Joints Made by RecA Protein in the Interior of Linear Duplex DNA: Effects of Single-Stranded Ends, Length of Homology, and Dynamic State†

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ABSTRACT: The importance of 3′ single-stranded ends in homologous recombination led us to reevaluate reactions of single strands at homologous sites in the interior of linear duplex DNA. As the length of homology increased, the yield of joints increased up to about 2 kb of homology, at which the apparent yields were the same at either 3′ or 5′ single-stranded ends, or away from ends, although such joints were qualitatively different. In the presence of RecA protein and ATP, joints that formed between any of these single strands and interior sequences in duplex DNA were in a dynamic state in which they constantly recycled. Consequently, their apparent yields at steady state were sensitive to conditions of reaction, such as the concentrations of DNA, salt, and ATP *vs* ADP, observations which rationalize conflicting reports in the literature. The dynamic state of joints in the interior of duplex DNA strengthens the conclusion [see Burnett *et al.* (1994)] that RecA protein dissociates joints when it cannot displace the 5′ end of a strand from the recipient duplex.

Escherichia coli RecA protein plays a central role in homologous genetic recombination *in vivo* and promotes homologous pairing of double-stranded DNA with single-stranded or partially single-stranded DNA molecules *in vitro* (Radding, 1982, 1988; Kowalczykowski, 1991; Roca & Cox, 1990). A commonly studied model system involves the pairing of circular single-stranded DNA with linear duplex DNA, which results in the formation of a nicked circular heteroduplex and a displaced linear single strand (Radding, 1988; Cox & Lehman, 1987). Studies on this model system have revealed that RecA protein carries out the strand exchange reaction in three distinct phases: (I) presynapsis, during which RecA protein binds cooperatively and stoichiometrically to single-stranded DNA and forms a right-handed helical nucleoprotein filament; (II) synapsis, in which naked duplex DNA first binds nonspecifically and weakly to sites on the nucleoprotein filament and then comes into homologous alignment; and (III) strand exchange, which produces a heteroduplex molecule and a displaced linear single strand. Another model system which is commonly studied involves the use of gapped duplex DNA and linear duplex DNA (West, 1992). These model systems have been useful in understanding the mechanism of the reactions carried out by RecA protein.

Fewer observations have been made, however, on the pairing of linear single strands with homologous sites located in the interior of duplex molecules of DNA, a combination of substrates that is frequently invoked to explain observations on recombination *in vivo*. In *E. coli*, various lines of evidence suggest that the 3′ ends of single strands are important in recombination, and it is commonly supposed that 3′ ends are involved in the initiation of recombination (Smith, 1988). In the RecBCD pathway, the RecBCD enzyme cuts DNA at χ sites more frequently than elsewhere. Cutting occurs on one strand, that containing the χ sequence 5′-G-C-T-G-G-T-G-G-3′, a few nucleotides to its 3′ end; subsequent DNA unwinding by the enzyme gives rise to single-stranded DNA with a 3′ end (Taylor *et al.*, 1985; Braedt & Smith, 1989). Similarly, 3′ ends are involved in the recombination pathways that are activated by *rec*BCD suppressor mutants, *sbc*A and *sbc*B. Certain strains of *E. coli* that give rise to *sbc*A mutants harbor a cryptic prophage designated as *rac*. In these mutants, the *rec*BCD defect is suppressed by the *rac*-encoded nuclease that specifically digests one strand of duplex DNA starting from a 5′ end, thus giving rise to DNA with 3′ tails (Low, 1973; Joseph & Kolodner, 1983; Kushner *et al.*, 1974). Unlike sbcA mutants which express a new nuclease, sbcB mutants are deficient in a nuclease activity, exonuclease I, which digests single-stranded DNA from the 3′ end. Thus, the *sbc*A mutation restores recombination in a *rec*BCD⁻ strain by eliminating an enzyme that degrades 3′ ends (Kushner *et al.*, 1972; Lehman & Nussbaum, 1964; Clark, 1973).

Recombination mediated by the Red pathway of bacteriophage λ may also involve the 3′ ends of single-stranded DNA. λ exonuclease digests double-stranded DNA from the 5′ ends, releasing 5′ mononucleotides and generating long single-stranded DNA tails with 3′ ends (Little, 1967). Similarly, 3′ ends appear to be important in eukaryotes in which they are produced by digestion of 5′ ends at double-strand breaks during meiotic recombination (Sun *et al.*, 1989, 1991; Bishop *et al.*, 1992).

Previous studies have shown that the strand exchange catalyzed by RecA protein is directional: the anticomplementary linear plus strand is displaced from duplex DNA in the 5′ to 3′ direction (Kahn *et al.*, 1981; Cox & Lehman, 1981). According to this polarity, one might expect a linear single strand coated with RecA protein to initiate an exchange *via* its 5′ end rather than the 3′ end to permit the strand exchange to proceed 5′ to 3′. Konforti and Davis (1987, 1990, 1991), however, found that the 5′ ends of single-stranded DNA are less effective in forming joints than the 3′ ends because of the directionality of the cooperative binding of RecA protein to single strands (Register & Griffith, 1985). In those studies, however, even single strands with homologous 3′ ends did not form joints unless the recipient duplex DNA was superhelical.

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Using oligonucleotides as the single-stranded substrate, Hsieh *et al.* (1992) found that in the presence of adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS)[1] there was no difference between 5′ and 3′ homologous ends. The present studies were carried out to understand better the specificity of RecA protein with respect to homologous single-stranded ends in the presence of the physiological cofactor ATP.

MATERIALS AND METHODS

RecA protein and single-stranded DNA binding protein (SSB) were purified according to the respective procedures of Shibata *et al.* (1981) and Lohman *et al.* (1986). The concentration of RecA protein was measured by the absorbance using a value of E_{277nm} = 6.33 (Tsang *et al.*, 1985). The restriction enzymes *Bam*HI, *Ban*II, *Cla*I, *Eco*RI, *Mlu*I, *Msc*I, *Nar*I, *Sal*I, and *Xho*I were purchased from New England Biolabs. Proteinase K was from EM Laboratories (Elmsford, NY).

