The Sep1 strand exchange protein from *Saccharomyces cerevisiae* promotes a paranemic joint between homologous DNA molecules

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Strand exchange protein 1 (Sep1) from the yeast *Saccharomyces cerevisiae* promotes the transfer of one strand of a linear duplex DNA to a homologous single-stranded DNA circle. Using a nitrocellulose filter binding assay and electron microscopy, we find that Sep1 promotes the pairing of homologous DNA molecules via a paranemic joint. In this joint there is no net interwinding of the parental DNA molecules, as in the standard plectonemic double helix. The paranemic joints form with as little as 41 bp of homology between the parental DNA molecules. The substrates used were a circular molecule (either single-stranded DNA or duplex supercoiled DNA) and a linear duplex with heterologous regions at both ends to bar duplex plectonemic intertwining. We excluded the possibility that the exonuclease activity of Sep1 exposes complementary single-stranded regions that constitute the joint. The paranemic joint is the key intermediate in the search for homologous DNA by the RecA protein of *Escherichia coli*. Our results imply that the search process in a eukaryote such as yeast can be mechanistically similar.

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The essential events in the homologous recombination of DNA are the synapsis, or apposition, of like sequences and the subsequent exchange of DNA between the parental molecules. Synapsis is the more challenging step because the efficiency of recombination depends on rapid and faithful pairing in the face of a vast excess of heterologous pairwise comparisons. In site-specific recombination, synapsis is brought about by the binding of a recombinase to each of a pair of specific sequences and the dimerization of the recombinase (Stark et al. 1992; Sadowski 1993). The synaptic complex is thus held together by the strong and specific forces of DNA-protein interactions and protein-protein interactions.

General recombination does not have the luxury of such a simple scheme, because the putative recombinase would have the conflicting demands of generality and specificity of binding. The only force known that reconciles these demands is hydrogen bonding between complementary bases. In general recombination, DNA-DNA interactions must replace the protein-DNA and protein-protein interactions in site-specific recombination.

Although base sequence complementarity can, in principle, solve the specific recognition problem of general recombination, formidable kinetic problems remain.

General recombination requires only a short region of sequence homology, on the order of 100 nucleotides. Thus, the number of such lengths is only a little less than the total number of base pairs in a genome. Even if some process generates a single-stranded region in one parent, it would take too long to search for homology in the other parent by sequential cycles of denaturation and attempted reannealing of candidate sequences.

The complete resolution of this difficulty is not known, but critical factors are probably the involvement of recombination proteins in the search for homology and the subdivision of the search into more manageable steps. A particularly informative model reaction for a portion of general recombination has been the transfer of one strand of a linear duplex DNA to a single-stranded DNA circle (Fig. 1). The product is a nicked circle with one strand from each parental DNA. The formation of such a heteroduplex could provide a committed step to recombination in which the search for homology has been concluded successfully.

Proteins capable of catalyzing the single-strand transfer reaction have been purified from numerous sources, but the RecA protein of *Escherichia coli* is the archetype and prototype (Kowalczykowski 1991; Kowalczykowski and Eggleston 1994; Radding 1991; Camerini-Otero and Hsieh 1993; Cox 1993). The critical role of RecA is attested to by the 10⁴-fold drop in conjugative recombina-

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Figure 1. Strand transfer via paranemic and plectonemic joint intermediates. (A) A duplex DNA (bold lines), a single-stranded circular DNA with the same sequence (thin line), and a strand transfer protein (vertically hatched oblong) are indicated. (B) A paranemic joint is formed. There is no net intertwining between the paired DNA molecules, but homologous sequences are juxtaposed. (C) A plectonemic joint is formed. A paranemic joint greatly increases the concentration of sequences in the single-stranded circular DNA that are homologous to the sequence near the end of the linear duplex DNA, thereby promoting the formation of a plectonemic joint in which the paired DNAs are intertwined. For simplicity, neither the strand transfer protein nor a paranemic joint is indicated. (D) The terminal heteroduplex of the plectonemic joint is extended by branch migration until a heteroduplex nicked circular DNA and a single-stranded linear DNA are generated.

tion in *recA*⁻ cells (Clark and Margulies 1965). In vitro RecA protein binds preferentially to single-stranded DNA and forms an extensive nucleoprotein filament at the expense of ATP. This filament is the active species in the search for homology in a duplex DNA partner.

