Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 2984–2988, April 1991
Biochemistry

Stable three-stranded DNA made by RecA protein

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Communicated by Howard A. Nash, December 31, 1990

ABSTRACT When RecA protein, in the form of a nucleoprotein filament containing circular single-stranded DNA (plus strand only), reacts with homologous linear duplex DNA, a directional transfer ensues of a strand from the duplex DNA to the nucleoprotein filament, resulting in the displacement of the linear plus strand in the 5′ to 3′ direction. The initial homologous synapsis, however, can occur at either end of the duplex DNA, or anywhere in between, and when homology is restricted to different regions of the duplex DNA, the joint molecules that form in each region show striking differences in stability upon deproteinization: distal joints > proximal joints >> medial joints. In the deproteinized distal joints, which are thermostable, 2000 nucleotide residues of the circular plus strand are resistant to P1 nuclease; both strands of the original duplex DNA remain resistant to P1 nuclease, and the potentially displaceable linear plus strand, which has a 3′ homologous end, remains resistant to *Escherichia coli* exonuclease I. These observations suggest that RecA protein promotes homologous pairing and strand exchange via long three-stranded DNA intermediates and, moreover, that, once formed, such triplex structures in natural DNA are stable even when RecA protein has been removed.

A model system that has proven useful in exploring the nature of recombination promoted by RecA protein consists of circular single-stranded DNA and duplex DNA, the latter usually in linear form (1, 2). Studies of this model system have revealed the overall course of the reaction and some mechanistic aspects. The reaction has three major phases: (*i*) presynapsis, in which RecA protein polymerizes on single-stranded DNA to form a right-handed helical nucleoprotein filament; (*ii*) synapsis, during 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 new heteroduplex molecule and a displaced linear single strand.

Strand exchange itself is directional, 5′ to 3′ with respect to the circular plus strand in the RecA filament or its displaced linear homolog, which defines a proximal and a distal end in the duplex DNA (Fig. 1; refs. 1 and 2). Many observations have been reported that show, however, that the initial pairing event that produces a joint molecule is not polarized but rather can occur at the proximal 5′ end of the plus strand, the distal 3′ end, or in the middle of a linear duplex molecule (Fig. 1; refs. 3–5). On the other hand, Konforti and Davis (6, 7), studying the pairing of linear single strands with superhelical DNA as assayed by gel electrophoresis, found that when homology was limited to the 5′ end of a linear plus strand, joints were scarcely detectable. Hsieh *et al.* (8) found that when homology was limited to very short sequences at the ends of duplex DNA, only distal joints, as defined above, were detectable by gel electrophoresis. Since strand exchange, which proceeds in the 5′ to 3′ direction, was previously observed to stabilize joint molecules (4, 9), these observations seemed paradoxical and suggested the need to examine further two possibly separate issues: the stability of homologous joints and the directionality of extensive strand exchange, both considered in relation to the location of ends in DNA. In this paper, we describe observations on the stability of joints formed at different locations and further characterization in particular of distal joints. Elsewhere, we describe further experiments on the polarity of strand exchange (35).

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METHODS

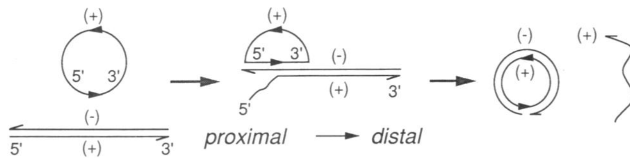
Materials. RecA protein and *Escherichia coli* single-stranded DNA binding protein were purified according to published protocols (10, 11). Restriction endonucleases (*Nco* I, *Kpn* I, *Xho* I, *Sac* I, *Mlu* I, and *Bam*HI) were purchased from New England Biolabs. S1 nuclease was purchased from Pharmacia LKB. P1 nuclease was from United States Biochemical. Proteinase K was from EM Laboratories (Elmsford, NY). Exonuclease I was a gift from S. Kushner (University of Georgia).

Preparation of DNA. Circular single-stranded DNA and superhelical duplex DNA were prepared as described (12, 13). The single-stranded DNA preparation contained <5% linear molecules as judged by gel electrophoresis. The fraction of nicked molecules in the superhelical duplex DNA preparation was <5% as assessed by the assay of Kuhnlein *et al.* (14).

