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Branch migration during Rad51-promoted strand exchange proceeds in either direction

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ABSTRACT **The *Saccharomyces cerevisiae* Rad51 protein is important for genetic recombination and repair of DNA double-strand breaks *in vivo* and can promote strand exchange between linear double-stranded DNA and circular single-stranded DNA *in vitro*. However, unlike *Escherichia coli* RecA, Rad51 requires an overhanging complementary 3′ or 5′ end to initiate strand exchange; given that fact, we previously surmised that the fully exchanged molecules resulted from branch migration in either direction depending on which type of end initiated the joint molecule. Our present experiments confirm that branch migration proceeds in either direction, the polarity depending on whether a 3′ or 5′ end initiates the joint molecules. Furthermore, heteroduplex DNA is formed rapidly, first at the overhanging end of the linear double-stranded DNA's complementary strand and then more slowly by progressive lengthening of the heteroduplex region until strand exchange is complete. Although joint molecule formation occurs equally efficiently when initiated with a 3′ or 5′ overhanging end, branch migration proceeds more rapidly when it is initiated by an overhanging 3′ end, i.e., in the 5′ to 3′ direction with respect to the single-stranded DNA.**

Saccharomyces cerevisiae Rad51, like *Escherichia coli* RecA, catalyzes joint molecule formation and complete strand exchange between circular single-stranded DNA (ssDNA) and linear double-stranded DNA (dsDNA) (1–3). Both require ssDNA binding protein and ATP to effect the strand exchange (1, 3, 4). However, unlike RecA, Rad51 requires an overhanging complementary 3′ or 5′ end on the dsDNA to initiate strand exchange; given that fact, we surmised (3) that the fully exchanged molecules resulted from branch migration in either direction depending on which type of end initiated the joint molecule. In that work, however, only joint and fully exchanged products were monitored, and the course and direction of branch migration were not measured directly.

Our present experiments confirm that heteroduplex DNA is formed rapidly, first at the overhanging end of the linear dsDNA's complementary strand and then more slowly by progressive lengthening of the heteroduplex region until strand exchange is complete. Furthermore, branch migration proceeds in either direction, the polarity depending on whether a 3′ or 5′ end initiates the joint molecules. Although joint molecule formation occurs equally efficiently when initiated with a 3′ or 5′ overhanging end, branch migration proceeds more rapidly when it is initiated by an overhanging 3′ end, i.e., in the 5′ to 3′ direction with respect to the ssDNA.

MATERIALS AND METHODS

Proteins. Yeast Rad51 was expressed in insect cells and purified as described previously (3); yeast replication protein A (RPA) was expressed in *E. coli* by using the plasmid pJM126 (provided by S. Brill and B. Stillman, Cold Spring Harbor Laboratory, NY) and purified as described (5). The concentrations of Rad51 and RPA were determined by using extinction coefficients of 1.26×10^3 and 8.8×10^4 at 280 nm, respectively.

DNA Substrates. The concentration of the DNA substrates is expressed as nucleotide equivalents. Circular (+) strand ssDNA and plasmid dsDNA of pBluescript SK(+) (Stratagene) and its derivatives were prepared as described (6). ^{32}P-labeled (+) strand pBluescript SK ssDNA was prepared as follows. Cells containing pBluescript SK(+) plasmid were grown to A_{590} of 0.1 in Luria–Bertani medium containing 25 μg/ml of carbecillin, washed twice with minimal medium containing 0.4 mM KH_2PO_4, and then suspended in 70 ml of the same medium. After infecting the cells with VCSM13 helper phage (Stratagene) (multiplicity of infection of 10) and addition of 10 mCi of inorganic phosphate, phage were collected 10 hr later by polyethylene glycol precipitation from the supernatant that had been cleared of cells. ^{32}P-labeled (+) strand ssDNA of pBluescript SK(+) was extracted from the phage with phenol followed by two cycles of preparative agarose gel electrophoresis. pB442 was constructed by inserting a 442-bp *Eco*RI–*Xho*I fragment of unrelated DNA into pBluescript SK(+) DNA in place of a segment bounded by these restriction sites. Linear pBluescript SK(+) DNA and its derivatives with different types of termini were prepared by cleavage with appropriate restriction endonucleases; linear dsDNA with 3′ overhanging termini were made with *Alw*NI, *Apa*I, *Kpn*I, *Nsp*I, or *Pst*I endonucleases; linear dsDNA with 5′ overhanging termini were generated with *Bsa*HI, *Eco*RI, *Ngo*MI, *Xho*I, or *Xma*I endonucleases.