A key intermediate in this search is a molecule in which donor and recipient DNA are synapsed via a paranemic joint (DasGupta and Radding 1982; Bianchi et al. 1983; Adzuma 1992) (Fig. 1B). The joint is called paranemic because there is no net intertwining of the paired DNA molecules, as in the standard plectonemic DNA double helix. A RecA-mediated paranemic joint can be formed between closed circular single-stranded

and duplex DNA molecules where the linking number between the circles must be zero (Cunningham et al. 1980). For most experiments, however, it is more convenient to use a linear duplex DNA containing a region of homology to a circular single-stranded partner bounded by heterology. The terminal heterology bars the formation of a plectonemic duplex in the homologous region.

As a consequence of the absence of net strand intertwining, the paranemic joint is unstable once the RecA protein is removed and probably includes non-Watson-Crick base pairs (Camerini-Otero and Hsieh 1993; Rao and Radding 1993). The paranemic joint should accelerate recombination by greatly increasing the local concentration of the homologous sequences that exchange. It has been demonstrated (Riddles and Lehman 1985) that the paranemic joint is formed more rapidly than the next intermediate in recombination, in which the donor molecules are synapsed via a plectonemic joint (Fig. 1C).

Formation of the plectonemic joint requires a DNA end in a homologous region to initiate intertwining. After the plectonemic joint is formed, branch migration can complete the formation of a heteroduplex DNA molecule (Fig. 1D). Branch migration with DNA alone can form a heteroduplex via a random walk. RecA facilitates heteroduplex formation by making migration unidirectional (Cox and Lehman 1981; Kahn et al. 1981; West et al. 1981). The energetic cost of unidirectionality is paid for by the hydrolysis of ATP. The RecA-mediated rate of branch migration of 2–10 bp/sec is still relatively slow, but the fork can traverse great distances and pass through long heterologous insertions (Bianchi and Radding 1983).

Intermediates in the search for homology have not been extensively characterized in eukaryotes, and that is the subject of this report. Eukaryotic proteins implicated in general recombination have been best studied in the yeast *Saccharomyces cerevisiae*. One class of yeast proteins, which includes Rad51, DMC1, and Rad57, shares sequence similarity with RecA (Bishop et al. 1992; Shinohara et al. 1992; Story et al. 1993). Rad51 binds DNA in an ATP-stimulated fashion (Shinohara et al. 1992), and the helical structure of the resulting nucleoprotein filament is remarkably like that formed by RecA (Ogawa et al. 1993). Although there is good genetic evidence for a role of these RecA sequence analogs in meiotic recombination (Bishop et al. 1992; Shinohara et al. 1992), there is as yet no biochemical evidence that these proteins carry out strand transfer or any reaction implicated in recombination.

Another class of proteins from a large number of eukaryotic species has been shown to carry out strand transfer in vitro (Kolodner et al. 1987; Sugino et al. 1988; Halbrook and McEntee 1989; Lowenhaupt et al. 1989; Fishel et al. 1990). These proteins have no amino acid sequence similarity to members of the RecA family, do not hydrolyze ATP, and form no discernible nucleoprotein filaments. The strand transfer proteins are better characterized biochemically than the eukaryotic RecA sequence homologs, but compelling genetic evidence for a role in recombination is absent.

The strand exchange protein 1 (Sep1, a.k.a. STP β) from yeast is the best studied of the eukaryotic strand transfer proteins (Kolodner et al. 1987; Dykstra et al. 1990; Kim et al. 1990; Johnson and Kolodner 1991). Sep1 is a large protein with a molecular weight of $\sim 175,000$. It has been purified to homogeneity, and its gene has been cloned, sequenced, and mapped (Larimer and Stevens 1990; Dykstra et al. 1991; Kersey and Kipling 1991; Tishkoff et al. 1991). Sep1 not only promotes strand transfer, as diagrammed in Figure 1, but also the renaturation of homologous single-stranded DNA molecules (Heyer et al. 1988). RecA has a similar annealing activity (Weinstock et al. 1979). Johnson and Kolodner (1991) have shown that in addition to these activities, Sep1 contains an intrinsic 5' $\rightarrow$ 3' exonuclease activity, which exposes a terminal single-stranded region in duplex linear DNA. They have found that such a region is necessary for strand transfer.

