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Torsional stress generated by RecA protein during DNA strand exchange separates strands of a heterologous insert

(nucleoprotein filament/genetic recombination/joint molecules/distal joints/triplex DNA)

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ABSTRACT **Previous studies have shown that the helical RecA nucleoprotein filament formed on a circular single strand of DNA causes the progressive, directional transfer of a complementary strand from naked linear duplex DNA to the nucleoprotein filament, even when the duplex contains a sizable heterologous insertion. Since RecA protein lacks demonstrable helicase activity, the mechanism by which it pushes strand exchange through long heterologous inserts has been a quandary. In the present study, a linear duplex substrate with an insertion of 110 base pairs in its middle yielded the expected products, whereas much less of the heteroduplex product was seen when the insertion was located at either end of the duplex substrate or 160 base pairs from the far end of the duplex substrate. In an ongoing reaction of the substrate with an insertion in its middle, P1 nuclease cleaved intermediates from the point of the insertion to various distal sites. Acting on a duplex substrate that contained a single nick located in the complementary strand just beyond the insertion, RecA protein formed joint molecules but failed to complete strand exchange. These data show that negative torsional stress is generated by distant homologous interactions that occur beyond the heterologous insertion and that such stress is essential for unwinding a heterologous insertion that otherwise halts strand exchange.**

RecA protein purified from *Escherichia coli* displays two major functions that are essential for genetic recombination, namely, homologous pairing and strand exchange. A model system consisting of circular single strands and linear duplex DNA provides an efficient reaction for exploring recombination promoted by RecA *in vitro*. RecA initially polymerizes on the single-stranded DNA to form a right-handed helical nucleoprotein filament. Following a search for homology, a complementary strand from the duplex DNA is transferred in the 3′-to-5′ direction into the nucleoprotein filament, which ultimately creates a nicked circular heteroduplex DNA molecule and a displaced linear single strand (1–3).

This strand exchange not only occurs between completely homologous sequences but also traverses pyrimidine dimers (4), mismatches (5), and heterologous insertions as long as 50–100 base pairs (bp) (6, 7), all with surprising efficiency. RecA can drive strand exchange through insertions of extra sequence located either in the single strand or in the duplex DNA. In the latter case, the yield of product strongly depends on the length of the insertion, presumably due to the increasing difficulty of unwinding long heterologous insertions.

Since RecA lacks any straightforward helicase activity, its ability to push strand exchange through long insertions has presented a long-standing quandary. Howard-Flanders *et al.* (8) proposed a model of strand exchange that seemed at odds with this ability of RecA, since the model invoked long homologous triplex regions as a concerted mechanism for taking apart the original duplex DNA and transferring hydrogen bonds to new heteroduplex pairs. Cox and coworkers (3, 9) proposed a mechanism in which the rotation of the nucleoprotein filament and duplex DNA about one another would unwind the duplex without regard to homology and thus permit heterologous insertions to be unwound in the course of homologous exchanges. Honigberg and Radding (10) showed that strand exchange is accompanied by rotation of the RecA nucleoprotein filament and duplex DNA about their long axes. These axial rotations are in principle capable of melding single-stranded and duplex DNA into a triplex intermediate, and of transferring a strand in a spool-like fashion from the parental duplex to a new heteroduplex molecule (2, 11). The experiments described here provide a synthesis of the foregoing proposals by showing that distant interactions in homologous DNA beyond a heterologous insert generate negative torsional stress that unwinds the insert.

METHODS

Enzymes. RecA protein (12) and single-stranded DNA-binding protein (SSB) (13) were purified as described. Restriction endonucleases were purchased from New England Biolabs or International Biotechnologies, T4 polynucleotide kinase and calf intestinal alkaline phosphatase were from Boehringer Mannheim. Creatine phosphokinase (type I) was obtained from Sigma, proteinase K from EM Laboratories (Elmsford, NY), and P1 nuclease from United States Biochemical.