Standard Reaction Conditions. Presynaptic filaments were formed by incubating 10 μM single-stranded DNA with 5 μM RecA protein and 0.83 μM SSB at 37 °C for 12 min in a reaction mixture containing 33 mM Pipes buffer (pH 6.5), 12 mM $MgCl_2$, 1.5 mM ATP, 8 mM phosphocreatine, creatine phosphokinase (10 units/mL), and BSA (100 μg/mL). The formation of joint molecules was initiated by the addition of labeled linear duplex DNA (5 μM).

Pairing Scheme. Joint molecules were formed between linear single-stranded DNA with homology limited to either end or to the middle, and linear duplex DNA in which the homology was present in the middle. Joint molecules were formed with substrates containing different lengths of homology, ranging from 48 bp to 6 kb of homology. To form the joint molecules with 1.7 kb homology, M13mp19-EG902 single-stranded DNA with homology (1.7 kb insert from the human β-globin cluster) restricted to the 3′ end, 5′ end, or to the middle was paired with pAγ linear duplex DNA in which the homology was present in the middle. To form the joint molecules with 6 kb of homology, M13G*ori*1 single-stranded DNA with homology (6 kb of M13 sequence) limited to its 3′ end, 5′ end, or to the middle was paired with M13mp8 linear duplex DNA in which the homology was present in the middle. Similarly, to form the joint molecules with 500 bp of homology, M13mp19-EG906 single-stranded DNA with homology at the 3′ end was paired with linear duplex DNA (p3′N10R) in which homology was present in the interior, away from either end. To form the joints at the 3′ end of the single-stranded DNA with 48 bp of homology, an oligonucleotide that had 48 nucleotides of homology at its 3′ end and a heterologous region of 24 nucleotides at the 5′ end was paired with a long linear duplex DNA that had the target sequence in the middle.

Preparation of DNA Substrates. Superhelical DNA from M13mp8, M13G*ori*1, pAγ, and p3′10NR and circular single-stranded DNA from M13, M13G*ori*1, M13mp19-EG902, and M13mp19-EG906 phages were prepared as described (Das-Gupta *et al.*, 1980; Cunningham *et al.*, 1981). pAγ is a derivative of pBR322 which has an insert of 3.3 kb of the human β-globin cluster (Collins & Weissman, 1984). p3′10NR is a derivative of pBR322 which has an insert of 0.5 kb of the human β-globin cluster (Kandpal *et al.*, 1990). M13G*ori*1 DNA contains the entire genome of M13 phage with an insert of 2216 bases from G4 phage (Kaguni & Ray, 1979). M13mp19-EG902 and M13mp19-EG906 are derivatives of M13mp19 with an insert of 1.7 and 0.5 kb fragments, respectively, of the human β-globin cluster.[2] The fraction of linear DNA in preparations of circular single-stranded DNA and the fraction of nicked molecules in preparations of superhelical DNA were less than 5% as judged by gel electrophoresis.

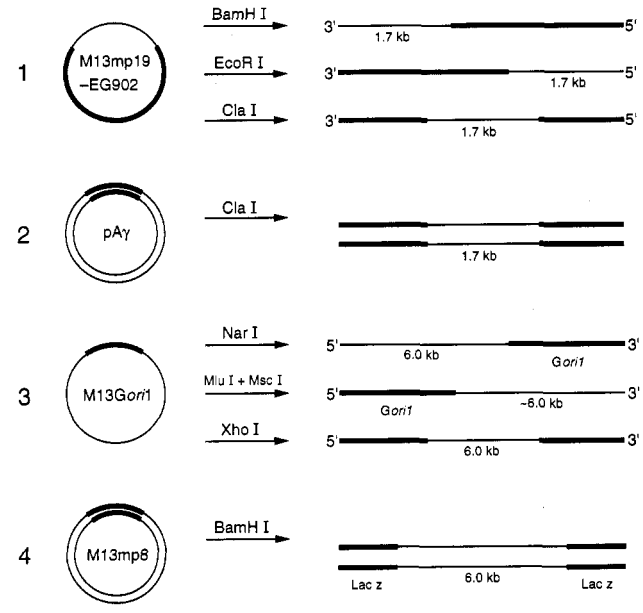

FIGURE 1: Substrates. Preparation of linear single-stranded DNA with the homology restricted to the 3′ end, to the 5′ end, or to the middle and linear double-stranded DNA with the homology limited to the middle (refer to Materials and Methods). Thick lines represent the region of heterology, and thin lines represent the region of homology.

Single-Stranded Linear DNA. Circular single-stranded DNA was annealed with a specific oligonucleotide to generate a unique restriction site and was then treated with restriction enzyme. Annealing of oligonucleotide to the single-stranded DNA was done by incubation of 600 μM single-stranded circular DNA and 105 μM oligonucleotide in 100 mM NaCl/10 mM Tris-HCl, pH 7.5, at 65 °C for 45 min, followed by slow-cooling to room temperature. Prior to incubation at 65 °C, samples were heated at 90 °C for 2 min. M13mp19-EG902 single-stranded DNA was linearized by *Bam*HI, *Eco*RI or by *Cla*I restriction enzymes to limit the homology to the 3′ end, the 5′ end, or to the middle of single-stranded DNA, respectively (Figure 1). Digestion with *Bam*HI, *Eco*RI, or *Cla*I restriction enzymes was done for 3 h, after adjusting the reaction conditions to those appropriate for the restriction enzyme to be used. Similarly, M13G*ori*1 circular single-stranded DNA was linearized by *Nar*I or by double digestion with *Mlu*I and *Msc*I or by *Xho*I restriction enzymes to restrict the homology to the 5′ end, the 3′ end, or to the middle, respectively (Figure 1). Digestion with *Nar*I or *Mlu*I and *Msc*I restriction enzymes was done for 4 h, after a 2-fold dilution of the sample and addition of 10 mM $MgCl_2$, 2 mM DTT, and 25 mM Tris-HCl, pH 7.5. The preparation of linear single-stranded DNA was treated with 100 μg/mL proteinase K/1% (w/v) SDS and purified on a Sepharose 2-B column. Preparation of linear single-stranded DNA made by the use of *Bam*HI, *Eco*RI, *Cla*I, and *Mlu*I+*Msc*I contained less than 5% circular DNA, and that made by cutting with *Nar*I less than 10% as judged by gel electrophoresis.

