Structure of Reaction Intermediates Formed During Saccharomyces cerevisiae Rad51-catalyzed Strand Transfer

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Summary

The process by which the *Saccharomyces cerevisiae* strand transfer protein, Rad51, seeks out homologous sequences in vivo can be modeled by an in vitro reaction between a single-stranded DNA circle and a double-stranded linear DNA. In addition to the substrates and products, electrophoresis of reaction mixtures resolves two groups of low-mobility bands. Here we show that the low-mobility bands formed during strand transfer by Rad51 (or *Escherichia coli* RecA) represent joint-molecules (JM) between the two substrates. One group, which we name JM1, is an obligatory reaction intermediate in which the complementary strand from the duplex substrate has been partially transferred to the single-stranded circle. Our assignment is based on pulse-chase and restriction enzyme digestion experiments and verified by electron microscopy. The slower moving group of bands, designated JM2, is formed by an unexpected reaction between JM1 and a second double-stranded linear substrate. Strand transfer of the second duplex initiates non-canonically from the end where the complementary strand is recessed. Thus JM2 is formed by two strand transfer reactions with the same single-stranded circular substrate but with opposite polarities. Finally, we show that the multiple sharp bands in JM1 and JM2 are due to substrate sequences that pause strand transfer.
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Introduction

During the initial steps of DNA double-strand break repair, a strand-transfer protein such as *Saccharomyces cerevisiae* Rad51 forms a filament along the 3' single-stranded tails generated from broken DNA ends (1). The resulting nucleoprotein filament then searches the genome for sequences homologous to the tail that can serve as templates for the repair synthesis needed to restore the continuity of the chromosome (2). Rad51 is called a strand transfer protein because it catalyzes the invasion of the single-stranded tail into the intact template duplex and transfers base pairing interactions from the identical strand of the template duplex to the tail. The 3' end of the transferred tail can then serve as a primer for repair synthesis (3). The mechanisms of the homology search and subsequent strand transfer are not well understood. Indeed, it is difficult to imagine a process that achieves both the speed and fidelity with which a sequence homolog is found in vivo.

The homology search process and the broader mechanism of strand transfer proteins have been studied using an in vitro strand transfer reaction that recapitulates the first steps of homologous recombination (4) (Fig. 1A). Whereas early work focused on the *Escherichia coli* RecA protein (5), the *Saccharomyces cerevisiae* Rad51 protein has been favored for studies of recombination in eukaryotes (6,7).

In the model strand transfer reaction, Rad51 coats a single-stranded circular (ssC) DNA to form a nucleoprotein filament that associates with a homologous double-stranded linear (dsL) DNA. Once the nucleoprotein filament has aligned the homologous partners in a paranemic joint, which involves no net intertwining of the substrate DNAs, strand transfer begins from one end of the dsL substrate (8). The strand of the dsL substrate that is complementary to the single-stranded circle, hereafter referred to as the complementary strand,
unwinds from the duplex substrate and anneals to the ssC substrate. Unidirectional branch migration, requiring ATP, continues along the dsL substrate until nicked circular (NC) and single-stranded linear (ssL) products are released (9,10). The yeast single-strand binding protein, RPA, is required for strand transfer both to facilitate Rad51 coating of the ssC substrate and to sequester the single-stranded DNA products (11,12). The two substrates and the two products are easily separated by agarose gel electrophoresis after deproteinization.

Rad51 is unique among strand transfer proteins in that it can catalyze branch migration in either the 3' to 5' or 5' to 3' direction (13-15). Namsaraev and Berg, however, reported that in vitro strand transfer requires that the duplex substrate have a single-stranded overhang that is complementary to the DNA in the filament (16). The reaction initiates at the complementary overhang, which can be a 5' or 3' end, and proceeds across the duplex substrate (16). Thus the polarity of the Rad51 strand transfer reaction is determined only by where initiation occurs.

In addition to the two products, strand transfer reactions produce two groups of low electrophoretic mobility species, each comprised of several sharp bands, as shown in Figure 1B and C. Although these species have been frequently observed for both RecA and Rad51 reactions (10,15,17-22), their structure and precise role in strand transfer have been unclear. Kinetic analysis of the yeast Rad51 reaction has shown that low-mobility species accumulate in an early fast phase and then dissipate during a second, slower phase as products accumulate (16). In this report we show that the low-mobility bands formed by yeast Rad51 represent joint molecules (JM) between the two substrates. Based on experiments that determine the stoichiometry and orientation of the dsL substrates within both JM groups, we conclude that the faster-migrating group, designated JM 1, is made up of obligatory reaction intermediates comprised of one ssC and one dsL substrate. The multiple sharp bands seen in the JM 1 and JM 2 groups and the accumulation of branch points measured by electron microscopy (EM) reveal positions in the
substrate that pause branch migration during strand transfer. The slower moving group, called JM2, contains complexes formed between one ssC substrate and two dsL molecules. We show that the second dsL substrate in JM2 reacts from the opposite end from the first and thus the two branch points migrate in opposite directions.

**Experimental Procedures**

**Protein Purification**

Rad51 was purified as described by Sung (10) except that the Sephacryl S200 column was omitted and the final MonoQ column was used twice, first to purify and then to concentrate the protein. Small aliquots of purified Rad51 were frozen in liquid nitrogen and stored at −80°C. Rad51 storage buffer contained 10 mM potassium phosphate (pH 7.5), 0.35 M KCl, 0.5 mM dithiothreitol, 0.5 mM EDTA, and 10% glycerol. Replication protein A (RPA) was purified from yeast over-expressing all three subunits (23,24). It was stored in small, single-use aliquots at −80°C in 25 mM Tris-HCl (pH 7.5), 0.35 M KCl, 0.5 mM dithiothreitol, 0.5 mM EDTA, and 10% glycerol.