DNAs. Unlabeled circular single-stranded DNA and duplex DNA of M13mp8 and its derivatives were prepared as described (14–16). Phage M13mp8-110 is a derivative of M13mp8 (17) into which a 110-bp segment of pBR322 DNA was cloned at the *Hin*cII site. The size and site of the insertion were checked by dideoxy sequencing (18). The insertion was variously located in the linear duplex DNA: at the proximal end by cleaving with *Bam*HI; in the middle by cleaving with *Sna*BI or other restriction enzymes as indicated in legends; and at the distal end by cleaving with *Pst* I. The duplex substrates shown in Fig. 3 were uniquely labeled with ^{32}P at the 5′ end of the complementary strand by an additional restriction cleavage of DNA labeled at both ends: by *Bsp*HI in the case of the medial insertion, or by *Bgl* I for the subterminal insertion substrate. The desired DNA was subsequently separated from the small fragment (30 bp) by Sepharose 2B gel filtration. Concentrations of DNA are reported in moles of nucleotide residues.

DNA with a Defined Nick in the Minus Strand Distal to the Insertion. Nicked circular duplex DNA (form II) was prepared by annealing circular single-stranded M13mp8-110 [(+)-strand] with denatured linear duplex DNA that had been prepared by cutting circular duplex DNA with *Pst* I at a site

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Abbreviation: SSB, single-stranded DNA-binding protein.

distal to the insertion sequence. Annealing was performed by successive dialyses against formamide (19). The form II DNA containing a nick in the (−)-strand was then separated from other DNA species by sucrose gradient sedimentation. The purified form II DNA had <5% form III DNA contaminants, and ≈70% of the form II molecules were ligatable (data not shown). The linear nicked duplex, which was derived by digestion of the form II DNA with *Sna*BI, was used as substrate in the experiment shown in Fig. 4.

Strand Exchange. The presynaptic complexes were formed by preincubating 4 μM circular single-stranded DNA and 6 μM linear duplex DNA with 4 μM RecA in a reaction mixture containing 31 mM Tris·HCl (pH 7.5), 12 mM $MgCl_2$, 6 mM phosphocreatine, 10 units of creatine phosphokinase per ml, 1.5 mM dithiothreitol, and 4% (vol/vol) glycerol at 37°C for 2 min. The reaction was initiated by the addition of 1.5 mM ATP and 0.4 μM SSB. After 90 min, 45 μl of reaction mixture was withdrawn and the reaction was terminated by addition of EDTA (20 mM), SDS (0.1%), and proteinase K (150 μg/ml), followed by incubation for 20 min.

Gel Electrophoresis and Hybridization. Electrophoresis was carried out in 0.8% agarose gels in 40 mM Tris acetate, pH 8.0/2 mM EDTA at 5 V/cm for 5 hr. The gels were dried on Whatman 3MM paper and soaked in distilled water to remove the paper backing. For detection of heteroduplex DNA containing an unpaired loop (Fig. 1), the dried gel, without prior transfer to filter paper, was hybridized for at least 4 hr with either a (+)-probe or a (−)-probe (20). The gel with the hybridized signal was examined by autoradiography. To examine the same DNA species in denatured form, the hybridization was repeated after the gel was soaked in alkali (21). Quantitative estimations of form II molecules or displaced strand were carried out by densitometry of autoradiograms (Visage 2000 gel scanner, Millipore).

Digestion by P1 Nuclease. Aliquots of 40 μl of the strand-exchange reaction mixture that were withdrawn at various times were mixed with 3.5 μl of 40 mM Pipes buffer (pH 6.1) to reach a final pH of 6.5. P1 nuclease (0.002 unit/pmol of single-stranded DNA) was added to each aliquot and incubated at 37°C for 5 min; the digestion was terminated by addition of EDTA (20 mM), SDS (0.1%), and proteinase K (150 μg/ml), followed by incubation for 20 min.

RESULTS

Assay for Separation of Strands of a Heterologous Insert During Strand Exchange. The chemical directionality of strand exchange, 5′ to 3′, in relation to the single strand that is within the RecA nucleoprotein filament defines unique proximal and distal ends of the duplex DNA substrate in the model system that is described here. Since the circular single strand, which comes from the small DNA phages, is always the viral (+) strand, exchange can proceed only from one unique end of the DNA to the other (see Introduction). Accordingly, one can describe the location of modifications of the linear DNA substrates, or events occurring during pairing and strand exchange, in relation to the proximal and distal ends.

