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The Formation of Paranemic and Plectonemic Joints between DNA Molecules by the recA and Single-stranded DNA-binding Proteins of *Escherichia coli**

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During the initial pairing events in the transfer of a strand from a linear duplex to a homologous single-stranded circular DNA by the recA and single-stranded DNA-binding proteins of *Escherichia coli*, two types of structure are formed that are distinguishable by their stability in the presence of protein denaturants. One type which is resistant to 5.2 M guanidinium chloride is most likely a D-loop that depends only on heteroduplex base pairing for its stability. These D-loops form rapidly when the ends of the linear duplex are homologous with the single-stranded DNA but do not form when the ends are heterologous. The second type appears to require protein, in addition to base pairing, for stability since it is rapidly dissociated by treatment with 5.2 M guanidinium chloride. These unstable structures form even when the ends of the duplex are not homologous with the circular single-stranded DNA. The stability and topological properties of the stable and unstable structures are consistent with those of plectonemic and paranemic joints, respectively (Bianchi, M., Das Gupta, C., and Radding, C. M. (1983) *Cell* 34, 931–939). The plectonemic joints can be generated *in situ* from paranemic joints by the addition of a restriction enzyme that cleaves in the region of homology, thus producing free homologous ends. Omission of single-stranded DNA-binding protein results in a large decrease in the rate of formation of both paranemic and plectonemic joints.

The exchange of strands between a linear duplex and a homologous single-stranded circular DNA promoted by the recA and single-stranded DNA-binding proteins of *Escherichia coli* is a useful model for the molecular analysis of homologous recombination (1–3). The initial step in the exchange is believed to be the formation of a complex between ssDNA[1] and recA protein (4–6). Approximately 1 recA protein monomer/4 nucleotides is required to saturate the ssDNA.[2] Although this stoichiometry is sufficient to produce maximal rates of pairing in the presence of SSB, a much higher level of recA is required in its absence (1, 6, 7). SSB very likely has multiple effects on the pairing reaction; thus, it stabilizes the recA-ssDNA complex (4, 6, 8), and it may bind to the displaced strand of the duplex DNA as strand exchange proceeds (4).

Radding and his colleagues have described a pairing event promoted by recA protein in the absence of SSB that does not require a free homologous end in the DNA duplex (11, 12). The paranemic joint so formed, in which the strands are not intertwined as in Watson-Crick duplex DNA, was suggested to be an intermediate in the overall pairing reaction (12). Such a paranemic joint probably contains ssDNA ((+)-strand) displaced from the duplex DNA to which SSB would be expected to bind.

In the studies reported here we have been able to resolve the reaction between ssDNA and a homologous DNA duplex into two components that have properties consistent with the paranemic and plectonemic joints described by Bianchi *et al.* (12). We have further observed that both paranemic and plectonemic joint formation is severely limited if SSB is omitted from the reaction.

EXPERIMENTAL PROCEDURES

Materials—M13*mp*8, M13*Gori*1, M13 wild-type, and ϕX ssDNAs, recA protein, and SSB were gifts from D. Soltis of this department. Restriction enzymes were from New England Biolabs, except for *Xho*I which was obtained from W. Segraves of this department and *Bam*HI from Boehringer Mannheim. ^{3}H-labeled DNAs were prepared and linearized with the appropriate restriction enzyme as indicated in the figure legends and in Fig. 1 (1, 4). Substantial nicking of ^{3}H-labeled supercoiled DNA, specific radioactivity of 100,000–250,000 cpm/μg, was observed within 3 days of preparation, as judged by the production of form II DNA; hence only freshly prepared DNA or DNA that had been rebanded in CsCl-ethidium bromide was used.

ATP was purchased from Sigma and tritiated nucleotides from Amersham Corp. Millipore filters (HAWP, 0.45-μm pore diameter) were washed consecutively with distilled water (2 ×) and 2.0 M NaCl, 0.15 M sodium citrate (2 ×) before use.

