Uptake and Processing of Duplex DNA by RecA Nucleoprotein Filaments: Insights Provided by a Mixed Population of Dynamic and Static Intermediates†

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Received January 20, 1995; Revised Manuscript Received April 26, 1995⊗

ABSTRACT: In the polarized strand exchange that is promoted by *Escherichia coli* RecA protein, when the initiating end of a duplex DNA molecule is blocked by heterology, the homologous distal end nonetheless forms a joint with single-stranded DNA, but strand exchange in that joint cannot be completed because the strand that would otherwise be displaced lacks a free 5′ end. Instead, 2/3 to 3/4 of such *distal joints* cyclically form and dissociate. Dissociation requires the hydrolysis of ATP (Burnett *et al.*, 1994). Observations on DNase protection revealed that consistent with their dynamic nature, these joints were heterogeneous in length, extending from the labeled distal end of the duplex up to 600 base pairs within the homologous region. Switching of base pairs was undetectable in this fraction of distal joints. However, the other 1/3 to 1/4 of distal joints, which did not cycle, were as long as the entire homologous region (6 kb), and underwent complete switching of base pairs. The formation of these static joints occurred at a rate in excess of 100 bp per second, without requiring hydrolysis of ATP. These and earlier observations suggest that the RecA filament containing single-stranded DNA rapidly incorporates duplex DNA into a coaxial three-stranded helix by a passive process, whereas additional energy is required to convert the three-stranded intermediate into products or back into substrates, both of which involve the unwinding of many turns of three-stranded helix.

Escherichia coli RecA protein is the prototype of a universal class of proteins that play essential roles in recombination and repair (Ogawa *et al.*, 1993; Story *et al.*, 1993; Shinohara *et al.*, 1993; Bezzubova *et al.*, 1993; Radding, 1993; Sung, 1994; Bishop, 1994; Benson *et al.*, 1994; Kowalczykowski, 1994; Haaf *et al.*, 1995). By polymerization on single-stranded DNA (ssDNA),[1] RecA protein forms a right-handed helical nucleoprotein filament that recognizes homology in duplex DNA and effects a switch of base pairs, creating heteroduplex DNA and displacing a strand from the original duplex (Figure 1). The polarity of the RecA filament establishes the polarity of strand exchange, which in turn defines the initiating or proximal end of homologous duplex DNA. Acting on a circular single strand and homologous linear duplex DNA, which are frequently used as model substrates, RecA protein drives displacement in the 5′ to 3′ direction by a process that is fueled at a late stage by ATP hydrolysis (Figure 2a) (Cox, 1994).[2]

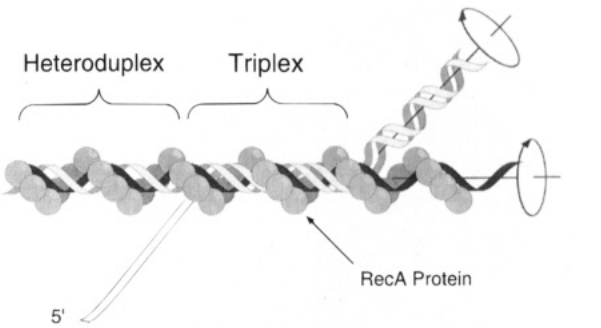

FIGURE 1: General model illustrating features of strand exchange mediated by RecA protein. The spheres represent monomers of RecA protein polymerized initially on the strand represented by a black ribbon. The filament takes up duplex DNA to form a triplex region within which base pairs are switched. The 5′ end of an unpaired strand exits from the filament, leaving behind a newly formed heteroduplex molecule.

In these complex reactions, whose mechanisms are still poorly understood, the nucleoprotein filament plays the key role, its helicity suited to the twin tasks of unwinding one double-helical molecule and making a new one. Howard-Flanders *et al.* (1984) postulated that the nucleoprotein filament takes up duplex DNA, creating an extensive triplex intermediate that mediates strand exchange (see Figure 1). Electron microscopic studies (Stasiak *et al.*, 1984) revealed interactions in which duplex DNA joined the RecA nucleoprotein filament some distance away from a second junction with single-stranded DNA, observations that were taken as evidence of a triple-stranded structure between the junctions. Subsequent electron microscopic studies of psoralen-photocross-linked intermediates provided further evidence for the close juxtaposition of three DNA strands over thousands of

† This study was supported by a grant from the National Institutes of Health (GM33504).

⊗ Abstract published in *Advance ACS Abstracts,* August 1, 1995.

[1] Abbreviations: ATPγS, adenosine 5′-*O*-thiotriphosphate; SSB, *E. coli* single-stranded DNA binding protein; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA.

[2] Recombination intermediates in which two parental molecules, or parts thereof, are joined by non-covalent bonds are commonly called *joint molecules*. That term has been adopted to designate the intermediates in the RecA reaction, whether protein is part of the complex or not. The term *joints* is used as shorthand for joint molecules, especially in compound terms such as *proximal joints* or *distal joints*. These terms respectively designate joint molecules in which only the proximal or distal ends of the duplex DNA are homologous to the single-stranded DNA in the RecA filament.

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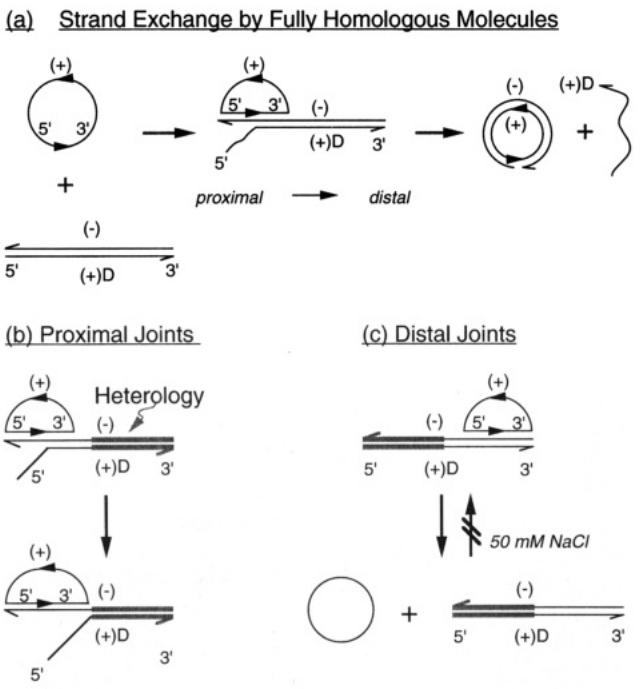

FIGURE 2: The model system. For simplicity, representation of RecA protein is omitted from all of these diagrams, which depict the state of DNA during the reactions of various model substrates. (a) Strand exchange between completely homologous duplex DNA and circular single strands. Strand exchange proceeds directionally 5′ to 3′ with regard to the polarity of the circular single strand and the identical strand from the duplex, termed the anticomplementary strand in this report. (b) Strand exchange that occurs when homology in duplex DNA is limited to its proximal end, forming proximal joints. (c) Cycling of distal joints that occurs when homology is limited to the distal end of duplex DNA.

base pairs (Bortner & Griffith, 1990; Griffith *et al.*, 1990; Umlauf *et al.*, 1990). Additional models of strand exchange or variations on the model of Howard-Flanders *et al.* (1984) have been proposed. Cox and colleagues (1994) proposed a model for strand exchange that couples ATP hydrolysis to DNA rotation. This coupling mechanism relies on hypothetical DNA binding sites on the exterior of the RecA filament to effect rotation of the DNAs about separate parallel axes. According to this model some distal portion of the duplex DNA must be on the outside of the filament. Burnett *et al.* (1994) proposed a variant of the Cox model in which ATP hydrolysis is coupled to a coaxial rotation of the three-stranded DNA within the RecA nucleoprotein filament. In this model, the entire duplex DNA can be located within the filament. In a recent model, Morel *et al.* (1994) suggested that only the proximal portion of the duplex DNA is taken up into the filament upon initial homologous contact.