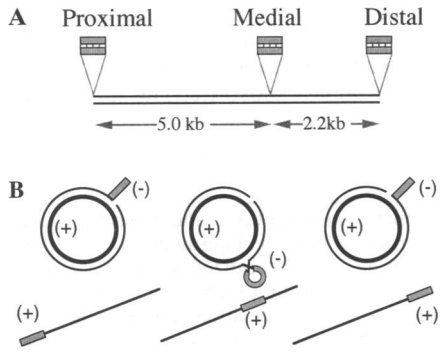

FIG. 1. Scheme for detection of traversal of heterologous inserts. (*A*) Location of 110-bp insertions. (*B*) Possible products from the respective inserts. kb, Kilobases.

We introduced a 110-bp restriction fragment into the M13mp8 vector, which, according to previous observations, should reduce strand exchange relative to that seen with completely homologous substrates but still yield about 25% of final products (6). The insertion was located at the proximal end, in the middle, or at the distal end of a linear duplex DNA substrate.

Reactions were analyzed by gel electrophoresis. To detect the unwinding of a heterologous insert that accompanies strand exchange through the insert, we hybridized a ^{32}P-labeled probe to gels that had either been soaked in alkali or not treated at all. In the absence of alkaline treatment, hybridization of a probe to nondenatured DNA in the gel can detect a single-stranded loop of heterologous sequence, such as that produced when RecA drives strand exchange through a heterologous insert (6, 20). The two probes used in this assay were oligonucleotides, each containing 24 residues corresponding to a sequence located in the middle of the insertion. The (+)-probe, derived from the viral (+)-strand, could be used to score the nicked circular duplex bearing the unpaired insertion sequence in the (−)-strand, while the (−)-probe allowed the detection of the other final product, a full-length displaced single strand containing the insertion sequence (Fig. 1).

Traversal of a Heterologous Insertion Requires Extensive Homology Distal to the Insertion. As expected, completely homologous substrates containing the insertion in both single-stranded and double-stranded DNA did not give any detectable signal upon hybridization of nondenatured DNA with the (+)-probe (Fig. 2*A*, lanes 3 and 4). Consistent with previous observations (6), the reaction involving a medial insertion yielded the final product, nicked circular duplex DNA (form II DNA), in which the insertion had been rendered single-stranded (Fig. 2*A*, lane 8). According to a densitometric scan of the autoradiogram, when the substrate had a medial insertion, about 30% of recovered counts in the gel appeared in form II product, whereas the distal insertion yielded only 10% (Fig. 2*B*, lanes 8 and 10), and the proximal insertion yielded only a trace of form II (lane 6).

When the (−)-probe was used to monitor the production of a displaced strand, at least 39% of recovered label appeared in the displaced strand (Fig. 2*C*, lane 8), whereas only 3–4% appeared when the insert was at either the proximal or the distal end.

Since longer heterologous inserts are decreasingly traversed by strand exchange (6), we interpreted the need for DNA beyond a heterologous insert to unwind it as a need for homologous DNA, and we further explored that requirement. Three different lengths of downstream homologous sequence were tested, 6000, 2200, and 160 bp, as permitted by available single restriction sites. The longest distal homology (6000 bp) led to a similarly productive reaction as that with 2200 bp of terminal homology (data not shown). By contrast, a substrate with only 160 bp of distal homology yielded undetectable amounts of form II product (Fig. 3, compare lanes 4 and 11).

Heterogeneous Cleavage of Duplex DNA by P1 Nuclease During Strand Exchange. The requirement for an extensive distal region of homologous duplex DNA to push strand exchange through a heterologous sequence led us to test the proposal that the distal interaction creates negative torsional stress that unwinds the heterologous insertion. P1 endonuclease was used to look for single-strandedness that might be produced in duplex DNA by a downstream homologous interaction. P1 nuclease, which acts preferentially on single-