Strand Exchange. Strand exchange was measured by using the modified agarose gel assay described previously (3). In the standard reaction, ^{32}P-labeled circular (+) strand pBluescript SK(+) ssDNA (20 μM) was preincubated with Rad51 (5.3 μM) in buffer containing 40 mM K-Mes (pH 6.5), 1 mM DTT, 5% glycerol, 2 mM $MgCl_2$, and 0.5 mM ATP for 10 min at 37°C. RPA (1 μM) then was added; 10 min later, 78 μM linear dsDNA, 12 mM $MgCl_2$, and 3 mM ATP were added, and the reaction was incubated at 30°C. After adding 0.5% SDS and 0.5 mg/ml of proteinase K, the mixture was incubated at 37°C for 30 min. The concentrations are given as final. The products of the reaction were analyzed by gel electrophoresis on 1% agarose gel in TAE buffer containing 40 mM Tris acetate (pH 7.5), 0.5 mM EDTA, visualized by autoradiography, and quantitated by using a PhosphorImager (Molecular Dynamics).

Analysis of Heteroduplex DNA. The extent of heteroduplex DNA formed during strand exchange was analyzed as follows. After terminating the reactions, 20-μl samples were freed of SDS by gel filtration on Micro Bio-Spin 30 columns equili-

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Abbreviations: dsDNA, double-standed DNA; ssDNA, single-stranded DNA; RPA, yeast replication protein A.

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brated with Tris-EDTA (pH 7.6), and after adjustment of the volume to 120 μl with the appropriate buffer, 20-μl aliquots were cleaved with pairs of endonucleases to generate specific fragments. Cleaved samples were loaded on a 1% agarose gel in TAE buffer, and electrophoresis was performed at 2 V/cm for 6 hr. The gel was dried and autoradiographed.

Kinetics of Heteroduplex DNA Formation with Linear dsDNA Having 3′ or 5′ Overhanging Ends. Linear dsDNAs with either 3′ or 5′ overhanging termini were prepared by cleaving pBluescript SK(+) dsDNA with either *Apa*I or *Eco*RI endonuclease. The extent and rate of strand transfer between linear dsDNAs with either 3′ or 5′ overhanging termini and circular ^{32}P-labeled (+)-strand pBluescript SK(+) ssDNA were analyzed by cleavage with appropriate restriction endonucleases and determining the amount of ^{32}P-label in the individual fragments. The pairs of restriction endonucleases were *Kpn*I and *Bsa*AI, *Kpn*I and *Bsa*HI, *Bam*HI and *Afl*III, or *Bam*HI and *Alw*NI (see Fig. 2).

Kinetics of Heteroduplex DNA Formation During Strand Exchange with Linear dsDNA Having a Single 3′ or 5′ Complementary End. The rate of branch migration in the 5′ to 3′ direction on the ssDNA was determined by selected cleavage of the reaction products produced from ^{32}P-labeled (+)-strand pBuescript SK(+) ssDNA and pB442 linear dsDNA, which contained 442 bp of heterologous DNA at either the 5′ or 3′ end of the complementary strand. The products of the strand exchange were cleaved with the following pairs of restriction endonucleases: *Kpn*I and *Bsa*AI, *Kpn*I and *Bsa*HI, *Bsa*HI and *Alw*NI, *Alw*NI and *Afl*III, or *Alw*NI and *Pvu*II. Similarly, the regions of heteroduplex DNA formed in the 3′ to 5′ direction on the ssDNA were determined by analyzing the restriction fragments generated by the following pairs of restriction endonucleases: *Bam*HI and *Afl*III, *Bam*HI and *Alw*NI, *Alw*NI and *Bsa*HI, *Bsa*HI and *Bsa*AI, or *Bsa*HI and *Pvu*II. These pairs of restriction endonucleases produce fragments that correspond to different regions of heteroduplex DNA (see Fig. 4).