The varying proposals in the models of strand exchange reflect uncertainty about the location of each of the three strands of DNA throughout the reaction. The difficulty of making this determination lies largely in the assays, each of which has limitations. The S1 nuclease assay, which detects the conversion of duplex DNA into single-stranded DNA, or *vice versa*, has been carried out under conditions that differ greatly from those in the RecA reaction. It detects the aggregate switching of base pairs in a population of molecules, but it does not reveal whether an unpaired strand is inside or outside of the filament. Electron microscopy, which has been used for that purpose, involves the preparation of samples under conditions that differ from those in solution, as well as the classification and interpretation of complex images. DNase protection assays, which assess the protection of DNA by protein, are empirical, but can be done under standard reaction conditions for RecA protein (Chow *et al.*, 1986; Pugh & Cox, 1987).

In the polarized strand exchange that is promoted by *E. coli* RecA protein, when the initiating end of a duplex DNA molecule is blocked by heterology, the homologous distal end nonetheless forms a joint with single-stranded DNA, but strand exchange in that joint cannot be completed because the strand that would otherwise be displaced lacks a free homologous 5′ end. Instead, the majority of such *distal joints* constantly recycle in a steady-state reaction (Figure 2C) (Burnett *et al.*, 1994). Homologous duplex DNA is taken up by the filament, and homologous naked duplex DNA is put out by the filament. If the switching of base pairs intervenes in distal joints, as it partially does (Wu *et al.*, 1982; Dutreix *et al.*, 1991), it must be reversed in the course of ejecting parental duplex DNA from the filament. Conditions were found that allow one to block the re-formation of joint molecules and thus to examine selectively the dissociation of distal joints. These findings led us to the DNase protection studies described here to understand better the interactions of duplex DNA with the recA nucleoprotein filament.

MATERIALS AND METHODS

Materials. 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 absorbance using a value of $E_{277mm} = 6.33$ (Tsang *et al.*, 1985). Restriction enzymes *Bam*HI, *Mlu*I, *Msc*I, *Kpn*I, *Xho*I, *Hpa*I, and *Sna*BI were purchased from New England Biolabs. Proteinase K was from EM Laboratories (Elmsford, NY). S1 nuclease was purchased from Sigma, and DNase I (RNase free) was purchased from Boehringer Mannheim (27 units/μL).

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 Tris-HCl (pH 7.5), 12 mM $MgCl_2$, 2 mM ATP, 8 mM phosphocreatine, creatine phosphokinase (10 units/mL), and BSA (100 μg/mL). Pairing was initiated by the addition of labeled duplex DNA (5 μM). Joint molecules were measured either by a gel electrophoresis assay or by a filter assay (see below).

Preparation of DNA Substrates. Superhelical DNA from M13G*ori1* and circular single-stranded DNA from M13 and M13G*ori1* phages were prepared as described (DasGupta *et al.*, 1980; Cunningham *et al.*, 1981). 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. Linear duplex DNA with 6 kb of M13 sequences limited to its distal end was prepared by cleaving M13G*ori1* superhelical DNA with *Msc*I and *Mlu*I restriction enzymes. The large fragment obtained after digestion of M13G*ori1* with *Msc*I and *Mlu*I contained about 6 kb of M13 sequences at the distal end (Figure 3A). Similarly, linear duplex DNA with 850 base pairs of M13 sequences limited to its distal end was prepared by cleaving M13G*ori1* with *Hpa*1 restriction enzyme. The small fragment obtained after digestion

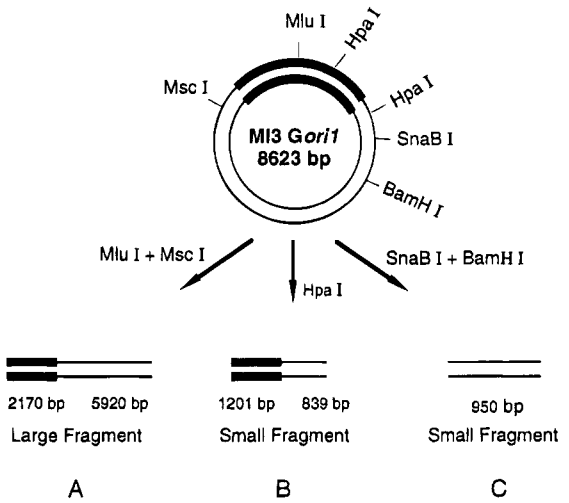

FIGURE 3: Preparation of linear duplex DNA substrates (see Materials and Methods). Thick lines represent regions of heterology, and thin lines represent regions of homology.

of M13G*ori1* with *Hpa*I contained about 850 bp of homology at the distal end (Figure 3B). Duplex DNA was labeled at the 5′ end with [γ-^{32}P]ATP by the use of polynucleotide kinase; similarly, duplex DNA was labeled at the 3′ end with [α-^{32}P]ddATP by the use of terminal transferase according to the procedures described in Maniatis *et al.* (1982). To obtain a uniquely labeled substrate with 6 kb of homology at the distal end, M13G*ori1* superhelical DNA was first digested with *Msc*I. The resulting linear duplex was end-labeled and was subsequently digested with *Kpn*I; the fragments were separated on a 5−20% neutral sucrose gradient. Similarly, to obtain a substrate with about 850 bp of homology at the distal end which was uniquely labeled at the 5′ end of the complementary strand, M13G*ori1* superhelical DNA was digested with *Hpa*I. The small fragment was electroeluted, end-labeled with kinase, digested with *Xho*I restriction enzyme, and subsequently passed through a Sepharose 2B column. For the completely homologous reactions, a 1-kb linear duplex DNA was used, which was obtained by digesting M13G*ori1* superhelical DNA with *Bam*HI and *Sna*BI (Figure 3C). To label this substrate uniquely at the 5′ end of the complementary strand, M13G*ori1* was initially digested with *Bam*HI, end-labeled, and digested with *Sna*BI. The resulting fragments were separated on a 5−20% neutral sucrose gradient.

Assay of the Joint Molecules. The formation of joint molecules was measured either by a filter assay (Burnett *et al.*, 1994) or by gel electrophoresis (Rao *et al.*, 1991). For the gel electrophoretic assay, aliquots were withdrawn at indicated times, deproteinized with 20 mM EDTA, 0.5% SDS, and 100 μg/mL proteinase K at 37 °C for 20 min, and loaded on the gel. Electrophoresis was done in 1% (w/v) agarose with 1× TBE (0.09 M Tris-borate/0.002M EDTA, pH 8.3) at 100 V/50 mA for 5−6 h at 4 °C. The gels were dried and examined by autoradiography after an overnight exposure; quantitation of the gels was done by a phosphorImager from Molecular Dynamics. In this assay, the level of joint molecules was measured by the appearance of a characteristic product during gel electrophoresis. For the filter assay, aliquots were withdrawn at indicated time points, added to 200 μL of 25 mM EDTA (pH 8.0), and kept on ice for 5 min. After this incubation, 5 mL of 10× SSC (1.5 M NaCl/0.5 M sodium citrate, pH 7.0) was added and the sample was passed through nitrocellulose filters.

Probing of the Joint Molecules with DNase I. Joint molecule formation was carried out under the standard reaction conditions. Aliquots (10 μL) were withdrawn at the indicated times and treated with 1 μL of DNase I for 1 min at 37 °C. The stock solution of DNase I (27 units/μL) was diluted appropriately so that 1 μL contained 0.1−1.0 unit of DNase I. The DNase I reaction was stopped by the addition of EDTA (25 mM) and vigorous mixing. Samples were deproteinized by the addition of SDS (0.5%) and 100 μg of proteinase K/mL and incubation at 37 °C for 20 min. The samples were treated at 100 °C for 2 min to denature the duplex DNA before loading on neutral 1% agarose gel. Unless noted, samples were analyzed on neutral 1% agarose gels.

