DNA Substrate Requirements for Stable Joint Molecule Formation by the RecA and Single-stranded DNA-binding Proteins of Escherichia coli*

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In reactions between linear single-stranded DNAs (ssDNAs) and circular double-stranded DNAs (dsDNAs), stable joint molecule formation promoted by the recA protein (RecA) requires negative superhelicity, a homologous end, and an RecA-ssDNA complex. Linear ssDNAs with 3'-end homology react more efficiently than linear ssDNAs with 5'-end homology. This 3'-end preference is explained by the finding that 3'-ends are more effectively coated by RecA than 5'-ends, as judged by exonuclease VII protection, and are thus more reactive. The ability of linear ssDNAs with 5'-end homology to react is improved by the presence of low concentrations of exonuclease VII. In reactions between ssDNAs and linear dsDNAs with end homology, stable joint molecule formation occurs more efficiently when the homology is at the 3'-end rather than at the 5'-end of the complementary strand. In addition, linear dsDNAs with homology at the 3'-end of the complementary strand react more efficiently with linear ssDNAs with 3'-end homology than with linear ssDNAs with 5'-end homology. The ability of linear ssDNAs with 5'-end homology to react, in the absence of single-stranded DNA-binding protein, is improved by adding 33-46 nucleotides of heterologous sequence to the 5'-end of the linear ssDNA. The poor reactivity of linear ssDNAs with 5'-end homology is explained by a lack of RecA at the 5'-ends of linear ssDNAs, which is a consequence of the polar association and dissociation of RecA.

The recA protein (RecA)-promoted strand exchange reaction proceeds via a number of kinetically distinct phases (for reviews, see Refs. 1-3). In the first phase of this reaction, RecA binds cooperatively and stoichiometrically to single-stranded DNA (ssDNA) to form a nucleoprotein filament (4, 5). The single-stranded DNA-binding protein (SSB) facilitates formation of this complex (4, 6-8). RecA binds to ssDNA as a sequence-independent DNA-binding protein and assembles primarily in the 5' to 3' direction (9). Once formed, this nucleoprotein complex becomes a sequence-specific DNA-binding entity. During the second phase, binding and homologous alignment of the two DNA molecules result in the formation of a protein-stabilized paranemic joint molecule (6, 10-13) in which the incoming strand is paired with its complement in the double-stranded DNA (dsDNA) but the two strands are not stably interwound. Conversion of a paranemic joint into a stably interwound plectonemic joint requires that the incoming ssDNA and its homologous partner are free to rotate around each other. Rotation and intertwining of strands require an end in the region of homology between the two DNAs (10, 13, 14). The final phase of strand exchange is branch migration, the exchange of the incoming ssDNA for the strand resident in the dsDNA molecule (8).

Strand exchange between linear dsDNA and circular ssDNA with a short hybridized fragment (15) or circular ssDNA (16) shows an end preference. Strand exchange occurs when the 3'-end of the complementary strand of the dsDNA pairs with the ssDNA circle, while the 5'-end of the identical strand of the dsDNA is displaced. Strand exchange does not occur when the 5'-end of the complementary strand of the dsDNA is available to pair with the ssDNA circle. Using completely homologous ssDNA circles and linear dsDNAs, Cox and Lehman (17) showed that the formation of heteroduplex DNA as monitored by the susceptibility to restriction enzyme cleavage occurs in a polar manner. Electron microscopy of reactions between ssDNA circles and linear dsDNAs with regions of nonhomologous DNA inserted at various positions from the end showed an accumulation of migrating branches at heterologous borders (18). Together, these data have been interpreted to suggest that RecA promotes branch migration in the 5' to 3' direction relative to the incoming ssDNA within the nucleoprotein complex.

The directionality of RecA-promoted branch migration suggests that the 5'-end of a linear ssDNA should be the initiating end in reactions with circular duplex DNA. Contrary to this prediction, in the presence of SSB, linear ssDNAs with 3'-end homology form stable joint molecules, whereas those with 5'-end homology do not (19, 20). Moreover, in the absence of SSB, linear ssDNAs with 3'-end homology are 5-10 times more reactive than those with 5'-end homology (20). These data suggest that the preference for 3'-end homology is intrinsic to RecA-promoted strand exchange.

The two sets of reactions described above differ most notably with regard to the structure of the interacting DNAs. Examining the ability of a variety of different sets of DNAs to participate in stable joint molecule formation has provided a set of rules that serve to predict the efficiency and end preference for any given set of DNA substrates.
Materials and Methods