Sep1 mutants have a complex phenotype and have been obtained in strikingly different ways (for review, see Kersey and Kipling 1991). They were isolated as knockouts of the gene encoding a DNA strand transfer protein (Dykstra et al. 1990; Tishkoff et al. 1991) and as a knockout of a gene that encodes a major exoribonuclease (Stevens and Maupin 1987). Sep1 mutants were also identified on the basis of their reduction of nuclear fusion (Kim et al. 1990) and the potentiation of the replication of plasmids with weak autonomously replicating sequences (ARSs) (Kipling et al. 1991). Mitotic recombination has been reported to be reduced fivefold in Sep1 mutants (Dykstra et al. 1991; Tishkoff et al. 1991). Because the protein is large and already known to carry out two very different reactions in vitro (strand transfer and exonuclease), multifunctionality may contribute to this pleiotropism. This is clearly the case for RecA, which is also a coprotease (Little and Mount 1982).

In this paper we establish that Sep1 efficiently forms a paranemic joint. The joint can be formed between two DNA molecules sharing only 41 bp of homology, between a single-stranded and a double-stranded DNA or even between two DNA duplexes. We have excluded the possibility that the exonuclease activity of Sep1 exposes complementary single-stranded DNA regions that compose the joint. Our results implicate Sep1 in general recombination because of the importance of the paranemic joint in the search for homology. They also imply that eukaryotic and prokaryotic strand transfer proteins may use similar intermediates in the search.

Results

Sep1 forms a paranemic joint between a single- and a double-stranded DNA

A simple yet quantitative assay for the formation of a paranemic joint that has been used extensively in studies of RecA protein involves the binding of DNA to a nitrocellulose filter (Riddles and Lehman 1985). After pretreatment with a high concentration of NaCl, single-

stranded DNA adsorbs to this material but duplex DNA does not. If ^3H -labeled duplex DNA is mixed with a homologous unlabeled single-stranded DNA and Sep1, ^3H label is retained by the filter only if a joint molecule is formed between the two DNAs. The pair of DNA molecules that we used to insure that a paranemic joint is detected is diagrammed at the top left of Figure 2. The single-stranded DNA is circular phage M13mp18 DNA (hereafter referred to as M13) and the linear duplex DNA is *Xho*I digested ^3H -labeled M13Gori 1 (hereafter referred to as MGori 1). In the latter DNA, the middle 6.4 kb of M13 sequences is surrounded by 1.0 and 1.2 kb sequences with no relation to M13. The identical, or homologous, sequences are shown as a thin line in this and subsequent figures, and the different, or heterologous, sequences are shown as a thick line. The terminal heterologies in the linear DNA prevent the formation of a standard plectonemic joint, and their large size blocks the weak exonuclease of Sep1 from digesting past them during the course of the experiments.

Within minutes at 37°C, Sep1 mediates the joining of one-third of the linear duplexes to the single-stranded M13 DNA (Fig. 2A). The joint is homology dependent, because no joint molecules were detected when the single-stranded M13 DNA was replaced by ϕX174 single-stranded DNA (hereafter referred to as ϕX DNA). The joint was abolished by treatment with 5.2 M guanidine hydrochloride, which demonstrates the need for the continued presence of Sep1. The paranemic joint formed by RecA has the same sensitivity to this protein denaturant (Riddles and Lehman 1985). Similar results were obtained with a moderately reduced amount of Sep1 and the yeast DNA-binding protein II (DBP II) (Fig. 2C). This protein spares the requirement in strand transfer for Sep1 and for another yeast DNA exchange protein, STP α (Hamatake et al. 1989; A. Sugino, unpubl.).

We measured the promotion of a plectonemic joint by Sep1 using the pair of DNA molecules diagrammed at the top right of Figure 2. In this case, the homologous M13 sequences (3.0 and 3.4 kb) are at the ends of the linear duplex DNA and the heterologous sequence is in the middle. With these substrates, 85% of the linear duplex DNAs form joint molecules (Fig. 2B). Similar results were again found in the presence of DBP II and less Sep1 (Fig. 2D). In contrast to the results with the paranemically joined molecules, one-third to two-thirds of the plectonemically joined molecules survived exposure to 5.2 M guanidine hydrochloride (Fig. 2B,D). Presumably, the disrupted joints were either paranemic or plectonemic but dissociated via branch migration. The plectonemic joint was homology dependent because replacement of single-stranded M13 DNA with single-stranded ϕX DNA led to no significant formation of joint molecules.