Methods—D-loop formation was assayed by a modification of our previously described procedure (1). Reaction mixtures contained 866 pM (molecules of DNA) circular ssDNA, 866 pM ^{3}H-labeled linear duplex DNA, and 4 mM ATP. Under these conditions recA protein was present at 1 monomer/3.0 nucleotides of ssDNA and SSB at 1 monomer/8.2 nucleotides. A control reaction containing ϕX ssDNA instead of the homologous M13 ssDNA was included in each experiment (heterologous control). The order of addition of the reactants was: recA protein, ssDNA, SSB and ATP together, and then duplex DNA which was added 5 min after incubation of the other components. At the times indicated, aliquots (10 or 20 μl) were withdrawn and mixed with GHCl and EDTA in 0.1 M Tris·HCl (pH 7.5) to give

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[1] The abbreviations used are: ssDNA, single-stranded DNA; SSB, *E. coli* single-stranded DNA-binding protein; ϕX, bacteriophage ϕX174; GHCl, guanidine hydrochloride.

[2] F. R. Bryant and I. R. Lehman, unpublished observation.

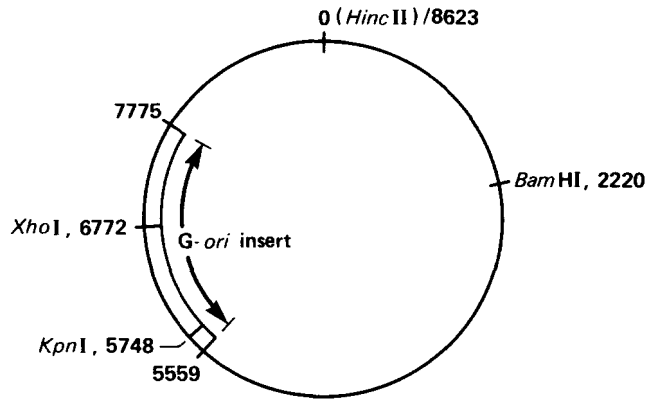

FIG. 1. **Map of M13G*ori*1 showing sites for restriction endonuclease cleavage relative to the G-*ori* insert (16).**

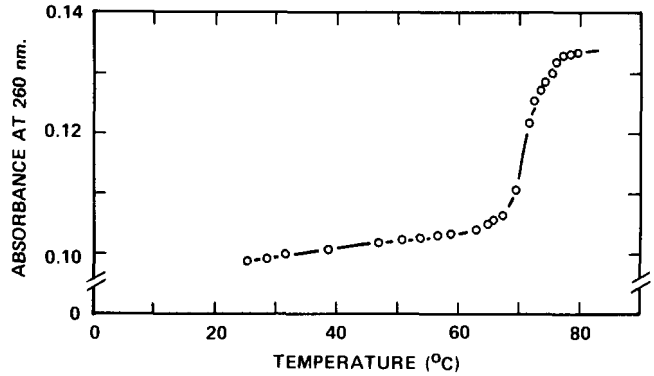

FIG. 2. **Temperature-induced melting of M13*mp*8 linear duplex DNA in 5.2 M GHCl.** In addition to GHCl, the solution contained 0.1 M Tris-HCl (pH 7.5), 22 mM EDTA, 0.1 mM dithiothreitol, 0.9 mM $MgCl_2$, and 0.45% (v/v) glycerol.

final concentrations of 5.2 M and 22 mM, respectively. After 10 min at the specified temperature, the mixture was diluted into 1 ml of 2.0 M NaCl, 0.15 M sodium citrate, applied to a nitrocellulose filter at room temperature, and washed twice with 2.0 M NaCl, 0.15 M sodium citrate (1 ml each) followed by three washes with 0.3 M NaCl, 0.02 M sodium citrate (1 ml each). After drying for 25 min, the amount of labeled duplex DNA bound to the filter was measured by scintillation counting.

KOH-treated nitrocellulose filters which bind protein but not ss- or duplex DNA were used to measure the binding of ssDNA to protein (13).