Enzymes and Chemicals—RecA was purified by the spemidine precipitation method (21) as modified by Kowalczykowski, and aliquots were stored at -70 °C in R-buffer (20 mM Tris-HCl, pH 7.5, 5 mM KCl, 5 mM MgCl2, 100 μg of bovine serum albumin/ml for 1 h at 30 °C. The reaction was stopped by the addition of SDS to 0.5%, Na2EDTA to 10 mM, and protease K to 100 μg/ml; incubated for 15 min at 37 °C and for 25 min at 65 °C; extracted twice with phenol/CHCl3 (1:1); precipitated with ethanol; and resuspended in 20 μl of 10 mM Tris-HCl (pH 7.6), 1 mM Na2EDTA. Relaxed dsDNA was prepared by treatment with calf thymus topoisomerase I or as a control I-relaxed DNA was prepared as follows. Supercoiled pMC874 DNA (10 μg) was incubated with 20 units of topoisomerase I in 20 mM Hepes/KOH (pH 8), 20 mM KCl, 5 mM MgCl2, 100 μg of bovine serum albumin/ml, and 5% glycerol (volume = 100 μl) for 40 min at 30 °C. The reaction was stopped and DNA was isolated as described above. Nicked plasmid DNA was prepared as follows. DNA (10 μg) was incubated with 6 μg of DNase in 50 mM Na2EDTA, 50 mM Tris-HCl, 10 mM MgCl2, and 0.35 mg of ethidium bromide/ml for 15 min at 37 °C. The mixture was then isolated as described above. Nicked DNA was labeled at the 3'-end by annealing the 18-mer used to generate ssDNA was annealed with a specific 77-mer that generated an EcoRI/HindIII fragment of M13mp18-lacZA ssDNA was annealed with specific oligonucleotide to generate a unique restriction site. After cleavage with the appropriate enzyme, labeled ssDNA was purified by gel electrophoresis through 1% low-gelling temperature SeaPlaque agarose in the presence of nonradioactive DNA. The ssDNA was isolated by phenol extraction, followed by ethanol precipitation. Specifically, circular M13mp18-lacZA ssDNA was annealed with a specific 16-mer, or 18-mer, which contains an EcoRI, BamHI, or HindIII site, respectively. Circular ssDNAs generated by HindIII digestion contained 1956 nucleotides of lacZ sequence at the 3'-end and 2720 nucleotides of M13mp18 sequence at the 5'-end. Linear ssDNAs generated by EcoRI digestion contained 1956 nucleotides of lacZ sequence at the 5'-end and 2720 nucleotides of M13mp18 sequence at the 5'-end. Linear ssDNAs generated by XmnI digestion contained 1956 nucleotides of lacZ sequence flanked by 3635 and 3585 nucleotides of M13mp18 sequence. Circular M13mp18-lacZA/PL ssDNA was annealed with a specific 77-mer that generated 14 unique restriction sites.

Susceptibility of End-labeled ssDNA to Exonuclease VII Digestion in Presence and Absence of SSB—Linear ssDNA was labeled at the 5'-end with T4 polynucleotide kinase and [γ-32P]ATP (24). Linear ssDNA was labeled at the 5'-end by annealing the 18-mer used to generate the end and filling in the recessed 3'-terminus with Klenow fragment and [α-32P]dCTP (24). In the presence of ATP, linear ssDNAs were preincubated for 10 min at 37 °C in the standard reaction buffer (see above) containing 4 mM MgOAc and RecA; the final concentration of MgOAc was increased to 10 mM, and SSB was added at a concentration of 25% RecA. In the presence of ATP-S, linear ssDNAs were preincubated for 10 min at 37 °C in the standard reaction buffer (see above) containing 4 mM MgOAc and SSB; RecA and ATP-S were then added, and the mixture was incubated for 10 min at 37 °C. The reaction was initiated by the addition of SSB, and the incubation was continued for 30 min at 37 °C. The reactions were stopped by the addition of Na2EDTA to 50 mM, ssDNAs to 1%, and protease K to 100 μg/ml and were incubated at 37 °C for 10 min. The agarose gel assay for stable joint molecules was conducted as described previously (20). Quantitation of the data was performed using a Molecular Dynamics Model 300A computing densitometer.

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**RESULTS**

Homology at 3'-End of Linear ssDNA Is Sufficient for Stable Joint Molecule Formation with Supercoiled DNA and Is Preferred over Homology at 5'-End—Our previous studies (19, 20) of joint molecule formation between linear ssDNAs and supercoiled DNAs demonstrated that in the presence of SSB, 3'-end homology is essential. Linear ssDNAs with 5'-end homology do not form stable joint molecules. In the absence of SSB, linear ssDNAs with 3'-end homology are still more reactive than those with 5'-end homology (20). The preference for a homologous 3'-end in the absence of SSB suggests that this is an intrinsic property of RecA-promoted strand exchange. To test the generality of these results, the new substrates schematically diagramed in Fig. 1A were assayed for stable joint molecule formation. In these experiments, the region of homology between the two DNAs is either lacZ or M13 and is limited to only the 5'- or 3'-end of the linear ssDNA.

Increasing amounts of RecA were titrated into the reactions in the absence or presence of SSB (Fig. 1B). In the presence of SSB, optimal joint molecule formation (58–62%) occurs at a concentration of one RecA monomer/three nucleotides of ssDNA and depends on homology at the 3'-end of the linear ssDNA. No stable joint molecules (<1%) are observed in reactions between linear ssDNAs with 5'-end homology and supercoiled DNAs. In the absence of SSB, the 3'-end is still preferred over the 5'-end at all of the RecA concentrations tested. Linear ssDNAs with 3'-end homology react 4–10 times more efficiently than linear ssDNAs with 5'-end homology.

These data demonstrate that homology at only one end of the linear ssDNA is sufficient for stable joint molecule formation with supercoiled DNA and that 3'-end homology is favored over 5'-end homology in the presence and absence of SSB. Moreover, the 4–10-fold 3'-end preference observed in these experiments is quantitatively comparable to the 5–10-fold preference previously published (20). These data are strengthened by the fact that the experiments are internally controlled. Specifically, linear ssDNAs with 5'-end lacZ homology and 3'-end M13 homology failed to react with pMC874A but did react with pBR322-M13(3'), and linear ssDNAs with 3'-end lacZ homology and 5'-end M13 homology formed joint molecules with pMC874A but did not react with pBR322-M13(5') to form stable joint molecules. These data suggest that the failure or ability of these substrates to react with a particular dsDNA is not due to nonspecific inhibitors or activators in either the ssDNA or dsDNA preparations. Furthermore, these experiments suggest that the 3'-end preference observed is not sequence-specific since the region of homology between the two DNAs is lacZ in one set of reactions and M13 in the other.