**DNA preparation**

Strand transfer reactions were performed with single-stranded circular (ssC) and double-stranded linear (dsL) forms of the pBluescript SK+ phagemid (pBS, Stratagene). Supercoiled DNA was purified from E. coli using two cesium chloride/ethidium bromide isopycnic bandings (25). Double-stranded linear DNA substrates were obtained by digesting supercoiled DNA with the restriction enzymes (NEB) indicated in Figure 3. The single-stranded circular phagemid DNA was rescued from XL1-Blue E. coli cells containing pBS as described (26). For reactions
with a heterologous insertion in one substrate, the ssC was prepared from a deletion derivative of pBluescript constructed by restriction enzyme digestion of the multiple cloning sequence with XhoI and NotI to excise a fragment of 74bp, treatment with the Klenow fragment of DNA polymerase I to yield blunt ends, and ligation with T4 DNA ligase.

**Strand Transfer Reactions**

Heterologous strand transfer reactions were performed using ssC DNA with a 74-base deletion and pBS dsL DNA without the deletion. Homologous reactions used ssC without the deletion. Throughout the paper we refer to the heterology as an insertion in the dsL, which is functionally equivalent to a deletion in the ssC. For the RecA strand transfer reactions (27), 4 µg of RecA was mixed with 0.12 µg of single-stranded DNA in 18 µl of 20 mM HEPES (pH 7.5), 10 mM MgCl₂, 1 mM dithiothreitol, 3 mM ATP, 10 mM phosphocreatine, and 10 U/ml creatine kinase. After 5 min at 37°C, 2 µg of E. coli SSB was added. After 10 min, 0.24 µg of homologous double-stranded DNA was added and the 25 µl reaction continued for the times indicated. The reaction was stopped by addition of 1/10 volume of 5% SDS and 50 mM EDTA.

The Rad51 strand transfer reaction was performed as described by Sung and Stratton (28). Seven µg of Rad51 was added to 0.12 µg of single-stranded circular DNA in 7.5 µl of 40 mM potassium MOPS (pH 7.2), 3 mM MgCl₂, 1 mM dithiothreitol, and 2.5 mM ATP. After 5 min at 37°C, 1.5 µg of RPA was added and incubation continued for 10 minutes. Finally, 0.24 µg of double-stranded linear DNA and spermidine to a final concentration of 4 mM were added for a total reaction volume of 13 µl. Incubation was continued for 30 min unless otherwise noted in the text. The reaction was stopped by adding one volume of 20 mM Tris-HCl (pH 7.5), 20 mM EDTA (pH 8.0), 1% SDS and 1 mg/ml proteinase K, and incubating another 30 min at 37°C. The
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DNA was resolved by electrophoresis through a 0.9% agarose gel in TBE buffer (11-19 V/cm for 600-850 V·h) and then visualized by staining with ethidium bromide or by Southern blotting. Quantification of Southern blots was done with a Fuji phosphorimager.

**Pulse-chase experiment**

The standard strand transfer protocol was followed using BsaI-cut dsL DNA. Seven minutes after addition of dsL DNA that had been end-labeled with $^{32}$P, the 13 µl reaction was mixed with 2 µl of 1.6 µg/µl unlabeled dsL DNA (14-fold excess). Samples of 2 µl were removed at times thereafter, diluted to 12 µl, stopped as usual, and analyzed by gel electrophoresis.

**Two-dimensional gel electrophoresis**

A standard strand transfer reaction was run in a TBE buffered gel as the first dimension. The lane was excised and rotated 90 degrees. For the second dimension, a new gel was poured around the excised lane, soaked in 0.5 N NaOH and 1 mM EDTA to denature the DNA, and run in the same NaOH solution at 4 V/cm for 20 hours at 4°C. The gel was re-equilibrated with TBE buffer before Southern blotting.

**Electron microscopy**

DNA bands in agarose gels were excised, extracted by electro-elution (29) or by the freeze-squeeze technique (30), and resuspended in 10 mM Tris (pH 7.5) and 1 mM EDTA (pH 8.0). The purified DNA was then incubated with *E. coli* single-strand binding protein (SSB) (Pharmacia) for 15 min at 37°C so that the coated single-stranded DNA could be distinguished from duplex DNA (31). The DNA-protein complex was then fixed with 0.02% glutaraldehyde for 15 min at 37°C. Unbound protein was removed by size exclusion chromatography on Sepharose CL-4B.
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(Pharmacia). Samples were then applied to a carbon grid using the poly-L-lysine technique (32), rotary shadowed with tungsten, and visualized with a JEOL electron microscope. Photographs were taken at 25,000X magnification and molecules were measured with a Numonics image digitizer.

**Results**

To determine whether formation of the two groups of low-mobility DNA species is a general property of strand transfer proteins, we performed parallel homologous strand transfer reactions with *S. cerevisiae* Rad51 and *E. coli* RecA (Fig. 1B). We find that the two distantly related strand-transfer proteins generate similar groups of JM bands, suggesting that the bands indeed represent structures that are fundamentally important to the strand transfer reaction. All subsequent experiments used Rad51 and RPA.

We next investigated the relationship between the two slow-moving sets of bands with a pulse-chase experiment. A strand transfer reaction was initiated by addition of $^{32}$P-labeled dsL DNA to a Rad51-coated ssC DNA. After a brief incubation, a 14-fold excess of unlabeled dsL DNA was added. Samples of the reaction were then taken at various times and analyzed by gel electrophoresis (Fig. 1C). At the first time point, immediately after the chase DNA was added, the reacted labeled DNA had formed almost exclusively JM1 bands, suggesting that these bands correspond to the simple reaction intermediate. Over the next fifteen minutes, the majority of the labeled DNA moved into the JM2 region, suggesting that these bands correspond to a more complex structure derived from JM1. The amount of JM2 species peaked at 30 minutes and declined thereafter. After 15 minutes, a portion of the labeled DNA had matured into NC product. Because both JM1 and JM2 turn over, it was not possible to distinguish whether the NC product came from one or both of the low-mobility species.
Three lines of evidence, described next, show that DNA in the JM1 region corresponds to a three-stranded intermediate where one dsL has initiated strand transfer with the ssC (Fig. 1A, middle diagram). We also show that the sharp bands in the JM1 region are due to the accumulation of branch points at sequences in the substrate that pause branch migration.