Standard Reaction Conditions. Presynaptic filaments were formed by incubating 10 μM circular single-stranded DNA with 5 μM RecA protein and 0.83 μM single-stranded DNA binding protein at 37°C for 12 min in a reaction mixture containing 33 mM Tris·HCl (pH 7.5), 12 mM $MgCl_2$, 2 mM dithiothreitol, 1.2 mM ATP, 8 mM phosphocreatine, creatine phosphokinase (10 units/ml), and bovine serum albumin (100 μg/ml) (nuclease free; Bethesda Research Laboratories). Pairing and strand-exchange reactions were initiated by adding linear [^{3}H]DNA at the concentrations indicated in the figure legends. Joint molecules were measured by the D-loop assay (15).

Deproteinization of Joint Molecules and Isolation by Gel Filtration. After the formation of joint molecules was initiated by the addition of linear duplex DNA to an otherwise complete reaction mixture, the reaction was carried out at 37°C for 15 min and stopped by adding EDTA to 20 mM, SDS to 0.5%, and proteinase K to 100 μg/ml, followed by incubation at 37°C for an additional 20 min. The sample was filtered through a column of Sepharose 2B at 4°C–10°C to recover deproteinized joint molecules. The concentration of joint molecules was expressed as mol of nucleotide residues in the duplex DNA part of the molecule.

Use of ELISA (16) to Assess Deproteinization of Isolated Joint Molecules. Microtiter wells (Immulon, Dynatech) were coated with 0.2 ml of various concentrations of antigen (RecA protein) in coating buffer (0.015 M Na_2CO_3, 0.035 M $NaHCO_3$, pH 9.6) by incubating at 37°C for 2 hr, followed by washing the wells five times with 0.2 ml of washing buffer per well (10 mM sodium phosphate buffer, pH 7.4/130 mM NaCl/0.05% Tween 20). Immunoglobulins (IgGs) were pre-



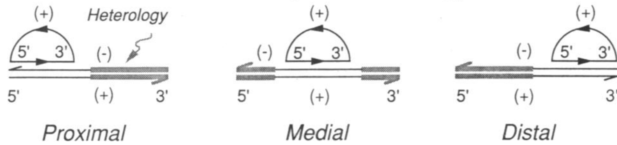


FIG. 1. Formation and designation of joints in relation to the polarity of strand exchange and the location of homology.

pared from normal rabbit serum or polyclonal rabbit RecA antiserum by affinity chromatography on protein A-Sepharose (17) and diluted to 12 μg of IgG per ml with the washing buffer containing 0.1% bovine serum albumin. The wells were filled with 0.2 ml of normal rabbit IgG or polyclonal rabbit anti-RecA IgG and incubated at 37°C for 1 hr, after which the wells were washed five times with washing buffer. Subsequently, 0.2 ml of horseradish peroxidase-linked goat anti-rabbit IgG (Bio-Rad; diluted 1:1000 with the washing buffer containing 0.1% bovine serum albumin) was added to each well and incubated at 37°C for 1 hr. Unbound double antibody was removed by washing the plate five times with the washing buffer. The peroxidase assay was performed by adding to each well 0.2 ml of freshly prepared assay mixture consisting of 0.04% (wt/vol) *o*-phenylene diamine (Sigma) and 0.04% (vol/vol) H_2O_2 in citrate phosphate buffer (pH 4.5). Citrate phosphate buffer was prepared by mixing 14 ml of 0.1 M citric acid, 11 ml of 0.1 M Na_2HPO_4 and 25 ml of water. The plate was incubated at 37°C for 20 min in the dark. The visual reading was confirmed by measuring absorbance at 492 nm in a Dynatech-MR5000 microplate reader.

RESULTS

The Formation of Joints at Either End of Duplex DNA and Their Deproteinization. M13G*ori*1 circular single-stranded DNA contains the entire genome of M13, 6407 bases, with an insert of 2216 bases from G4 (18). With the use of appropriate restriction endonucleases, G4 form I [^{3}H]DNA was linearized so that most of the above-mentioned 2216-base-pair (bp) region of G4 DNA was positioned at either the proximal or the distal end of the duplex molecule* (Fig. 2 *a* and *b*). Therefore, reactions of circular single-stranded M13G*ori*1 DNA with these two substrates provide comparisons of pairing when identical sequences are positioned at one or the other end of duplex DNA. Joints formed similarly at either end: in the reaction at the proximal end, ≈80% of duplex DNA was converted into joint molecules; at the distal end, 70% of duplex DNA was converted into joint molecules.