As RecA is increased beyond the optimal one RecA monomer/three nucleotides of ssDNA, the efficiency of joint molecule formation decreases 2–3-fold in reactions with linear ssDNAs with 3'-end homology, whereas it remains approximately the same in reactions with linear ssDNAs with 5'-end homology in the absence and presence of SSB. As a consequence, in the absence of SSB, the difference in reaction efficiency between linear ssDNAs with 5'- or 3'-end homology is less dramatic (~4-fold) at high RecA concentrations (greater than two RecA monomers/three nucleotides of ssDNA). The inhibition of linear ssDNAs with 3'-end homology at high RecA concentrations in the absence and presence of SSB has been previously observed (20).

Superhelicity Is Required for Stable Joint Molecule Formation between Linear ssDNAs with 3'-End Homology and Covalently Closed dsDNAs—In addition to 3'-end homology, reactions between covalently closed circular dsDNAs and linear ssDNAs require superhelicity (Fig. 2A). Linear ssDNAs with either 5'- or 3'-end homology do not form stable joint molecules with topoisomerase I-relaxed dsDNA. This conclusion is true over a wide range of RecA concentrations (0.5–10-fold excess, assuming a binding stoichiometry of one RecA monomer/three ssDNA nucleotide residues) and at various times of incubation (0–60 min) (data not shown). Moreover, linear ssDNAs with either 5'- or 3'-end homology do not form stable joint molecules with DNase I-nicked DNA or dsDNAs in which the region of homology is flanked by heterology (data not shown). The ability of topoisomerase I-relaxed DNA to support stable joint molecule formation between linear ssDNA and supercoiled DNA is discussed in detail elsewhere. In the presence of SSB, linear ssDNAs with 3'-end homology are still more reactive than those with 5'-end homology (20). The preference for a homologous 3'-end in the absence of SSB suggests that this is an intrinsic property of RecA-promoted strand exchange. To test the generality of these results, the new substrates schematically diagramed in Fig. 1A were assayed for stable joint molecule formation. In these experiments, the region of homology between the two DNAs is either lacZ or M13 and is limited to only the 5'- or 3'-end of the linear ssDNA.

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Fig. 2. Stability of joint molecules formed between linear ssDNAs with end homology and relaxed or supercoiled DNAs. A, stable joint molecule formation between linear ssDNAs with 5'-or 3'-end homology and topoisomerase I-relaxed or gyrase-supercoiled DNAs in presence and absence of SSB. *Thick lines*, lacZ sequences; *shaded regions*, M13mp18 sequences, and *thin lines*, nonhomologous sequences. Standard reactions were performed using uniformly 32P-labeled linear ssDNAs with 5'- or 3'-end lacZ homology. Topoisomerase I-relaxed pMC874Δ and gyrase-supercoiled pMC874Δ were prepared as described under “Materials and Methods.” In these reactions, the region of homology between the two DNAs is 2 kb of lacZ sequence. The reactions were performed under standard reaction conditions, which contained RecA and SSB (R, S) or lacked either RecA (−R) or SSB (−S). Stable joint molecule formation was assayed as previously described (20). B, stability of stable joint molecules formed between uniformly 32P-labeled linear ssDNA with 3'-end homology and supercoiled DNAs upon linearization. Stable joint molecules formed between linear ssDNAs with 3'-end lacZ homology and supercoiled pMC874Δ were untreated (UNCUT) or linearized by HindIII digestion at the site indicated by arrow (CUT) in the presence of RecA and SSB. The resulting product is a linear dsDNA with 2 kb of lacZ sequence flanked on either side by 5.5 and 0.6 kb of heterology. Stable joint molecules were analyzed as previously described (20). The percent product formed in this assay was defined as the percentage of total ssDNA that was converted to joint molecules.

DNA with 3'-end homology and covalently closed dsDNAs is restored in reactions in which the supercoils are reintroduced into topoisomerase I-relaxed dsDNA by the action of gyrase (Fig. 2A).

In reactions between linear ssDNAs with end homology and supercoiled dsDNAs, stable joint molecule formation could be driven by the release of positive free energy as superhelical turns are removed (25). In contrast, in reactions between linear ssDNAs with end homology and relaxed (linear with internal homology, nicked, or covalently closed) dsDNAs, stabilization of the joint molecule and further strand uptake would not be driven by a favorable increase in entropy (25). It is possible that joint molecules are formed in reactions between linear ssDNAs with end homology and relaxed dsDNAs but are unstable to deproteinization and/or agarose gel electrophoresis. To address the problem of stability, joint molecules were formed between supercoiled DNA and linear ssDNAs with 3'-end homology (Fig. 2B). After 20 min at 37 °C, the reaction was divided into two aliquots. One aliquot was digested for 1 min at 37 °C with HindIII, which cleaves uniquely once in the region of nonhomology to generate a linear dsDNA with 5.5 and 0.6 kb of heterology on either side of the lacZ homology; and the other aliquot was untreated. Digestion for 1 min at 37 °C with HindIII was sufficient to linearize ~100% of the joint molecules formed as measured by ethidium bromide staining. The products were analyzed by agarose gel electrophoresis. Less than 10% of the joint molecules originally formed remained stable after linearization of the dsDNA with HindIII. This percentage did not vary significantly (<2-fold) with the type of enzyme used (PstI, SalI), the enzyme concentration (0.1–2.5 units/μl), or the length of digestion (1–5 min) (data not shown). In these experiments, the total amount of radioactivity before and after linearization remained constant. Removal of RecA and SSB by phenol/CHCl₃ (1:1) extraction prior to digestion with HindIII had a <2-fold effect on the percentage of joint molecules that remained stable to linearization. These data point to the importance of superhelicity in stabilizing the initial joint molecule formed. Moreover, these data suggest that dissociation of joint molecules through RecA-promoted and/or spontaneous branch migration could in part, explain why reactions between linear ssDNAs with end homology and relaxed dsDNAs are relatively inefficient in comparison to those with supercoiled DNAs.