[1] Abbreviations: ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); SSB, *E. coli* single-stranded binding protein; SDS, sodium dodecyl sulfate.

[2] Efim Golub, personal communication.

Linear Duplex DNA. Cleavage of pAγ superhelical DNA with *Cla*I restriction enzyme generated linear duplex DNA that had long flanking sequences on either side of the region of homology (1.7 kb from the β-globin cluster). M13mp8 superhelical DNA digested with *Bam*HI restriction endonuclease generated a linear duplex in which the region of homology (6 kb of M13 sequences) was flanked by LacZ sequences. p3′N10R superhelical DNA was cleaved with SalI restriction enzyme to generate a linear duplex DNA that contained the region of homology (500 bp fragment from β-globin cluster) in the middle. M13G*ori*1 superhelical DNA was digested with *Ban*II restriction enzyme to restrict the homology (M13 sequences) to the proximal end, and with *Mlu*I and *Msc*I restriction enzymes to limit the homology to the distal end of the resulting linear duplex DNA, and was purified over a neutral sucrose gradient.

Assay of the Joint Molecules. Detection of the joint molecules without deproteinization was done according to the procedure of Bianchi *et al.* (1983). In this assay, aliquots were withdrawn at the indicated time and put directly into 5 mL of cold 10 × SSC (1.5 M NaCl/0.15 M sodium citrate, pH 7.0), and the sample was filtered immediately. To detect the formation of joint molecules that are stable after deproteinization, we used an assay described by Rao *et al.* (1991). In this assay, joint molecules formed during the reaction were measured by withdrawing aliquots at indicated times and deproteinizing with 20 mM EDTA, 0.5% SDS, and 100 μg/mL proteinase K treatment at 37 °C for 20 min. After deproteinization, 5 mL of cold 10 × SSC was added, and the sample was filtered. In the gel electrophoresis assay, the level of joint molecule formation was detected by the appearance of a characteristic product during gel electrophoresis. At indicated times, an aliquot of the reaction mixture was withdrawn and subjected to deproteinization with 20 mM EDTA, 0.5% SDS, and 100 μg/mL proteinase K treatment for 20 min at 37 °C and then loaded directly onto a gel. Gel electrophoresis was done in 0.8% (w/v) agarose using 1 × TBE at 100 V/50 mA for 5–6 h at 4 °C. The gel was dried and examined by autoradiography after overnight exposure.

RESULTS

Experimental Design and Terminology. To study the effects of single-stranded ends on the formation of joint molecules by RecA protein, we employed the substrates that are diagrammed in Figure 2, for which we adopted the following terminology: A joint formed in the interior of linear duplex DNA by a single strand in which homology is restricted to the 3′ end is a 3′ joint (Figure 2g). A joint formed by a single strand with homology limited to its 5′ end is a 5′ joint (Figure 2e), and one formed by a single strand with homology limited to its middle is a medial joint (Figure 2f).

The formation of joint molecules and their stability after deproteinization were determined by several assays. The total level of joint formation was measured by the filter assay described by Bianchi *et al.* (1983). This particular assay, which does not involve any intended deproteinization, was originally devised to detect unstable "paranemic" or medial joints. This assay can detect the conversion of virtually all of the duplex DNA into joint molecules, including those whose stability depends on the continued attachment of RecA protein (Riddles & Lehman, 1985; Bianchi *et al.*, 1983). However, this assay cannot be used to determine the level of joint formation when oligonucleotides are used as substrates, in which case we used the restriction protection assay described by Hsieh *et al.* (1992).

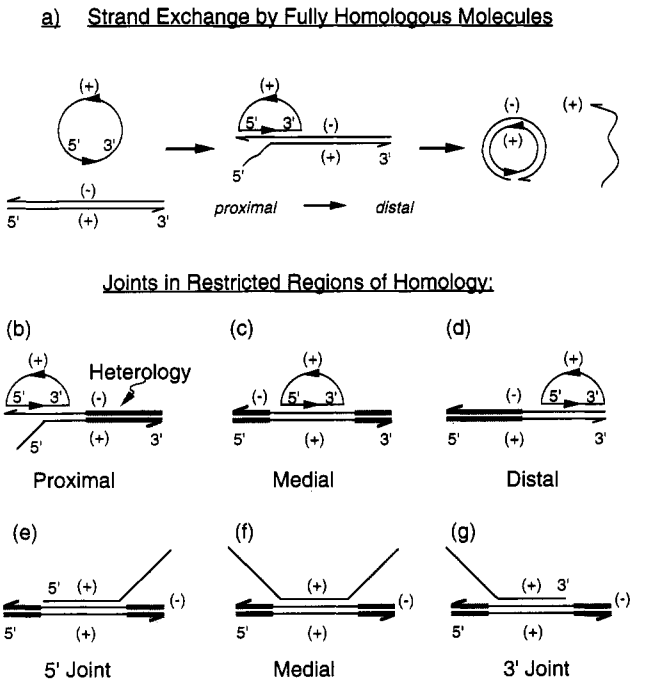

FIGURE 2: Experimental design and terminology. (a) A model system that illustrates the polarity of strand exchange promoted by RecA protein; (b–d) substrates in which homology is restricted to different locations in duplex DNA; (e–g) substrates used in the present study in which homology in linear single-stranded DNA was restricted to different locations and homology in linear duplex DNA was located in its middle.