To study naked, protein-free joint molecules, we formed them in a standard RecA reaction mixture for 15 min and then treated them with proteinase K in the presence of EDTA and SDS (see *Methods*). To assess the efficiency of deproteinization, we analyzed the treated joints by ELISA. As shown in Fig. 3*C*, the assay detects as little as 5 ng of RecA protein and possibly even less. We therefore analyzed 0.27 pmol of

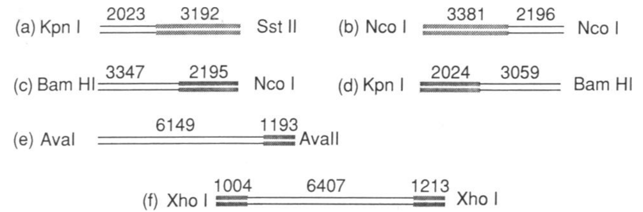


FIG. 2. Linear duplex substrates with limited regions of homology. Stippled lines represent heterologous DNA. The left end of each diagram represents the proximal end as defined in Fig. 1. (*a* and *b*) G4 DNA. (*c–f*) M13G*ori*1 DNA.

deproteinized distal joints and 0.29 pmol of deproteinized proximal joints separately in duplicate for the presence of immunoreactive material using polyclonal anti-RecA antibody (see legend to Fig. 3*C* for details). If, after deproteinization, there were even 1 residual molecule of RecA protein per joint molecule, these amounts of joints should have contained 10–11 ng of RecA protein, which is well above the lower limit of detection by the immunoassay. However, both distal joints (wells E3 and E4) and proximal joints (wells F3 and F4) showed only the background signals seen in normal serum controls (A2–A11 and B2–B11) or controls lacking RecA protein (C2 and D2). The absorbance values read by a Dynatech microplate reader were 0.037 for the control lacking RecA protein (wells C2 and D2) and 0.407 for 5 ng of RecA protein (wells C3 and D3), whereas the values for deprotein-

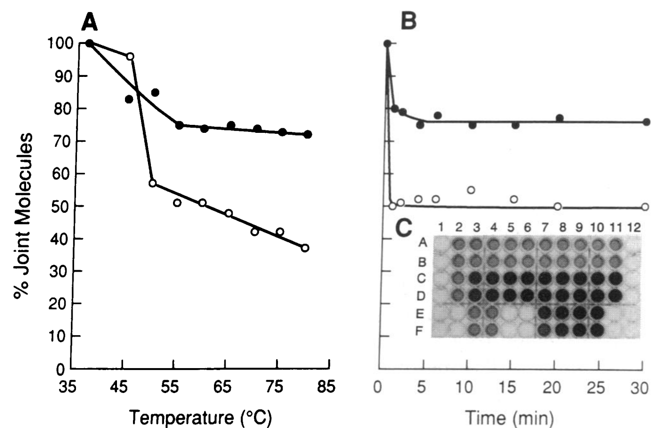


FIG. 3. Thermal stability of proximal and distal joints and assessment of their deproteinization. (*A*) After 20 min of a pairing reaction, joint molecules were deproteinized, and duplicate aliquots were withdrawn, exposed to different temperatures for 5 min, and assayed for D loops. ○, Proximal joints; ●, distal joints. (*B*) As in *A*, but the entire deproteinized reaction mixture was shifted to 55°C. ○, Proximal joints; ●, distal joints. (*C*) ELISA of deproteinization. Microtiter plate wells 2–11 were coated with 0, 5, 10, 20, 30, 50, 80, 120, 200, and 400 ng of RecA protein, respectively, in four separate rows (A, B, C, and D). Antigen wells in rows A and B underwent reaction with normal control rabbit serum, whereas those in rows C and D underwent reaction with polyclonal rabbit RecA antiserum. Wells 3 and 4 in row E were each coated with 0.27 pmol of deproteinized distal joints; wells 3 and 4 in row F were coated with 0.29 pmol of deproteinized proximal joints. Isolated joint molecules showed ≈71% of the ^{3}H duplexes in D loops for distal joints and 90% of the ^{3}H duplexes in D loops for proximal joints. The concentration of joints was expressed as the mol of ^{3}H duplex molecules in D loops. As internal controls, 10, 30, 50, and 80 ng of each RecA protein (wells 7, 8, 9, and 10, respectively) were added to 0.273 pmol of deproteinized distal joints (row E) or to 0.29 pmol of deproteinized proximal joints (row F) and then the wells were coated as described. Antigen wells in rows E and F underwent reaction with polyclonal rabbit RecA antiserum. All the wells were then treated with peroxidase-linked double antibody followed by the colorimetric assay for the peroxidase bound to the plate.