When the samples were to be analyzed on a polyacrylamide gel, the DNase I reaction was stopped by extracting with phenol followed by chloroform. The DNA was precipitated by adding 100 μg of tRNA/mL, sodium acetate (0.2 M), and 3 vol of ethanol and storing overnight at −20 °C. The pellets were washed 6 times with 70% ethanol and were then diluted in 5 μL of loading buffer (90%, v/v, deionized formamide, 10 mM EDTA, 0.25% w/v, bromophenol blue, and 0.25% w/v, xylene cyanol). The Cerenkov counts of each sample were measured. The samples were heated at 95 °C for 5 min, and equal numbers of counts were loaded in each lane of an 8% denaturing polyacrylamide gel containing 8 M urea (38 × 42 × 0.04 cm). Electrophoresis was performed with the temperature maintained between 65 and 70 °C (1800 V/90 W). The gels were dried and analyzed by autoradiography.

Probing of Joint Molecules with S1 Nuclease. The extent of base pair switching was measured by determining the fraction of ^{3}H-labeled duplex DNA that became sensitive to S1 nuclease (Wu *et al.*, 1982). Since only one strand of the labeled duplex can become sensitive to S1 nuclease, the amount of ^{3}H-labeled duplex DNA that became sensitive to S1 nuclease was multiplied by 2 and divided by the fraction of duplex DNA engaged in joint molecules, to calculate the mean extent of base pair switching per joint molecule. At various times during the formation and processing of joint molecules, 25-μL aliquots were withdrawn and mixed with 2.2 μL of 10% SDS and stored on ice. All the collected samples were diluted into 0.5 mL of S1 nuclease digestion mixture (0.5 M NaCl, 50 mM sodium acetate, pH 4.5, 1 mM zinc acetate, and 0.12 unit of S1 nuclease) and incubated at 37 °C for 40 min. To each sample, 5 μL of sonicated carrier DNA (calf thymus DNA at 5 μg/μL) was added with thorough mixing, followed by the addition of 60 μL of 100% trichloroacetic acid. A 250-μL aliquot was withdrawn from each sample for counting the total radioactivity. The samples were stored on ice for 20 min and then centrifuged at 15600*g* for 15 min at 4 °C, and 250-μL aliquots from the supernatant were counted.

RESULTS

Experimental Design and Terminology. When a single plus strand in the RecA nucleoprotein filament reacts with fully homologous duplex DNA, the complementary minus strand from the latter ultimately remains in the filament in a new heteroduplex molecule, whereas the plus strand from

the duplex becomes a displaced single strand. As explained above, when homology is limited to the distal end of a duplex molecule, a joint is formed, but the two strands of the duplex cannot completely separate; the fate of these strands is the subject of the present study. To name the strands from the duplex, without specifying whether the strand is inside or outside the filament, we designate the minus strand from the duplex as the *complementary strand* and the plus strand from the duplex as the *anticomplementary strand* (see Figure 2A); both strands from the original duplex molecule are named in relation to the single plus strand initially present in the nucleoprotein filament.

In the experiments described below, we formed joint molecules either from M13G*ori1* circular single-stranded DNA and a 1-kb fragment of fully homologous linear duplex DNA or from M13 circular single-stranded DNA and a chimeric duplex DNA (M13G*ori1*) in which homology was limited to the distal end. In both cases, the duplex DNA was uniquely labeled at the 5′ end of the complementary (minus) strand except as noted. The formation of joint molecules was measured by gel electrophoresis and by an assay based on nitrocellulose filters (see Materials and Methods). To explore the interaction of duplex DNA with the nucleoprotein filament, we probed joint molecules with pancreatic DNase I in the continued presence of RecA protein. Samples that were treated with DNase I were denatured before loading on a neutral agarose gel.

Protection of the Complementary Strand in Joint Molecules. A reaction was set up between a RecA nucleoprotein filament containing circular single-stranded M13G*ori1* DNA and a 1-kb fragment of fully homologous linear duplex DNA. The linear duplex DNA was obtained after *Sna*BI and *Bam*HI digestion of M13G*ori1* superhelical DNA and was uniquely labeled at the 5′ end of the complementary strand (Figure 3C). Strand exchange was carried out under standard conditions (see Materials and Methods). At each time point, two aliquots were withdrawn: one aliquot was used for the estimation of joint molecules, and the other was used for digestion of the joint molecules by DNase I at 37 °C for 1 min. The DNase I reaction was stopped by the addition of 25 mM EDTA and vigorous mixing. The samples treated with DNase I were heated for 2 min at 100 °C to denature the DNA before loading on a neutral agarose gel.

The formation of joints, assessed by gel electrophoresis, followed a normal time course, with yields of 21%, 44%, 69%, 90%, and 94% at 1, 2, 3, 6, and 8 min, respectively (Figure 4 and Table 1). DNase I probing of the joints in the presence of RecA protein revealed both full-length and partial protection of the complementary strand at each time point: 5′-labeled fragments of the complementary strand were of two types, full-length protected fragments and a heterogeneous smear of partially protected fragments, most of which were larger than fragments produced by digestion of naked duplex DNA. All of the input label was recovered in the full-length band plus the heterogeneous fragments. At 1 min, 48% of complementary strands in the joints was protected from DNase I digestion over the entire length of homology. Although the yield of joints increased for the first 8 min of the reaction, and the absolute amount of DNA in the protected band increased, the fraction of fully protected complementary strand in joints remained relatively constant until quite late in the reaction (Table 1). In the controls, when heterologous substrates were used, when single-stranded DNA was omitted, or when RecA protein was omitted, the duplex DNA was completely sensitive to DNase I (Table 1 and data not shown). Full-length protection of the complementary strand was observed within 1 min even when substrates that were 8 kb in length were used (data not shown).

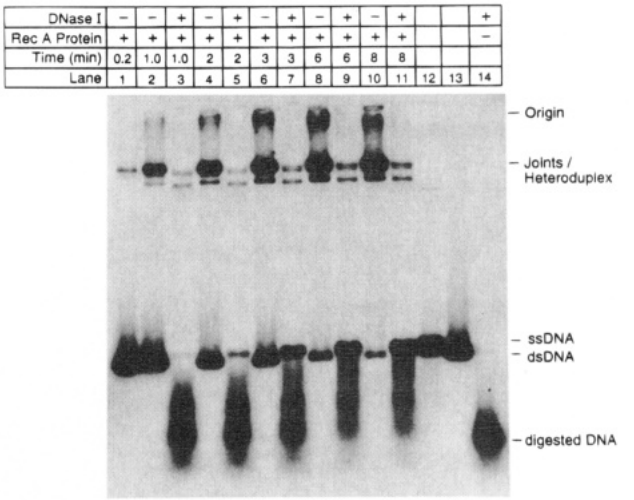

FIGURE 4: Time course of protection of the complementary strand in fully homologous joint molecules. Joint molecules were formed under standard conditions (see Materials and Methods) from M13G*ori1* circular single-stranded DNA coated with RecA protein and a 1-kb linear duplex DNA (Figure 3C) that was uniquely labeled at the 5′ end of the complementary strand. Two aliquots, 10 μL each, were taken from the reaction mixture at the indicated times. One aliquot was simply deproteinized and stored on ice prior to loading on a 1% neutral agarose gel (lanes 1, 2, 4, 6, 8, and 10). The other aliquot was treated with 1 μL of DNase I (0.1 unit) at 37 °C for 1 min. The DNase I reaction was stopped by the addition of EDTA (25 mM) with vigorous mixing, deproteinized, heated at 100 °C for 2 min, quick chilled, and then loaded on the gel (lanes 3, 5, 7, 9, and 11). Note that all the samples treated with DNase I were subsequently denatured. Deproteinization was done by the addition of EDTA (25 mM), SDS (0.5%), and 100 μg of proteinase K/mL and incubation at 37 °C for 20 min. An aliquot (10 μL) of a control reaction lacking RecA protein was treated with 1 μL of DNase I (0.1 unit) (lane 14). Lanes 12 and 13 contained single-stranded DNA and duplex DNA markers, respectively. Duplex DNA marker (lane 13) is the 1-kb linear duplex used in this experiment (See Figure 3C), and single-stranded DNA marker was obtained by heat denaturation of this linear duplex DNA. Since the duplex DNA is only 1 kb in length, the relative movement of joint molecules and the heteroduplex product cannot be differentiated in a 1% agarose gel.

