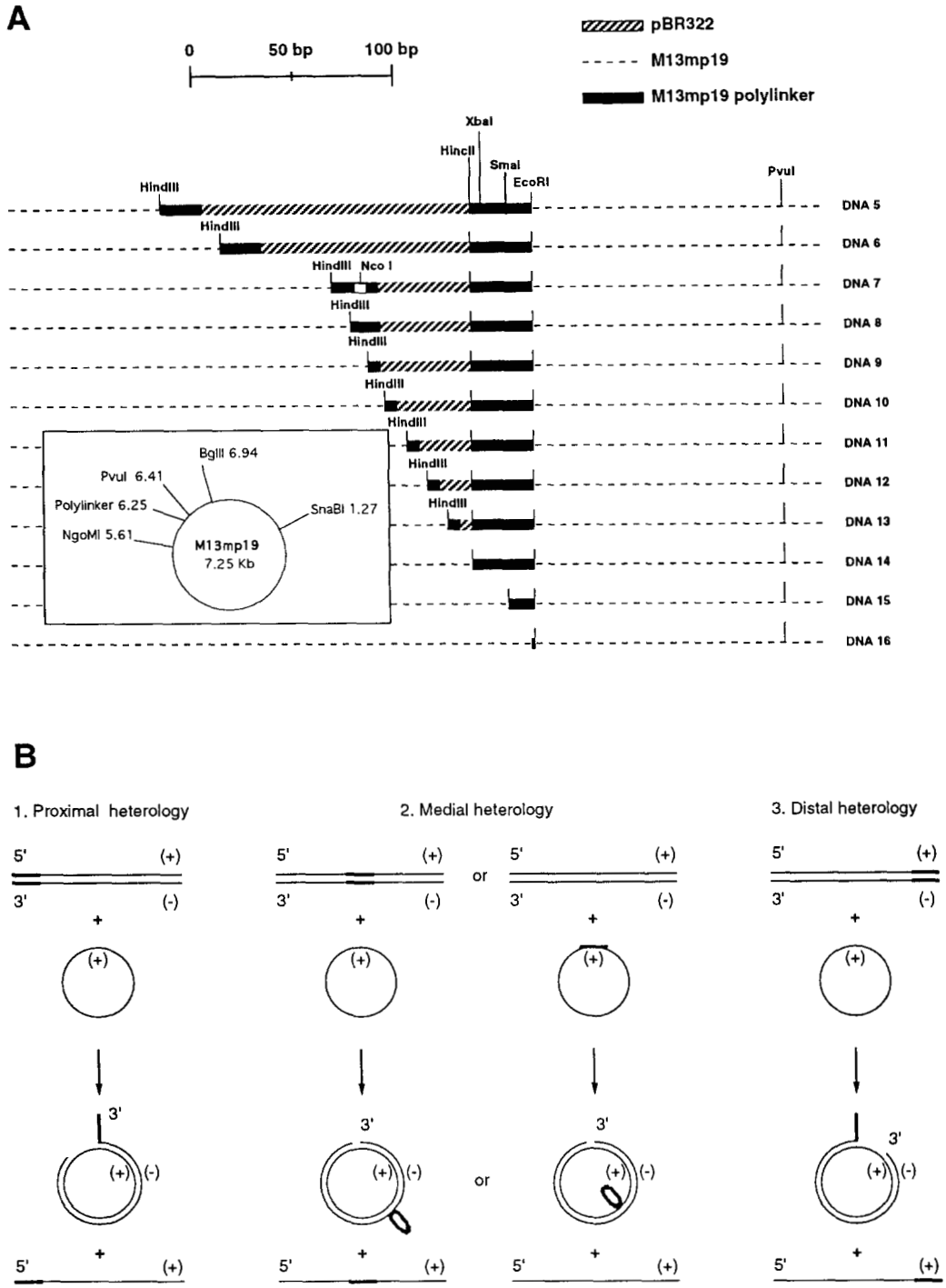


FIG. 1. *A*, Heterology between DNA substrates used in the experiments. In experiments with proximal heterologies (Fig. 2), DNA 1 to 4 (not shown, see "Experimental Procedures") were used as ssDNA. The length of heterology corresponds to the sequence carried by the dsDNA between *Hin*dIII and *Hin*cII when the single-stranded substrate was DNA 1 (ssDNA 1), between *Hin*dIII and *Sma*I with ssDNA 2, between *Hin*dIII and *Eco*RI with ssDNA 3, and between *Hin*dIII and *Pvu*I with ssDNA 4. In experiments with medial inserts, the length of heterology corresponds to the extra polylinker and/or pBR322 sequence carried either by the dsDNA (Fig. 3*A*) or the ssDNA (Fig. 3*B*). The M13mp19 map gives the position of the restriction sites used to place the inserts at various distances from the ends of the linear duplex DNA. Except for homologous controls, only DNA 1 was used as single-stranded substrate in experiments with distal inserts (Fig. 4). In this case, the length of heterology corresponds to the polylinker and pBR322 sequences carried by the dsDNA between the *Hin*dIII and *Xba*I sites. *B*, expected structure of the possible products of strand exchange between partially heterologous substrates. Heterologous inserts are represented by *thick lines*. *Kb*, kilobase pairs.

Labeling of Linear dsDNA Substrates—To obtain linear duplex molecules with heterologies at various distances from the ends, the form I DNA was linearized with *Hin*dIII for proximal heterologies, with *Xba*I for distal heterologies and with either *NgoM*I, *SnaB*I, *Bgl*II, or *Pvu*I for medial heterologies. After extraction with phenol-chloroform and ethanol precipitation, the duplexes were labeled at the 3′ end with Sequenase or Klenow or at the 5′ end with T4 polynucleotide kinase. Unincorporated nucleotides were eliminated by filtration through a Chroma-Spin column (Clontech).

Strand Exchange Reactions—Reaction mixtures contained, in 20 μl, 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 2 mM dithiothreitol, 2 mM ATP, 100 μg/ml bovine serum albumin (Life Technologies, Inc.), 6 mM creatine phosphate (Sigma), 10 units/ml creatine kinase, 7.5 μM each circular ssDNA and labeled linear dsDNA, 3 μM RecA, and 0.5 μM SSB. Incubation was at 37 °C for 60 min unless otherwise indicated. The reactions were stopped by addition of EDTA, pH 8, to a concentration of 25 mM