Electron microscopy of Sep1-mediated paranemic joints between single- and double-stranded DNAs

The filter-binding assay is rapid and quantitative, but electron microscopy is needed to observe the position

Figure 2. Detection of Sep1-mediated paranemic and plectonemic joints between homologous DNA molecules by nitrocellulose filter binding. Quantities of 0.07 pmole of a ^3H -labeled linear duplex DNA (MGori I) and 0.07 pmole of single-stranded circular DNA (M13) were mixed with either 14 pmoles of Sep1 (A,B) or 7 pmoles of Sep1 plus 30 pmoles of DBP II (C,D), the yeast DNA-binding protein. The mixture was then passed through a nitrocellulose filter, which retains only single-stranded DNA. Thus, ^3H -labeled DNA is retained only if a joint is formed between the single-stranded and duplex DNA molecules. The reaction mixtures were either added directly to the filter (■) or were treated first with 5.2 M guanidine hydrochloride at 37°C for 10 min to denature proteins (□). In the control reactions (●), M13 single-stranded DNA was replaced by ϕX single-stranded DNA, which has no sequence homology to the ^3H -labeled duplex DNA. (A,C) Paranemic joint formation. Both ends generated by *Xho*I digestion of the ^3H -labeled duplex MGori I DNA are homologous to M13 circular single-stranded DNA, diagramed at top left. The thin and thick lines refer to homologous and heterologous regions, respectively. (B,D) Plectonemic joint formation. Both ends generated by *Bam*HI digestion of the ^3H -labeled duplex MGori I DNA are homologous to the M13 circular single-stranded DNA, diagramed at top right.

and extent of the joints. The DNA molecules examined were the same ones analyzed by filter binding, namely M13 single-stranded DNA and linear duplex MGori I DNA (see diagram, Fig. 3, top). We chose to examine joint molecules formed after only 3 min to reduce the multimerization of the DNAs. The DNA was spread out by binding to a cytochrome *c* film and viewed without addition of a protein-cross-linking agent such as glutaraldehyde. About 70–80% of the DNA molecules were in oligomeric forms, which were difficult to interpret. The remainder were mostly dimers containing either one molecule of circular single-stranded M13 DNA and one molecule of the linear duplex DNA or two linear duplex DNAs. We saw no oligomers containing only single-stranded DNA circles and very few monomers of either DNA. Examples of joints between the circular and linear DNAs are shown in Figure 3. The single-stranded DNA could often be distinguished from the duplex by its crinkly form, and its circularity was unmistakable. The two molecules were usually joined at only one place. The extent of the joint ranged from 100 to 1000 bp, and the majority were ~200–300 bp in length.

The distribution in the position of the joint measured from the nearest end of the duplex DNA is plotted in Figure 3. The pattern is remarkably uniform except for the absence of joints in the first 10% of the duplex DNA, the region of heterology. These results suggest that the paranemic joint requires sequence homology but has no obvious sequence preference.

Minimum homology requirement for paranemic joint formation

To provide a more stringent test of the homology requirement of the paranemic joint, we limited the homology in the linear duplex DNA parent to just 41 bp of a ϕX sequence. As diagramed in Figure 4A, we positioned the 41 bp only 20 bp from one end of the molecule. If the intrinsic 5' $\rightarrow$ 3' exonuclease of Sep1 were to digest the linear duplex DNA, it would remove the strand that is complementary to the single-stranded ϕX . Thus, if a joint is observed, it cannot have resulted from exonuclease resection of the duplex and annealing to the single-stranded circular DNA. Using electron microscopy,

Figure 3. Detection of Sep1-mediated paranemic joints by electron microscopy. MGori I DNA was cleaved by *Xho*I restriction endonuclease to generate a linear duplex DNA with a 6.4-kb region that is identical to circular single-stranded M13 DNA and that is bordered by 1.0 and 1.2 kb of heterologous sequences (see diagram, top left). Duplex DNA (0.07 pmole) was mixed with single-stranded DNA (0.07 pmole) in the presence of 14 pmoles of Sep1. After 3 min at 37°C, the molecules were spread for electron microscopy by a cytochrome *c*-formamide method. Electron micrographs of examples of joint molecules are shown. A schematic tracing of the molecule is shown in which the M13 circular DNA is indicated by a thin line and the MGori I duplex linear DNA by the bold line. The position of the joint in all scorable molecules is plotted in the bar diagram.

we observed that 70% of the dimeric molecules had circular single-stranded ϕ X DNA joined to one end of the linear duplex DNA, the predicted location (Fig. 4A).