To examine the further fate of the complementary strand once it is taken up into the filament, we probed the joint molecules with DNase I at various time points between 10 and 60 min. Complementary strands that were protected over the full length of homology and fragments of partially protected complementary strands persisted until about 30 min. Between 30 and 40 min, fully protected strands disappeared and smaller partially protected fragments were seen. By 50 min, complementary strands were as sensitive to DNase I as naked duplex DNA in the control reaction (Table 1).

The persistence of DNase protection to 30 min appeared to contradict the results of Chow *et al.* (1986) who observed that the complementary strand was sensitive to DNase I, after 20 min of strand exchange. However, the concentration of phosphocreatine used in the present study was 8 mM, whereas Chow *et al.* (1986) used 3 mM phosphocreatine. To check whether these differences in conditions could

Table 1: Time Course of Protection and Loss of Protection of the Complementary Strand[a]

expt	time (min)	joint (%) molecules	full-length uptake: % of total duplex DNA	full-length uptake: % of joint molecules
1: homologous DNA	1	21	10	48
	2	44	12	27
	3	69	21	30
	6	90	35	38
	8	94	39	41
2: homologous DNA	10	94	38	40
	20	96	44	46
	30	95	34	36
	40	94	04	4.2
	50	96	.009	.009
3: heterologous DNA	1	0	0	0
	20	0	0	0

[a] A fully homologous reaction was carried out with the substrates described in Figure 3C. The duplex DNA was uniquely labeled at the 5′ end of the complementary strand. Joint molecules formed between 1 and 50 min were probed with DNase I and analyzed in neutral 1% agarose gels as described in Materials and Methods. Quantitation of the gels was done with a PhosphorImager (Molecular Dynamics); the gel for experiment 1 is shown in Figure 4. The level of joint molecules was measured from the appearance of a characteristic band that moves slower than the duplex DNA in an agarose gel. Percent of total counts present in this band was taken as the percent of joint molecules (column 3). Probing of the joints with DNase I revealed both full-length and partial protection of the complementary strand at each time point: when the duplex DNA is taken up into the filament along its entire length, all of the complementary strand is protected from DNase I, and the protected complementary strand migrates to the same position as that of single-strand DNA marker. Similarly, when only part of the duplex DNA is present inside the filament, partial protection of the complementary strand is seen, and the protected complementary strand moves as a heterogeneous smear. We have designated full-length protection of the complementary strand as full-length uptake. Counts present in the full-length protected bands were calculated either as percent of total input label (column 4) or as percent of counts present in joint molecules (column 5).

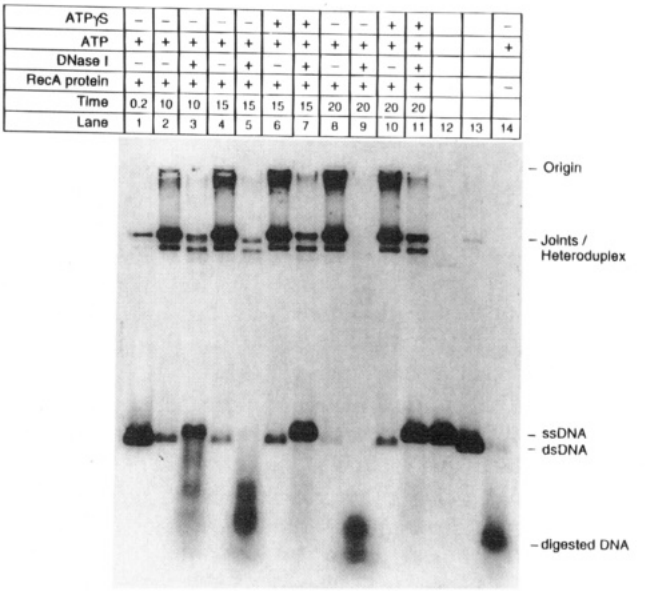

FIGURE 5: ATPγS inhibits loss of protection of the complementary strand in joint molecules. Joint molecules were formed with the substrates described in Figure 3C for 10 min in a reaction mixture that contained ATP and 3 mM phosphocreatine for the regeneration system. At 10 min the joint molecules (lane 2) were probed with DNase I (lane 3). Then the reaction mixture was split into two parts; 1 mM ATPγS was added to one part, and an equivalent volume of buffer was added to the other part. The reaction mixtures were incubated at 37 °C for another 10 minutes. At 15 and 20 min, 10-μL aliquots were withdrawn from both the reactions mixtures; one was used for the estimation of joints, and the other was treated with 1 μL of DNase I (0.1 unit) as described in Figure 4. A control reaction lacking RecA protein was subjected to DNase I treatment (lane 14). Lanes 12 and 13 contained single-stranded DNA and duplex DNA markers, respectively. Duplex DNA marker is the 1-kb linear duplex used in this experiment (See Figure 3C), and single-stranded DNA was obtained by heat denaturation of this linear duplex DNA. Samples treated with DNase I were denatured at 100 °C for 2 min prior to loading on a 1% neutral agarose gel (see legend to Figure 4).

account for the observed difference in the sensitivity of the complementary strand to DNase I, we used 3 mM phosphocreatine for the ATP regeneration system and probed the complementary strand between 5 and 60 min of strand-exchange reaction. Full-length protection of the complementary strand was seen only until 10 min, and at 20 min the complementary strand was completely sensitive to DNase I (data not shown). The formation of joint molecules, however, was not affected by the differences in the conditions. Thus, these experiments reveal that the time at which the complementary strand becomes accessible to DNase I during strand exchange depends on the strength of the regeneration system. In related studies, Pugh and Cox (1987) also observed that the time at which duplex DNA becomes sensitive to DNase I depends on the strength of the regeneration system.

ATPγS Inhibits Loss of Protection of the Complementary Strand in Joint Molecules. We formed joint molecules for 10 min in the presence of ATP in a reaction mixture that contained 3 mM phosphocreatine in the regeneration system. At 10 min, the yield of joint molecules was 92%, and in 35% of joint molecules the entire length of the complementary strand was protected from digestion by DNase I (Figure 5). The reaction mixture was then divided into two parts: to one part was added 1 mM ATPγS, and to the other was added an equivalent volume of buffer. Incubation was continued for another 10 min at 37 °C. Two aliquots were withdrawn from each reaction mixture at 15 and 20 min, one for the estimation of joint molecules and the other for DNase I probing. Whereas the complementary strand became completely sensitive to DNase I at 20 min in the presence of ATP, it remained fully resistant in the presence of ATPγS (Figure 5).

From the experiments described above, we conclude that in reactions of fully homologous molecules leading to strand exchange a large fraction of the complementary strand from the duplex partner is rapidly converted to a protected form within the RecA nucleoprotein filament. The duration of this protection depends on the strength of the ATP regeneration system, and protection is prolonged by the nonhydrolyzable analog ATPγS.