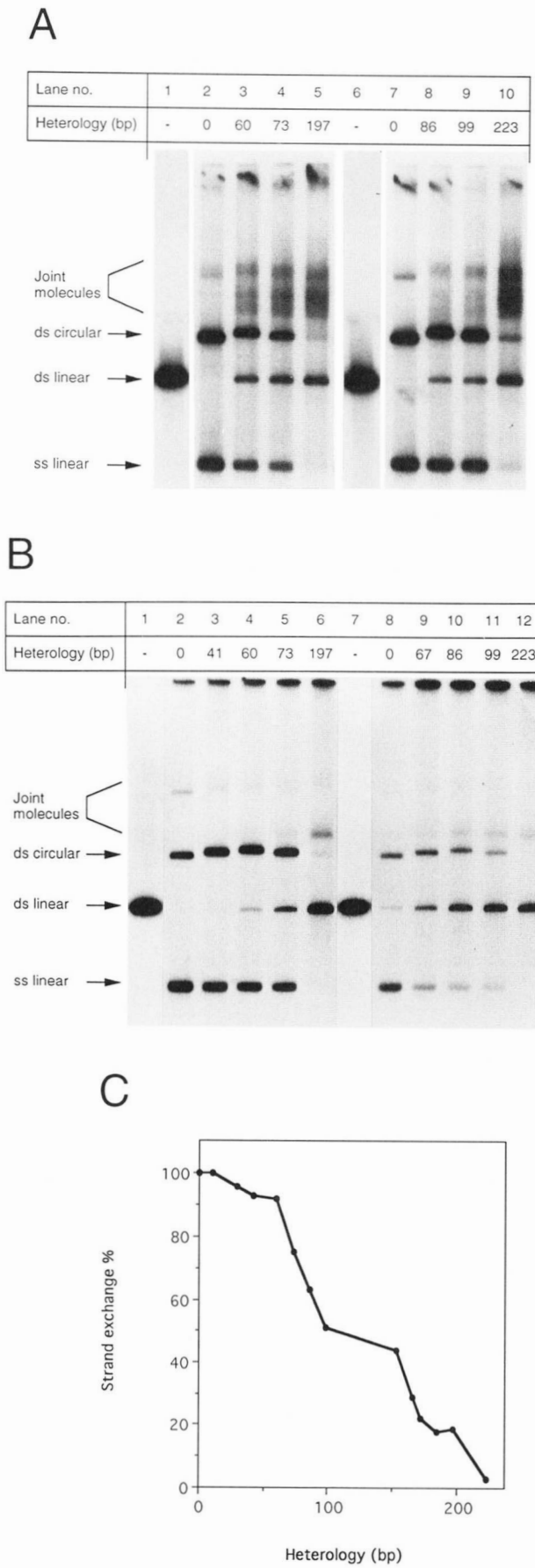


FIG. 2. **RecA-mediated strand exchange through proximal inserts.** The double-stranded linear DNA was labeled at the 3′ end (*A*) or at the 5′ end (*B*). The autoradiograms are representative of the data obtained with a number of combinations of substrates. In *A*, dsDNA 10 (*lanes 2–5*) and dsDNA 7 (*lanes 7–10*) were reacted with homologous ssDNA 10 and ssDNA 7 (*lanes 2* and *7*), ssDNA 2 (*lanes 3* and *8*), ssDNA 3 (*lanes 4* and *9*), and ssDNA 4 (*lanes 5* and *10*). In *B*, dsDNA 10 (*lanes 2–6*) and dsDNA 7 (*lanes 8–12*) were reacted with homologous ssDNA 10 and ssDNA 7 (*lanes 2* and *8*), ssDNA 1 (*lanes 3* and *9*), ssDNA 2 (*lanes 4* and *10*), ssDNA 3 (*lanes 5* and *11*), and ssDNA 4 (*lanes 6* and *12*). Input radioactivity was determined on a separate gel subjected to the same exposure and is shown in *lanes 1* and *6* for *A* and in *lanes 1* and *7* for *B*. In *C*, the yield of strand exchange as a function of insert length is expressed as the fraction of label in the displaced strand relative to that in the homologous control taken as 100%.

and SDS to a concentration of 0.5%.