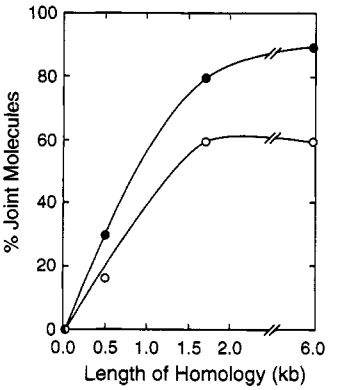

FIGURE 3: Effect of length of homology on the yield of 3′ joints. The 3′ joints were formed under standard reaction conditions for 30 min, with substrates containing different lengths of homology. Joint molecules with 48 bp, 500 bp, 1.7 kb, and 6.0 kb of homology were formed with the substrates described under Materials and Methods and in Figure 1. The yield of joint molecules that were formed with 500 bp, 1.7 kb, and 6.0 kb of homology was measured by the filter assay with (○) and without (●) prior deproteinization, as described by Rao *et al.* (1991) and Bianchi *et al.* (1983), respectively. The formation of the 3′ joints at the smallest length of homology (48 bp) was sought by the assay described by Hsieh *et al.* (1992), and by gel electrophoresis of the deproteinized product.

To detect joints that survive deproteinization, we used both gel electrophoresis and a filter assay described by Rao *et al.* (1991). In both assays, aliquots were deproteinized by incubation for 20 min at 37 °C in 20 mM EDTA, 0.5% SDS, and 100 μg of proteinase K per mL.

Effect of Length of Homology on the Formation and Stability of 3′ Joints. We used single-stranded DNA substrates with homologous regions ranging from about 50 bp to 6 kb at its 3′ end to form joint molecules (refer to Materials and Methods). As shown in the Figure 3, the yield of the 3′ joints, assayed either with or without deproteinization, depended on the length of homology. While the formation

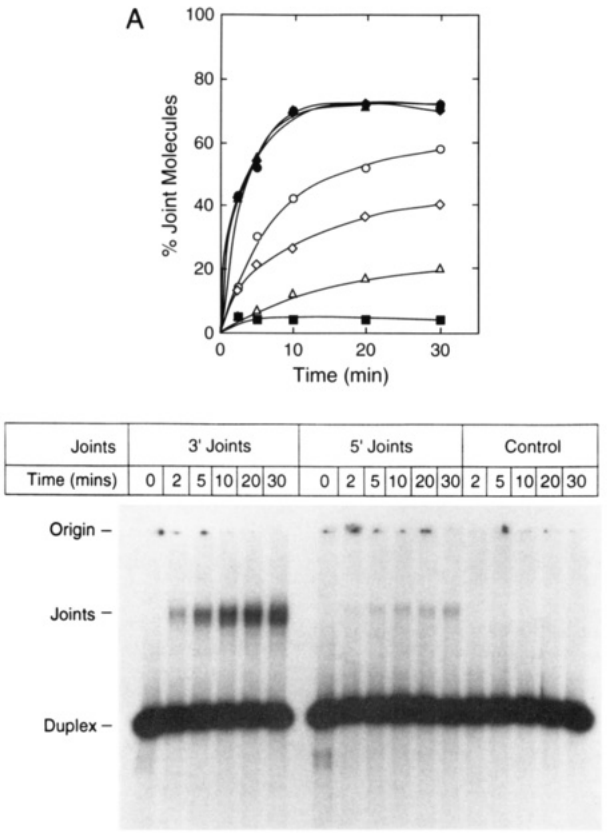

FIGURE 4: (A) Time course of formation of joint molecules with 1.7 kb of homology. The 3′, 5′, and medial joints were formed under standard reaction conditions with M13mp19-EG902 linear single-stranded DNA in which the homology was limited to either end or to the middle and pAγ linear duplex DNA in which the homology was present in the middle (see Materials and Methods). Aliquots were withdrawn at the indicated times and assayed by the filter assay with (○, 3′ joints; △, 5′ joints; and ◇, medial joints) and without (●, 3′ joints; ▲, 5′ joints and ◆, medial joints) prior deproteinization. A control reaction was done under the same conditions but for the omission of single-stranded DNA (■). (B) Formation of 3′ and 5′ joint molecules with 1.7 kb of homology as measured by gel electrophoresis. The 3′ and 5′ joint molecules were formed with the substrates described in panel A. Reactions were performed under standard conditions described under Materials and Methods. At various time intervals, samples were treated with 20 mM EDTA, 0.5% SDS, and 100 μg/mL proteinase K for 20 min at 37 °C and analyzed by electrophoresis in agarose gels as described under Materials and Methods. A control reaction was done under the same conditions but with heterologous single-stranded DNA.