Both Strands of Duplex DNA in Distal Joints Are Protected from DNase I. Strand exchange in the RecA reaction is polarized; it proceeds 5′ to 3′ with regard to the strand from duplex DNA that is displaced from the nucleoprotein filament. Joints, however, can form wherever homology exists, as for example at the distal end of a duplex molecule whose proximal end is heterologous (Figure 2C). These have been called distal joints. Because the homologous 5′ end of the anticomplementary strand is tied down by a covalent connection to the upstream heterologous DNA, distal joints cannot undergo strand exchange in the full sense, which

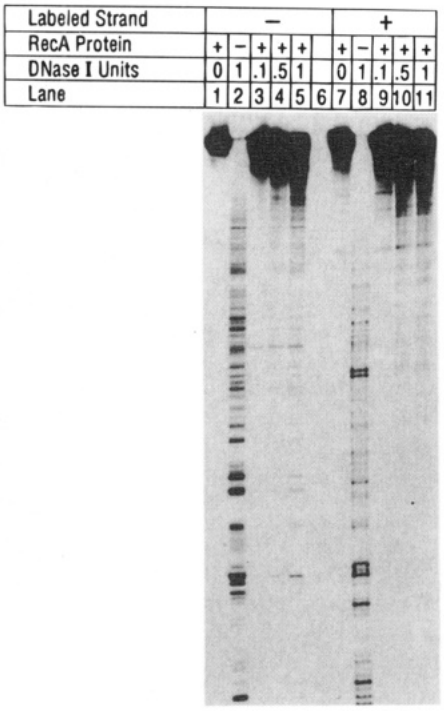

FIGURE 6: Both strands of duplex DNA in distal joints are protected from DNase I. Distal joints were formed for 20 min under standard reaction conditions with M13G*ori1* linear duplex DNA uniquely labeled at the 5′ end of the complementary strand or at the 3′ end of the anticomplementary strand (see Figure 3A and Materials and Methods). At 20 min, joint molecules were probed with different amounts of DNase I, 0.1, 0.5, or 1.0 unit. Stock solution of DNase I (27 units/μL) was diluted appropriately so that 1 μL would contain the different concentrations of DNase I mentioned above. Lanes 2 and 8 contained naked DNA digested with 1 μL of DNase I (1.0 unit). Lanes 1 and 7, respectively, contained the 5′ and 3′ end labeled DNA used for the formation of joint molecules. The samples were analyzed on an 8% denaturing polyacrylamide gel containing 8 M urea.

includes exchange of base pairs and complete separation of the anticomplementary strand. Distal joints, however, undergo partial base pair switching (Dutreix *et al.*, 1991), and the anticomplementary strand is in a changed configuration (Rao *et al.*, 1993; Bedale, 1993; Chiu *et al.*, 1993). Previous observations have shown that distal joints represent a steady-state product created by repeated cycles of formation and dissociation (Wu *et al.*, 1982; Burnett *et al.*, 1994). The dissociation of distal joints shares several properties with strand exchange, including a similar time course and inhibition by ATPγS (see below) (Burnett *et al.*, 1994). Thus distal joints provide an opportunity to explore how duplex DNA is taken up by the RecA nucleoprotein filament and then disgorged.

Distal joints with nearly 6 kb of homology were formed for 20 min under standard conditions (See Figure 3A and Materials and Methods). The linear duplex DNA was uniquely 5′ or 3′ end labeled. After 20 min, joints were incubated with different amounts of DNase I at 37 °C for 1 min in the continued presence of RecA protein. Analysis of the products of DNase I treatment on a denaturing polyacrylamide gel showed that both the complementary and anticomplementary strands were protected from DNase I digestion, whereas under the same conditions naked duplex DNA was extensively digested (Figure 6). At 20 min, 80% of duplex DNA was present in distal joints as measured by the filter assay (See Materials and Methods) (Table 2 and

Table 2: Rapid Uptake of the Duplex DNA in Distal Joints[a]

time (min)	joint molecules (%) by filter assay	joint molecules (%) by gel assay	full-length uptake % of total duplex DNA	full-length uptake % joints by filter assay	full-length uptake % joints by gel assay
1.0	48	10.5	13.5	28	128
2.5	60	12	14.5	24	120
5.0	67	13	19	29	146
10	80	29	23	28.5	80
20	80	39	22	28	57

[a] Data from the experiment described in Figure 7 are shown here. Quantitation and calculations were done as described in Table 1. In this experiment, joint molecules were measured by both filter assay and gel electrophoresis assay. Counts present in the full-length protected band were calculated either as percent of total input label (column 4) or as percent of counts present in joint molecules (column 5) measured by filter assay or as percent of joint molecules (column 6) measured by gel assay. A fraction of distal joints dissociate upon electrophoresis; thus there is a lesser yield of joints by this assay compared to filter assay. This results in numbers above 100% in column 6.

data not shown). In the samples digested with various amounts of DNase I, most of either 5′ or 3′ terminal label was present in fragments that were sufficiently large to remain near the top of an 8% polyacrylamide gel, which means that they were larger than 600 base pairs.[3] In controls, when the single-stranded DNA was heterologous, when RecA protein was omitted, or when single-stranded DNA was omitted, the duplex DNA was completely sensitive to DNase I (data not shown).

Time Course of Uptake of Duplex DNA into Distal Joints. We formed distal joints with 6 kb of homology under standard reaction conditions (See Materials and Methods) and observed the formation of joints and the protection of the complementary strand from attack by DNase I. The reaction was monitored by the filter assay for joint molecules and by gel electrophoresis in 1% agarose. The general procedure for probing with DNase I and analyzing the samples was the same as described above (Protection of the Complementary Strand in Joint Molecules). Within a minute of the start of the reaction, joints formed which contained homologous DNA that was protected from the labeled 5′ end of the complementary strand to points near the junction with the proximal heterologous region (Figure 7). Thus, for these molecules, which contained 6 kb of homology, the initial rate of uptake of duplex DNA equalled or exceeded 100 bp/s. The steady-state level of joints was achieved in about 10 min. As determined by a scan of the gel with a PhosphorImager (Molecular Dynamics), the fraction of duplex DNA in joints that was protected from DNase I digestion over its full length of homology was constant at 24−29%, from 1 to 20 min of the RecA reaction (Figure 7 and Table 2).

Uptake of Duplex DNA Does Not Require Hydrolysis of ATP. A side by side comparison of reactions in the presence of ATP *vs* ATPγS showed that the formation of distal joints and the consequent protection of complementary strands occurred equally well in both reactions (Figure 8), indicating that ATP hydrolysis is not required for the uptake of the duplex DNA into the nucleoprotein filament. In the experi-

[3] ϕ*X174* form I DNA digested with *Hae*III was electrophoresced on an 8% denaturing polyacrylamide gel containing 8 M urea. Whereas fragments less than 600 bp could be separated from each other, fragments longer than 1 kb remained at the top of the gel.

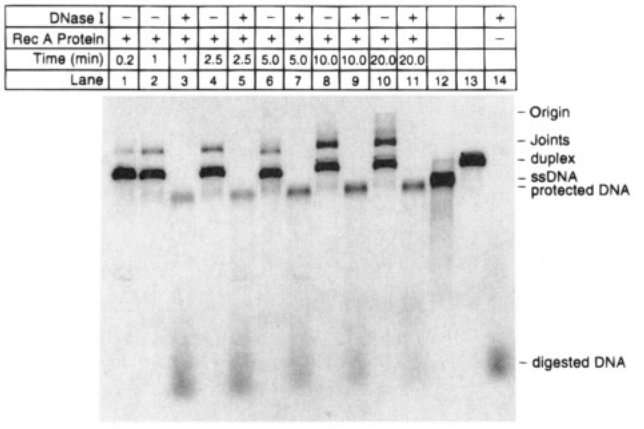

FIGURE 7: Time course of uptake of duplex DNA in the distal joints. Distal joints with 6 kb of homology were formed under standard reaction conditions (see Figure 3A and Materials and Methods). Double-stranded DNA was uniquely labeled at the 5′ end of the complementary (−) strand. Aliquots of the joint molecules were probed with DNase I at various time points between 1 and 20 min as described in Figure 4. Aliquots (10 μL) of the reaction mixture were treated with 1 μL of DNase I (0.8 unit) for 1 min and denatured by heating prior to loading on a neutral 1% agarose gel. Lanes 12 and 13 contained single-stranded DNA and duplex DNA, respectively. Duplex DNA marker is the 8-kb linear duplex used in this experiment (see Figure 3A), and single-stranded DNA was obtained by heat denaturation of this linear duplex DNA. A control reaction lacking RecA protein was subjected to DNase I treatment (lane 14). After the completion of electrophoresis, agarose gels are dried under vacuum; during this process a DEAE paper (Whatman DE51) is placed beneath the gel to capture small fragments of DNA obtained after DNase I digestion. In this experiment, DEAE paper was not used, resulting in loss of counts in some lanes.