The melting profile of M13*mp*8 duplex DNA, linearized with *Pst*I, was determined using a Cary 118 recording spectrophotometer fitted with a thermostatted cuvette holder. The reaction mixture, diluted into GHCl, was identical to that used in the D-loop assay except that ATP, recA protein, and SSB were omitted.

The term *plectonemic joint* will be used to describe pairing of ssDNA and linear duplex DNA at an end such that intertwining of strands leads to Watson-Crick base pairing (12). This is essentially the "D-loop" as defined earlier (1, 14), the criteria for which are (i) requirement for homology, (ii) requirement for an end, (iii) stabilization through base pairing after deproteinization, and (iv) binding to nitrocellulose through ssDNA. The term *paranemic joint* will be used to describe pairing in which the strands are not intertwined as, for example, when plectonemic joint formation is topologically restricted (12). The criteria in this case are (i) requirement for homology, (ii) requirement of protein for stabilization, and (iii) binding to nitrocellulose through ssDNA and/or protein. The general term *D-loop* will be reserved for pairing events where this distinction is not made.

RESULTS

Use of GHCl for the Removal of Protein from recA Protein-ssDNA Complexes—GHCl (5.2 M) is an effective protein denaturant that does not significantly alter the stability of duplex DNA. As shown in Fig. 2, the melting temperature of linear duplex M13*mp*8 DNA in the presence of 5.2 M GHCl was 71 °C, a value similar to that observed in the absence of GHCl. Table I shows the effect of treating complexes of recA protein and ssDNA which are formed in the presence of SSB and ATP with solutions of 5.2 M GHCl at various temperatures. KOH-treated filters bind only a residual amount of ssDNA (5.3%) under these conditions, and this value is, therefore, taken as 100% deproteinization. Since the KOH-treated filters do bind protein (13), presumably 100% deproteinization results from treatments that dissociate recA and/or SSB from the ssDNA before filtration and also prevent denatured protein bound to the nitrocellulose from rebinding ssDNA. As indicated in Table I, treatment at 32 °C was sufficient to deproteinize recA protein-ssDNA complexes made in the presence of SSB and ATP and of SSB alone. Consequently, 5.2 M GHCl at 32 °C for 10 min was chosen as the condition that would deproteinize DNA but minimize

TABLE I

Removal of protein by GHCl from ssDNA-protein complexes

ssDNA-protein complexes were formed as for a standard reaction (see "Methods") in the absence of duplex DNA. ^{3}H-labeled ϕX ssDNA was used instead of M13. After treatment for 10 min at the designated temperature, the mixtures were diluted into 2 M NaCl, 0.2 M sodium citrate, washed with the same solution (twice with 1 ml) and 0.3 M NaCl, 0.02 M sodium citrate (three times with 1 ml).

Treatment	^{3}H-labeled DNA retained by filter		
	recA protein, SSB, ssDNA, ATP	SSB, ssDNA	ssDNA
	%		
2 M NaCl, 0 °C	94.6		
5.2 M GHCl, 0 °C	9.8	8.3	
5.2 M GHCl, 25 °C	8.2	8.0	
5.2 M GHCl, 32 °C	6.4	5.0	5.3
5.1 M GHCl, 37 °C	4.6	5.7	

melting of duplex DNA and/or random branch migration. After treatment with 2 M NaCl at 0 °C for 10 min, most of the ssDNA was retained by the filter, indicating that deproteinization did not occur to any significant extent with these conditions.

Identification of Two Types of D-loops—When 2 M NaCl at 0 °C was used to treat the products of a reaction between M13*mp*8 ss- and duplex DNAs, the apparent rate of D-loop formation was faster than that observed following treatment of the products with GHCl at 32 °C (Fig. 3*A*). The same result was obtained with M13G*ori*1 ss- and duplex DNAs (Fig. 3*B*). In all cases, the equivalent heterologous control (ϕX ssDNA) showed no reaction over the same time period, demonstrating an absolute requirement for homologous sequences. Inasmuch as 2 M NaCl does not disrupt protein-ssDNA complexes at 0 °C, the paired structures observed after this treatment are very likely stabilized by protein in addition to heteroduplex base pairing. Paired structures that persist after the GHCl treatment should be exclusively plectonemic joints. The difference between the two treatments, therefore, determines the amount of protein-stabilized D-loops present at any given time.