As further proof of the sequence specificity of the paranemic joint, we moved the 41 bp of homology to one-third of the way from one end of the linear duplex DNA. We again observed joint molecules, but now 64% of the joints were located one-third of the way from the end of the linear duplex DNA, the region of homology (Fig. 4B). The two experiments in Figure 4 provide controls for each other. They also show that only 41 bp is sufficient for formation of a paranemic joint. The DNA molecules seemed to just touch at the joints (Fig. 4). The joints were clearly shorter than those formed between more extensive regions of homology (Fig. 3).

Formation of a paranemic joint between two duplex molecules

Thus far we have demonstrated that Sep1 forms a

paranemic joint between a single-stranded circular DNA and a homologous region of a linear duplex DNA. This is a paradigmatic reaction for RecA, a protein that is activated by single-stranded DNA. Sep1, however, binds to duplex DNA almost as well as to single-stranded DNA (Heyer et al. 1989). Therefore, we tested whether Sep1 could mediate a paranemic joint between two duplex DNAs. For these experiments, we used a supercoiled plasmid, pPiaI, and a linear duplex DNA with heterology at both ends (diagramed in Fig. 5, top). Observation of a supercoiled DNA molecule at a joint ensures that the DNA has not been nicked and resected by the nuclease activity of Sep1.

Electron microscopy showed efficient joint formation between the two duplex DNA molecules. The 900-bp homologous region stretches from 4% to 16% from one end of the duplex DNA, and the attachments between the supercoiled and linear duplex DNAs were spread relatively uniformly over this interval (Fig. 5). Only a background level of joints was found elsewhere. The electron micrographs verified that the plasmid DNA part of the joint molecule is still supercoiled. Thus, the paranemic joint does not relax a supercoiled parental DNA, unlike the situation with RecA protein (Wu et al. 1983; Christiansen and Griffith 1986; Schutte and Cox 1988). The Sep1-mediated joint was sharply defined, usually a point contact. Supercoiled DNA molecules seemed about as likely to form a joint molecule with another supercoiled molecule as with linear DNA, as judged by the appearance of pPiaI oligomers. Half of the supercoiled molecules shown in Figure 5 that are joined to a linear duplex DNA are also joined to another supercoiled DNA.

To test for the stringency of the homology requirement for formation of a paranemic joint between two duplex DNA molecules, we used as one parent the duplex linear DNAs with 41 bp of homology to ϕ X that we used in the experiments shown in Figure 4. The other parent was supercoiled ϕ X DNA rather than single-stranded DNA (diagramed in Fig. 6A and B, top). Electron microscopy showed that the joints were primarily at the sites of homology. When the homology was at one end of the duplex DNA (Fig. 6A), 52% of the joints were at this position. With the 41 bp of homology one-third of the way in from one end (Fig. 6B), 54% of the joints were at this position. In the remainder of the joint molecules, the position of the joined supercoiled DNA was spread uniformly over the duplex DNA. Thus, the paranemic joint between duplex DNA molecules is also homology dependent. The electron micrographs indicate again that the ϕ X DNA was still supercoiled and that the joint region is small.

Discussion

We studied the formation of homology-dependent synthesis of DNA molecules by the Sep1 strand exchange protein of yeast. First, we present our major conclusions and the experimental evidence for them. Subsequently, we discuss the possible structure of the Sep1-mediated

Figure 4. Sep1-mediated formation of a paranemic joint molecule between circular ϕ X single-stranded DNA and linear duplex DNA sharing only 41 bp of homology. The linear duplex DNA has a 41-bp sequence that is also found in the + strand of ϕ X DNA. After restriction of pRKG27 with *KpnI* endonuclease, the homologous region is 20 bp from the 5' end of the – strand of the duplex DNA (A). After restriction with *ScaI* endonuclease, it is 1022 bp from the 5' of the (–) end of the duplex DNA (B). The input DNA pairs are diagrammed in the inserts. If the 5' $\rightarrow$ 3' exonuclease activity of Sep1 digested the 5' end of the (–) strand, then the exposed single-stranded region would be the + strand, which is identical to and thus not complementary to the + circular single-stranded ϕ X present. Electron micrographs of some of the joint molecules are shown, along with a histogram of the position of the joint along the linear duplex DNA.

paranemic joint and compare the properties of Sep1 and RecA.