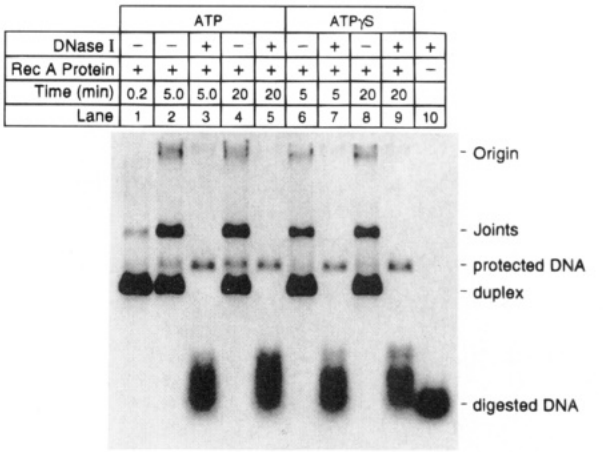

FIGURE 8: Uptake of duplex DNA does not require ATP hydrolysis. Distal joints were formed under standard reaction conditions (see Materials and Methods) with substrates described in Figure 3B, in the presence of ATP or ATPγS. Aliquots (10 μL) were withdrawn from both reaction mixtures and probed with 1 μL of DNase I (0.1 unit) as described in Figure 4. A control reaction lacking RecA protein was treated with DNase I (lane 10).

ment shown in Figure 9, the linear duplex DNA contained only 850 bp of homology at the distal end. When the same experiment was done with the duplex that contained about 6 kb of homology at the distal end, both the formation of joints, as judged by the filter assay, and the full-length protection of the complementary strand were the same in the presence of ATP or ATPγS, as for the shorter joints (data not shown).

Time Course of Loss of Protection in Distal Joints. Burnett *et al.* (1994) observed that the yield of distal joints decreased when one of several reagents (NaCl or ADP) that block the formation of joints was added to a reaction at steady state. NaCl at 50 mM, which is sufficient to cause net dissociation of distal joints, has little or no effect on the binding of RecA protein to single-stranded DNA and the hydrolysis of ATP (Cox & Lehman, 1982; Tsang *et al.*, 1985; Gonda & Radding, 1986; Honigberg *et al.*, 1986; Rould *et al.*, 1992). This relatively gentle treatment thus provides a way to examine selectively the dissociation of distal joints.

Accordingly, we formed distal joints with 6 kb of homology for 20 min and then added 50 mM NaCl to examine dissociation. Aliquots were withdrawn at various time points after the addition of NaCl to measure both the level of distal joints and the sensitivity to DNase I. Although joints began to dissociate as soon as NaCl was added, and residual joints reached some basal level in about 20 min (Figure 10A), the fraction of DNA that was protected over its entire length of homology did not appear to change until more than 15 min later (Figure 9). Forty minutes after the addition of NaCl, some decrease in the amount of the fully protected DNA occurred, and the average size of partially protected fragments diminished (Figure 9). We attribute the loss of protection to accumulation of ADP. The presence of 50 mM NaCl did not affect the activity of DNase I on duplex DNA (data not shown).

Joints That Do Not Recycle Have Extensively Switched Base Pairs. The observations just described showed that protection of the full length of homologous DNA in distal joints was associated with a fraction of the joints that did not dissociate when 50 mM NaCl was added (compare Figures 9 and 10A). Indeed, the fraction of molecules that did not dissociate after the addition of NaCl and the fraction of complementary strands in the joints that were resistant to DNase were both about 1/3 of the DNA in joints (Figures 7 and 10A and Table 2).

To look for further evidence of heterogeneity in the population of joint molecules, we examined the sensitivity of distal joints to S1 nuclease during the formation and dissociation of distal joints. Sensitivity to S1 nuclease at acid pH has been extensively used in the past to monitor strand exchange, or more precisely the switching of base pairs. Resistance or sensitivity of DNA in this assay reflects the transition of the anticomplementary strand from parental DNA to a single-stranded state. As the assay was performed on deproteinized samples, it provides no information on the location of the anticomplementary strand inside or outside the filament.

We formed distal joints with 6 kb of homology as described in Materials and Methods. The formation of joints and the sensitivity of [^{3}H]DNA to S1 nuclease were determined at various time points. At 20 min, the reaction mixture was split into three parts: 50 mM NaCl, 1 mM ATPγS, or an equivalent volume of buffer, respectively, was added to each part, and the reaction was continued for another 30 min. The yield of joint molecules and the sensitivity to S1 nuclease were determined at various time points after addition of NaCl, as described in Materials and Methods.

Consistent with previous observations (Dutreix *et al.*, 1991), during the formation of distal joints the amount of DNA that became sensitive to S1 nuclease was equivalent to 40% of the homologous region of the anticomplementary strand (Figure 10A). Strikingly, when the addition of NaCl

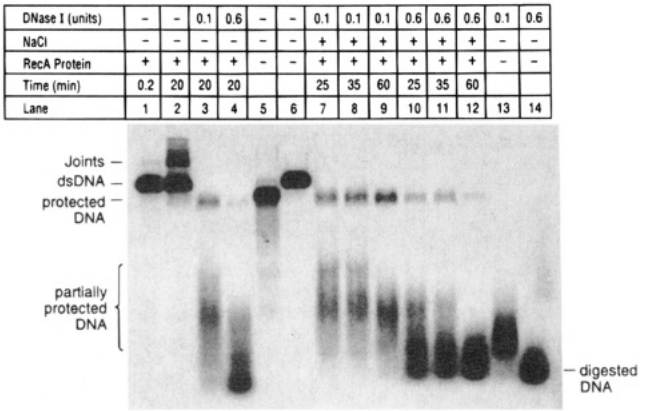

FIGURE 9: Time course of loss of protection of the complementary strand in distal joints after addition of 50 mM NaCl. Distal joints were formed for 20 min under the standard conditions (see Materials and Methods) with the substrates described in Figure 3A. Two aliquots (10 μL) of the reaction mixture were withdrawn at 20 min; one was treated with 1 μL (0.1 unit), and the other was treated with 2 μL (0.6 unit), of DNase I. After 20 min of joint formation, 50 mM NaCl was added to the reaction to block the re-formation of joints. Aliquots (10 μL) were removed 5, 15, and 40 min after the addition of NaCl and were probed with 1 μL (0.1 unit) or with 2 μL (0.6 unit) of DNase I. Digestion of duplex DNA was with 0.1 unit (lane 13) or 0.6 unit of DNase I (lane 14) in the absence of RecA protein. Lanes 5 and 6 contained single-stranded DNA and duplex DNA markers, respectively. Duplex DNA marker is the 8-kb linear duplex used in this experiment (See Figure 3A), and single-stranded DNA was obtained by heat denaturation of this linear duplex DNA.

provoked the net dissociation of 70% of distal joints, the sensitivity to S1 nuclease did not diminish, but rather remained nearly constant in absolute amount, becoming equivalent finally to 90% of the homologous region of the anticomplementary strand of the remaining nondissociable joints. When ATPγS was added along with NaCl, it prevented the dissociation of distal joints, as observed previously (Burnett *et al.*, 1994), and the S1 sensitivity remained at about 35% per anticomplementary strand per joint (see Discussion).

A control experiment tested the possibility that the joints that did not dissociate after the addition of salt resulted from nibbling of the 3′ end of the anticomplementary strand by a contaminating exonuclease: we formed distal joints in which the duplex was uniquely labeled at the homologous 3′ end of the anticomplementary strand (See Materials and Methods). After 20 min of formation of joints, the reaction mixture was split into two parts; 50 mM NaCl was added to one, an equivalent amount of buffer was added to the other, and the reactions were continued for 40 min. Aliquots were withdrawn at various times to determine the yield of joints and to detect any digestion of the labeled 3′ end of the anticomplementary strand of the duplex DNA. No digestion was detected throughout the reaction (Figure 10B).