Electrophoresis and Autoradiography—DNA samples were analyzed by electrophoresis through 0.8% agarose gels (Sea Kem GTG) in 40 mM Tris acetate buffer at 50 V/30 mA for 18 h. The DNA was visualized by ethidium bromide staining and, for labeled DNA, by direct exposure of dried gels to x-ray films (Fuji RX). Autoradiograms were analyzed by densitometry (Gel Reader, NCSA for MacIntosh).

RESULTS

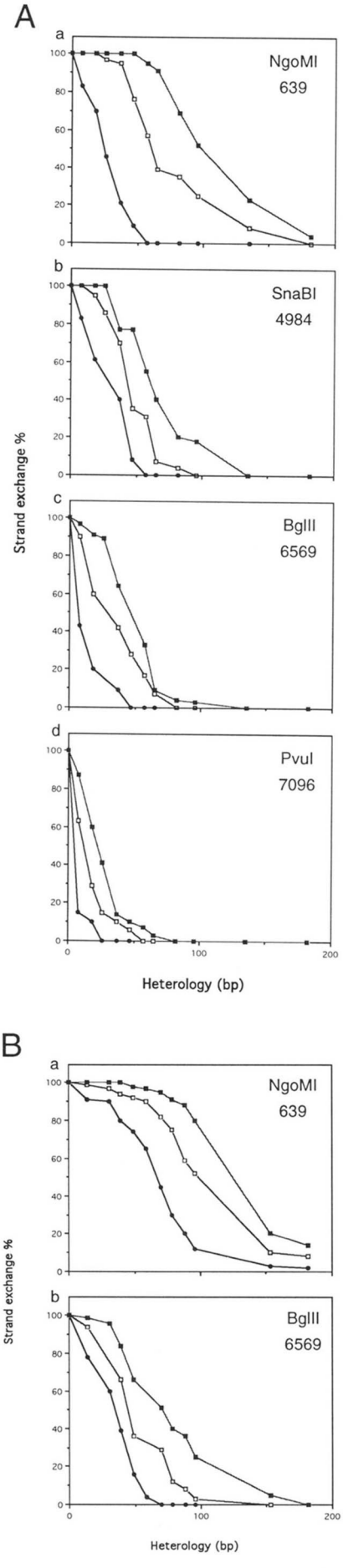
Influence of Proximal Heterology on Strand Exchange—To obtain increasing lengths of heterology (0–309 bp) at the proximal end of the linear duplex, the substrates designated dsDNA 13, 10, 7, and 5 linearized with *Hin*dIII were combined with homologous ssDNA or ssDNA 1 to 4. The representative results, obtained with dsDNA 10 and 7 labeled at the 3′ or at the 5′ end, are shown in Fig. 2, *A* and *B*. Strand exchange, measured in each case against the homologous control, was almost unaffected for proximal inserts ≤60 bp and then decreased gradually and was practically blocked for inserts of about 200 bp. The appearance of a band corresponding to the starting linear double-stranded substrate (form III), the intensity of which increased with the length of the insert, probably reflects the instability of deproteinized paranemic joints (Riddles and Lehman, 1985) formed when large proximal inserts block strand exchange.