*For the substrate cleaved by *Kpn* I and *Sst* II (Fig. 2*a*), 193 bp of homology was removed in a small fragment that was separated by gel filtration. For the substrate cleaved with *Nco* I (Fig. 2*b*), 20 bp of G4 DNA was located at the proximal end, which is too short a region of homology at that end to form stable joint molecules (8, 19).

ized proximal (wells F3 and F4) and distal (wells E3 and E4) joints were 0.036 and 0.042, respectively. Additional controls showed that when known amounts of RecA protein were added to either the deproteinized distal or proximal joints, the assay revealed signals (E7–E10 and F7–F10) similar to the corresponding RecA standards (rows C and D; see legend). These data showed that deproteinization was effective: the least amount of RecA protein that we measured in a standard curve was 5 ng, which corresponded to 1 molecule of RecA protein per 2 joint molecules in our test samples. Since the values for the latter were 10 times lower, and were indistinguishable from the blanks, the samples of joint molecules contained <1 molecule of residual RecA protein for every 2 joint molecules.

Thermal Stability of Protein-Free Proximal vs. Distal Joints. The thermal stability of deproteinized joints was assessed by incubation at various temperatures from 37°C to 80°C for 5 min (Fig. 3*A*). At 80°C, <40% of proximal joints survived heating, whereas at that temperature 75% of deproteinized distal joints survived. Preparations of both proximal and distal joints contained an unstable component that dissociated within 1 min at 55°C and a stable component that did not dissociate even when held at 55°C for as long as 30 min (Fig. 3*B*).

The assay that we use for joint molecules (15) detects the retention by nitrocellulose filters of duplex DNA when the latter is attached to single-stranded DNA. Thus, sufficient contamination by exonucleases could give the false impression that a stable joint had formed. To exclude that artefact in the case of 3′ or distal joints, we filled in the 3′ ends of the duplex substrate with [α-^{32}P]dGTP by using the Klenow fragment of *E. coli* DNA polymerase I. We confirmed that the labeling had occurred at the 3′ termini of the duplex DNA, and not internally, by treating the DNA with *Sph* I, which revealed that all the ^{32}P label was associated with only those DNA fragments 126 and 41 bp long that were released from the respective termini of the original duplex molecule; no label was detected in the 5410-bp-long middle fragment (data not shown). We used the intact, terminally labeled duplex substrate and monitored the release of ^{32}P label into the acid-soluble fraction during the pairing reaction. This experiment revealed that even after 30 min of incubation, only 7% of terminal ^{32}P label was released into the acid-soluble fraction, whereas ≈70% of duplex DNA was in deproteinized joint molecules (data not shown).

Previous observations had led us to believe that little or no genuine strand exchange occurs in the 3′ to 5′ direction and hence that distal joints were like medial joints in which a region of homology is flanked on both sides by heterologous sequences (refs. 3 and 4; see Fig. 1). Medial joints (one form of paranemic joint) are, however, completely unstable after deproteinization (4, 5). To confirm that distal joints are more stable than medial joints, we compared directly the stability of joints during deproteinization at 37°C. Both proximal and distal joints, made with substrates c and d (Fig. 2), decayed 25–30% in 5 min and remained at that level for 20 min, whereas medial joints made with substrate f (Fig. 2) decayed >80% in the same time (data not shown).