The mobility of JM1 bands depends on the position of a heterology

We used a substrate plasmid with an inserted 74-base pair heterology to stop branch migration at a defined point (22). The heterology-containing plasmid was digested with one of six restriction enzymes to yield substrates with the heterology at the beginning, middle, or end of the molecule. Enzymes that yielded either 3' or 5' complementary overhangs were chosen to test strand transfer in both polarities (Fig. 2A). Homologous reactions, where the ssC DNA also contained the inserted sequence, yielded NC product for all six dsL substrates, showing that the substrates were competent for strand transfer (as in Fig. 2B, Hom. lane). Unique and reproducible sets of JM1 bands appeared in both homologous and heterologous strand transfer reactions.

The first result supporting our proposed structure of the JM1 species was a clear correlation between the position of the heterology within the dsL substrate and the electrophoretic mobility of the predominant JM1 band in each heterologous reaction (Fig. 2C). The mobility of the JM1 molecules diminished as the heterology was placed farther from the initiating overhang.

This JM1-mobility result is easily explained by our proposed structure for the three-stranded intermediate. Neither the total molecular weight nor the total length of duplex can change during branch migration, and thus neither can be responsible for the differences in electrophoretic mobility. The duplex region, however, shifts from a linear to a circular form as
the reaction continues. Just as NC DNA migrates more slowly than dsL DNA of the same molecular weight, intermediates that have nearly completed strand transfer and have mostly circular duplex DNA should migrate more slowly than intermediates that have stopped strand transfer early and have mostly linear duplex DNA.

The curve in Figure 2C was used to map two branch-migration pause sites in pBluescript. The mobilities of the one or two minor bands in each JM1 region were measured and, based on the standard curve, the locations of the pauses were interpolated. JM1 groups with zero or one minor band corresponded to dsL substrates where at least one pause site was beyond the heterology (i.e. NgoMIV or NspI).

When the six substrates were aligned by the position of the heterology and the initiating end, as in Fig. 2A, the interpolated pauses clearly clustered around two sites. The interpolated positions in each cluster were averaged, yielding pause sites at roughly 20% and 70% from the heterology 5’ along the complementary (plus) strand of pBluescript (Fig 2A, gray bars). The mobility of minor JM1 bands is plotted against the position of the averaged pause sites for each substrate in Figure 2C (gray squares). These points are horizontally offset from the curve because the pause site positions were averaged after interpolation. The inferred positions of the pause sites were confirmed by EM studies described below.

**Restriction digests of purified JM1 species support the proposed structure of the three-stranded intermediate**

The second line of evidence for the JM1 structure is the agreement between the predicted restriction digestion products of a three-stranded intermediate and bands seen after digestion of purified JM1 DNA (Fig. 3). The complementary strand in the three-stranded intermediate is paired to two partners, the circular DNA at its proximal end and the linear DNA at its distal
end. If branch migration proceeds beyond restriction sites close to the either end of the complementary strand, digestion with appropriate restriction enzymes will yield information about the structure by linearizing the circle or cutting only one of the strands of the duplex.

After the joint molecules are deproteinized, the heterology prevents branch migration forward into products. Branch migration backward, however, can cause the joint molecules to fall apart if the branch point reaches the end of the complementary strand. Molecules that fall apart before digestion will yield substrates and not be informative. Molecules that fall apart after digestion, however, will yield the diagnostic fragments shown in Figure 3A.

JM1 DNA from a heterologous strand transfer reaction with $^{32}$P end-labeled dsL DNA was purified from an agarose gel, digested with XmnI and AlwNI that cut approximately 500 base pairs from the proximal and distal ends of the dsL, respectively, and analyzed again by gel electrophoresis (Fig. 3B). Besides the expected band produced by digestion of the free linear duplex (cut dsL), two new bands appeared with mobilities that were consistent with the predicted products of digestion of the three-stranded intermediate. The upper band migrated at the expected position of a dsL DNA with a 500-base single-stranded tail at the proximal end. The lower band matched the other structure predicted from the three-stranded intermediate, the 500-base proximal fragment of the complementary strand annealed to the end of the linearized ssC substrate.

Direct visualization of the three-stranded intermediate by EM

The third and most direct line of evidence linking bands in the JM1 region to the three-stranded intermediate is the direct visualization of purified JM1 bands by EM. Joint molecule DNA from preparative heterologous strand transfer reactions was isolated from agarose gels and
coated with *E. coli* single strand binding protein (SSB). SSB thickens single-stranded DNA so that it is visually distinct from double-stranded DNA.

Figure 4 shows representative images of JM1 molecules from heterologous reactions along with schematic traces of the DNA paths. These images clearly show the expected three-stranded intermediate structure with the duplex region split between linear and circular paths. The displaced single-stranded tail and unreacted portion of the ssC, coated by SSB, are also clearly visible. Of several hundred molecules that we identified as being JM1, over 90% could be traced as a duplex linear tail and a partially duplex circle. The remainder was composed mostly of linear duplex reacting with linear single-stranded DNAs, which may correspond to complexes with broken circles or to reactions between the ssL product and a duplex substrate.

The position of branch points observed with electron microscopy confirmed that branch points in JM1 accumulate at the heterology. Table 1 lists measurements from EM images from reactions of the six substrates in Figure 2A. Because SSB-coated single-stranded DNA variably appears two-to-three-fold shorter than double-stranded DNA of the same length (31), we used only the more accurate length of the double-stranded DNA in our quantitative analysis. The extent of branch migration was calculated as the fraction of duplex DNA that was in the circle. For the predominant band in each heterologous reaction there was excellent agreement between the fraction of strand transfer and the location of the heterology relative to the complementary overhang of the dsL substrate. In particular, we find no molecules where the branch point is beyond the heterology, confirming that it cannot be bypassed.

We purified the lower JM1 band from a reaction with AflIII-cut dsL DNA separately from the upper two JM1 bands. We found the fraction of the circle that was duplex in molecules from the lower band indicated an average branch migration of 14±8%, consistent with the position of the first predicted pause site at 16%. Molecules in the upper bands showed a bimodal
distribution with averages of 64±8%, consistent with the predicted position of the second pause site at 66%, and 83±4%, consistent with the expected position of the heterology at 86% (Table 1).

Having established the structure of the JM 1 species, we turned to determining the structure of the JM 2 intermediate.