We conclude that the joints that did not dissociate had undergone complete switching of base pairs to produce a stable heteroduplex joint in which the third strand was intact (Figure 11).

DISCUSSION

In brief, we have used the cyclical formation and dissociation of distal joints as a model system to explore further the uptake and processing of duplex DNA by RecA nucleoprotein filaments that already contain a homologous single strand. The results provide further biochemical support for the idea proposed by Howard-Flanders *et al.* (1984) that duplex DNA is rapidly assimilated to form an extensive coaxial three-stranded filament. Still further, the evidence of a coaxial three-stranded filament presented here supports the proposal that the energy of ATP hydrolysis fuels the unwinding of the triplex structure into a single strand and either parental duplex or recombined heteroduplex DNA (Burnett *et al.*, 1994).

The experiments described here show that both the complementary and anticomplementary strands of duplex DNA are protected from the action of DNase I when the duplex is taken up by a RecA nucleoprotein filament that initially contains a single strand. However, when the duplex DNA is fully homologous or is homologous only at its proximal end, switching of base pairs and progressive displacement of the anticomplementary strand promptly ensue; and because RecA protein in the reaction mixture also binds the displaced single strand (Chow *et al.*, 1986, 1988), probing with DNase I does not reveal when there are two vs three strands within the RecA filament. On the other hand, in the case of distal joints the determination by DNase I probing of what is inside or outside the filament is simplified because in most molecules the two strands of the parental duplex do not separate from one another, and duplex DNA leaves the filament in a sufficiently protein-free state to form new joint molecules.

In our present observations on distal joints, DNase protection was conferred on both strands of the duplex DNA labeled at their distal ends. Protection was not conferred on heterologous duplex DNA, or in the absence of either single strands or RecA protein. These results indicate that homologous DNA is taken up into the helical filament to form a three-stranded coaxial helix. Since protection is based on homology, and the single strand in the filament follows the helical path of the protein (Leahy & Radding, 1986; Egelman & Yu, 1989), a linear path of uniformly protected duplex DNA on the outside of the filament is excluded. A helical homologous path on the outside of the filament is also excluded given the diameter of the RecA filament, the maximal extensibility of the sugar−phosphate backbone of DNA, and the wide separation that would be imposed between homologous duplex and the single strand in the filament. As calculated previously (Leahy & Radding, 1986), the maximal diameter of the helical path of the DNA within the filament is 4.0 nm, whereas the outer diameter of the filament is 9−10 nm.

Distal joints, however, comprised two distinct subpopulations, one that contained the majority of joints and the other that contained 25−30% of the joints (Figure 11). The distal joints that completely assimilated the 6-kb region of homology belonged to the minor class. All of these had undergone virtually complete switching of base pairs, as judged by the S1 nuclease assay, and were stuck inside the RecA nucleoprotein filament. They did not dissociate in the presence of 50 mM NaCl as did the rest of the distal joints, and protection of labeled complementary strand remained until late in the reaction when ATP was becoming exhausted and RecA was presumably dissociating from DNA.

The switching of base pairs in the minor class of distal joints leads one to ask whether there were indeed three strands inside the filament in those joints. It is possible that

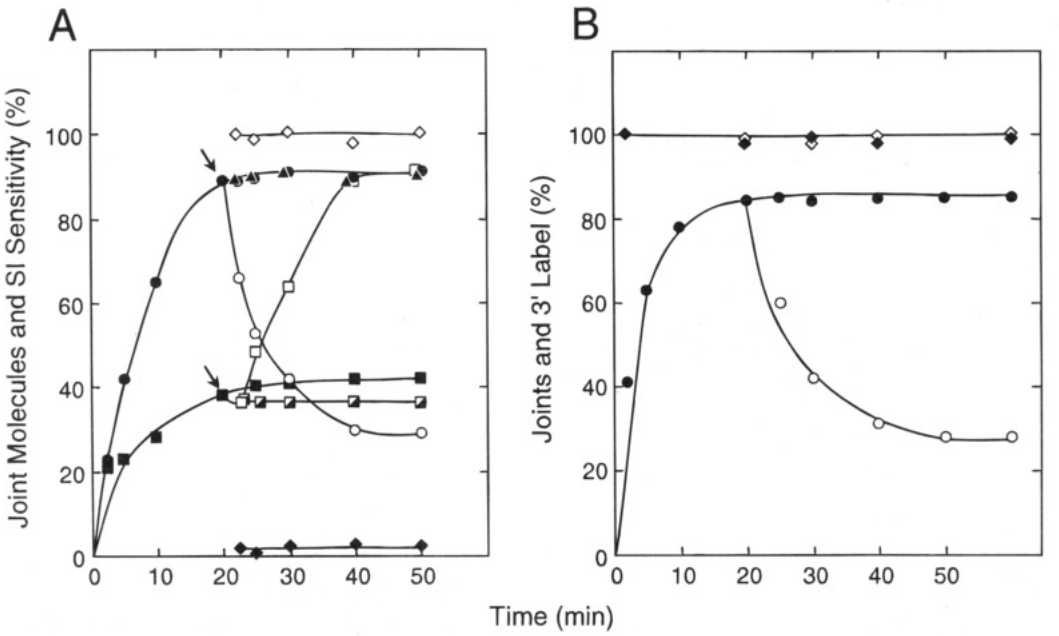

FIGURE 10: (A) S1 nuclease assay of distal joint molecules before and after the addition of 50 mM NaCl. Distal joints were formed under the standard conditions (see Materials and Methods) with the substrates described in Figure 3A. The duplex DNA used in this experiment was uniformly tritium labeled. The formation of joints (●) and the sensitivity of [^{3}H]DNA to S1 nuclease per joint (■) were determined at various times, as described in Materials and Methods. The sensitivity to S1 nuclease was normalized to the fraction of joint molecules at each time point and the fraction of homology in the duplex substrate (See Materials and Methods). After 20 min, the reaction mixture was split into three parts; either 50 mM NaCl, 1 mM ATPγS, or an equivalent volume of buffer was added to each: yield of joints after addition of 50 mM NaCl (○); yield of joints after addition of 50 mM NaCl and ATPγS (▲); sensitivity to S1 nuclease per joint molecule after addition of 50 mM NaCl (□); sensitivity to S1 nuclease after addition of NaCl and ATPγS (◪). Controls for S1 assay: single-stranded DNA (◇) and double-stranded DNA (◆). (B) The anticomplementary strand in distal joints is intact. Distal joints with 6 kb of homology were formed under standard reaction conditions (see Figure 3A and Materials and Methods). Double-stranded DNA was uniquely labeled at the 3′ end of the anticomplementary (+) strand. After 20 min, the reaction mixture was split into two parts: either 50 mM NaCl or an equivalent volume of buffer was added to each. The reation was continued for another 40 min. At indicated time points an aliquot of 10 μL was withdrawn for the estimation of joints. Another aliquot of 50 μL was withdrawn for the estimation of acid insolubility of the 3′ label; sonicated calf thymus DNA (100 μg/mL) and trichloroacetic acid (10%) were added, and the samples were stored on ice for 30 min. Half the sample was used for totals, the other half was centrifuged at 15600*g* for 15 min at 4 °C, and the supernatant was counted for radioactivity: yield of joints after addition of 50 mM NaCl (○) and without 50 mM NaCl (●); acid insolubility after addition of NaCl (◇) and without NaCl (◆).