The kinetics of formation of plectonemic joints showed a small lag before the maximal rate of reaction was achieved, which was somewhat longer in the pairing of the M13G*ori*1 than the M13*mp*8 DNAs (Fig. 3, *A* and *B*). To determine whether this lag was due to incomplete complex formation or some other process requiring equilibration, the time of preincubation before adding duplex was varied from 1 to 8 min. As

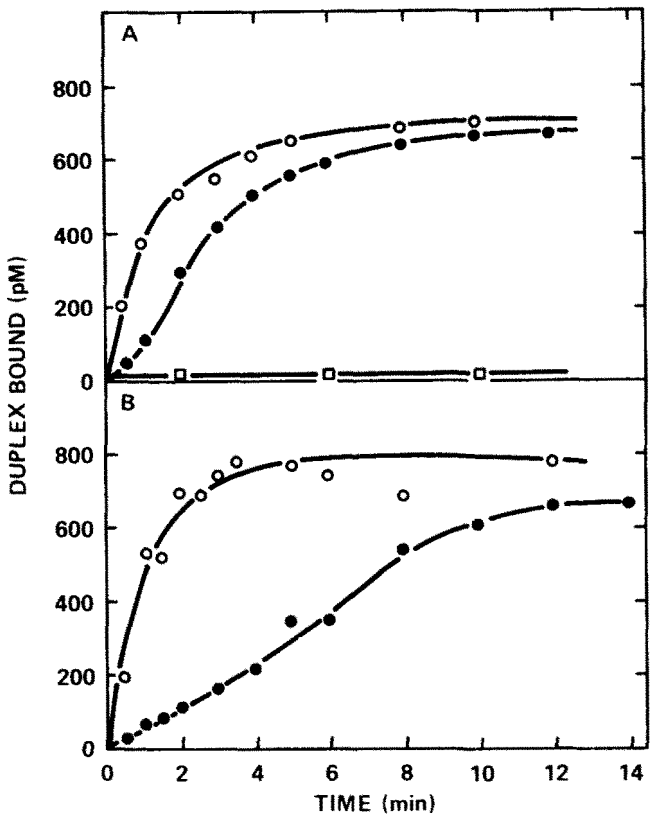

FIG. 3. **The effect of treatment with 2 M NaCl or 5.2 M GHCl on the binding of products to nitrocellulose filters.** *A*, M13*mp*8 ssDNA and M13*mp*8 duplex DNA linearized with *Pst*I. *B*, M13G*ori*1 ssDNA and M13G*ori*1 duplex DNA linearized with *Kpn*I (see Fig. 1). Concentrations of components and order of addition are described under "Methods." Pretreatment was in 2 M NaCl (no GHCl) for 10 min, 0 °C (○); 5.2 M GHCl, 32 °C (●). φX ssDNA and M13*mp*8 duplex DNA (□).

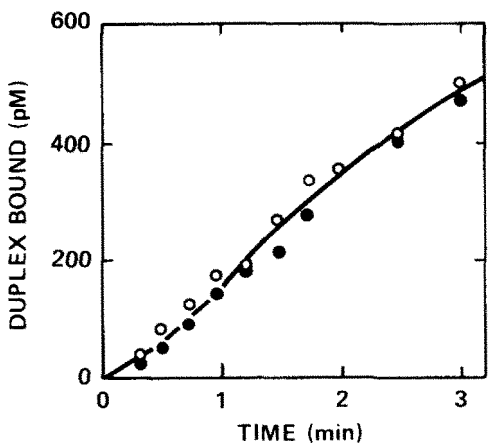

FIG. 4. **Effect of preincubation on kinetics of reaction between M13*mp*8 ssDNA and M13*mp*8 duplex DNA linearized with *Pst*I.** Reactions were performed as described under "Methods"; preincubation was for 1 min (○) and 8 min (●).

shown in Fig. 4 there was no effect of preincubation on the pairing of M13*mp*8 DNAs; hence, the lag must be due to some other feature of the reaction. For example, it may represent the time required to form a stable plectonemic joint (one with a sufficient number of base pairs to survive subsequent treatment). A lag would also be expected if the reaction required the rate-limiting formation of an intermediate prior to the plectonemic joint, as in a series first-order reaction (15).