ssDNA Circles Form Stable Joint Molecules with Linear dsDNAs More Efficiently if Homology Is at 3'-End Rather Than at 5'-End of the Complementary Strand—Stable joint molecule formation requires a homologous end. The homologous end could be provided by the linear ssDNA, as is the case in reactions between ssDNAs and supercoiled DNAs, or by the complementary strand of the dsDNA, as is the case in reactions between ssDNAs and dsDNAs with end homology. Linear duplex DNAs with end homology and ssDNA circles efficiently form stable joint molecules in cases where the 3'-end of the complementary strand of the dsDNA is paired with the ssDNA circle (Fig. 3, A and B) (16). In the presence of SSB, the reaction efficiency is 55–62% at all RecA concentrations tested. In the absence of SSB, the reaction efficiency is reduced to 10–20% (Fig. 3B). RecA forms few joint molecules by transferring the 5'-end of the complementary strand of the dsDNA to the ssDNA circle (Fig. 3, A and B) (16). In the presence of SSB, the reaction efficiency is 1–5%, whereas in the absence of SSB, no (<1%) stable joint molecules are formed (Fig. 3B). This end preference is observed whether the region of homology between the two DNAs is lacZ (Fig. 3) or M13mp18 (data not shown).

Linear ssDNAs with 5'-End Homology Form Few Stable Joint Molecules in Reactions with dsDNAs in Which Homology Is at 3'-End of the Complementary Strand—The structural requirements for stable joint molecule formation in reactions between linear ssDNAs and dsDNAs with homology at the 3'-end of the complementary strand were further addressed in the following experiment. Linear ssDNAs with 3'-end homology, 5'-end homology, or internal homology were allowed to react with dsDNAs in which the homology is at the 3'-end of the complementary strand (Fig. 4A, i–iii, respectively). In these reactions, a homologous 3'-end is provided by the complementary strand of the duplex linear DNA. If a homologous 3'-end is the only structural requirement for stable joint molecule formation in these reactions, then all three linear ssDNAs should react with equal efficiency.

In the presence of SSB, linear ssDNAs with 3'-end homology form stable joint molecules with an efficiency of 50–62% (Fig. 4, B and C). Linear ssDNAs with internal homology
In the absence of SSB, the reaction efficiency of linear ssDNAs with 3'-end homology and linear ssDNAs with internal homology is reduced 2-3-fold (Fig. 4C). Nonetheless, linear ssDNAs with 3'-end homology react 6-10 times more efficiently than linear ssDNAs with 5'-end homology over the range of RecA concentrations tested.

Note that the inhibition of joint molecule formation observed at high RecA concentrations in reactions between linear ssDNAs and supercoiled DNAs (Fig. 1B) is not observed in reactions between ssDNAs and linear dsDNAs with end homology. This finding is consistent with the idea that RecA and/or SSB binds more readily to supercoiled DNA than to relaxed linear dsDNA and in so doing decreases or eliminates the superhelicity required for stable joint molecule formation (Fig. 2A) (27, 28).

These data demonstrate that when pairing of the 3'-end of the complementary strand of the dsDNA initiates within the ssDNA (Fig. 4A, i and iii), efficient stable joint molecule formation occurs. In contrast, few stable joint molecules form when pairing of the 3'-end of the complementary strand of the dsDNA initiates at the 5'-end of the linear ssDNA (Fig. 4A, ii). These conclusions are qualitatively the same when ATPγS is substituted for ATP, suggesting that the 3'-end preference observed in reactions between linear ssDNAs with end homology and linear dsDNAs with homology at the 3'-end of the complementary strand does not depend on ATP hydrolysis (data not shown).

Activation of Linear ssDNAs with 5'-End Homology—Because RecA polymerizes in the 5' to 3' direction onto the ssDNA, 3'-ends are more likely to be coated with RecA than are 5'-ends (9). In fact, 5'-ends are often complexed with SSB (9). The presence of SSB at the 5'-ends of the majority of nucleoprotein filaments could explain why linear ssDNAs with 5'-end homology fail to form stable joint molecules (Figs. 1B and 4, B and C). In the absence of SSB, however, linear ssDNAs with 5'-end homology still form few (1-5%) stable joint molecules (Figs. 1B and 4C). The poor reaction efficiency of linear ssDNAs with 5'-end homology in the absence of SSB could be explained by the requirement for a RecA-ssDNA complex in addition to a homologous end. In reactions between linear ssDNAs with end homology and supercoiled DNAs, the homologous end and the RecA-ssDNA complex would be provided by the linear ssDNA. In contrast, in reactions between linear ssDNAs and dsDNAs with end homology, the homologous end would be provided by the complementary strand of the linear dsDNA, whereas the RecA-ssDNA complex would be provided by the ssDNA.