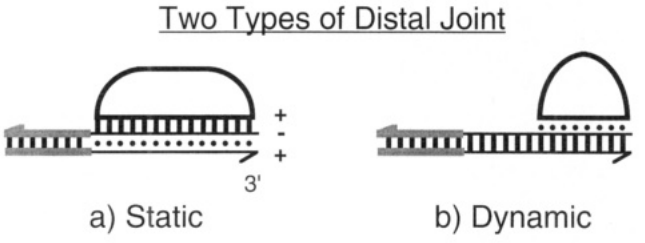

FIGURE 11: Proposed states of the two subpopulations of distal joints: static and dynamic. (a) Static distal joints in which extensive switching of base pairs has occurred, but the anticomplementary strand has not been fully displaced. (b) Dynamic distal joints comprising the majority, which constantly turn over, and in which switching of base pairs, if it occurs, is limited to less than 10% of the joints. Vertical bars denote Watson−Crick hydrogen bonds; dots denote additional hypothetical bonds that mediate the formation of base triplets (Chiu *et al.*, 1993; Zhurkin *et al.*, 1994; Rao & Radding, 1994).

the unpaired strand was outside of the filament. The sensitivity of the plus strand to exonuclease I in a fraction of distal joints favors the conclusion (Bedale *et al.*, 1993). On the other hand, indirect functional evidence argues against the existence of displaced plus strands as branches of distal joints: under conditions in which displaced anticomplementary strands in proximal joints were active in pairing with other molecules, distal joints did not produce similarly active displaced strands (Chow *et al.*, 1988). The rest of the distal joints, 2/3 to 3/4 of the total, showed no evidence of having undergone base pair switching since the dissociation of these joints resulted in no change in the number of switched base pairs as determined by the S1 nuclease assay. The accuracy of these assays does not allow us to conclude that no switching at all occurred in the dissociable joints; we could have failed to detect under 5−10% of switching, and a small extent of base pair switching could play an important role in stabilizing these intermediates, for example, when they are deproteinized (Burnett *et al.*, 1994). If, at steady state, there is some small extent of base pair switching in joints that are turning over, it must be reversed when the joint dissociates.

In the major class of distal joints, which were "unswitched" and dynamic, DNase protection was conferred on fragments of smaller and more heterogeneous size than in the minor class, which was "switched" and static (Figure 11); the smaller size and heterogeneity is consistent with the expectation for a steady-state population of joints that is turning over. Although the size of fragments cannot be well assessed from electrophoresis on agarose (Figure 9), electrophoresis in 8% polyacrylamide indicates that the protected fragments were more than 600 base pairs in length.

Our observation that base pair switching occurred in about a fourth of distal joints and our conclusion that the majority of distal joints contain coaxial triplex regions are in good agreement with a recent electron-microscopic study of psoralen cross-linked distal joints by Jain *et al.* (1995). In

a fourth of distal joints they found no cross-linking of the anticomplementary strand to either strand of the heteroduplex DNA, whereas in the rest of the joints there were extensive regions involving cross-linking of all three strands.

The reason that distal joints comprise two subpopulations (Figure 11) is not clear. We do not know whether the formation of long coaxial three-stranded joints is the cause or the effect of extensive base pair switching, but plausibly extensive switching of base pairs might prevent dissociation of distal joints because one of the strands of the duplex is bound to the strand in the filament by thousands of Watson–Crick base pairs. Indeed, that is precisely the situation in normal strand exchange, in proximal joints or fully homologous joints, where heteroduplex DNA remains in the filament until ATP is exhausted. In normal strand exchange, however, as contrasted with distal joints, the free 5′ end of the anticomplementary strand allows displacement of that strand to occur.

The switching of base pairs that occurs in a minority of distal joints has also been observed in paranemic joints in which the complete physical separation of the third strand is impossible (Adzuma, 1992), and paranemic joints, like distal joints, appear to consist of a major population that is dynamic and a minor population that is static (Reddy *et al.*, 1994).

In the experiments described here, the uptake of duplex DNA into the filament did not require the hydrolysis of ATP, since it occurred equally well in the presence of the nonhydrolyzable analog ATPγS. That observation is consistent with earlier observations that homologous pairing and limited base pair switching can take place in the presence of ATPγS (Honigberg *et al.*, 1985; Menetski *et al.*, 1990; Rosselli *et al.*, 1990). Previous observations have shown by contrast that the dissociation of distal joints is an active process that requires the hydrolysis of ATP (Burnett *et al.*, 1994).

The initial rate of uptake of duplex DNA into distal joints was greater than 100 bp/s (Figure 7). Thus, uptake occurs fast enough for the product of this step to be a precursor to strand exchange. A similar rate of uptake of duplex DNA was observed when completely homologous substrates of 8 kb were used (data not shown). We infer therefore that an extensive coaxial triplex forms rapidly in fully homologous joints, or in proximal joints, where it is a likely intermediate in strand exchange.

To explain certain observations on strand exchange, Stasiak and colleagues recently proposed a new model (Stasiak & Egelman, 1994; Morel *et al.*, 1994) which reverses the conventional view of polarity in strand exchange and proposes among other things that the distal end of a linear duplex molecule is not incorporated into the RecA filament. The observations described here argue against that model. In our experiments on distal joints in which label was present only at the distal end of duplex DNA, most of the duplex was protected from DNase I (Figure 6). Homologous duplex DNA was rapidly protected along its entire length in 1/4 to 1/3 of joints and at terminal regions of heterogeneous size in the rest (Figures 6 and 9). Morel *et al.* (1994) also proposed that base pair switching takes place immediately after homologous recognition in all of the joints. Our experiments on distal joints suggest that extensive base pair switching takes place only in a fraction of the joints (Figure 10A). Furthermore, in our experiments with fully homologous substrates we have shown that the duplex DNA remains protected from DNase I long after strand exchange has gone to completion (Figure 4B); this result agrees well with Chow *et al.* (1986) and Pugh and Cox (1987) but clearly disagrees with the model proposed by Morel *et al.* (1994) in which they propose that RecA dissociates from the newly formed heteroduplex.

Other experiments support the view that there is an intermediate that is common to strand exchange and the cyclical formation and dissociation of distal joints. When fully homologous joints or proximal joints are deproteinized and isolated before strand exchange is completed, exchange can be made to resume instantly by addition of RecA protein in the presence of a concentration ratio of ADP to ATP that inhibits the binding of RecA protein to single-stranded or duplex DNA (Rao *et al.*, 1990). Similarly, when distal joints are deproteinized and isolated by gel filtration, their dissociation can be initiated instantly by adding back RecA protein at the same concentration ratio of ADP to ATP (Burnett *et al.*, 1994). A site for which RecA protein has a high affinity, one that is resistant to the dissociating effect of ADP, is present in both kinds of joints. Furthermore, the high-affinity site in isolated proximal joints decreases in direct proportion to the completion of base pair switching, and the high-affinity site in distal joints decreases in proportion to the dissociation of those joints (Burnett *et al.*, 1994). Since the intermediate in the dissociation of distal joints is a coaxial triplex, this same intermediate is arguably the one that binds RecA protein in the presence of a high ratio of ADP to ATP, and that mediates strand exchange.

We previously proposed a model according to which the energy of ATP hydrolysis is used to expel one DNA moiety from a coaxial triplex within the RecA filament: in proximal or fully homologous joints, the expelled moiety is an anticomplementary strand that is a product of base pair switching; in distal joints, the expelled moiety is a parental duplex molecule (Burnett *et al.*, 1994). Several aspects of the present observations on DNase protection in distal joints support that model. Virtually all of the homologous duplex DNA that is taken up into distal joints is protected against DNase from the distal end of the duplex DNA to internal sites; and one may infer, as indicated above, that this DNA is in a coaxial triplex. Evidence of coaxial triplex supports a key feature of the proposed model. Evidence that the distal end of the duplex DNA is usually included in these joints supports another important feature of the model, which posits that the expulsion of duplex DNA from distal joints has the same directionality as expulsion of the anticomplementary strand in normal strand exchange. Inclusion of the distal end of duplex DNA in coaxial triplexes of heterogeneous length means that there exists a nested set of joints that is consistent with the proposed polarity.

ACKNOWLEDGMENT

We thank Ning Ye and Zhufang Li for technical assistance and Jan Zulkeski for secretarial help.

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BI950131Q