**JM 2 species contain two dsL molecules per ssC substrate**

The kinetic studies of the maturation of JM 1 (Fig. 1C) suggest that JM 2 is derived from JM 1 but many possible structures are consistent with that pathway. For example, JM 2 species may be comprised of two circles reacting with a single dsL DNA, or to a structure where the displaced ssL tail has reacted with a second dsL or with a second JM 1. We distinguished among the possibilities with two biochemical experiments and EM of purified JM 2 structures.

First, we examined the effect of dsL substrate concentration on the amount of JM 2 formed. Strand transfer reactions were performed with the usual amount of ssC but between a 1:1 and 4:1 molar ratio of dsL to ssC (Fig. 5A). Whereas the amount of JM 1 increased less than 1.5 fold across the titration, the amount of JM 2 increased 4.4 fold, suggesting that JM 2 formation involves multiple dsL molecules per ssC DNA.

Second, we determined the stoichiometries of the dsL to ssC substrates within the JM 2 species. We examined a standard homologous strand transfer reaction by two-dimensional gel electrophoresis in which the first dimension was run in standard TBE buffer and the second in denaturing conditions where all joint molecules separate into constituent ssC and ssL DNAs (Fig. 5B). Averaging three experiments, which were each normalized to a 1:1 ratio of ssL to ssC for the NC product, we found that JM 1 contained 2.1±0.5 ssL strands for each ssC DNA and that JM 2 contained 3.3±0.7 ssL strands per ssC DNA. The observed ratio for JM 1 met the expectation
for the simple strand transfer intermediate described above. The observed ratio for JM 2 supported our conclusion of a JM 2 structure with more than one dsL per ssC. The precision of the results did not allow us to distinguish between a structure with three linear single strands per ssC, a structure with four linear single strands per ssC (two dsL molecules per circle), or a mixture of the two.

We decided among these alternatives by visualizing gel-purified JM 2 species by EM (see Fig. 7). Although the structures are complex, we were able to confidently identify molecules with two duplex tails emanating from a circular DNA. We therefore conclude that the JM 2 species are structures in which a second dsL DNA has reacted with the circular portion of a JM 1 to give a complex strand transfer intermediate with two branch points.

**Two possible models for the JM 2 structure**

Given the structure of JM 1 molecule, there are two possible ways in which a second dsL can react to form JM 2, distinguished by which end of the second dsL initiates strand transfer as diagrammed in Figure 6A. Both possibilities are interesting as they require reactions that have not been identified previously for Rad51. In the first possibility, the second dsL (blue and black) uses the complementary overhang to initiate strand transfer at the same site as the first dsL DNA (Fig. 6A, #2). For this to occur, the annealed strand of the first dsL substrate must somehow be unwound from the ssC before the identical strand from the second dsL substrate can replace it. As the reaction continues, the second branch point will follow the first around the circle (Fig. 6A, #3), replacing duplex made from the first complementary strand with duplex made from the second. An analogous reaction, called a 4-strand exchange, has been reported to occur with RecA, albeit inefficiently (33), but has never been shown to occur for Rad51. We hereafter refer to this model of JM 2 structure as strand replacement.
If the second dsL instead initiates strand transfer from the opposite end as the first (Fig 6A, #4), then the two branch points will proceed around the circle in opposite directions (Fig. 6A, #5). This model takes advantage of the unique ability of Rad51 to promote strand exchange in both the 3' to 5' and 5' to 3' direction (13). However, whereas Namsaraev and Berg have reported that strand transfer initiates only from the complementary overhang (16), in this model the second dsL substrate must initiate via the end where the complementary strand is recessed. We refer to this model for JM2 formation as reverse initiation.

**Rad51 can initiate strand transfer without a complementary overhang**

Because the reverse initiation model contradicts the conclusions of Namsaraev and Berg, we tested for ourselves whether Rad51 requires a complementary overhang to initiate strand transfer. Plasmid DNA was linearized with enzymes that leave 5' or 3' complementary overhangs immediately adjacent to either side of the heterology such that for each kind of overhang, one substrate was blocked at the proximal end and could not initiate at the complementary overhang while the other was blocked at the distal end and could initiate as usual (Fig. 6B).

Homologous strand transfer reactions yielded both JM1 and NC product, showing that all four substrates were competent for strand transfer (as in Fig. 6C, Hom. lane). Heterologous reactions with the two distally-blocked dsL substrates, BamHI and ApaI, yielded the expected JM1 bands. Surprisingly, a small amount of JM1 also formed in the two reactions with the proximally-blocked XhoI- and PstI-cut dsL substrates. The proximally-blocked JM1 were ~8% the intensity of the distally-blocked JM1.

We conclude that the JM1 species formed with proximally-blocked substrates likely correspond to reverse initiation events for two reasons. First, Rad51 strand transfer is strongly impeded by nine-base heterologies and blocked by heterologies more than fourteen bases long.
(22). The 74-base heterology used here should completely block strand transfer. Second, if the heterology were somehow bypassed, the positions of pause-site-derived bands would match between the proximally- and distally-blocked reactions with the same overhang. Instead, the pause-site derived JM 1 bands from the proximally-blocked substrates match those of distally-blocked substrates with the opposite overhang. For example, the JM 1 bands in the PstI reaction, which has 3′ overhangs on the dsL substrate, match the JM 1 bands of the BamHI reaction, which has 5′ overhangs on the dsL substrate.

Because Rad51 can initiate strand transfer, albeit at low frequency, from a dsL end where the complementary strand is recessed, the reverse initiation model for JM 2 formation remains a possibility. We distinguished decisively between the two models for JM 2 structure by electron microscopy and diagnostic restriction digests described below.

**EM of JM 2 structures favor the reverse initiation model**

JM 2 DNA from homologous strand transfer reactions with three different dsL substrates, AflIII, NspI, and AlwNI, was purified by agarose gel electrophoresis, coated with *E. coli* SSB to accentuate single-stranded regions, and examined by EM. Figure 7 shows four of the JM 2 structures from NspI-cut DNA with schematic traces of the DNA-strand paths.

Both models for JM 2 formation predict complex structures with partially duplex circles and ssL and dsL tails. The EM images were difficult to trace unambiguously, but we found thirty-three molecules that we believed were JM 2 structures. All of these had two dsL tails emanating from a partially duplex circular DNA.