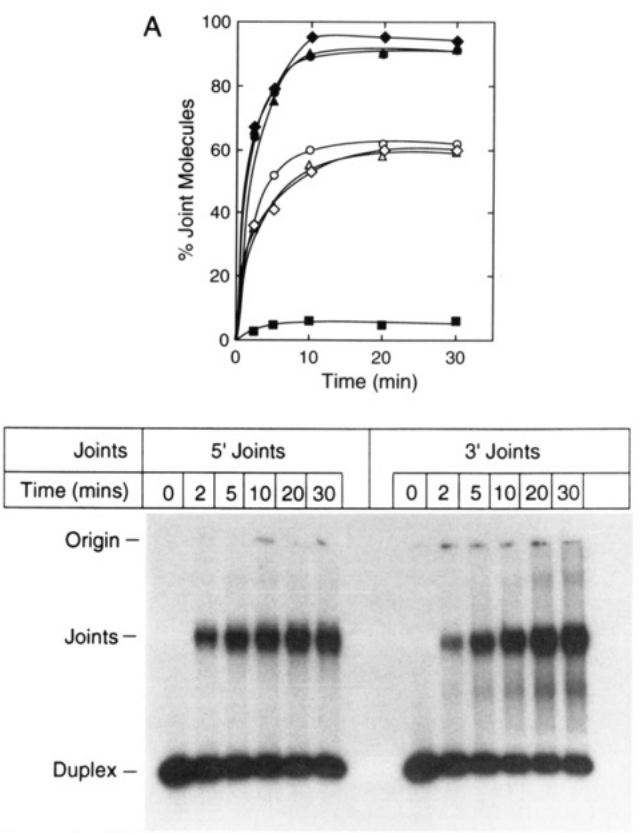

FIGURE 5: (A) Time course of formation of joint molecules with 6.0 kb of homology. The 3′, 5′, and medial joints were formed under standard reaction conditions with M13*Gori1* linear single-stranded DNA in which the homology was limited to either end or to the middle and M13mp8 linear duplex DNA in which the homology was present in the middle (see Materials and Methods). Aliquots were withdrawn at the indicated times and assayed by the filter assay with (○, 3′ joints; △, 5′ joints, and ◇, medial joints) and without (●, 3′ joints; ▲, 5′ joints, and ◆, medial joints) prior deproteinization. A control reaction was done under the same conditions except for the omission of single-stranded DNA (■). (B) Formation of 3′ and 5′ joint molecules with 6.0 kb of homology as measured by gel electrophoresis. The 3′ and 5′ joint molecules were formed with the substrates described in panel A. Reactions were performed under standard conditions described under Materials and Methods. At various time intervals, samples were treated with 20 mM EDTA, 0.5% SDS, and 100 μg/mL proteinase K for 20 min at 37 °C and analyzed by electrophoresis in agarose gels as described under Materials and Methods.

of joints at 1.7 and 6 kb of homology was 70% and 90%, respectively, the yield of the joints after deproteinization was about 60% in both cases. However, both the formation and the stability of the joints fell off sharply when the length of homology was less than 1.7 kb.

Effect of Single-Stranded Ends on Joint Molecules Involving 1.7 vs 6 kb of Homology. When the region of homology was 1.7 kb in length, joint molecules formed with equal efficiency whether homology was restricted to either end or to the middle of the single strand: In all three cases, the yield of joint molecules exceeded 70% (Figure 4A). However, upon deproteinization by SDS and proteinase K, the yield of joint molecules fell to 57%, 40%, and 20%, respectively, for 3′, medial, and 5′ joints. The differential stability of the 3′ joints *vs* 5′ joints was also seen when joint molecules were assayed by gel electrophoresis (Figure 4B).

When the length of homology was increased from 1.7 to 6 kb, the yield of joints increased from 70% to 90% for all three single-stranded substrates whether homology was located at either end or in the middle of single-stranded DNA (Figure 5A). A more striking effect of a longer region of homology was seen, however, in the yield of joints following deproteinization. Whereas at 1.7 kb of homology the yield of deproteinized joints differed greatly according to the location of homology, at 6 kb of homology, two-thirds of all joints survived deproteinization without regard to the location of homology (Figure 5A). Stability to deproteinization of both the 5′ and 3′ joints that were formed with substrates having 6 kb of homology was seen by gel electrophoresis as well (Figure 5B).

Dynamic State of 5′, 3′, and Medial Joints. In a reaction mixture, in the presence of RecA protein and ATP, the level of distal joints (see Figure 2d) reflects a steady state in which joints constantly dissociate and re-form (Wu *et al.*, 1982; Burnett *et al.*, 1994). Several experimental conditions reveal the recycling: The addition of either NaCl or ADP, both of which inhibit the re-formation of joints, results in the net dissociation of distal joints; and the addition of unlabeled linear duplex DNA chases labeled duplex DNA out of distal joints. By contrast, in the case of proximal joints (see Figure 2b), RecA protein carries out strand exchange, which includes the

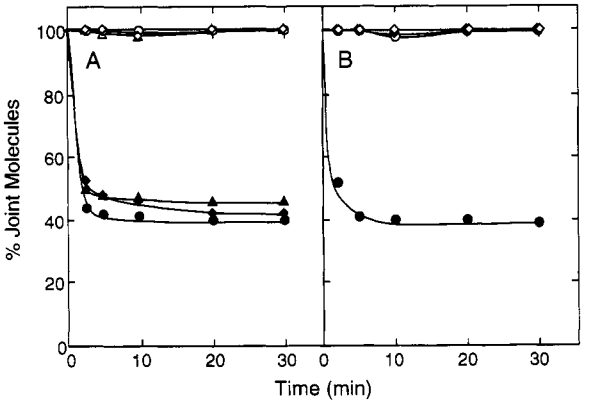

FIGURE 6: (A) Effect of addition of 50 mM NaCl to ongoing reactions of 3′, medial, and 5′ joint molecule formation. (A) The 3′, 5′, and medial joint molecules with 1.7 kb of homology were formed under standard reaction conditions. After 20 min of joint formation, the reaction mixture was divided into two parts: to one part was added 50 mM NaCl (●, 3′ joints; ▲, 5′ joints and ♦, medial joints), and an equivalent volume of buffer was added to the other (○, 3′ joints; △, 5′ joints and ◇, medial joints). The reaction was continued for another 30 min. At the indicated times, aliquots of reaction mixtures were withdrawn and added directly into 5 mL of cold 10 × SSC and filtered immediately. (B) Effect of addition of unlabeled DNA to the ongoing reaction of 3′ and proximal joints. The 3′ joints and proximal joints were formed under the standard reaction conditions. After 20 min of joint formation, the reaction mixture was divided into two parts: to one part was added 10 times excess of unlabeled form III DNA (●, 3′ joints, and ♦, proximal joints) and an equivalent volume of buffer was added to the other (○, 3′ joints, and ◇, proximal joints). At the indicated time intervals, aliquots were withdrawn and added directly into 5 mL of 10 × SSC and filtered immediately.