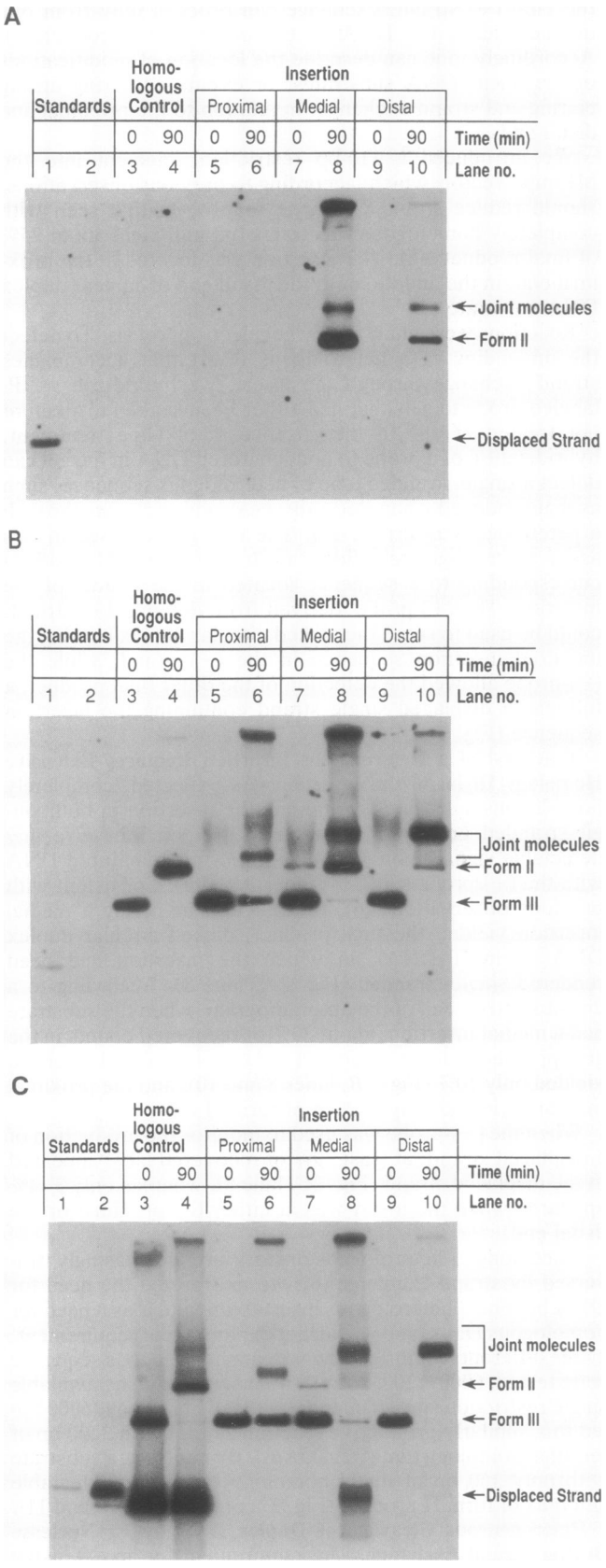

FIG. 2. Analysis of DNA in gels by hybridization *in situ*. (*A*) Hybridization with the (+)-probe, without prior denaturation of DNA in the gel. Lane 1, denatured M13mp8-110 duplex; lane 2, M13mp8-110 circular single-stranded DNA; lanes 3 and 4, homologous control reaction; lanes 5–10, reactions containing M13mp8 circular single-stranded DNA and M13mp8-110 linear duplex DNA with an insert located as indicated. (*B*) Same gel as *A* but subjected to alkaline denaturing conditions and hybridized again with the (+)-probe to visualize both the intermediates and the products. (*C*) A denatured gel as in *B*, hybridized with the (−)-probe to detect (+)-strand displaced from duplex DNA containing the insert (see Fig. 1). The band migrating in the position of the displaced strand in lanes 3 and 4 is attributable at least in part to excess M13mp8-110 circular single-stranded DNA used in the reaction of completely homologous substrates. In lane 7 of both *B* and *C*, the form II band seen at zero time resulted from incomplete restriction digestion during the preparation of the form III substrate.