Enzymatic Characterization of Proximal vs. Distal Joints. Previously, enzymatic characterization of joints formed during strand exchange has revealed that between the creation of nascent heteroduplex DNA and the full separation of the third strand, there exists an intermediate that, when deproteinized, still contains three strands of DNA in intimate association over a region that is several kilobases long (15). Since extensive or complete strand exchange does not occur in distal joints (3, 9), we postulated that distal joints might have a three-stranded structure representing a trapped intermediate that RecA protein cannot further process by completing an exchange of strands. To test this hypothesis, the following enzymic characterizations were done.

In all of the following experiments, deproteinized joints were further purified by gel filtration to remove proteinase K and permit us to study the action of other enzymes on these joints.

P1 Nuclease. P1 nuclease is similar to S1 nuclease in its specificity for cleavage of single strands, but it has the advantage of acting at neutral pH (20, 21). P1 nuclease has been used as an enzymic probe to detect various structural or conformational changes in duplex DNA (21–24). We previously used P1 nuclease to detect three-stranded intermediates formed by RecA protein during strand exchange (15). In the present experiments, we separately assessed the sensitivity of each strand in distal joints to the action of P1 nuclease.

To estimate the length of a distal joint, we paired uniformly ^{3}H-labeled circular single-stranded DNA with duplex DNA that was ^{32}P-labeled at 5′ ends. The pairing reaction was done with an excess of linear duplex DNA so that all of the

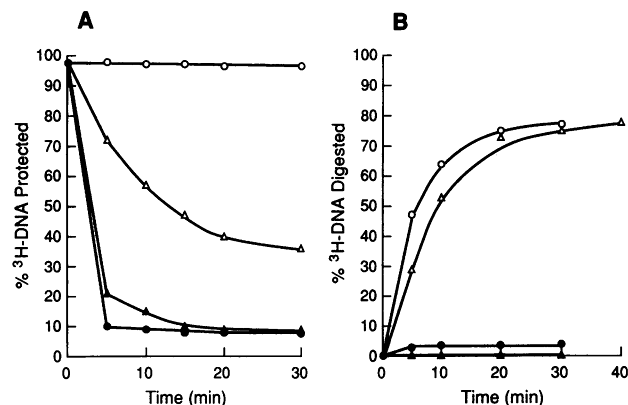


FIG. 4. Resistance of DNA in distal joints to digestion by P1 nuclease and *E. coli* exonuclease I. (*A*) Joint molecules were formed between M13 circular single-stranded [^{3}H]DNA (8 μM) and 5′ ^{32}P-labeled chimeric duplex large fragment (20 μM) derived from the *Kpn* I/*Bam*HI double digestion of M13G*ori*1 form I DNA (Fig. 2*d*). The deproteinized joint molecules, which were obtained after filtration through Sepharose 2B, showed 73% of ^{32}P duplex DNA in D loops. The joint molecules (5.22 μM; 300 μl) were digested with P1 nuclease (5 × 10^{-2} units/nmol as defined by the supplier) in 30 mM Tris·HCl, pH 7.0/0.5 mM EDTA, pH 8.0/4 mM zinc acetate/50 mM NaCl at 37°C. At different time points, aliquots were withdrawn to measure the percentage of ^{3}H-labeled M13 plus strand that remained acid precipitable (△). Control digestions included joints denatured at 95°C for 2 min, followed by quick chilling (▲), M13 circular single-stranded [^{3}H]DNA (2.1 μM) from a mock reaction mixture lacking duplex DNA (●), and M13 linear duplex [^{3}H]DNA (5.22 μM) (○). (*B*) Joint molecules were formed between M13G*ori*1 circular single-stranded DNA (20 μM) and G4 linear duplex [^{3}H]DNA (15 μM, cut by *Nco* I as shown in Fig. 2*b*). The isolated protein-free joint molecules contained 71% of [^{3}H]DNA in D loops. The joint molecules (7 μM; 100 μl) were digested with 0.15 unit of *E. coli* exonuclease I at 37°C in a buffer containing 30 mM Tris·HCl (pH 8.0), 0.4 mM EDTA, 3 mM $MgCl_2$, 2 mM dithiothreitol, and bovine serum albumin (100 μg/ml). At different time intervals, aliquots were withdrawn to measure the [^{3}H]DNA rendered acid soluble. The percentage of homologous G4 plus strand digested was calculated as (% acid soluble [^{3}H]DNA × 2)/ (fraction of [^{3}H]DNA in joint molecules × fraction of homologous region in the duplex) (●). In all of the following controls, % [^{3}H]DNA digested refers simply to the percentage of total radioactivity. Controls included exonuclease digestion of joint molecules that were heat denatured (○), ^{3}H-labeled M13G*ori*1 linear plus strand (3.5 μM) (generated by linearizing the circular single-stranded DNA with *Nar* I after annealing to a 30-mer oligonucleotide), which has the same G4 sequences at the 3′ end as plus strand in the joints (△), and M13 linear duplex [^{3}H]DNA (7 μM) (▲).