The idea that stable joint molecule formation between ssDNAs and linear dsDNAs with end homology depends on the presence of an RecA-ssDNA complex directly opposite the homologous end provided by the complementary strand of the dsDNA is tested in the following experiment. Various defined lengths of heterology (6-66 nucleotides) were added to the 5'-end of the linear ssDNA, and the ability of these linear ssDNAs to form stable joint molecules with linear dsDNAs in which homology is at the 3'-end of the complementary strand in the absence of SSB was examined. A schematic diagram of the expected joint molecules and the results are shown in Fig. 5. The reaction efficiency of ssDNA circles and linear dsDNAs with homology at the 3'-end of the complementary strand in the absence of SSB serves as a positive control (20-30%) (Fig. 3A). The reaction efficiency of linear ssDNAs with 5'-end homology (actually, four nucleotides of heterology at the 5'-end; see Fig. 4 legend) and linear dsDNAs with homology at the 3'-end of the complementary strand (Fig. 4A, ii) is the base-line level of joint molecule
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FIG. 4. Stable joint molecule formation between linear ssDNAs and linear dsDNAs with homology at 3'-end of complementary strand. A, schematic of predicted joint molecules. Thick lines, lacZ sequences; shaded regions, M13mp18 sequences; thin lines, nonhomologous sequences. Region of homology between the two DNAs is 2 kb of lacZ sequence. In these reactions, the 3'-end of the complementary strand of the linear dsDNA is paired with the ssDNA, whereas the 5'-end of the identical strand of the dsDNA is displaced. The three linear ssDNAs derive from circular M13mp18-lacZΔ ssDNA, which is cleaved to generate linear ssDNAs with 3' or 5' end or internal lacZ homology as described under “Materials and Methods.” Linear dsDNAs with lacZ homology at the 3'-end of the complementary strand were prepared by digesting pMC874α with EcoRI. In reactions between linear dsDNAs with lacZ homology at the 3'-end of the complementary strand and linear ssDNAs with 3'-end lacZ homology (i) or internal lacZ homology (ii), pairing of the 3'-end of the complementary strand of the dsDNA initiates within the ssDNA. In reactions between linear dsDNAs with homology at the 3'-end of the complementary strand and linear ssDNAs with 5'-end lacZ homology (iii), pairing of the 3'-end of the complementary strand of the dsDNA initiates four nucleotides away from the 5'-end of the linear ssDNA. This is due to removal of four nucleotides from the dsDNA by EcoRI digestion. B, stable joint molecule formation between linear dsDNAs with lacZ homology at 3'-end of complementary strand and uniformly 32P-labeled linear ssDNAs with 3'-end (i), 5'-end (ii), or internal (iii) lacZ homology. The RecA/ssDNA nucleotide ratio was varied from 0 to 43 in the presence of SSB under standard reaction conditions. Stable joint molecule formation was assayed as previously described (20). C, percent efficiency of stable joint molecule formation between linear dsDNAs with homology at 3'-end of complementary strand and linear ssDNAs with 3'-end (○ and ●), 5'-end (□ and ▲) or internal (△ and ■) lacZ homology as function of increasing RecA concentration either in absence (○, □, △, and ■) or presence (●, ▲, and △-SSB) of SSB under standard reaction conditions. The percent product formed in this assay was defined as the percentage of the total ssDNA that was converted to joint molecules.

FIG. 5. Stable joint molecule formation between linear ssDNAs with various lengths of 5'-end heterology and linear dsDNA with homology at 3'-end of complementary strand in absence of SSB. Thick lines, lacZ sequences; shaded regions, M13mp18 sequences; thin lines, nonhomologous sequences. The region of homology between the two DNAs is 2 kb of lacZ sequence. The length of heterology added to the 5'-ends of the linear ssDNAs varied from 6 to 66 nucleotides as schematically shown. Linear ssDNAs were generated by annealing circular M13mp18-lacZΔ/PL ssDNA with a specific 77-mer, which generated 14 unique restriction sites. The circular ssDNA with the annealed 77-mer was separately digested with Smal, BamHI, PstI, HindIII, SacI, XhoI, BglII, EcoRV, ClaI and EcoRI to generate linear ssDNAs with 6, 13, 21, 35, 39, 46, 50, 55, 59, and 66 nucleotides of heterologous sequences, respectively, at the 5'-end. Linear dsDNA with lacZ homology at the 3'-end of the complementary strand was prepared by digesting pMC874α with EcoRI. Each of the linear ssDNAs was incubated with linear dsDNA with homology at the 3'-end of the complementary strand under standard reaction conditions (one RecA monomer/three nucleotides of ssDNA) in the absence of SSB. Stable joint molecule formation was assayed by agarose gel electrophoresis as previously described (20), followed by Southern hybridization (23) with a probe specific for M13mp18-lacZΔ/PL DNA. The percentage of product formed in this assay was defined as the percentage of the total ssDNA that was converted to joint molecules. The percent joint molecules formed between linear ssDNAs with 5'-end homology (actually, four nucleotides of heterology at the 5'-end; see Fig. 4 legend) and linear dsDNA with homology at the 3'-end of the complementary strand served as a negative control. Percent joint molecules formed between ssDNA circles and linear dsDNAs with homology at the 5'-end of the complementary strand served as a positive control. Predicted structures of the joint molecules formed and the percent reactivity of each set of substrates are shown.