Fig. 2*C* summarizes the data obtained with many combinations of substrates, reacted with RecA under standard conditions (37 °C for 60 min). Comparable results were obtained with reactions carried out for different lengths of time (20, 30, 40, 50, 90, and 120 min, not shown). In addition, some of the above experiments were performed with two different RecA preparations (see West *et al.* (1982b))[2] and with several single-stranded binding (SSB) protein preparations, with identical results.

Traversal of Medial Heterologous Inserts—To examine the effect of medial heterology on strand exchange, dsDNA 5 and 7 were linearized with *Ngo*MI, *Sna*BI, *Bgl*II, or *Pvu*I instead of *Hin*dIII, 3′ end-labeled, and reacted with various single-stranded substrates. Depending on the restriction enzyme, inserts of various sizes were placed at 639 (*Ngo*MI), 4984 (*Sna*BI), 6569 (*Bgl*II), or 7096 (*Pvu*I) bp from the proximal end of the M13mp19 vector (Fig. 1*A*). The results are shown in Fig. 3. The ability of RecA to traverse heterologies decreased with the length of the insert and the distance between the insert and the proximal end of the duplex. However, in all cases, the extent of strand exchange increased with the reaction time.

Remarkably similar results (Fig. 3*B*) were obtained when the insert was in the ssDNA, which is formally equivalent to a deletion in the duplex. With the combinations of substrates used, the deletion in the dsDNA was flanked by the same homologous regions upstream and downstream as the inserts in Fig. 3*A*, *a* and *c*. The extent of exchange was only slightly higher than with inserts in the dsDNA. Also, as observed for medial inserts in the dsDNA, the efficiency of strand exchange decreased with the length of heterology and the distance from

[2] S. C. West and E. Cassuto, unpublished results.



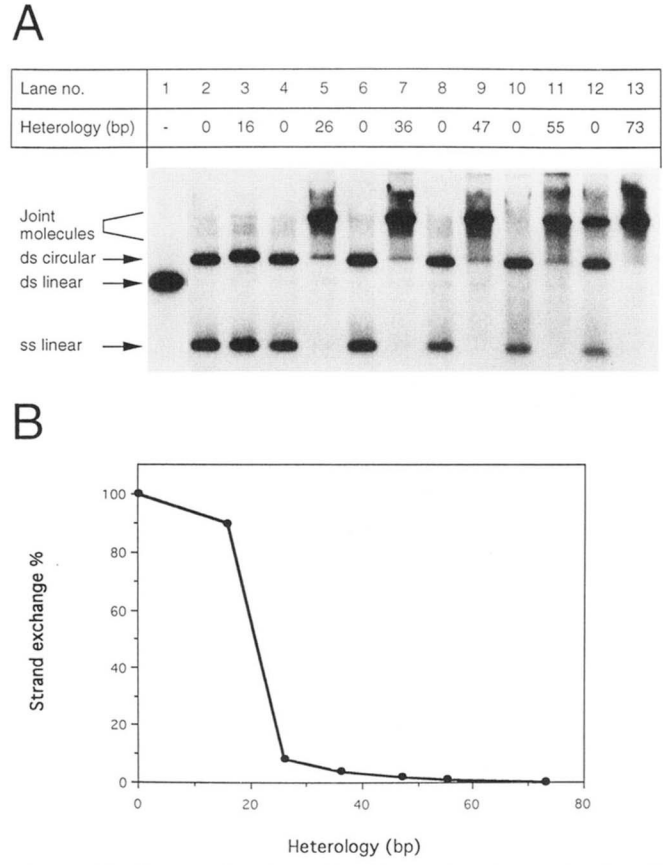


FIG. 4. **Blockage of RecA-mediated strand exchange by distal heterologous inserts.** In *A*, dsDNA 13, 12, 11, 10, 9, and 7 were reacted with homologous ssDNA (*lanes 2, 4, 6, 8, 10*, and *12*) or with ssDNA 1 (*lanes 3, 5, 7, 9, 11*, and *13*). Input radioactivity was determined for each dsDNA on a separate gel subjected to the same exposure. It was virtually identical for all of the duplex substrates and is shown in *lane 1*. In *B*, the yield of strand exchange is expressed as in Fig. 2*C*.

the deletion to the proximal end of the duplex and increased with the time of incubation.