single-stranded [^{3}H]DNA was incorporated into joints (details are in legend in Fig. 4A). The time course of digestion of deproteinized and purified joints by P1 nuclease revealed that ≈30% of labeled single-stranded DNA, equivalent to 2000 nucleotide residues, was resistant under conditions that caused complete digestion of circular single-stranded [^{3}H]DNA from RecA filaments that had gone through the same protocol of deproteinization and gel filtration (Fig. 4A). Another control experiment showed that the single-stranded [^{3}H]DNA in the deproteinized joint molecules became fully sensitive to P1 nuclease after heat denaturation of these joint molecules (Fig. 4A).

The action of P1 nuclease on proximal and distal joints was compared for each strand of the parental duplex DNA. For each chimeric duplex used to form either distal or proximal joints, each strand was uniquely labeled with ^{32}P at the 3′ or 5′ end in the joint (see legend to Fig. 5). Purified joints and heat-denatured control samples were titrated with P1 nuclease under identical conditions. Samples were denatured prior to electrophoresis and analyzed in denaturing gels. A single cleavage by P1 nuclease anywhere in each polynucleotide strand should release terminal label from the band of high molecular weight DNA and cause the label to appear in a smear of smaller molecular weight material. Controls lacking any P1 nuclease show that most of the label in the denatured material was in high molecular weight DNA and therefore was at or near the termini of intact strands rather than at internal nicks (Fig. 5).

In the case of distal joints, 75% of ^{32}P was in stable joints after deproteinization and gel filtration and migrated as a distinct band during electrophoresis in a nondenaturing agarose gel (Fig. 5A). The autoradiographic analysis of joints digested by P1 nuclease and run on denaturing gels revealed that, in the distal joint, both strands contributed by the parental duplex molecule were resistant to P1 nuclease, whereas under the same conditions of digestion, the denatured single-stranded DNA controls were sensitive (Fig. 5 B and C). Under the same conditions, control joints incubated without P1 nuclease remained stable without any detectable loss in the total level of joint molecules in the samples (data not shown).

In the case of proximal joints, 91% of label was recovered in joint molecules after deproteinization and gel filtration and migrated as a single species during electrophoresis in a nondenaturing agarose gel (Fig. 5D). After P1 nuclease digestion and electrophoresis in a denaturing gel, label in a duplex control and label at a 3′ terminus of proximal joints were seen to be resistant to P1 nuclease, whereas label at a 5′ terminus was as sensitive as that in DNA that had been denatured before treatment with P1 nuclease. The observations on proximal joints were those expected on the basis of all previous studies that have shown that the 5′ proximal end of the plus strand is displaced early in the exchange reaction (9, 26).

Exonuclease I. Exonuclease I from *E. coli* acts processively on single-stranded DNA in the 3′ to 5′ direction (27); it should therefore reveal any displacement of the noncomplementary plus strand contributed by the parental duplex molecule in a distal joint. When deproteinized distal joints were treated with exonuclease I, only about 3–4% of the homologous plus strand became sensitive to the 3′ exonuclease, while the rest of the strand remained protected even after incubation for 30 min (Fig. 4B). In a parallel incubation of the same sample without the exonuclease I, there was no loss in the total level of joint molecules, indicating that the material resistant to exonuclease I was in joint molecules. A control experiment showed that when the joints were heat denatured before nuclease digestion, the DNA strands became sensitive to exonuclease I. Another positive control consisted of a ^{3}H-labeled linear plus strand that was incubated in a mock