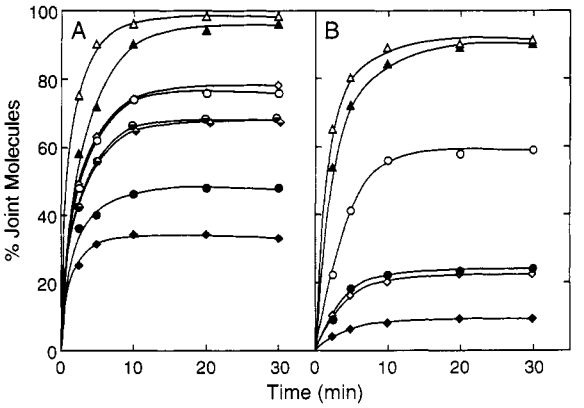

FIGURE 7: Concentration of RecA nucleoprotein filaments *versus* the formation of proximal, 3′, and 5′ joint molecules. (A) Without deproteinization. The ratio of RecA nucleoprotein filaments to molecules of duplex DNA was varied from 1:1 to 4:1, while the ratio of molecules of RecA protein to nucleotide residues of single-stranded DNA remained constant at 1:2, and that of SSB to single-stranded DNA nucleotides remained at 1:12.5. Nucleoprotein filaments were formed as described under Materials and Methods. The reactions were started by the addition of 5 μM duplex DNA, and the joint molecules were assayed without deproteinization as described by Bianchi *et al.*, (1983). Ratio of single-stranded DNA to duplex DNA: proximal joints (△, 4:1; ▲, 1:1), 3′ joints (○, 4:1; ⊖, 2:1; ●, 1:1), and 5′ joints (◇, 4:1, ♦, 2:1, ♦, 1:1). (B) Deproteinized joints. The reactions were performed as described in panel A with the exception that the joint molecules were assayed after deproteinization. Ratio of single-stranded DNA to duplex DNA: proximal joints (△, 4:1; ▲, 1:1), 3′ joints (○, 4:1; ●, 1:1), and 5′ joints (◇, 4:1; ♦, 1:1).

switching of base pairs and the extrusion of the displaced third strand from its 5′ end. This strand exchange in proximal joints does not appear to be reversible, as proximal joints do not recycle.

To characterize further 5′, 3′, and medial joints, we examined their turnover in the presence of RecA protein, ATP, and an ATP regeneration system. We formed 3′, 5′, and medial joint molecules with the substrates containing 1.7 kb of homology, under standard conditions for 20 min. Then the reaction was split into two parts: to one part was added 50 mM NaCl, and to another part was added an equivalent volume of buffer. Addition of 50 mM NaCl led to the dissociation of more than half of the 3′, 5′, or medial joint molecules (Figure 6A). In another experiment, addition of excess unlabeled competitor DNA to an ongoing reaction involving 3′ joints led to loss of label from about 60% of the joints, whereas a control revealed no loss of label from proximal joints (Figure 6B).

These experiments reveal that with or without ends, when single-stranded DNA forms joints in the interior of duplex DNA, such joints exist in a dynamic state: RecA protein catalyzes a reversible or steady-state reaction in which it constantly forms and dissociates these joints (see Figure 8C).

Conditions That Affect the Apparent Yield of the 3′ and 5′ Joint Molecules. The observed dynamic state of joints formed in the interior of duplex DNA led us to examine the influence of reaction conditions on the steady state or equilibrium level of joints. As a control, we simultaneously examined the levels of proximal joints, previously shown not to turn over (Burnett *et al.*, 1994). Joints, (3′, 5′, and proximal) were formed by varying the ratio of linear single-stranded DNA to linear duplex DNA from 1:1 to 4:1. The ratio of RecA protein to single-stranded DNA was kept constant at 1 molecule of RecA protein to 2 residues of nucleotide, and the ratio of SSB to single-stranded DNA was kept constant at 1 molecule of SSB per 12.5 nucleotide residues. Homologous pairing was initiated by the addition of a fixed concentration of linear double-stranded DNA, and the formation of joint molecules was assayed with or without deproteinization (see Materials and Methods).

Consistent with our previous observations (Burnett *et al.*, 1994), optimal formation of proximal joints was achieved at a 1:1 ratio of single-stranded DNA to double-stranded DNA, with little additional yield resulting at a ratio of 4:1. By contrast, the yield of both the 3′ and 5′ joints, with or without deproteinization, depended on the ratio of single-stranded to double-stranded DNA. The yield was poor at a ratio of 1:1 and increased markedly at a ratio of 4:1 (Figure 7A,B).

Wu *et al.* (1982) and Burnett *et al.* (1994) observed that when ADP was allowed to accumulate in a RecA reaction, distal joints formed early in the reaction and then dissociated, whereas proximal joints reached some level and persisted. We observed similarly in the present experiments that when ADP was allowed to build up in the reaction mixture, the yield of both the 5′ and 3′ joints reached a maximum level and then declined (data not shown), further suggesting that both these joints belong to the same class of dynamic joints as distal joints (see Figure 8C).

DISCUSSION

To gain a better understanding of the role of ends in the specificity of RecA protein, we studied the pairing of linear single strands with linear duplex DNA in which the region of homology was far from either end of the duplex. Using filter assays and gel electrophoresis, we found that the formation and properties of joints depend on both the length and location of homology.

Single-stranded DNA with homology limited to its 3′ end formed joint molecules with nonsuperhelical duplex DNA, as judged both by filter assays and by gel electrophoresis. The yield of 3′ joints, with or without deproteinization, depended on the length of homology (Figure 3). Using the nonhydro-