Arrest of Strand Exchange by Distal Heterology—To obtain distal inserts ranging from 16 to 73 bp, dsDNA 7 and dsDNA 9 to 13 were linearized with *Xba*I, 3′ end-labeled, and incubated with either homologous ssDNA or ssDNA-1 (Fig. 4). Here, a distal insert of 16 bp was passed with close to 100% efficiency relative to the homologous control. With longer inserts, strand exchange was virtually blocked, and joint molecules accumulated. The results were not significantly affected by the time of incubation (not shown).

Traversal of Heterologous Regions Is Not Caused by Contamination of RecA and/or SSB—In order to ascertain the validity of our results, it was imperative, even with protein preparations routinely tested for nucleases (see "Experimental Procedures"), to exclude potential contaminating activities that might have been a serious hazard in experiments involving heterologous inserts. Any ssDNA exonuclease would prevent the recovery of the displaced (+) strand, which is clearly not the

Fig. 3. **RecA-mediated strand exchange through medial heterologous insertions.** Inserts were in the dsDNA (*A*) or in the ssDNA (*B*). The enzymes used to linearize the duplex substrate are indicated at the *top right*. The *numbers* refer to the distance from the heterology to the proximal end of the duplex. Incubation was for 30 min (*black circles*), 60 min (*white squares*), or 120 min (*black squares*). In *A*, dsDNA 7 was reacted with ssDNA 7 (homologous control) and with ssDNA 8 to 16, and dsDNA 5 was reacted with ssDNA 5 (homologous control) and with ssDNA 14 and 16. In *B*, dsDNA 16 was reacted with ssDNA 16 (homologous control) and with ssDNA 5 to 15. The yield of strand exchange is expressed as in Fig. 2*C*.

case in our experiments. This rules out strand exchange caused by contamination with exonuclease I, which requires complete degradation of the duplex (+) strand for the formation of the heteroduplex (Bedale *et al.*, 1993). Contamination by double-stranded exonucleases would eliminate the label from the 3′ or the 5′ end-labeled dsDNA substrates, whereas in Fig. 2, *A* and *B*, the overall radioactivity remained constant in all lanes. In addition, the fraction of input label recovered in the form II and linear ssDNA products was practically identical in reactions with inserts <60 bp, which have little effect on strand exchange (Fig. 2*A*, *lane 3* and Fig. 2*B*, *lanes 3* and *4*) and in the homologous controls (Fig. 2, *A* and *B*, *lane 2*). The effect of endonucleolytic cleavage on the (+) strand of the duplex downstream of the insert (Bedale *et al.*, 1991) would be expected to vary with the type of end labeling (3′ or 5′) of the duplex substrate. With 3′ end labeling, cleavage on the (+) strand would not affect the distribution of the radioactivity in the products, whereas with 5′ end labeling, the same cleavage would place all the input label in the form II heteroduplex and produce an unlabeled displaced strand. The data in Fig. 2*B* exclude this possibility because the radioactivity was always equally distributed between the strand exchange products. In addition, with double-stranded substrate labeled at the 3′ or 5′ end, we did not detect any cleavage product in denaturing gels (data not shown). Finally, contamination by a helicase is unlikely because such an activity would not discriminate between the ends of the duplex and therefore would lead to successful strand exchange for distal inserts as well as for proximal ones.