RESULTS

Rad51-Mediated Strand Exchange Is More Efficient with Linear Duplex DNA Having Complementary 3′ Overhanging Ends. Rad51 promotes formation of joint molecules and completely strand exchanged product only between circular ssDNA and linear dsDNA with a complementary overhanging end (3). Accordingly, it seemed likely that Rad51, unlike its prokaryotic counterpart, RecA, promotes branch migration in both directions and that the polarity of branch migration depends on whether a 3′ or 5′ overhanging end initiates the strand exchange. However, with M13 and φX174 DNA as substrates we were unable to follow each step of the strand exchange reaction because joint molecules were the major product with M13 DNA substrates and the completely exchanged product, nicked circular dsDNA, predominated with φX174 DNA substrates (3). To overcome that limitation, we used substrates derived from pBluescript SK(+) DNA. pBluescript SK(+) dsDNA, after a single cleavage, provided the linear dsDNA substrate and ^{32}P-labeled pBluescript SK(+) circular (+) ssDNA served as the other participant in the strand exchange. ^{32}P-labeled joint molecules and completely strand-exchanged nicked circular dsDNA can easily be separated by agarose gel electrophoresis and detected by autoradiography (Fig. 1).

With these DNA substrates, Rad51 promotes the formation of joint molecules and complete strand exchange with either 3′ or 5′ overhanging complementary ends at rates that are easily measurable (Fig. 1 *B* and *C*). The reaction has two phases: rapid formation of joint molecules and a slow accumulation of the fully exchanged product. After 20 min, about 35–40% of the circular ssDNA is converted to joint molecules irrespective of whether the linear dsDNA had 3′ or 5′ overhanging ends (Fig. 1*C*). The formation of the completely strand-exchanged