1. Sep1 promotes a paranemic joint between two DNA molecules. There are two pieces of evidence for this conclusion. First, electron microscopy shows that the joint is formed in a region of homology in a linear duplex that is surrounded by long stretches of heterology. Second, filter-binding experiments showed that unlike the pleconemic joint, the joint is abolished by the protein denaturing agent guanidine hydrochloride.
2. The paranemic joint forms between homologous sequences; 41 bp of identical sequence suffice. A minimum estimate of the sequence specificity can be made from the data in Fig. 4, for which homology is limited to 41 bp. Two-thirds of the joints are in the homology region. Assuming that all joints outside the homology region are nonspecific, and normalizing for the length ratio of the heterologous and identical sequences (2987/41), there is a 150-fold preference for a sequence-specific joint.

3. The paranemic joint does not require exonucleolytic processing by the intrinsic exonuclease activity of Sep1 to expose single-stranded DNA in the region of homology. The exonuclease activity is feeble under our conditions, and the region of homology in the paranemic joint can be kilobases from the end. Moreover, with the linear DNA substrate used in Figure 4A, 5' $\rightarrow$ 3' exonuclease digestion would destroy the complementary strand in the homologous region.
4. The joints form between duplex linear DNA and either single- or double-stranded closed circular DNA. We observed chains of supercoiled DNA rings by electron microscopy, but in the absence of a sequence-specific landmark, we do not know whether these complexes are homology dependent.

The Sep1-mediated paranemic joint

It is worthwhile to speculate on the possible structure of the Sep1-mediated paranemic joint. When the region of homology was large, the size of the joint between the

Figure 5. Sep1-mediated paranemic joints between a supercoiled plasmid DNA and a linear duplex DNA. The plasmid pPiaI and M13 RF DNA cut with *Bgl*II endonuclease share 900 bp of homology. There are 300 and 6000 bp of heterology at the ends of the linear duplex DNA (see diagrammatic insert). The molecules were spread for electron microscopy. Electron micrographs of examples of the joint molecules are shown along with the distribution of the position of the supercoiled DNA along the linear DNA. Note that in three of the examples shown, a supercoiled pPiaI is joined both to a linear DNA and to another supercoiled pPiaI.

single-stranded and duplex DNAs averaged a few hundred base pairs. The extent of the joint between a supercoiled and a linear duplex DNA was even less. Because of its sequence specificity, the joint is most likely maintained by hydrogen bonds between bases on the parental DNA molecules.

The fact that the joint is paranemic strongly limits its possible structure. Consider, for example, the pair of molecules examined in Figure 5, a linear duplex DNA with 900 bp of homology and a supercoiled plasmid. We assume that the heterologous regions flanking the region of homology in the duplex DNA do not unwind and rewind during the reaction. This seems highly likely on kinetic grounds, given the large size of the heterologous region and the lack of a requirement for ATP hydrolysis. With this assumption, the region of homology can be treated topologically as if it were a closed single-stranded circle, even though it is a linear duplex. The linking

number between this virtual circle and either or both strands of the supercoiled plasmid must be zero because no DNA breakage and reunion occurs during Sep1 treatment. This condition must be met even if one end of the duplex threads through the plasmid (West 1992). Therefore, if the paranemic joint contains a plectonemic region, there must be an exact topological compensation in twist or writhe.

For example, if the paranemic joint contains a B-type DNA duplex between the complementary strands, this positive twist could be compensated by an equivalent negative writhe. However, this is unlikely on energetic grounds. The writhe must not be of either parental DNA molecule alone but of an axis between these molecules, because the twist is intermolecular. This writhing would have to be stabilized by binding to Sep1. We take the core nucleosome as our model of (–) writhe stabilized by DNA–protein interactions (Richmond et al. 1984). In the nucleosome, there are ~140 bp bound per unit change in linking number. Thus, for every turn of the postulated plectonemic portion of the paranemic joint, there would have to be 140 nucleotides from each parental DNA wound in a superhelix around Sep1. This requires substantial DNA bending energy. Moreover, 130 of the 140 bp would have to be juxtaposed without the benefit of forming a plectonemic double helix. A highly organized Sep1 structure would be required to direct and stabilize this rather bizarre structure, and there is no evidence for any Sep1 higher-order architecture.