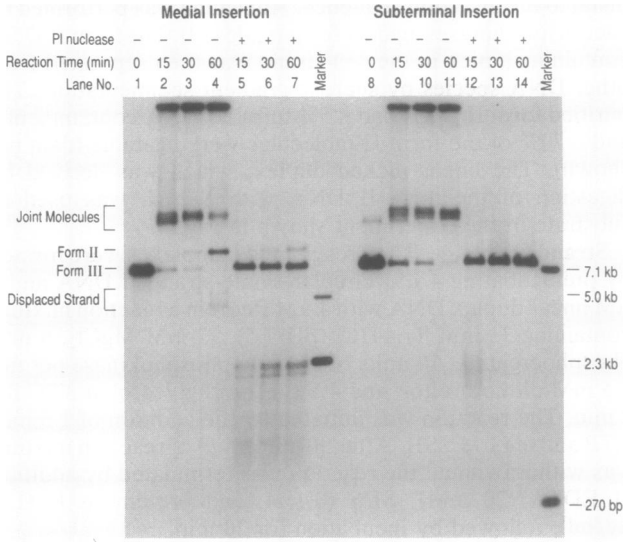

FIG. 3. Strand-exchange intermediates of medial insertion reaction are sensitive to P1 nuclease. The linear duplex DNAs in both reactions were uniquely labeled at the 5′ end of the complementary strand. The medial insertion was located 2200 bp from the distal end; the subterminal insertion was 160 bp from the distal end. The markers were made by an additional restriction cut (*Bam*HI) of the linear duplex substrate at the proximal junction of the insertion. The gel was dried on DEAE paper to prevent loss of DNA fragments shorter than 270 bp.

stranded DNA (22), also cleaves negatively supercoiled DNA because of the tendency of the latter to generate single-stranded regions and has been used successfully to detect the unwinding or opening of duplex DNA during replication and repair *in vitro* (23, 24).

Concentrations of P1 nuclease were chosen that degrade both naked single-stranded DNA and RecA/SSB nucleoprotein filaments but leave duplex DNA intact. Aliquots containing strand-exchange intermediates were withdrawn from a reaction mixture at intervals and subjected to a short incubation with P1 nuclease at pH 6.5. The single-stranded DNA-dependent ATPase activity of RecA had been shown to be unaffected in the pH range from 6 to 9 (25, 26), indicating that the binding of RecA to single-stranded DNA might not be seriously affected by the slightly acidic pH that was selected here for efficient P1 digestion. The linear DNA substrates were uniquely labeled at the distal 5′ end of the complementary strand. The strand-exchange intermediates bearing the medial insertion were sensitive to P1 nuclease (Fig. 4, lanes 5–7). They were converted into distinctive bands ≈2.3 kb in length, which mapped certain frequent sites of P1 sensitivity to the proximal junction of the insertion sequence. However, in addition to the discrete bands, there was a smear of material of lower molecular weights, which would correspond to cuts made by P1 nuclease between the proximal end of the heterologous insertion and the labeled distal end of the duplex substrate. The smear appeared at 15 min of a strand-exchange reaction, when little or no nicked circular duplex molecules had yet formed, indicating that the

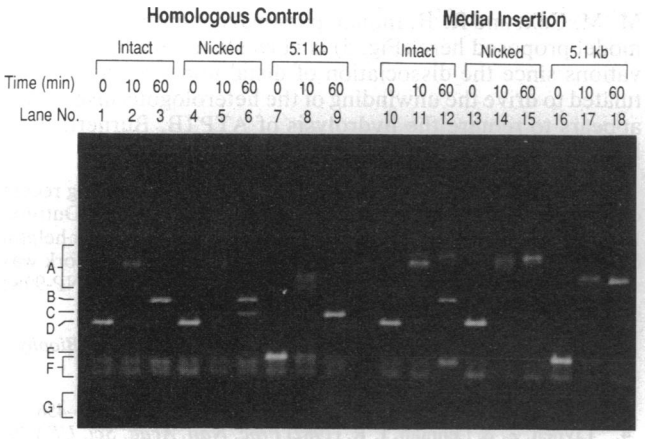

FIG. 4. A nick at the distal junction of a medial insertion inhibits the traversal of heterology. The linear duplex used in each reaction was either 7.3-kb full-length DNA without any nick (intact), full-length DNA containing a specific nick (nicked), or a 5.1-kb fragment generated by *Pst* I cleavage at the site of the nick (5.1 kb). Strand-exchange reactions were performed under standard conditions with 10 μM duplex DNA for full-length substrate or 7 μM for 5.1-kb truncated substrate, 10 μM single-stranded DNA, 3.3 μM RecA, and 0.83 μM SSB. Lanes 1, 4, 7, 10, 13, and 16 give the positions of starting substrates, single-stranded DNA (band F) and duplex DNA (band D or E). Reactions at 10 min showed bands presumed to contain reaction intermediates (band A). Heteroduplex DNA products migrated at distinctive positions (band B or C), whereas the displaced strand in the full-length reaction was inseparable from single stranded DNA (band F), or migrated faster (band G) in the case of the 5.1-kb reaction. Single-stranded DNA moved as a doublet as sometimes seen (27, 28).

finished product, nicked circular duplex DNA, was not the source of P1 sensitivity. Moreover, the extent of the smear indicated that P1-sensitive sites were located far beyond the heterologous insert. By contrast, the subterminal insertion reaction containing limited downstream homology 160 bp long did not display comparable sensitivity to P1 nuclease at or beyond the insertion, which would otherwise give a corresponding smear beginning from 270 bp, the length of duplex DNA from the proximal junction of the subterminal insertion to the distal terminus (lanes 12–14).