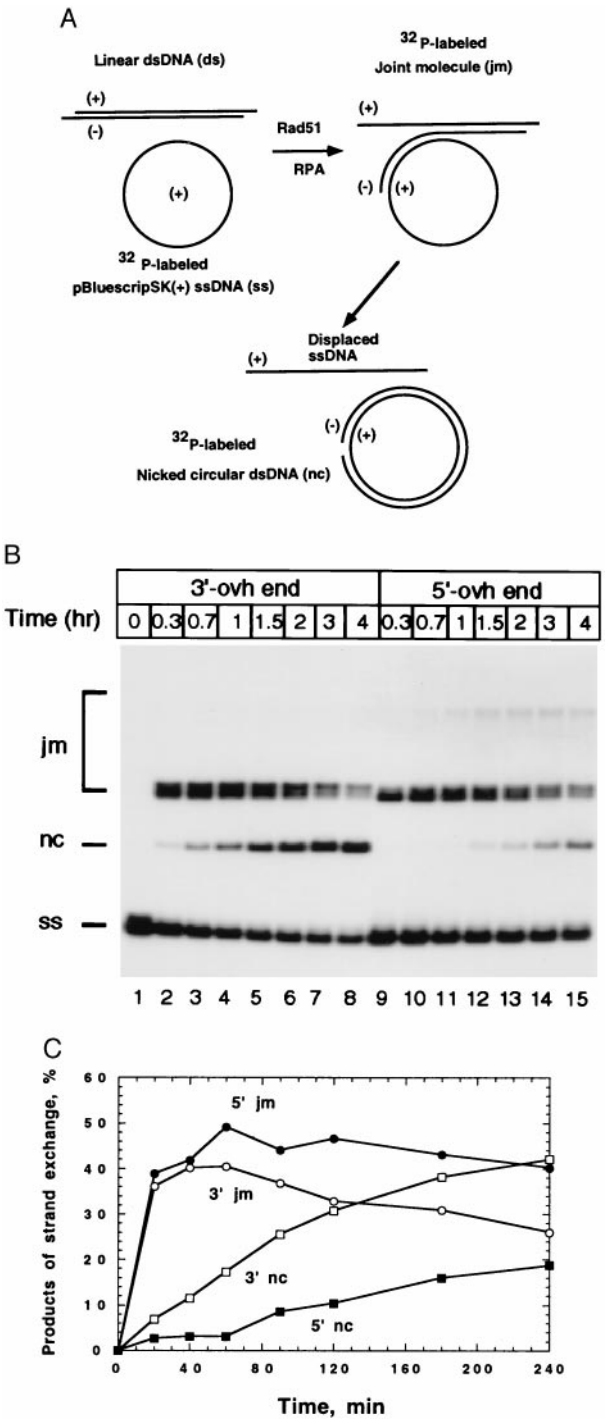

FIG. 1. Rad51-promoted strand exchange between circular ssDNA and homologous linear dsDNA. (*A*) DNA substrates, joint molecule, and completely strand-exchanged product (nicked circular dsDNA). ss, circular ssDNA; ds, linear dsDNA; jm, joint molecules; nc, nicked circular dsDNA. (*B* and *C*) Kinetics of strand exchange. After preincubation of ^{32}P-labeled pBluescript SK(+) circular ssDNA with Rad51 and RPA, the reaction was started by the addition of homologous linear dsDNA with either 3′ or 5′ complementary overhanging ends prepared by cleavage of the pBluescript SK(+) DNA with either *Apa*I or *Eco*RI restriction endonuclease. At the indicated times, samples (6 μl) were removed, and the DNA products were analyzed by agarose gel electrophoresis followed by autoradiography (B); quantitation of these data are shown in *C*. Joint molecules formed by linear dsDNA with 3′ (○) or 5′ (●) overhanging ends; nicked circular dsDNA formed by linear dsDNA with 3′ (□) or 5′ (■) overhanging ends.

product is considerably slower in both cases, but the rate is considerably faster (about three times) when strand exchange is initiated by a 3′ overhanging end compared with that with a 5′ overhanging end. To examine the possibility that a sequence near the 3′ end of the dsDNA influences the rate at which the final product is produced, linear dsDNA having different 3′ or 5′ complementary overhanging ends were produced by cleavage of the pBluescript SK(+) dsDNA at different sites. With each of the linear dsDNAs, the formation of final product was more efficient when initiated with linear dsDNA having a 3′ overhanging end than with a 5′ overhanging end. The preference is not affected by temperature or whether RPA is replaced by ssDNA binding protein (data not shown).

Heteroduplex DNA Forms First at Overhanging Complementary Ends of Linear dsDNA. Identifying the products of strand exchange by gel electrophoresis leaves open the question of where heteroduplex DNA forms first and how it is extended. Consequently, we determined the order and rate at which the dsDNA's complementary strand forms heteroduplex DNA with the circular ssDNA. Because joint molecules are formed predominantly in the first 20 min and maximal accumulation of the full strand-exchanged product occurs after 4 hr, appearance of the two products was analyzed at these two times (Fig. 1 *B* and *C*). Samples taken at 20 min and 4 hr were digested with proteinase K and SDS for 30 min, and after removal of the detergent and proteinase, the buffer was changed to Tris-EDTA by centrifugation in small gel filtration columns directly into tubes containing Mg^{2+} to avoid spontaneous branch migration (7). The samples then were incubated with pairs of restriction endonucleases to generate specific fragments, the presence of which are indicative of which parts of the dsDNA's complementary strand have entered into heteroduplex with the ssDNA (Fig. 2). Thus, restriction with *Kpn*I and *Bsa*AI endonuclease yields a 425-bp fragment (431 bp from the 3′ end of the complementary strand of dsDNA) and cleavage with *Kpn*I and *Bsa*HI produces a 1,029-bp fragment (1,035 bp from the 3′ end); similarly, restriction with *Bam*HI and *Afl*III yields a 434-bp fragment (452 bp from the 5′ end) and with *Bam*HI and *Alw*NI a 845-bp fragment (865 bp from the 5′ end). The amount of ^{32}P in each fragment was determined after electrophoretic separation and normalized to the amount of circular ssDNA converted into strand-exchanged products. Fig. 3*A* shows that when strand exchange is carried out with linear dsDNA having a complementary 3′ overhanging end, it is that end that is converted to heteroduplex DNA first (the 425- and 1,029-bp fragments) after 20 min; however, the 5′ end of that strand does not accumulate in heteroduplex DNA until 4 hr (the 846- and 434-bp fragments). Similarly, when linear dsDNA with a complementary 5′ overhanging end is used to initiate strand exchange (Fig. 3*B*), heteroduplex DNA is formed first from that end (the 434- and 846-bp fragments) and the 3′ end appears in heteroduplex considerably later (1,029- and 425-bp fragments). Clearly, in both cases, the sequences that enter into heteroduplex structure earliest are those closest to the end that initiates the strand transfer, whether it is a 3′ or 5′ end.