The reverse initiation model predicts that the two dsL tails will be separated by both duplex and single-stranded regions of the circle. Fourteen of the thirty-three (42%) molecules
rigorously met this criterion: each dsL tail could be clearly traced to a four-way junction with single-stranded and double-stranded regions of the circle and a single-stranded tail (Fig. 7a-c).

The strand replacement model predicts, in addition to one tail emanating from a four-way junction as above, either one or two dsL tails emanating from within the duplex region of the circle, depending on whether there is annealing of the two single-strands displaced at the second branch point. On thermodynamic and kinetic grounds, two complementary single-stranded DNAs tethered close together in space are expected to anneal. Although we found no molecules with the expected two tails, two of the thirty-three molecules (6%) appeared to have a single dsL tail emanating from a duplex region of the circle (Fig. 7d, arrow).

Seventeen molecules (51%) were considered inconclusive because the exact junction of at least one dsL tail with the circle was obscured by the SSB-coated single-stranded DNA. Most of these molecules, however, favored the reverse initiation model because the dsL tails did not appear to originate from within the duplex region of the circle.

Seven of the thirty-three molecules appeared to have a completely duplex circle. Both models are consistent with this structure. The reverse initiation model predicts this when the two forks meet. The strand replacement model predicts this only if the first fork completes strand transfer and the two displaced single-stranded tails anneal. A corollary of the strand replacement model, however, is that molecules with three dsL tails should be observed if the first fork has not completed strand transfer. None of the observed thirty-three molecules had three dsL tails, arguing against the strand replacement model.

**Restriction-enzyme digests of purified JM 2 confirm the reverse initiation model**

Because the two models differ by which end of the second dsL substrate initiates strand transfer, we readily distinguished between them by digestion of purified JM 2 with enzymes that
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cut at either end of the dsL DNA (Figure 8A). A strand transfer reaction was initiated with an excess of unlabeled dsL DNA to saturate the ssC substrate. After a brief incubation to allow JM1 formation, an equal amount of 5'-$^{32}$P labeled dsL substrate was added to generate JM2 in which only the second dsL DNA was labeled. The formation of JM2 with both dsL DNAs labeled was minimal, because only a minute fraction of the radioactive DNA formed JM1 (data not shown). The JM2 molecules were purified by gel electrophoresis, digested with AlwNI, XmnI, or both, and analyzed by agarose gel electrophoresis (Fig. 8B).

The predictions of the two models differ in three ways. The first difference is in the predicted electrophoretic mobilities of the cleaved JM2 molecules (Fig. 8A, cut JM2). The strand replacement model predicts JM2 mobility shifts similar to those seen for JM1 (Fig. 3B): digestion with XmnI will linearize the circular part of the joint molecule which, for JM1, decreases mobility. Digestion with AlwNI will remove part of a duplex tail and thus increase mobility. Digestion with both enzymes should result in an intermediate mobility where the effect of opening the circle balances that of removing duplex DNA. None of these predictions was fulfilled (compare Fig. 8B, "JM2" with Fig. 3B, "JM1"). Instead, we found that JM2 molecules in the two single digests have similar mobility and JM2 in the double digest migrates significantly faster than either the uncut or singly-cut JM2. These results perfectly match the predictions of the reverse initiation model. Both single-enzyme digests should cut the circular portion of the molecule and remove part of the duplex tail, yielding structures of almost identical mobility. The double digest should dramatically increase the mobility, as observed, by removing two additional segments of duplex DNA.

Second, cut JM2 molecules can branch migrate to yield smaller fragments, some of which are diagnostic (Fig. 8A). The strand replacement model predicts a DNA composed of the proximal fragment of the complementary strand annealed to the linearized ssC substrate (same
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as Fig. 3B, lower band). Digestion of JM 2 does not yield a band of the expected mobility (Fig. 8B). The reverse initiation model predicts a dsL DNA with a single-stranded tail in the XmnI digest (Fig. 3B, upper band), and a shorter tailed DNA fragment in the double digest. Bands consistent with both predicted products appear in the expected lanes (Fig. 8B).

Third, and most convincingly, the reverse initiation model predicts that digestion with both enzymes will yield a 11 kb, non-radioactive DNA derived from the initiating ends of the two complementary strands annealed to the circle. A band of this size was apparent only after Southern-blotting, and only in the double digest (Fig 8B, bottom).

Discussion

Here we described the structures of the two joint molecule intermediate species seen in the in vitro Rad51 strand transfer reaction. JM 1 is an intermediate where one dsL and one ssC DNA have begun but not completed transfer whereas JM 2 has a more complex structure in which two dsL substrates have initiated transfer with the same ssC DNA but opposite directions (Fig. 6A, JM 1 as #1, JM 2 as #5).

JM 1 was predicted as a strand-transfer intermediate based primarily on studies of RecA (17,20,34-37). The structure we have deduced for JM 1 is based on three lines of evidence. First, the three-stranded structure was clearly visualized in EM of JM 1. Second, diagnostic restriction digest products predicted for the three-stranded intermediate agree with those observed for purified JM 1 DNA (Fig. 3). Third, for all six circularly permuted substrates (Fig. 2A), both the electrophoretic mobilities of the JM 1 bands (Fig. 2C) and the branch-point distributions seen by EM (Table 1) agree with the position of the heterology.

Several lines of evidence show that the JM 2 intermediate is formed by the reaction of a second dsL with JM 1. The pulse-chase experiment in Figure 1C shows that JM 2 derives from
Structure of Rad51 Strand Transfer Intermediates

JM 1. The two-dimensional gel in Figure 5B shows that JM 2 contains approximately two dsL substrates per ssC. Additionally, the substrate titration in Figure 5A shows that increasing dsL concentration preferentially favors JM 2 formation and, we conclude, the conversion of JM 1 into JM 2.

But what is the structure of JM 2? Originally we assumed that JM 2 must use the complementary overhang and thus form via the strand replacement model (13). There were, however, two problems with this interpretation. First, it was not clear why the annealed end of the first duplex should denature to allow the second duplex to react (Fig. 6A, #2). Second, the two single-stranded tails or second duplex predicted by this model (Fig. 6A, #3) were never observed by EM. The restriction enzyme analysis of purified JM 2 species shown in Figure 8 led us to question the strand replacement model and retest whether Rad51 could initiate from a recessed end. Upon finding that strand transfer could initiate without a complementary overhang (Fig. 6C), we reconsidered the reverse initiation model. We now conclude, based on both analysis of JM 2 EM images (Fig. 7) and diagnostic restriction enzyme digests (Fig. 8), that JM 2 forms primarily via the reverse initiation model (Fig. 6A, ( #4) and ( #5)).