In all cases, P1 digestion also produced a major band of linear duplex DNA (form III molecules), which presumably resulted from trimming the single-stranded portions of joint molecules, including the displaced strand and the part of the circular nucleoprotein filament that had not yet been converted into heteroduplex DNA.

A Nick Distal to the Medial Insertion Blocks the Traversal of a Heterologous Insertion. The P1 sensitivity of duplex DNA distal to a medial insertion suggested a build-up of negative torsional stress coupled with the process of strand exchange. To examine this more closely, we introduced a specific nick in the complementary strand of form III DNA at the distal boundary of the medial insertion. Seventy percent of the nicked substrate was ligatable, indicating the integrity of the substrate. This nick can act as a swivel to release any of the postulated negative torsional stress and thus prevent such stress from reaching the heterologous insert. When the substrates were completely homologous, a nick at the same place in the duplex DNA led to a high yield of the normal product of the reaction, which is nicked circular (form II) DNA (Fig. 4, lanes 4–6). As predicted, however, in the reaction of substrates in which a medial insertion was present a nick caused the accumulation of joint-molecule intermediates and blocked the production of any form II molecules (lanes 13–15). These observations support the notion that the transmission of negative torsional stress is required for unwinding a heterologous insertion located upstream.

Intact form III DNA (Fig. 4, lanes 1–3 and 10–12) and a truncated linear duplex containing a 5.1-kb fragment extending from the proximal end to the site of the nick (lanes 7–9 and 16–18) were used for comparison in the same experiment in which we examined the influence of the nick on the traversal of a medial heterologous insertion. The heterologous insertion was at the distal terminus of the 5.1-kb fragment. The failure of this substrate to be converted into a final product (compare lanes 9 and 18) further confirms the need for distal homology to traverse a heterologous insertion.

DISCUSSION

Strand exchange through a 110-bp heterologous insertion produced a nicked circular duplex product containing an unpaired loop of the heterologous sequence that was detected by hybridization of native product with a probe directed to the loop (see ref. 6). The accompanying generation of a full-length displaced strand, monitored by the use of the (−)-probe, confirmed that the two strands within the insertion sequence were unwound as a result of strand exchange and recovered intact (see Fig. 1).

Recently, Bedale *et al.* (29) described an alternative mechanism for dealing with a heterologous barrier. They observed that when a heterologous sequence was placed at the proximal end of duplex DNA, a strand break occurred very close to the insertion junction in the displaced strand and led to the completion of strand exchange. In that case, strand exchange went around the heterologous insert rather than through it. In our experiments on a proximal insertion of similar size, we did not observe comparable formation of a product that migrated like form II DNA in 0.8% agarose gels (Fig. 2*B*, lane 6). In other experiments involving end-labeled duplex DNA with a medial insert, we precisely followed the conditions of Bedale *et al.*, but we did not detect in a denaturing gel the specific truncated displaced strand that should have been produced by the same cleavage phenomenon, nor evidence of any other cleavage products (data not shown). More recent findings from that laboratory indicate that cleavage was due to an extrinsic nuclease activity (M. M. Cox, personal communication). Although such a cleavage could be an important feature of a recombination pathway *in vivo*, our experiments show that it is not an obligatory path since RecA *in vitro* possesses the ability to separate the strands of a heterologous insertion and thus to push strand exchange through the insertion.