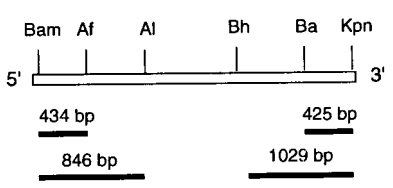

FIG. 2. Anticipated fragments produced by cleavage of the fully exchanged product of transfer of the complementary strand of linear pBluescript SK(+) dsDNA to ^{32}P-labeled circular ssDNA with pairs of restriction endonucleases. The following pairs of restriction endonucleases were used to generate the fragments shown: *Bam*HI (Bam) and *Afl*III (Af) (434 bp), *Bam*HI and *Alw*NI (Al) (846 bp), *Kpn*I (Kpn) and *Bsa*AI (Ba) (425 bp), or *Kpn*I and *Bsa*HI (Bh) (1,029 bp).

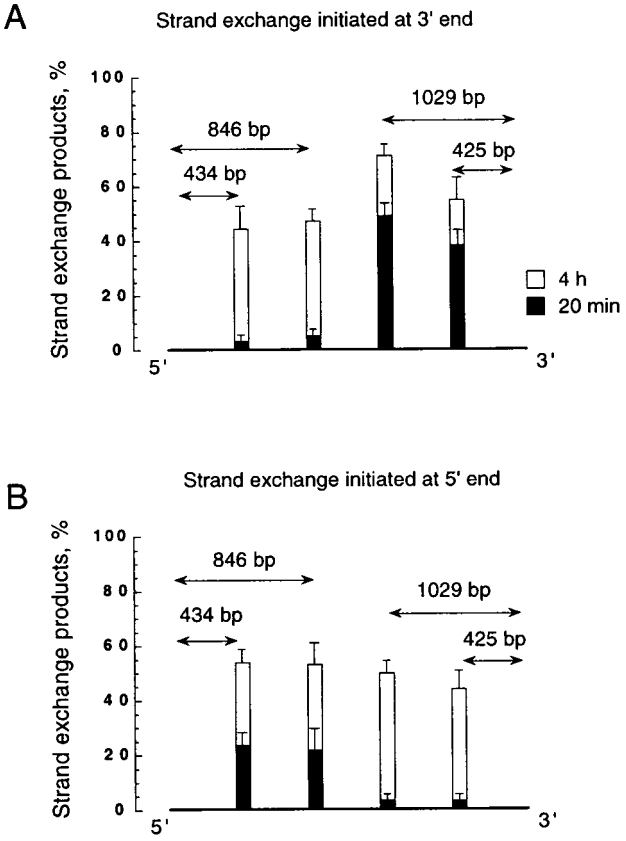

FIG. 3. Kinetics and nature of heteroduplex DNA formation when strand exchange is initiated at either a 3′ (*A*) or 5′ (*B*) overhanging end of the linear dsDNA's complementary strand. ^{32}P-labeled pBluescript SK(+) circular ssDNA was incubated with Rad51 and RPA, and then the reaction was initiated by the addition of linear dsDNA with either 3′ or 5′ overhanging ends. After 20 min and 4 hr, the extent of heteroduplex DNA formed was determined from the amount of ^{32}P-label in the specific dsDNA fragments produced by cleavage with the restriction endonucleases indicated in Fig. 2. The ordinate represents the mol percent of circular ssDNA that has been incorporated into each of the fragments, and the abscissa represents the linear "map" of the DNA.