We suggest an upper limit of roughly 5% for the frequency of JM 2 forming by strand replacement. This calculation is based on the two putative strand replacement structures seen by EM and the failure to detect by autoradiography diagnostic restriction digest products in Figure 8. JM 2 intermediates formed by reverse initiation can mature to product if either of the dsL substrates completes branch migration around the circle. Unlike JM 1, however, JM 2 is likely not a necessary intermediate in the strand transfer reaction.

One apparent inconsistency remained to be explored in the formation of JM 2 by reverse initiation: the efficiency of JM 2 formation is much greater than expected given the poor reactivity of the recessed end. In a standard reaction, where ssC and dsL substrates are
equimolar, JM 2 species usually account for 10-30% of reacted DNA. At the high end of our dsL titration (Fig. 5A), JM 2 accounted for roughly 70% of the total joint molecule population. However, when only the recessed end was available, Rad51 formed just 8% as many joint molecules (Fig. 6C). When both ends of the dsL DNA were available, only 4% of the total JM 1 population formed from the recessed end (Fig. 3B faint band of tailed-duplex in the AlwNI lane).

We can explain the discrepancy by noting a fundamental difference between the reactions of the first and second dsL substrates with the ssC: whereas the first dsL DNA anneals to a completely single-stranded DNA, the second dsL DNA anneals adjacent to a duplex region (Fig. 9). Previous studies have shown that Rad51 preferentially transfers DNA that is next to a double-strand junction (38). We propose that the duplex from the first complementary strand provides a double-strand junction that makes the adjacent single-stranded region more reactive and enables Rad51 to overcome the difficulty of initiating from a recessed complementary strand (Fig. 9, b).

The polarity of Rad51 strand transfer has been a source of concern because the apparent 3' to 5' preference (along the single-stranded substrate) is inconsistent with the in vivo goal of annealing and priming repair synthesis from the 3' end of the single-stranded tail generated at the DNA break (12,13). It had been thought that Rad51 strand transfer polarity was determined solely by the complementary overhang of the duplex substrate (13). But no overhang is available in vivo, where the duplex substrate is an essentially endless chromosome (Fig. 9, c). Here we have shown that the presence of a duplex region on the invading single-stranded substrate promotes strand transfer without a complementary overhang. Thus, strand invasion in vivo may initiate at the highly-reactive single-strand/double-strand junction and proceed to the 3' hydroxyl terminus (Fig. 9, d) as has been proposed (38).
reported for Rad51 would then have no import for in vivo function; the structure of the in vivo substrate dictates the polarity of strand invasion.

The JM1 and JM2 regions of the gel do not contain a smear representing molecules at all stages of strand transfer but instead include several sharp bands (Fig. 2B). These bands are the result of two sites in pBluescript at which branch migration has paused. The two major pause sites were localized by EM (Table 1) and electrophoretic mobility (Fig. 2C).

What is the nature of the pause sites? The same JM1 bands are present in reactions with RecA and Rad51 (Fig. 1B), implying that the pauses are inherent to the DNA sequence. Direct sequencing of the dsL and ssC substrates ruled out point mutations which are known to pause branch migration (16,21,39). Nor do pause sites correspond to obvious GC-rich sequences that could slow denaturation of the duplex.

Two non-exclusive explanations for the pauses suggest themselves. First, secondary structures in the single-stranded DNA substrate could block branch migration. The putative secondary structure would have to be stable enough to survive denaturation by both Rad51 and RPA. We did find that in a strand transfer reaction without RPA, a larger fraction of JM1 DNA was in pause-based bands than in the band corresponding to the heterology (data not shown), suggesting that RPA diminishes pausing. Second, RecA and Rad51 preferentially associate with GT-rich DNA sequences (40,41). High affinity binding to particular sequences in pBluescript could cause strand transfer to pause at the observed sites. Similar sites in vivo could result in stalled strand transfer and possibly manifest themselves as hotspots for recombination.
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Figure Legends

**Figure 1.** Two sets of low electrophoretic mobility DNA species are generated during strand transfer.

A) Schematic of the strand transfer reaction. A single-stranded circular (ssC) DNA substrate coated with Rad51 anneals to a double-stranded linear (dsL) DNA substrate and begins transfer of the complementary strand to form joint molecules. The simplest joint molecule is shown. Joint molecules can mature by branch migration to nicked-circular (NC) and single-stranded linear (ssL) DNA products. DNA strands identical in sequence to the ssC substrate are shown in black while complementary DNA strands are shown in red.

B) Joint molecules formed in strand transfer reactions by *S. cerevisiae* Rad51 and *E. coli* RecA. The negative of an ethidium bromide stained agarose gel is shown. Reaction times, in minutes, are indicated above the gel. The electrophoretic mobility of the dsL substrate, two classes of joint molecules (JM1 and JM2), and NC product are indicated at the left. The region of the gel containing the ssC substrate and ssL product is omitted.

C) Kinetics of the formation and maturation of JM1. Radioactive dsL DNA was mixed with ssC, Rad51, and RPA to form JM1 and then chased with excess unlabeled dsL DNA for the times indicated at the top. An autoradiogram of an agarose gel is shown.
Figure 2. Correlation of JM1 electrophoretic mobility with the position of the heterology along the dsL substrate

A. Schematic of the dsL substrates used. The plasmid pBluescript (SK+), with a 74-base heterology (black square), was linearized with the indicated restriction enzymes to give circularly permuted dsL substrates with 3’ or 5’ overhangs. The substrates are aligned by the position of the heterology and oriented such that the Rad51 reaction proceeds from left to right. The complementary strand (red) is decorated with an arrow at the 3’ end and a dot at the 5’ end. The distance along each substrate from the initiating end to the heterology is given as a percentage of substrate length. Gray brackets mark the locations of predicted pause sites between the initiating end and the heterology.