formation (1–5%) (Fig. 4C). At an optimal RecA concentration of one RecA monomer/three nucleotides of ssDNA, extending the 5'-end of the linear ssDNA by ~33 nucleotides of heterology is sufficient to allow half the maximal amount of stable joint molecule formation (~15%). Maximal joint molecule formation (~28%) occurs when ~46 nucleotides of heterology are added to the 5'-end of the linear ssDNA. In the presence of SSB, >66 nucleotides are required for stable joint molecule formation (data not shown). One interpretation of these data is that under optimum strand exchange conditions, 33–46 nucleotides of heterology at the 5'-end of the linear ssDNA are necessary and sufficient to allow RecA to bind to the ssDNA directly opposite the 3'-end of the complementary strand of the linear dsDNA and thus provide the RecA/ssDNA complex required for plectonemic joint molecule formation.

If linear ssDNAs with 5'-end homology form few joint molecules simply because they lack RecA, then removal of
the "naked" 5'-tail should increase the efficiency of reactions between linear ssDNAs with 5'-end homology and supercoiled DNAs. These experiments are complicated by the fact that strand exchange is a dynamic process in which RecA and SSB are associating and dissociating from the linear ssDNA (for review, see Ref. 3). With this caveat in mind, the reactions between linear ssDNA with 5'-end homology and supercoiled DNA were repeated in the presence of low concentrations of exonuclease VII, which degrades ssDNA from both 3'- and 5'-termini (29, 30). In the absence of SSB, exonuclease VII at a concentration of 20-40 units/ml increases the reaction efficiency between linear ssDNAs with 5'-end homology and supercoiled DNA 2-3-fold (Fig. 6). In the absence of SSB, the effect of exonuclease VII at the same concentrations is more dramatic (Fig. 6). Linear ssDNAs with 5'-end homology react with supercoiled DNA 5-10-fold more efficiently in the presence of exonuclease VII than in its absence. In contrast, in reactions between linear ssDNAs with 3'-end homology and supercoiled DNAs, exonuclease VII at a concentration of 20-40 units/ml did not significantly affect (<2-fold) the efficiency of joint molecule formation in the presence or absence of SSB. Note that in the absence and presence of SSB, the stimulatory effect of exonuclease VII on stable joint molecule formation between linear ssDNAs with 5'-end homology and supercoiled DNAs did not reach saturation (Fig. 6). The stimulatory effect of exonuclease VII could be explained by the exonucleaseolytic digestion of naked 5'-tails to generate a population of linear ssDNAs in which the 5'-end is coated with RecA and thus could participate in stable joint molecule formation.

RecA Coats 3'-Ends More Effectively Than 5'-Ends—To more directly test the idea that the 5'-ends of linear ssDNAs lack RecA whereas the 3'-ends are coated, the susceptibility of 5'- or 3'-end-labeled linear ssDNA to brief digestion with exonuclease VII in the absence or presence of SSB was examined. The length of time of exonuclease VII digestion sufficient to remove ~90% of the end label in the absence of RecA and SSB was determined for each of the end-labeled linear ssDNAs. The amount of end label remaining after exonuclease VII treatment in the absence of RecA and SSB (5-10%) is defined as 0% protection. The percent protection of 3'- or 5'-end-labeled ssDNA with increasing amounts of RecA in the presence and absence of SSB is shown in Fig. 7. In the presence of SSB, only 10-13% of the 5'-end-labeled ssDNA is protected from digestion by exonuclease VII, whereas 57-88% of the 3'-end-labeled ssDNA is protected. In the absence of SSB, 10-13% of the 5'-end-labeled ssDNA is protected from exonuclease VII digestion, whereas 36-66% of the 3'-end-labeled ssDNA is protected.

Since the structure of the RecA-ssDNA complex formed in the presence of ATPγ-S is indistinguishable from that formed in the presence of ATP (5, 31, 32, 34), the percent protection of 3'- or 5'-end-labeled ssDNA in the presence of ATPγ-S and SSB was determined. At a concentration of one RecA monomer/three nucleotides of ssDNA, only 10% of the 5'-end-labeled ssDNA is protected from exonuclease VII digestion, whereas 60-80% of the 3'-end-labeled ssDNA is protected (data not shown).

The susceptibility (~90%) of the 5'-end-labeled ssDNA in the presence of ATP or ATPγ-S argues that if SSB is com-

![Image](https://example.com/image1.png)

**Fig. 6.** Stable joint molecule formation between α-P-labeled linear ssDNAs with 5'-end homology and supercoiled DNA in the presence of exonuclease VII. Thick lines, lacZ sequences; shaded region, M13mp18 sequences; thin lines, nonhomologous sequences. The region of homology between the two DNAs is 2 kb of lacZ sequence. Linear ssDNAs with 5'-end lacZ homology and supercoiled pMC874A DNA were prepared as described under "Materials and Methods." The region of homology between the two DNAs is 2 kb of lacZ sequence. The reactions were performed under standard reaction conditions in the absence (○ and ■) or presence (● and ♦) of SSB with increasing amounts of exonuclease VII. Stable joint molecule formation was assayed as previously described (29). The percent product formed in this assay was defined as the percentage of the total ssDNA that was converted to joint molecules.