A B-type plectonemic region of the paranemic joint compensated by negative twist would also be energetically costly. We assume that the joint is between only one of the two strands of each duplex. The free energy of formation of such a joint would be roughly that needed to denature the 100- to 200-bp of the joint in each duplex DNA parent plus the penalty at the junction between the – and + twist regions, minus the energy gained from rewinding only half of the DNA. Without invoking a remarkable interaction with Sep1, a plectonemic joint made of left-handed and right-handed double helices also seems unlikely.

We conclude that a significant duplex plectonemic portion of a paranemic joint is unlikely because of the substantial energy needed to stabilize the compensatory twist or writhe.

A more likely alternative is a periodic bonding by side-by-side DNA molecules. Because the circular parent of the paranemic joint can be either single-stranded or double-stranded, then either the single-stranded DNA is folded between the interaction regions to maintain phasing or the duplex(es) is unwound. The hydrogen bonds between the bases that maintain the joint are probably non-Watson–Crick and must be if the duplex DNA is not unwound.

Although we have emphasized the role of DNA–DNA interactions in providing sequence specificity, Sep1 plays a continuing role in maintaining the joint. Denaturation of Sep1 by guanidine hydrochloride destroys the joint. We can imagine several possible roles for Sep1: increasing local DNA concentration; shielding repulsive

Figure 6. Sep1-mediated formation of a paranemic joint between supercoiled ϕ X DNA and a linear duplex DNA that shares only 41 bp of homology. The experiment was the same as that described in Fig. 4, except that supercoiled ϕ X RF DNA was used instead of circular ϕ X single-stranded DNA.

charge interactions between the parental backbones; and distorting the DNA into a specific structure that favors homologous non-Watson-Crick interactions (Sikorav and Church 1991).

Past experiments imply that a single-stranded end of a duplex DNA is necessary for forming a plectonemic joint by Sep1 (Johnson and Kolodner 1991). Under conditions in which the intrinsic exonuclease activity of Sep1 is inhibited, a terminal single-stranded region is required. Our data show convincingly that resection of the homologous region is not necessary for a paranemic joint by Sep1.

Comparisons of Sep1 and RecA

A comparison of Sep1 with RecA is instructive. Both proteins promote the formation of plectonemic and paranemic joints and bring about efficient DNA strand transfer and renaturation. They differ in that RecA forms a signature helical filament and hydrolyzes ATP during strand transfer, and in that Sep1 is also an exonuclease. The differences may not be so significant. Kowalczykowski and colleagues have shown that ATP hydrolysis is not needed for strand transfer by RecA. (Menetski et al. 1990), and Cox has argued persuasively that the RecA filament has evolved for its role in repair but perhaps not in recombination (Cox 1993).

The genetic data implicating RecA in conjugative re-

combination are impressive. Absence of the protein causes a 10^4 -fold drop in recombination (Clark and Margulies 1965) and a 40- to 100-fold decrease in plasmid recombination (Laban and Cohen 1981). The genetic data on Sep1 are complex and not yet convincing for a role in recombination. A fivefold drop in mitotic recombination has been observed (Dykstra et al. 1991; Tishkoff et al. 1991), but it is not clear that this is a direct effects, because Sep1 mutants display a highly pleiotropic phenotype.

We believe, however, that our finding of formation of a paranemic joint by Sep1 enhances the likelihood that Sep1 has a role in recombination, and that the paranemic joint is an important intermediate in strand exchange. Sep1 is the first eukaryotic strand transfer protein that has been demonstrated to form a paranemic joint.

Materials and Methods

Proteins

Sep1 was purified as described (Dykstra et al. 1990). The final preparation was >95% pure as judged by SDS-polyacrylamide gel electrophoresis. As reported by Johnson and Kolodner (1991), Sep1 has an exonuclease activity. Under our strand exchange conditions, it is relatively weak. Incubation of 14 pmoles of Sep1 with 0.07 pmole of a 5' 32 P-terminally labeled MGori I linear duplex DNA and 0.07 pmole of circular M13 single-stranded DNA released 4% of the label in 3 min at 37°C, the

incubation time used in our electron microscopy studies. With a blunt-ended linear duplex the activity was even less. No endonuclease activity was detected under the same conditions using a supercoiled substrate DNA. Restriction endonucleases *Bam*HI, *Xho*I, *Sca*I, *Kpn*I, and *Bgl*II were from New England Biolabs. DBP II was a gift from A. Sugino.