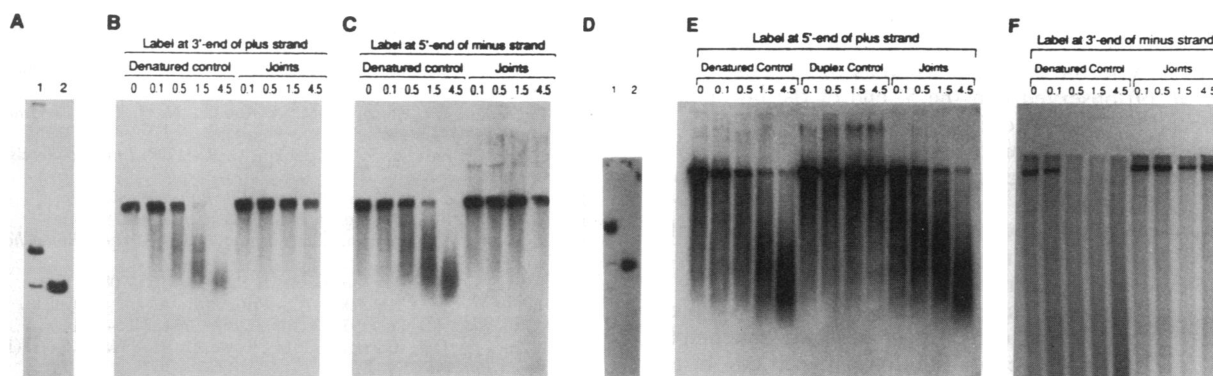


FIG. 5. Insensitivity to P1 nuclease of the two strands contributed to a distal joint by the original duplex DNA. M13 circular single-stranded DNA (10 μM) was paired with ^{32}P-end-labeled chimeric duplex large fragment (8 μM) derived from the *Kpn* I/*Bam*HI double digestion of M13G*ori*1 form I DNA (see Fig. 2d), the distal end of which was singly 3′-labeled by Klenow fragment of *E. coli* DNA polymerase I or 5′-labeled by T4 polynucleotide kinase. This was achieved by labeling both ends of full-length M13G*ori* duplex DNA after the first cut with *Bam*HI, followed by the second cut with *Kpn* I and purification of the large fragment. The deproteinized joint molecules after filtration through Sepharose 2B showed 75% of ^{32}P duplexes in D loops. (*A*) The joint molecules were analyzed by electrophoresis on 0.8% nondenaturing agarose gel at 4 V/cm with 40 mM Tris acetate (pH 8.0) and 2 mM EDTA (pH 8.0), after which the gel was dried and exposed for autoradiography. Lanes: 1, joint molecules; 2, standard duplex substrate. (*B* and *C*) Aliquots (40 μl) of the singly ^{32}P-labeled joint molecules (3 μM) in 30 mM Tris·HCl, pH 7.5/0.5 mM EDTA, pH 8.0/4 mM zinc acetate/50 mM NaCl were treated with various levels of P1 nuclease (0.1, 0.5, 1.5, or 4.5 × 10^{-3} unit) at 37°C for 20 min, followed by quenching the reactions with 20 mM EDTA (pH 8.0) and 100 mM NaOH. Parallel control digestions were performed after heat denaturing the joint molecules at 95°C for 2 min, followed by quick chilling. All the samples were analyzed by electrophoresis on 1% Seakem GTG agarose (FMC) under alkaline gel conditions as described (25), after which the gel was dried and exposed for autoradiography. Proximal joints were formed by pairing M13 circular single-stranded DNA (10 μM) with uniquely ^{32}P-end-labeled chimeric duplex large fragment (8 μM) derived from the *Ava* I/*Ava* II double digestion of M13G*ori*1 form I DNA, which has M13 sequences at the proximal end and G4 sequences at the distal end (see Fig. 2e). The *Ava* I ends of the duplex DNA were either 3′-labeled by Klenow fragment of *E. coli* DNA polymerase I or 5′-labeled by T4 polynucleotide kinase, followed by *Ava* II digestion and purification of the large fragment away from the other small fragments by gel filtration through Sephacryl S1000. The deproteinized joint molecules, after filtration through Sepharose 2B, showed 91% of ^{32}P duplexes in D loops. (*D*) The joint molecules were analyzed by electrophoresis in a nondenaturing 0.8% agarose gel as described in *A*. Lanes: 1, joint molecules; 2, standard duplex substrate. (*E* and *F*) Aliquots of the singly ^{32}P-labeled joint molecules were treated with various levels of P1 nuclease followed by electrophoretic analysis under alkaline gel conditions. The conditions of P1 nuclease digestion as well as the subsequent analysis were as described for *B* and *C*.

reaction mixture lacking duplex DNA and subsequently deproteinized and filtered through Sepharose 2B. Digestion with exonuclease I converted ≈75% of radioactivity into acid-soluble material, which is close to the limit previously described for the action of exonuclease I on single-stranded DNA (Fig. 4*B*; ref. 27).