Branch Migration Proceeds More Rapidly When Joint Molecules Are Initiated at a 3′ Complementary End. To follow the kinetics of branch migration more closely, we used linear dsDNA that contained a 3′ or 5′ complementary overhang at one end and a nonhomologous sequence at the other end. In such a reaction, joint molecules can be formed only from one end, and branch migration can proceed in only one direction. pB442 DNA contained a heterologous 442-bp insertion so that a single cleavage with *Apa*I endonuclease created a linear dsDNA with a complementary 3′ overhanging end and a nonhomologous sequence at the 5′ end; cleavage of the same plasmid with *Eco*RI endonuclease produced a comparable linear dsDNA with a complementary 5′ overhanging terminus and nonhomology at the other end. Strand-exchange reactions were carried out with each of these linear dsDNAs and ^{32}P-labeled pBluescript SK(+) circular ssDNA for 20 min and 4 hr. Joint molecules were formed at about equal rates with each of the linear DNAs and, as expected, fully strand-exchanged products were not detected (data not shown).

Cleavage of the products with pairs of restriction endonucleases yielded the fragments shown in Fig. 4; each fragment identifies a region of heteroduplex DNA and is diagnostic of the extent of branch migration after strand transfer is initiated

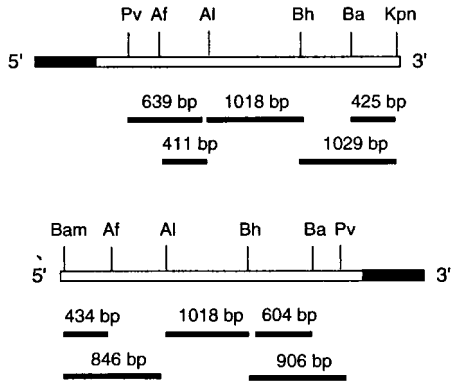

FIG. 4. Anticipated fragments produced by cleavage of the joint molecules formed by transfer of pB422 dsDNA's complementary strand to the ^{32}P-labeled circular ssDNA. Cleavage of pB422 dsDNA with *Apa*I produces a linear dsDNA with a complementary 3′ overhanging end and a nonhomologous 5′ end. Cleavage of the same plasmid with *Eco*RI endonuclease produces a linear dsDNA with a complementary 5′ overhanging end and a nonhomologous 3′ end. The extent of heteroduplex DNA formed from either type of linear dsDNA was determined from the fragments produced by cleavage with pairs of endonucleases. With linear dsDNA having a single complementary 3′ end these were *Kpn*I and *Bsa*AI (425 bp), *Kpn*I and *Bsa*HI (1,029 bp), *Bsa*HI and *Alw*NI (1,018 bp), *Alw*NI and *Afl*III (411 bp), and *Alw*NI and *Pvu*II (639 bp); with linear dsDNA having a single complementary 5′ end, the cleavages were produced with *Bam*HI and *Afl*III (434 bp), *Bam*HI and *Alw*NI (845 bp), *Alw*NI and *Bsa*HI (1,018 bp), *Bsa*HI and *Bsa*AI (604 bp), and *Bsa*AI and *Pvu*II (906 bp). Af, *Afl*III; Al, *Alw*NI; Bam, *Bam*HI; Ba, *Bsa*AI; Bh, *Bsa*HI; Kpn, *Kpn*I; and Pv, *Pvu*II.