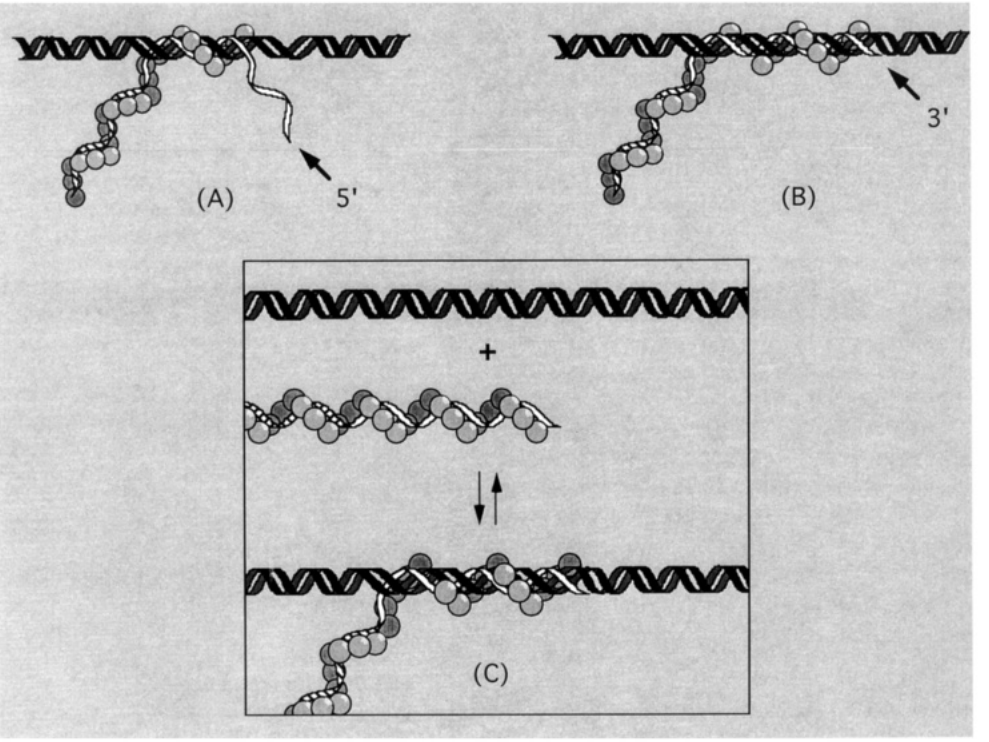

FIGURE 8: Model for the structure of the 5′ and 3′ joint molecules and their dynamic state. Depiction of a 5′ joint (A), a 3′ joint (B), and the dynamic nature of the joints formed in the interior of duplex DNA by linear single-stranded DNA (C). The dynamic nature of the reaction as diagrammed in (C) was observed for 5′, 3′, and medial joints. Cross-hatched ribbons in single-stranded DNA represent heterology.

lyzable analog ATPγS as cofactor, oligonucleotides with homology at the 3′ or 5′ end, and superhelical recipient DNA, Hsieh *et al.* (1992) had observed that RecA protein does not exhibit polarity in the initiation of pairing. However, in the presence of the physiological cofactor ATP and linear recipient duplex DNA, we could find no joints with an oligonucleotide that had 48 residues of homology at its 3′ end, either by the restriction assay of Hsieh *et al.* (1992) or by any of the assays described above. When we increased the length of homology to 1.7 kb, we found that joints formed equally efficiently without regard to the location of homology in relation to ends, yet those joints differed qualitatively, as seen by differences in survival upon deproteinization. When deproteinized, 3′ joints were the most stable; medial joints were less stable, consistent with earlier observations (Bianchi *et al.*, 1983; Riddles & Lehman, 1985); and 5′ joints were the least stable.

Whereas at 1.7 kb of homology the stability of deproteinized joints was clearly influenced by the nature of the ends (Figure 4A,B), by another criterion, namely, the tendency to recycle, 5′, 3′, and medial joints were all of the same kind. We have previously found that when homology is limited to the ends of a double-stranded substrate, two distinct kinds of joint are formed: proximal joints (see Figure 2b) that undergo strand exchange and do not turn over at all, and distal joints (see Figure 2d) that do not complete strand exchange, but constantly dissociate and reassociate (Wu *et al.*, 1982; Rao *et al.*, 1991, 1992; Burnett *et al.*, 1994). We have suggested that when a displaceable 5′ end does not exist and strand exchange cannot be completed, RecA protein uses the energy derived from the hydrolysis of ATP to dissociate triplex joints by the same mechanism that it otherwise uses to separate the products of strand exchange (Burnett *et al.*, 1994). The present observations support the view that recycling is simply an alternative to completion of strand exchange: When a joint is formed in the middle of duplex DNA, the plus strand from the duplex, which is covalently linked to flanking DNA on both sides of the joint, cannot be separated from the joint (Figure 8C). The equal propensity of 5′, 3′, and medial joints to recycle suggests that all of these joints are structurally similar. In all of these joints, there is also an inhomogeneity that is revealed by the significant fraction of joints that fail to recycle, for which we have no explanation (see Figure 6A,B). The joints that fail to recycle in the presence of 50 mM NaCl may represent a new equilibrium value in the presence of NaCl. However, even in the presence of 10 times excess cold competitor linear duplex DNA, the joints did not recycle completely, suggesting that there are two kinds of joints, some dissociable and some not.

At 6 kb of homology, all joints formed equally efficiently, had the same stability after deproteinization (Figure 5A), and recycled equally, when the region of homology in single-stranded DNA had a free 5′ end, a free 3′ end, or no free homologous end. The disappearance of any influence of free homologous ends on the properties of joints formed by long regions of homology suggests that all such joints were located away from the ends of the homologous regions and therefore completely equivalent.

All of the foregoing observations on the formation and stability of joints can be rationalized by supposing that the major influence of free homologous ends in single-stranded DNA is indirect: when the region of homology is big enough, there is no effect of ends; as the region of homology gets shorter, the presence or absence of free homologous ends affects the size and hence the stability of the joint. Single strands with homologous 3′ ends are best because the region of homology is fully coated by RecA protein and the length of the resulting joint is maximized (Figure 8B), single strands without free homologous ends form less stable joints perhaps because topological constraints tend to shorten the joints, and homologous 5′ ends are the worst because RecA protein tends not to coat the 5′ end (Register & Griffith, 1985; Konforti & Davis, 1987, 1990), leading to shorter joints (Figure 8A).

The present observations offer an explanation for a disagreement that exists in the literature concerning the ability

of RecA protein to form 5′ joints in either superhelical or nonsuperhelical DNA, and to form 3′ joints in nonsuperhelical DNA in the presence of ATP. Examples exist for (Wu *et al.*, 1982; Dutreix *et al.*, 1991) and against (Konforti & Davis, 1987, 1990, 1991) the proposition that such joints can form, including the favorable examples provided in this paper. The discrepancies are not attributable to differences in the assays used, since we can detect either 5′ or 3′ joints in nonsuperhelical DNA by gel electrophoresis as well as by filter assays. The observations reported here may reconcile these conflicting findings: The level of joints formed in the interior of duplex DNA, like the level of distal joints (Burnett *et al.*, 1994), represents a steady state or equilibrium that is known to be sensitive to several reaction parameters, such as the ratio of single-stranded DNA to duplex DNA,the concentration of salt, and the accumulation of ADP (Wu *et al.*, 1982; Burnett *et al.*, 1994). A measurable steady-state level of 3′ joints is not observed at all if the ATP regeneration system is lacking or insufficient [see also Wu *et al.* (1982) and Burnett *et al.* (1994)]. We note that in the observations of Konforti and Davis (1991) the ratio of single-stranded DNA to duplex DNA was 1:1, which in our experiments resulted in a low steady-state level of 3′ joints, and a still lower level of 5′ joints.