![Image](https://example.com/image2.png)

**Fig. 7.** Susceptibility of 5'- or 3'-end-labeled linear ssDNA to exonuclease VII digestion in the presence of RecA or RecA and SSB. 3' (△ and ●) or 5' (○ and ■)-end-labeled ssDNA was preincubated with RecA for 10 min at 37 °C. SSB was added (△, ●, and ♦), and the DNA was digested with 50 units/ml exonuclease VII for 2 min at 37 °C. The reaction was stopped by the addition of SDS to 0.5% and Na2EDTA to 10 mM. The products were analyzed by agarose gel electrophoresis. The percent label remaining after 2 min of exonuclease VII digestion in the absence of RecA and SSB is defined as 0% protection. The percent protection is defined as the amount of label remaining after exonuclease VII digestion divided by the amount of label initially added to the reaction, prior to exonuclease VII digestion, multiplied by 100.
plexed to the 5'-end, it does not protect the terminal phosphate group from exonuclease VII digestion. In fact, the limit products of exonuclease VII action are oligonucleotides predominantly in the range of tetramers to dodecamers (30). This observation suggests that RecA and/or SSB is absent from at least 4–10 nucleotides at the 5'-ends of linear ssDNAs. These data demonstrate that 3'-ends are coated by RecA and/or SSB more efficiently than 5'-ends in the presence of ATP or ATPγS, as measured by exonuclease VII protection.

ssDNAs and dsDNAs with Homology at 5'-End of the Complementary Strand Form Few Stable Joint Molecules—In reactions between ssDNAs and linear dsDNAs with homology at the 5'-end of the complementary strand (Fig. 8, A and B), few (1–5%) stable joint molecules form despite the fact that a homologous end is provided by the linear dsDNA and an RecA-ssDNA complex is provided by the ssDNA. These findings are true over the range of RecA concentrations tested. These data demonstrate that a homologous end and an RecA-ssDNA complex are not sufficient for efficient stable joint molecule formation between ssDNAs and linear dsDNAs in which homology is at the 5'-end of the complementary strand.

**DISCUSSION**

The ability of various sets of DNA substrates (schematically diagrammed in Fig. 9) to form stable joint molecules depends on the presence of a homologous end and an RecA-ssDNA complex. Stable joint molecules refer to those joint molecules that are stable to deproteinization and agarose gel electrophoresis.

In reactions between linear ssDNAs and supercoiled dsDNAs (Fig. 9, A and D), the homologous end and the RecA-ssDNA complex are provided by the linear ssDNA. Linear ssDNAs with 3'-end homology react more efficiently than linear ssDNAs with 5'-end homology (Fig. 1B). This is most readily explained by the association (9) and dissociation (35) of RecA in the 5' to 3' direction relative to the incoming ssDNA. As such, 3'-ends are coated with RecA and thus intertwine to form stable joint molecules. On the other hand, 5'-ends are either complexed with SSB and/or lack RecA and thus fail to form stable joint molecules. Note that in these reactions, the observed 3'-end preference is adequately and solely explained by the association and dissociation of RecA in the 5' to 3' direction without the need to invoke the notion of polar RecA-driven branch migration.

The ability of linear ssDNAs with 5'-end homology to form stable joint molecules with supercoiled dsDNAs is stimulated by low concentrations of exonuclease VII, a single-strand-specific exonuclease that acts at both 5'- and 3'-ends (Fig. 6) (29, 30). One explanation for the 5'-end activation is that exonuclease VII digests the naked 5'-tail, resulting in an RecA-ssDNA complex at the very 5'-end of the linear ssDNA that is capable of forming a stable joint molecule.

In addition to the requirements for a homologous end and an RecA-ssDNA complex, stable joint molecule formation between linear ssDNAs with end homology and covalently closed dsDNAs depends on negative superhelicity (Fig. 2A). The role of superhelicity in RecA-promoted pairing has been previously established (27, 28). The data described here confirm and extend this observation by demonstrating that topoisomerase I-relaxed DNA (Fig. 2A), DNase I nicked DNA, and linear dsDNAs with internal homology do not support stable joint molecule formation with linear ssDNAs with either 5'- or 3'-end homology. Negative superhelicity could act to drive ssDNA uptake since the removal of superhelical turn releases the positive free energy of supercoiling (25). The free energy of supercoiling is derived predominantly from the difference in entropy between relaxed circular DNA and a more compact and constrained superhelical DNA (36). Note...
Joint molecules formed between linear ssDNAs with 3'-end homology and supercoiled DNAs are unstable upon linearization in the presence or absence of RecA and SSB (Fig. 2B). Previous studies (37) of spontaneous ssDNA uptake by superhelical DNA showed that random but limited DNase I treatment allows the strands of the dsDNA to rotate freely and to displace the ssDNA by branch migration. The instability of spontaneously formed joint molecules upon DNase I treatment is probably similar to that observed with RecA-promoted joint molecules upon linearization. In contrast, cleavage of the displaced strand by single strand-specific endonucleases prevented displacement of the ssDNA by branch migration (38). It is unclear whether linear ssDNAs with end homology fail to form joint molecules with relaxed (linear with internal homology, nicked, or covalently closed) dsDNAs or whether joint molecules are formed but are unstable to deproteinization and/or agarose gel electrophoresis.