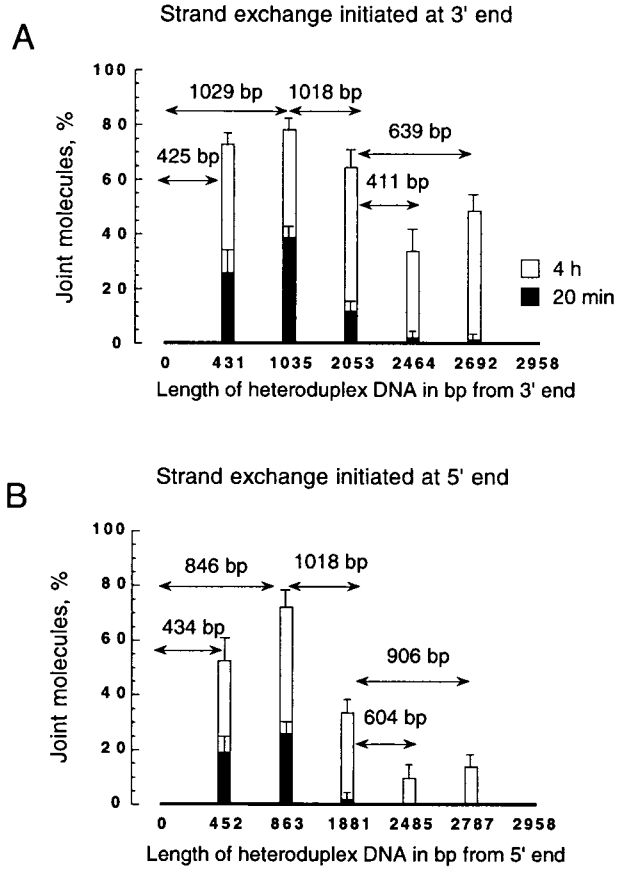

FIG. 5. Kinetics and nature of the heteroduplex DNA formation when single-strand exchange is initiated from a single 5′ or 3′ complementary overhanging end. ^{32}P-labeled pBluescript SK(+) circular ssDNA was incubated with Rad51 and RPA, and then pB422 linear dsDNA with a homologous 3′ or 5′ overhang at one end and a heterologous sequence at the other end of the complementary strand was added, and the reaction was sampled at 20 min and 4 hr. The direction and rate of heteroduplex DNA formation was determined by analyzing the specific fragments shown in Fig. 4 as described in *Materials and Methods*. The results are shown as the mol percent of circular ssDNA that appears in the fragments of the indicated sizes as they are located along the length of the heteroduplex DNA.

from either the initiating complementary 3′ or 5′ overhanging end. Fig. 5*A* shows that after 20 min most of the joint molecules that initiated at the linear dsDNA's complementary 3′ end contain heteroduplex DNA about 1.5 kbp in length (the 425-, 1,029-, and 1,018-bp fragments); very little heteroduplex DNA is longer than about 2.5 kbp (the 411- and 639-bp fragments). After 4 hr, the heteroduplex DNA extends to lengths longer than 2.7 kbp in about half of the joint molecules. Similar measurements of strand exchange initiated at the 5′ complementary end of the linear dsDNA (Fig. 5*B*) show that about 1 kbp of heteroduplex DNA (the 434- and 846-bp fragments) is formed by 20 min and only after 4 hr has the strand transfer reached 2.7 kbp (the 604- and 906-bp fragments). In each case, it takes only 20 min for heteroduplex formation to reach a length about 1.2 kbp and more than 10 times longer for the heteroduplex to extend to 2.7 kbp.

These experiments provide strong support for our earlier suggestion (3) that strand exchange mediated by Rad51 is initiated by either 3′ or 5′ complementary overhanging ends of linear dsDNA and that the ensuing branch migration proceeds in either the 3′ to 5′ or 5′ to 3′ direction on the single strand. Furthermore, once formed, the rate at which the length of the heteroduplex DNA is extended by branch migration is about three times faster when joint molecules are initiated by a 3′ end than with a 5′ end. Thus, considering that the formation of completely exchanged product is about 3-fold faster when the strand exchange is initiated by a 3′ complementary overhanging end compared with a 5′ complementary overhanging end, we surmise that the rate of branch migration is the limiting step in the strand-exchange reaction.