These observations *in vitro* indicate that although RecA protein is well suited to using the 3′ single-stranded ends that are important *in vivo* (see the introduction) the action of an endonuclease or a topoisomerase is presumably required for further processing of 3′ joints. *In vitro*, however, RecA protein is able to deal with any single-stranded region, particularly if the length of such a region is large. It is of further interest and possible biological importance that RecA protein itself dissociates the very three-stranded joints it makes if strand exchange does not supervene [see also Burnett *et al.* (1994)]. Thus, *in vivo*, if further processing of a joint fails for any reason, RecA protein may participate in undoing the mischief that would be done by leaving a three-stranded intermediate in place.

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REFERENCES

Beattie, K. L., Wiegand, R., & Radding, C. M. (1977) *J. Mol. Biol. 116*, 783–803.
Bianchi, M., DasGupta, C., & Radding, C. M. (1983) *Cell 34*, 931–939.
Bishop, D. K., Park, D., Xu, L., & Kleckner, N. (1992) *Cell 69*, 439–456.
Braedt, G., & Smith, G. R. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 871–875.
Burnett, B. P., Rao, B. J., Jwang, B., Reddy, G., & Radding, C. M. (1993) *J. Mol. Biol. 238*, 540–554.
Clark, A. J. (1973) *Annu. Rev. Genet. 7*, 67–86.
Collins, F. S., & Weissman, S. M. (1984) *Prog. Nucleic Acid Res. Mol. Biol. 31*, 315–462.
Cox, M. M., & Lehman, I. R. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 6018–6022.
Cox, M. M., & Lehman, I. R. (1987) *Annu. Rev. Biochem. 56*, 229–262.
Cunningham, R. P., Wu, A. M., Shibata, T., DasGupta, C., & Radding, C. M. (1981) *Cell 24*, 213–223.
DasGupta, C., Shibata, T., Cunningham, R. P., & Radding, C. M. (1980) *Cell 22*, 437–446.
Dutreix, M., Rao, B. J., & Radding, C. M. (1991) *J. Mol. Biol. 219*, 645–654.
Hsieh, P., Camerini-Otero, C. S., & Camerini-Otero, R. D. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 6492–6496.
Joseph, J. W., & Kolodner, R. (1983) *J. Biol. Chem. 258*, 10418–10424.
Kaguni, J., & Ray, D. S. (1979) *J. Mol. Biol. 135*, 863–878.
Kahn, R., Cunningham, R. P., DasGupta, C., & Radding, C. M. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4786–4790.
Kandpal, P. R., Ward, D. C., & Weissman, S. M. (1990) *Nucleic Acids Res. 18*, 1789–1795.
Konforti, B. B., & Davis, R. W. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 690–694.
Konforti, B. B., & Davis, R. W. (1990) *J. Biol. Chem. 265*, 6916–6920.
Konforti, B. B., & Davis, R. W. (1991) *J. Biol. Chem. 266*, 10112–10121.
Kowalczykowski, S. C. (1991) *Annu. Rev. Biophys. Biophys. Chem. 20*, 539–575.
Kushner, S. R., Nagaishi, H., & Clark, A. J. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1366–1370.
Kushner, S. R., Nagaishi, H., & Clark, A. J. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 3593–3597.
Lehman, I. R., & Nussbaum, A. L. (1964) *J. Biol. Chem. 239*, 2628–2636.
Little, J. W. (1967) *J. Biol. Chem. 242*, 679–686.
Lohman, T. M., Green, M. J., & Beyer, R. S. (1986) *Biochemistry 25*, 21–25.
Low, K. B. (1973) *Mol. Gen. Genet. 122*, 119–130.
Radding, C. M. (1982) *Annu. Rev. Gen. 16*, 405–437.
Radding, C. M. (1988) in *Genetic Recombination* (Kucherlapati, R., & Smith, G. R., Eds.) pp 193–229, American Society for Microbiology, Washington, D.C.
Radding, C. M., Beattie, K. L., Holloman, W. K., & Wiegand, R. C. (1977) *J. Mol. Biol. 116*, 825–839.
Rao, B. J., Dutreix, M., & Radding, C. M. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 2984–2988.
Rao, B. J., Chiu, S. K., & Radding, C. M. (1992) *J. Mol. Biol. 229*, 328–343.
Riddles, P. W., & Lehman, I. R. (1985) *J. Biol. Chem. 260*, 165–169.
Roca, A. I., & Cox, M. M. (1990) *Crit. Rev. Biochem. Mol. Biol. 25*, 415–456.
Shibata, T., Cunningham, R. P., & Radding, C. M. (1981) *J. Biol. Chem. 256*, 7557–7564.
Smith, G. R. (1988) *Microbiol. Rev. 52*, 1–28.
Sun, H., Schultes, N. P., & Szostak, J. W. (1989) *Nature 338*, 87–90.
Sun, H., Treco, D., & Szostak, J. W. (1991) *Cell 64*, 1155–1161.
Taylor, A. F., Schultz, D. W., Ponticelli, A. S., & Smith, G. R. (1985) *Cell 41*, 153–163.
Tsang, S. S., Muniyappa, K., Azhderian, E., Gonda, D. K., Radding, C. M., Flory, J., & Chase, J. W. (1985) *J. Mol. Biol. 185*, 295–309.
West, S. C. (1992) *Annu. Rev. Biochem. 61*, 603–640.
Wu, A. M., Kahn, R., DasGupta, C., & Radding, C. M. (1982) *Cell 30*, 37–44.