DISCUSSION

In early *in vitro* work on RecA, proximal heterology was found to completely block strand exchange (West *et al.*, 1981a; Kahn *et al.*, 1981). The duplex substrates carried large proximal inserts (≥150 bp), and the methods of detection would not have revealed low levels of exchange. The difference between these and the present results is therefore more apparent than real. There are, however, discrepancies between this work and a recent report (Jwang and Radding, 1992) in which the authors used a unique 110-bp insert located in the middle or at either end of the duplex and concluded that traversal of a heterologous region required homology upstream and downstream of the insertion. In addition, our data are at odds with an earlier report (Bianchi and Radding, 1983) in which single-stranded inserts ranging from 33 to 1300 nucleotides all had the same moderate effect on strand exchange. There is no obvious explanation for the reported differences. Our results, obtained with numerous inserts of different size and location (more than 90 combinations) and with several incubation times, appear to be internally consistent and to be due to genuine properties of RecA. Furthermore, there is a remarkable analogy between our data with medial inserts flanked by long homologous regions (Fig. 3*A*, *b*) and those of Hahn *et al.* (1988) with duplex DNA carrying similarly located inserts reacting with gapped DNA. The authors found that in a four-strand reaction, RecA could drive strand exchange through a 38-bp insert but not through 120 bp. Because four-strand and three-strand reactions are known to proceed in the same way in terms of speed and polarity (West *et al.*, 1981b, 1982a), this analogy suggests that the traversal of heterologies may occur by the same mechanism in both types of reactions.

To explain the ability of RecA to traverse proximal insertions and the polar effect of the location of medial inserts, we propose to introduce two modifications in the model of Howard-Flanders *et al.* (1984).

In the original model, the whole duplex DNA was incorporated into the synaptic complex, in accordance with EM pictures of reactions lacking SSB (Stasiak *et al.*, 1984). We propose that upon initial homologous contact between the presynaptic filament and the protein-free duplex, only the proximal portion of the duplex (with respect to the point of initial contact) is

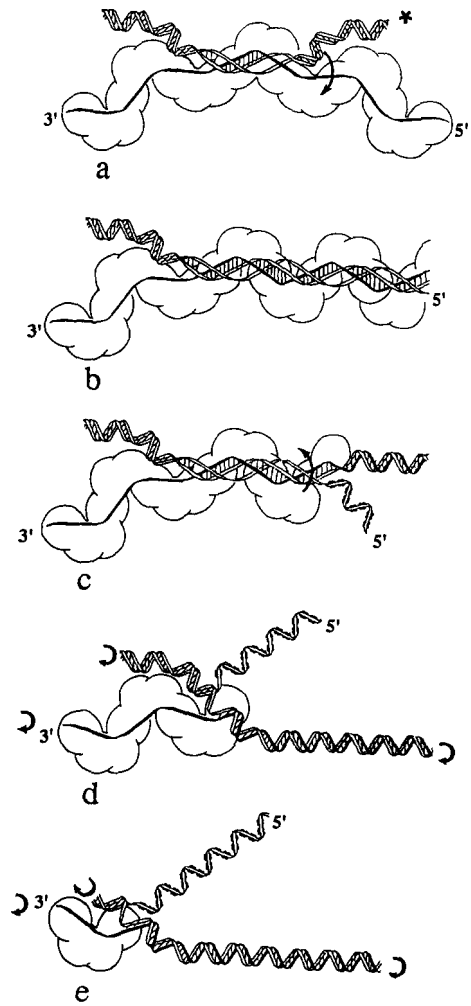


FIG. 5. **Strand exchange between homologous double-stranded and single-stranded substrates.** The strands of the duplex substrate are represented in *white*, the ssDNA in the nucleoprotein complex in *black*. RecA protomers in the presynaptic and synaptic complexes are drawn as *contours*. Coaxial alignment of DNA molecules, their extension, and partial unwinding within RecA-DNA complexes are as proposed by Howard-Flanders *et al.* (1984). SSB is represented in tetrameric form (Williams *et al.*, 1984). The *asterisk* indicates the proximal end of the linear duplex. *a*, initiation of strand exchange. Initial pairing of the interacting DNA molecules can occur at any homologous site. Shortly after homologous recognition, strand exchange commences at the site of initial contact and progresses toward the proximal end of the duplex. Note that within the synaptic complex, base pairing connects the strands that will form the new duplex molecule. The *arrow* indicates the direction of envelopment of the duplex into the synaptic complex. *b*, strand exchange in the proximal region of the linear duplex. The dsDNA is progressively enveloped into the synaptic complex in the 3′ to 5′ direction relative to the resident single strand until the proximal end of the duplex is reached. *c*, spatial separation of the displaced strand. Starting from its 5′ end, the displaced strand unravels from coaxial arrangement with the newly formed heteroduplex and is bound by SSB while RecA dissociates from the proximal portion of the duplex DNA. The direction of unraveling is indicated by the *arrow*. *d*, strand exchange without coaxial alignment of homologous regions. In the distal portion of the duplex, strand exchange and strand displacement are coupled and proceed toward the 3′ end of the displaced strand, which is bound by SSB. RecA helps to anneal the strands of the nascent heteroduplex. Here and in *e*, the *arrows* give the direction of axial rotation of the DNA. *e*, termination of strand exchange. When the distal end of the duplex is reached, the product molecules can separate. The displaced strand is coated with SSB as RecA dissociates from the recombinant duplex.