DNA substrates

Unlabeled circular single-stranded DNA M13mp18 (designated M13) and ϕ X, as well as supercoiled M13 RF (replicative form) and ϕ X RF, were from New England Biolabs. 3 H-labeled MGori I was prepared as described (Kaguni and Ray 1979). To minimize the amount of nicked 3 H-labeled MGori I RF DNA, only freshly prepared DNA was used in the filter-binding experiments.

Plasmid pRKG27 is a 2987-bp plasmid that contains 41 bp of homology to ϕ X (nucleotide positions 3500–3540). It was constructed by cloning the oligonucleotide 5'-AATTGGAGG-TATATGCACAAA-TGAGATGCTTGCTTATCAACAGA-3' and its complement with *Eco*RI and *Hind*III sticky ends, into the *Eco*RI and *Hind*III restriction sites of pBluescript SK(-) (Stratagene). Digestion of pRKG27 with *Kpn*I generates a linear duplex DNA with the 41 bp of homology to ϕ X 20 bp from one end. After digestion with *Sca*I, the homologous region is 1022 bp from one end of the duplex DNA.

pPial is a 3656-bp plasmid that was constructed by inserting a 970-bp fragment into the *Eco*RI site of pUC19. There is 900 bp of homology between pPial and M13. On the linear M13 DNA the homologous region is contiguous, but on pPial it is disrupted by the 970-bp insertion. Digestion of the RF form of M13 (7.2 kb) with *Bgl*II generates a linear duplex with the homologous region to pPial located 300 bp from one end.

Reaction conditions

The standard reaction mixture for formation of joint molecules contained in a volume of 50 μ l, 20 mM Tris-HCl (pH 7.9), 10 mM $MgCl_2$, 2 mM dithiothreitol, 0.1 mg/ml of bovine serum albumin, 0.07 pmole of the single-stranded DNA parent, 0.07 pmole of the duplex DNA parent, and 14 pmoles of Sep1. DNA concentrations are expressed in terms of molecules and not nucleotide equivalents. In the reactions in Figure 2, C and D, only 7 pmoles of Sep1 were used plus 30 pmoles of DBP II. The reactions were performed at 37°C for the times indicated in Figure 2 or for 3 min in the experiments described in Figures 3–6.

Nitrocellulose filter-binding assay

The nitrocellulose filters (Schleicher & Schuell, 0.45 μ m) were washed extensively with distilled water and soaked in 2.0 M NaCl and 0.15 M sodium citrate at 4°C for at least 1 hr before use. At the times indicated, samples (10 μ l) were withdrawn, diluted into 1 ml of chilled 2.0 M NaCl and 0.15 M sodium citrate, and applied to a nitrocellulose filter at room temperature. The filters were washed twice with 1 ml of 2.0 M NaCl and 0.15 M sodium citrate at 4°C and three times with 1 ml of 0.3 M NaCl and 0.02 M sodium citrate at the same temperature. After drying for 40 min, the amount of 3 H-labeled duplex DNA bound to the filter was measured by a liquid scintillation spectrometer. For the treatments with guanidine hydrochloride, the samples were mixed with the denaturant and EDTA in 0.1 M Tris-HCl (pH 7.5) to give final concentrations of 5.2 M and 22 mM, respec-

tively. After 10 min at room temperature, the samples were applied to a filter and processed as described above. The determinations were done in triplicate.

Electron microscopy

After forming joint molecules in a standard reaction mixture at 37°C for 3 min, the mixture was diluted to a DNA concentration of 0.1–1 μ g/ml in a spreading solution containing 30% formamide, 0.1 M Tris-HCl (pH 8.5), 0.01 M EDTA, and 120 μ g/ml of cytochrome c. This sample was then spread over a hypophase of 10 mM Tris-HCl (pH 8.4), 1 mM EDTA, and 10% formamide, picked up on parlodion grids, stained in uranyl acetate, and shadowcast with tungsten (Davis et al. 1970). Micrographs were taken with a JOEL 100 CX.

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