Our experiments show further that the ability of RecA to separate the strands of a heterologous insertion requires homology on both sides of the insert. The role of downstream homology is particularly interesting. It appears that the segment of downstream homology must be long. A distal segment that was half again as long as the insert did not suffice, whereas several kilobase pairs of distal homology did suffice. This long segment of distal homology was associated with persistent sensitivity to P1 nuclease that extended from the proximal junction of the heterologous insert to within perhaps 500 bp of the distal end of the molecule. Since the sensitivity was detected by gel electrophoresis, without denaturing treatment at any time, both strands of the duplex intermediate must have suffered nearby cuts. These experiments were stimulated in part by the precedents set by observations on transcription (30, 31) and DNA excision repair (24), which revealed the partition of DNA into negatively and positively superhelical domains. In the present case, underwinding generated by homologous interactions beyond a heterologous insert played an essential role in traversing a heterologous insertion, as inferred from the

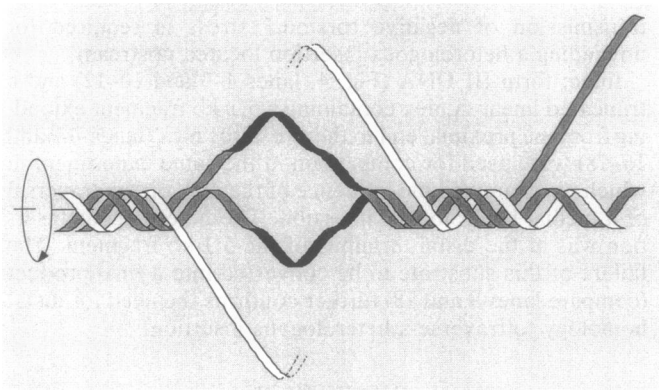

FIG. 5. Model for the unwinding of a heterologous insertion during strand exchange. The representation of RecA protein has been omitted, but the white ribbon represents the single strand originally incorporated in the right-handed helical nucleoprotein filament. The proximal joint, shown on the left, which had been moving from left to right, is stalled at the heterologous insertion. The distal joint, shown on the right, may be formed by the same or another nucleoprotein filament. The heterologous insertion is denoted by the black portion of the ribbon.

dramatic effect of a single nick at the border of the insert and the downstream homology.

Our observations support the suggestion of Cox and colleagues (3, 9) that the unwinding of a heterologous insertion is effected in a retrograde fashion by interactions that occur beyond the insertion. Since the distal interaction is a homologous one, we conclude that the other partner is the RecA nucleoprotein filament, which, however, need not be the same filament that is engaged upstream of the heterologous insertion (Fig. 5). Cox and coworkers (3, 9) suggested that the downstream interaction involves a side-by-side rotation of the nucleoprotein filament and duplex DNA. None of our data exclude that model, but some other observations from our laboratory suggest an alternative explanation. Distal triplex joints several kilobases in length are formed when a proximal heterologous insertion blocks the initiation of strand exchange (32); moreover, such distal joints turn over in the presence of ATP (ref. 33; B. Burnett, B. J. Rao, and C.M.R., unpublished observations). If, as we suppose, the distal joint is genuinely a triple-helical structure, its dissociation during the turnover may be responsible for generating negative superhelicity in the domain of DNA that is transiently trapped between a stalled proximal joint and a mobile distal joint (Fig. 5). We assume that dissociation of the distal joint has the same directionality as strand exchange, moving from left to right in Fig. 5, and that stalling of the proximal joint at the heterology results in a cessation in rotation of the proximal end of the complex. Progression of strand exchange and its rotational component are linked (ref. 10, and see Introduction). One must assume further that in the absence of the active rotation associated with the progression of exchange, the static proximal portion of the complex is tethered by its contacts with other molecules in the nucleoprotein network (10). Since both single-stranded and duplex DNA in the nucleoprotein filament have a greater pitch than free duplex DNA (34–37), the release of duplex DNA between a static proximal joint and a dissociating distal joint, as well as the continuing rotation of the distal joint, would contribute to the creation of an intervening segment that is transiently underwound relative to B-form DNA (Fig. 5). Once the heterologous segment is unwound and folded out of the way, strand exchange can resume. Since a net loss of base pairs occurs, the traversal of a heterologous insert requires energy that is provided by the hydrolysis of ATP, as demonstrated recently by two laboratories (ref. 38; J. I. Kim, M. M. Cox, and R. B. Inman, personal communication). The model proposed here (Fig. 5) is consistent with those observations since the dissociation of distal joints, which is postulated to drive the unwinding of the heterologous insert, also appears to require the hydrolysis of ATP (B. Burnett, B. J. Rao, and C.M.R., unpublished observations).

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