incorporated into the synaptic complex, as suggested by EM observations of reactions carried out with RecA and SSB (Stasiak and Egelman, 1988). Consequently, strand exchange in this portion of the double-stranded DNA occurs within the synaptic complex where the ATPase activity of RecA can fully aid the ongoing reaction. In contrast, strand exchange in the distal portion proceeds by a different mechanism where SSB (rather than the ATPase activity of RecA) drives branch migration by binding to the displaced strand (Fig. 5, *d–e*).

The second modification concerns the state of the three strands within the synaptic complex. The model of Howard-Flanders *et al.* (1984) considered the possibility that a long triple-stranded structure was formed in the synaptic complex and later converted into products in a reaction starting from the proximal end of the duplex and progressing in the 5′ to 3′ direction with respect to the ssDNA. We propose that as soon as homologous recognition is established, strand separation is initiated internally and proceeds in the 3′ to 5′ direction with respect to the resident ssDNA. With homologous DNA molecules, the opening of every base pair in the duplex substrate leads to immediate formation of a new base pair in the duplex product (Fig. 5, *a* and *b*). This hypothesis, strongly supported by recent work from Adzuma (1992), which demonstrated a postexchange arrangement of the strands within synaptic complexes, does not appear to favor the formation of stable, long triplex structures as intermediates in strand exchange (Hsieh *et al.*, 1990; Umlauf *et al.*, 1990; Rao *et al.*, 1991).

When strand switching reaches the proximal end of the duplex, the strand to be displaced can unravel without topological difficulties from coaxial arrangement with the newly formed heteroduplex (Fig. 5, *c*). Then, upon encountering the point of initial contact, strand switch and strand displacement become coupled and proceed 5′ to 3′ in relation to the single strand (Fig. 5, *d* and *e*).

For proximal heterology, the strands in the insert would only have to be kept separated for a short time to allow unraveling of the displaced strand. For medial heterology, completion of strand exchange requires, in addition to strand separation, lateral slippage of the exchanging strand within the RecA-DNA complex in order to reestablish the alignment of the proximal homologous regions. Such a mechanism is expected to operate in a similar way whether the insert is in the duplex or in the ssDNA, as confirmed by the results in Fig. 3.

The point of initial recognition seems to be randomly located in joint molecules (Stasiak and Egelman, 1988). The closer an insert is to the proximal end of the duplex, the greater its chances of being taken into the synaptic filament. The extreme case is the distal insertion, which should never be incorporated into the complex and should therefore block strand exchange. The results in Fig. 4, where strand exchange is arrested by a 22-bp insert, are in good agreement with this prediction. The efficient bypass of the 16-bp distal insert could be caused by a helicase (Bianchi *et al.*, 1985) or a helix destabilizing activity of RecA and/or SSB on very short duplexes.

Even in the most favorable case, the ability of RecA to open heterologous insertions is limited to 150–200 bp (Fig. 2*C*). Keeping complementary strands apart requires a high stability of the RecA-DNA complex. However, after each cycle of ATP hydrolysis, RecA protomers lose their affinity for ssDNA (Kowalczykowski, 1987). In regions where several adjacent protomers are affected, separated strands can snap back, limiting the size of inserts that could be processed by RecA.

In summary, regardless of the location of the insert in the single-stranded or double-stranded substrate, we found that far from being required, homology upstream of a heterologous insert decreases the efficiency of strand exchange, mainly because initial homologous contacts in this region will trap RecA and the DNA substrates into nonproductive complexes. The adverse effect on strand exchange is, as expected, proportional to the length of proximal homology. Downstream of an insert, homology is required to establish productive initial contacts, which may be its major (and possibly only) role.

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