DISCUSSION

Our earlier work indicated that Rad51 promotes strand exchange only between circular ssDNA and linear dsDNA having complementary overhanging ends. Accordingly, we reasoned that once initiated, branch migration can proceed in either direction depending on which end is used to create the joint molecule (3). In the present work, we made direct measurements of the kinetics of both the initiation step and the subsequent formation of heteroduplex DNA during the strand-exchange reaction. This work was facilitated by replacing the M13 and ϕ174 DNA-based substrates used previously with pBluescript SK(+) linear DNA and complementary ^{32}P-labeled circular ssDNA. This change facilitated the detection of the strand-exchange products and readily identified two kinetically distinguishable phases: a rapid formation of joint molecules and a slow accumulation of completely exchanged product. Furthermore, strand transfer of the linear dsDNA's complementary strand to the ^{32}P-labeled circular ssDNA creates ^{32}P-labeled heteroduplex DNA that can easily be analyzed by restriction analysis.

Irrespective of whether the linear dsDNA's overhanging end is 3′ or 5′, joint molecules are formed with equal efficiency (Fig. 1 *B* and *C*). However, the appearance of fully exchanged product is considerably slower, but about three times faster when the joint molecules are initiated by a linear dsDNA with 3′ overhanging ends. This finding suggests that once strand transfer is initiated, branch migration in the 5′ to 3′ direction on the ssDNA is favored. Interestingly RecA, the functional

homologue of Rad51, promotes branch migration in only one direction, the 5′ to 3′ on the ssDNA (8, 9).

In the course of the strand exchange, the linear dsDNA's complementary strand is transferred to the circular ssDNA, forming a heteroduplex DNA. To follow the course of that transfer the extent of heteroduplex formation was followed after 20 min and 4 hr of incubation by examining the restriction fragments produced by digestion of the strand-exchange products with pairs of restriction endonucleases. Our results show that at the early time, when most of the product is in the form of joint molecules, heteroduplex DNA is formed from the region closest to the dsDNA's complementary overhanging end (Fig. 3). As strand exchange proceeds, the extent of heteroduplex DNA increases to include the other end of the complementary strand. With linear DNA containing a stretch of 442 bp of nonhomologous sequence at either the complementary strand's 3′ or 5′ end, it was possible to follow branch migration in only one direction. The results show that after 20 min the joint molecules contain heteroduplex lengths up to 1.5 kbp of DNA when strand exchange is initiated by either 3′ or 5′ overhanging ends, and that the formation heteroduplex DNA proceeds mostly to completion (2.7 kbp) after about 4 hr (Fig. 5). Here, too, it is apparent that Rad51 promotes branch migration in the 5′ to 3′ direction of ssDNA more effectively than in the opposite direction. Thus, although branch migration during Rad51-promoted strand exchange can proceed in either direction there is a preference for the 5′ to 3′ direction on the single strand.

Although both Rad51 and RecA promote strand exchange between circular ssDNA and linear dsDNA, there are notable differences in the mechanism of strand exchange, the most distinctive being that in the presence of ATP the branch migration and extensive heteroduplex formation promoted by RecA is unidirectional (8, 9). Bidirectional branch migration is, however, a characteristic of RecA-promoted strand exchange in the presence of nonhydrolyzable ATP analogues (10, 11) and with the ATP-hydrolysis deficient mutant RecA K72R in the presence of dATP (12, 13). It is interesting that strand exchange promoted by Rad51 is accompanied by low rates of ATP hydrolysis compared with that of RecA; indeed, Rad51 may require ATP only for activation of the protein and DNA binding. Notable in this regard is that with RecA K72R in the presence of dATP, whose hydrolysis occurs with low efficiency, branch migration is characteristic of Rad51, i.e., bidirectional. Our findings of the mechanistic behavior of Rad51 performed with the purified protein should be interpreted cautiously, however, because Rad51 acts in recombination together with Rad52, Rad54, Rad55, Rad57, and possibly others, and these may alter the characteristics of the strand exchange (14–16).

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