In reactions between single-stranded DNAs and linear dsDNAs with homology at the 3'-end of the complementary strand (Fig. 9, G–I), the homologous end is provided by the dsDNA, whereas the RecA-ssDNA complex is provided by the ssDNA. Linear ssDNAs with 5'-end homology form few stable joint molecules with linear dsDNAs in which the homology is at the 3'-end of the complementary strand in the presence of ATP (Figs. 4C and 4J). In these reactions, the 3'-end of the complementary strand is available to pair with the 5'-end of the linear ssDNA, which either remains complexed with SSB or is devoid of RecA in the absence of SSB as a consequence of the association (9) and dissociation (35) of RecA in the 5' to 3' direction with regard to the ssDNA. As a result, stable joint molecule formation is inefficient. In the presence of ATPγS, the failure to detect stable joint molecules in reactions between linear ssDNAs with 5'-end homology and linear dsDNAs with homology at the 3'-end of the complementary strand (data not shown) could also be explained by the polymerization of RecA in the 5' to 3' direction onto ssDNA and the requirement for the complementary strand of the dsDNA to pair with a RecA-ssDNA complex. In reactions between linear dsDNAs with homology at the 3'-end of the complementary strand and ssDNA circles (Fig. 9G), linear ssDNAs with 3'-end homology (I), and linear ssDNAs with 5'-end homology (J); and between linear dsDNAs with homology at the 5'-end of the complementary strand and single-stranded circles (K), linear ssDNAs with internal homology (L), linear ssDNAs with 3'-end homology (M), and linear ssDNAs with 5'-end homology (N).

that the amount of heteroduplex DNA in the joint molecule is a function of the superhelical density of the dsDNA. Once the negative superhelical turns are removed and the dsDNA is relaxed, further strand uptake, which would generate positive superhelical turns, is energetically unfavorable and therefore limits the amount of heteroduplex DNA formed to the amount of negative superhelicity originally contained in the dsDNA. This limited amount of heteroduplex DNA could account for the relative instability of linearized joint molecules formed between linear ssDNAs and supercoiled DNAs (Fig. 2B) as compared to joint molecules formed between ssDNAs and linear dsDNAs with end homology (Fig. 9, G–I), which have the potential to form longer regions of heteroduplex DNA. Whereas the amount of heteroduplex DNA formed is limited to less than the total amount of homology available in joint molecules formed between linear ssDNAs and supercoiled DNAs, it is not known whether a population of joint molecules exists in which the amount of heteroduplex DNA remains constant but its location within the region of homology varies due to spontaneous and/or RecA-promoted branch migration.
5). This finding is quantitatively comparable to the results of Lindsey and Cox (40) showing that ssDNA gaps or tails between 34 and 50 nucleotides are sufficient to stimulate RecA binding to dsDNA. Together, these data suggest that a minimum length of ssDNA (~50 nucleotides) is required for the efficient association of RecA onto DNA and that the binding of RecA to the localized region that corresponds to the end of the complementary strand of the dsDNA is required for efficient stable joint molecule formation. Most important, these data suggest that the failure to detect stable joint molecules in reactions with linear ssDNAs with 5′-end homology is due to the lack of RecA at the 5′-ends of linear ssDNAs.

Direct evidence that 5′-ends are devoid of RecA whereas 3′-ends are covered comes from the relative susceptibility of 5′- or 3′-end-labeled ssDNAs to brief exonuclease VI1 digestion (9) and dissociation (35) of RecA. Likewise, in the presence of ATPyS, RecA, and SSB, 3′-end-labeled linear ssDNAs resist brief exonuclease VII digestion more effectively than 5′-end-labeled linear ssDNAs (data not shown). These data are consistent with the idea that the poor reaction efficiency of linear ssDNAs with 5′-end homology in the presence of ATP or ATPyS is due to the lack of RecA at the 5′-ends of the linear ssDNAs under both these conditions. In the presence of ATP, the lack of RecA at the 5′-ends of linear ssDNAs is likely a consequence of the polar association (9) and dissociation (35) of RecA. In contrast, the dissociation of RecA from ssDNA is blocked in the presence of ATPyS (33), the lack of RecA at the 5′-ends of linear ssDNAs is likely a consequence of the polar association of RecA.

In reactions between ssDNAs and linear dsDNAs with homology at the 5′-end of the complementary strand (Fig. 9, K–N), few (1–5%) stable joint molecules form (Fig. 8, A and B) despite the fact that a homologous end is provided by the linear dsDNA and an RecA-ssDNA complex is provided by the ssDNA. These data demonstrate that a homologous end and an RecA-ssDNA complex are not sufficient for stable joint molecule formation in reactions between ssDNAs and linear dsDNAs with 5′-end homology. Whereas these data appear to contradict those of Wu et al. (6), which showed efficient joint molecule formation between ssDNA circles and linear dsDNAs with homology at the 5′-end of the complementary strand, the nicked.cculeus filter binding assays in that study were performed without prior protein removal by SDS. As such, paneramic as well as plectomonic joint molecules should be retained. Since protein-stabilized paneramic joint molecules form efficiently without any ends (11, 12), the findings of Wu et al. (6) demonstrate that paneramic joint molecule formation occurs with equal efficiency between ssDNA circles and linear dsDNAs with homology at either the 5′- or 3′-end of the complementary strand.

The polar association (9) and dissociation (35) of RecA readily explains the preference for linear ssDNAs with 3′-end homology over those with 5′-end homology in forming stable joint molecules with supercoiled DNAs or linear dsDNAs with homology at the 3′-end of the complementary strand. Whether the directional association and dissociation of monomers from filament ends could also explain the preference for homology at the 3′-end rather than the 5′-end of the complementary strand of the dsDNA in reactions with ss-DNAs remains to be directly addressed.

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