B. Comparison of the electrophoretic mobility of JM1 bands from Rad51 strand transfer reactions with the substrates shown in A. An autoradiogram of a Southern blot is shown. Lane 1 is a control reaction between the Bsam cut-dsL DNA shown in A and a completely homologous (Hom.) ssC DNA to test if the substrates are competent for strand transfer.

C. Plot of the relative electrophoretic mobility of JM1 bands in B versus the distance of the heterology or pause site from the initiating end of the dsL, shown in A. Black circles correspond to the predominant JM1 bands in each reaction, which are all blocked at the heterology. The curve fit to these points was used to extrapolate the position of pause sites along the substrate from the electrophoretic mobility of minor JM1 bands (gray squares), which are blocked before the heterology. The pause positions clustered into two groups, which were each averaged to yield pause sites at 20% and 70% from the heterology. This averaging resulted in an offset of the gray points from the line. Relative mobility is defined here as the distance from the well to the JM1 band divided by the distance from the well to the center of the dsL band.
**Figure 3. Determination of JM1 structure by restriction enzyme digestion**

The ssC and 5'-32P labeled dsL heterologous substrates were reacted with Rad51 and RPA. Resulting JM1 species were purified from an agarose gel and digested with neither, one, or both of the restriction enzymes AlwNI and XmnI.

A. Products expected from digestion of JM1. The predicted structure of uncut JM1 is shown at the top left with the cleavage sites of the two enzymes indicated. Stars at the ends of the dsL substrate denote the 5'-32P label. The column at the left lists the restriction enzymes used in each row. The middle column (cut JM1) shows the predicted JM1 structure after digestion. Small fragments released from the unreacted end of the dsL DNA are shown in parentheses. The right column shows the labeled DNA species that are released when the branch point in the cut JM1 migrates to the end of the complementary strand.

B. Autoradiogram of the restriction enzyme digests of purified JM1 DNA. Enzymes used in each lane are indicated at the top. Bands corresponding to uncut dsL DNA and dsL DNA cut with one enzyme are indicated at left; dsL DNA cut with both enzymes is not radio-labeled. The small fragments released from the unreacted end are too small to be seen on this gel. Bands corresponding to the diagnostic products shown in A are indicated at the left.
Figure 4. Electron Microscopy of JM1 molecules

JM1 species were formed by reacting Rad51 and RPA with ssC DNA and heterology-containing dsL DNA linearized at various positions as described in Figure 2. The JM1 DNA was purified from an agarose gel, treated with E. coli SSB protein to coat the single-stranded regions, and visualized with an electron microscope. Three micrographs of JM1 molecules with schematic interpretations are shown. Single-stranded DNA appears thicker than duplex DNA in the micrograph because of the SSB-protein coat but is represented by a single black line in the schematic. The scale bar is 200 nm. The three images were chosen to show typical intermediates with increasing fractions of strand transfer. The complementary (red) strands in the traces are decorated with an arrow at the 3' end and a dot at the 5' end, with the orientation based on assuming branch migration up to the heterology. A. Molecule from the lower JM1 band of a reaction with NspI-cut dsL DNA. The heterology is 14% along this dsL substrate. B. JM1 molecule from a reaction with DraIII-cut dsL DNA. The heterology is 85% along the dsL substrate. C. Molecule from the upper JM1 bands of a reaction with an AflIII-cut dsL substrate. The heterology is 84% along the dsL substrate.
Figure 5. Formation and structure of the JM2 species

A. Effect of dsL substrate concentration on the formation of JM2 species. Rad51 strand transfer reactions were performed with constant amounts of ssC substrate and 1, 2, 3, or 4 molar equivalents, from left to right, of AflIII-cut, heterology-containing dsL DNA. The reaction was analyzed by agarose gel electrophoresis; an ethidium bromide stained gel is shown.

B. Two-dimensional gel electrophoresis of a Rad51 strand transfer reaction. A homologous reaction with BsaI-cut dsL DNA was run in the first dimension, a 1% agarose gel in TBE buffer. The first dimension is shown at left as the negative of an ethidium bromide stained gel. The second dimension, run in denaturing conditions, separated the ssC and ssL strands of each DNA species shown at the left. The right panel shows a Southern blot of the denaturing gel. The direction of electrophoresis and the position of the ssC and ssL bands are indicated at the top.
**Figure 6. Two possible structures for JM2**

A. The two possible models for JM2 formation. JM2 is formed by a reaction between JM1 and a second dsL DNA (#1). The complementary strand of the second dsL DNA is shown in blue. The two models for JM2 formation differ by which end of the second dsL DNA initiates strand transfer. In the strand replacement model (left), the second dsL initiates from the complementary overhang (#2) and follows the first dsL around the circle (#3). In the reverse initiation model (right), the second dsL initiates from the opposite end of the dsL, where the complementary strand is recessed (#4), and proceeds around the circle in the opposite direction as the first dsL (#5). Ends of the complementary strands are marked with arrows and dots for clarity, but JM2 can form with substrates of either overhang.

B. Diagram of the substrates used to test the requirement for a complementary overhang for initiation of strand transfer. A heterology-containing plasmid was linearized with the indicated restriction enzymes to yield dsL substrates with 5' or 3' overhangs and with the heterology (black box) adjacent to the complementary overhang or at the opposite end of the dsL. The substrates are oriented such that the Rad51 reaction proceeds from left to right. The 5' end of the complementary strand is marked with a dot and the 3' end with an arrow.