The results show that in the deproteinized distal joints, the entire linear plus strand contributed by the parental duplex molecule resisted both exonuclease I, as well as P1 nuclease digestion. In the case of exonuclease I in particular, in which virtually all joints were resistant to digestion, the amount of residual RecA protein estimated by ELISA (see above) was insufficient to account for the observed resistance.

DISCUSSION

A comparison of the stability of joint molecules formed by circular single-stranded RecA nucleoprotein filaments when homology was restricted to the middle of duplex DNA, to its proximal end, or to its distal end, revealed three distinct interactions: The most unstable interaction was that between a circular single strand and a region of homology flanked by heterologous DNA on both sides. As observed earlier, the joints formed in this case dissociated rapidly upon deproteinization at 37°C (4, 5). Proximal joints were next in order of increasing stability, and such molecules appeared to consist of an equal mixture of molecules that dissociated above 45°C and molecules that were stable up to 80°C. By contrast, 75–80% of distal joints were in the most stable category of joints. The high stability of distal joints may partially explain why Konforti and Davis observed a strong bias favoring the detection of corresponding joints formed by single strands with homologous 3′ ends and superhelical DNA (6, 7).

Earlier experiments with circular single strands and linear duplex DNA as substrates had indicated that the proximal and distal ends of linear duplex DNA interact differently with circular single strands in the reaction promoted by RecA protein, with the 5′ end of the linear plus strand being displaced early in the reaction, whereas the 3′ end is not. Kahn *et al.* (9) observed that the 5′ end of the linear plus strand became sensitive to *E. coli* exonuclease VII even in the presence of RecA protein, whereas the 3′ end was sensitive neither to exonuclease VII nor exonuclease I. More recently, Chow *et al.* (26) found that the 5′ end of the linear plus strand reinitiated new pairing reactions soon after the start of a first round of pairing and strand exchange, whereas the 3′ end did not. A recent reinvestigation of the polarity of strand exchange further supports the view that a complete strand exchange leading to displacement of the noncomplementary plus strand proceeds only from the 5′ end of the strand (35).

The observations cited above and the extraordinary stability of distal joints, even after RecA protein was removed, suggested that distal joints have a different structure than proximal joints. Enzymatic probing of deproteinized distal joints suggests that these consist of long three-stranded structures that at neutral pH lack significant single-stranded character in any of the three strands.

There are two aspects of these conclusions: (*i*) As a part of its mechanism of homologous pairing and strand exchange, RecA protein makes three-stranded intermediates, as indicated earlier by the pairing of molecules that lack free ends (4, 28), model building (29), electron microscopy (30–32), and physical and enzymic characterization of intermediates (8, 15). (*ii*) When distal joints made by RecA protein are deproteinized, they reveal an exceptional thermal stability attributable in theory to their three-stranded nature—i.e., to the existence of additional hydrogen bonds by which homologous recognition is accomplished. This observation agrees with the findings and interpretation of Hsieh *et al.* (8), who found a similar stability in very short distal joints. We observed in addition that a large fraction of proximal joints are much less stable, which we attribute to spontaneous branch migration operating on fully deproteinized branched intermediates (33, 34). Our observations also indicate that stable protein-free three-stranded joints in natural DNA can be very long, of the order of several kilobases at least, indicating that the triplex RecA nucleoprotein precursor can also be very long (15, 29–31).

We thank Clara Wong for technical assistance and Lydia Romanik for preparing the manuscript. This work was sponsored by grants from the American Cancer Society (NP-90P) and the National Institutes of Health (GM33504-06). M.D. was supported in part by the Centre National de la Recherche Scientifique and the North Atlantic Treaty Organization.

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