C. JM1 formation with the substrates in B. An autoradiogram of a Southern blot is shown. The left lane is a homologous positive control reaction between Apal-cut dsL DNA and ssC DNA that also contains the heterology.
Figure 7. Electron Microscopy of JM2 molecules

JM2 species were formed in a reaction between Rad51, RPA, ssC DNA, and NspI-cut dsL DNA without a heterology. JM2 DNA was purified from an agarose gel, treated with SSB to mark the single-stranded region, and visualized by electron microscopy. Four micrographs of purified JM2 molecules with schematic interpretations are shown. Single-stranded DNA appears thicker than duplex DNA in the micrograph because of the SSB-protein coat but is represented by a single black line in the schematic. The scale bar is 200 nm. A-C are molecules consistent with reverse initiation because they clearly show dsL tails emanating from junctions of single- and double-stranded regions of the circle. The assignment of red and blue to the two duplex tails in the trace is arbitrary. For clarity, the complementary strands of the two dsL substrates are diagrammed meeting at the center of the duplex region of the circle, even though this may not be consistent with the relative lengths of the duplex tails. D is a molecule consistent with strand replacement because of the duplex tail emanating (arrow) from a fully duplex region of the circle.
Figure 8. Determination of JM2 structure by restriction enzyme digestion

JM2 molecules in which only the second dsL DNA was $^{32}$P-labeled were generated by reacting Rad51 and RPA with ssC DNA and a two-fold molar excess of unlabeled dsL DNA to saturate the ssC. After a seven-minute incubation to allow JM1 formation, an equal amount of 5'-$^{32}$P-labeled dsL DNA was added. The labeled JM2 DNA was purified from a gel, digested with neither, one, or both of the restriction enzymes AlwNI and XmnI, and analyzed by gel electrophoresis.

A. Diagram of the products expected from restriction enzyme digestion for the two models of JM2 structure. The strand replacement model is diagrammed in the upper four rows, the reverse initiation model in the lower four rows. Stars at the ends of the dsL substrate denote the 5'-$^{32}$P label. The left column lists the restriction enzymes used in each row. The second column (cut JM2) shows the labeled digestion product that will migrate in the JM2 region of the gel. The third column shows the DNA species that are released when branch points in the cut JM2 structure migrate to the end of the duplex regions. Only species that are diagnostic of either model are shown.

B. Analysis of restriction enzyme digests of purified JM2 DNA. Enzymes used in each lane are indicated at the top. The top two-thirds of the agarose gel are presented as an autoradiogram showing the $^{32}$P-labeled DNA species whereas the bottom third is a Southern blot showing size standards and a small, unlabelled fragment. Bands corresponding to uncut dsL DNA and dsL DNA cut with one enzyme are indicated at left. The expected positions of three diagnostic structures and the short, unlabeled linear fragment shown in A are indicated at left. Bands corresponding to linear DNA markers of 16 kb, 10 kb, and 800 bases are indicated along the Southern blot.
Figure 9. Model for preferential initiation from a duplex junction

Diagrams of strand transfer initiation. The diagrams at top, (a) and (c), show initiation from a completely single-stranded substrate. The diagrams below, (b) and (d), show initiation from a single-stranded substrate adjacent to a duplex region (indicated by a dotted circle) and the subsequent maturation of strand transfer. The column on the left shows reactions in vitro, specifically reverse initiation to form JM 2. The column on the right suggests analogous events in vivo, where the single-stranded tail generated at a double-strand break must invade a continuous chromosome. The invasion reaction is slow for single-stranded DNA (thin, dashed arrow in c) but promoted by the duplex junction (thick arrow in d). Dashed lines on the end of DNAs indicate that they continue indefinitely.
### Table 1. Electron microscopy analysis of JM 1 from heterologous reactions

The observed and expected extent of strand exchange seen for JM 1 molecules. The first column lists the restriction enzymes used to generate the dsL DNA in each JM 1 species (as in Fig. 2A). The second column specifies which gel-purified bands were examined from each reaction. The number of molecules measured for each sample is in parenthesis. The third column lists the average observed extent of branch migration, measured as the percent of the dsL that had been transferred to the circle, plus or minus one standard deviation. Note that the distributions were not symmetrical but instead biased heavily toward the pause or heterology and trailed off toward the initiating end of the dsL. The fourth column lists the expected end point of branch migration: the distance to the heterology from the initiating end of the dsL as a percent of the dsL length.

| dsL substrate linearized with: | Sample (n) | percent of double-stranded DNA in circle |
|-------------------------------|------------|----------------------------------------|
|                              | Observed   | Expected                               |
| AflIII                        | JM 1 lower bands (19) | 14 ± 8                                 |
|                               | JM 1 upper bands (45) | 64 ± 8; 83 ± 4                         |
| Bsal                          | W hole reaction (54) | 44 ± 10                                |
| NgoM IV                       | JM 1 (24)   | 10 ± 5                                 |
| DraIII                        | JM 1 (26)   | 70 ± 12                                |
| AlwNI                         | W hole reaction (31) | 29 ± 10                                |
| NspI                          | JM 1 lower band (13) | 13 ± 3                                 |
|                               | JM 1 upper band | ND                                     |
### Table A

| Digest | Cut Products | Radio-Labeled Branch Migration Products |
|--------|--------------|----------------------------------------|
| None   | ![Diagram](none.png) | ![Diagram](radio-labeled.png) |
| XmnI   | ![Diagram](xmn.png) | ![Diagram](radio-labeled.png) |
| AlwNI  | ![Diagram](alw.png) | ![Diagram](radio-labeled.png) |
| Double Digest | ![Diagram](double.png) | ![Diagram](radio-labeled.png) |

### Diagram B

- **uncut**: uncut gel lane
- **XmnI**: gel lane after XmnI digestion
- **AlwNI**: gel lane after AlwNI digestion
- **Double Digest**: gel lane after double digestion

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![Image](JM1.png)

- **JM1**: Radio-labeled branch migration products
- **uncut dsL**: Uncut double-stranded ladder
- **cut dsL**: Cut double-stranded ladder
### A. Strand Replacement

| digest       | Strand Replacement | diagnostic branch migration products |
|--------------|--------------------|---------------------------------------|
| none         | ![Diagram](image1) | ![Diagram](image2)                     |
| Xmnl         | ![Diagram](image3) | ![Diagram](image4)                     |
| AlwNI        | ![Diagram](image5) | ![Diagram](image6)                     |
| double digest| ![Diagram](image7) | ![Diagram](image8)                     |

### B. Reverse Initiation

| digest       | Reverse Initiation | diagnostic branch migration products |
|--------------|--------------------|---------------------------------------|
| none         | ![Diagram](image9) | ![Diagram](image10)                    |
| Xmnl         | ![Diagram](image11) | ![Diagram](image12)                    |
| AlwNI        | ![Diagram](image13) | ![Diagram](image14)                    |
| double digest| ![Diagram](image15) | ![Diagram](image16)                    |
Structure of reaction intermediates formed during *Saccharomyces cerevisiae* Rad51-catalyzed strand transfer

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