The stability in 5.2 M GHCl at 56 °C of the products of a reaction at 3.5 min (M13*mp*8) and 5.0 min (M13G*ori*1) is shown in Fig. 5. The reaction was stopped at an early stage to maximize the difference between protein-stabilized D-loops and plectonemic joints. In each case, the time course showed a first-order decrease (see *inset*) in D-loops. The zero time point obtained by interpolation of the linear plots represents the amount of plectonemic joints at 3.5 min (M13*mp*8) and 5 min (M13G*ori*1) (Fig. 5). Excellent agreement was obtained with the value determined independently by treatment at 32 °C for 10 min (Fig. 3, *A* and *B*). These findings are consistent with the existence of two types of D-loops, each with a different stability to GHCl. The protein-stabilized D-loops are instantaneously dissociated, whereas the plectonemic joints show a slower decay which may represent random branch migration at the elevated temperature (see "Discussion"). The apparent first-order rate constant ($k = 0.9 \pm 0.1$ min^{-1}) was independent of the time at which the reaction was stopped (not shown).

Formation of Paranemic Joints in the Absence of Plectonemic Joint Formation—The reaction between *Xho*I-cleaved M13G*ori*1 (see Fig. 1) and wild-type M13 circular ssDNA should yield paranemic joints exclusively (12). Since each end of the linear duplex contains heterologous sequences (1213 and 1003 bp) (16), it is unable to form plectonemic joints but should generate paranemic joints. As shown in Fig. 6*A*, the kinetics of D-loop formation (product treated with 2 M NaCl at 0 °C) are similar to those seen with the fully homologous duplex (compare Fig. 6*A* with Fig. 3*B*). In contrast, the formation of plectonemic joints (treatment with GHCl at 32 °C) was markedly reduced. This result strongly suggests that a majority of the protein-stabilized D-loops are very likely paranemic joints that persist after treatment with 2 M NaCl at 0 °C. As shown in Fig. 3, *A* and *B*, paranemic joints form before plectonemic joints, and whereas paranemic joint formation is similar for both DNAs (*cf.* M13*mp*8 with M13G*ori*1), plectonemic joints form more slowly with M13G*ori*1 than M13*mp*8.

Effect of SSB on Paranemic and Plectonemic Joint Formation—When SSB was omitted from the reaction where plectonemic joint formation was prevented by heterologous terminal sequences, the rate of formation of paranemic joints fell by approximately 10-fold (Fig. 6*B*). Thus, SSB very likely plays a role in the formation and/or stabilization of paranemic structures. Furthermore, when SSB was omitted from a reaction in which plectonemic joint formation could occur (M13*mp*8, Fig. 6*C*), formation of both paranemic and plectonemic joints was decreased by >10-fold. Thus, if SSB acts only by stabilizing paranemic joints, then there is an apparent dependence of plectonemic on paranemic joint formation. This finding taken together with the kinetics of the two reactions suggests strongly that the paranemic joints are essential intermediates in strand exchange between a linear duplex and circular ssDNA.

The residual plectonemic joint formation shown in Fig. 6*A* could result from a small amount of nicking within the duplex DNA, or it could be due to the ability of recA to unwind stretches of DNA that are not homologous to the circular ssDNA as has been suggested by Bianchi and Radding (17). To confirm whether plectonemic joint formation requires an end, the restriction enzyme *Bam*HI was added *in situ* to a reaction containing M13 wild-type ssDNA and *Xho*I-linearized M13G*ori*1. *Bam*HI cleaves M13G*ori*1 within the region of homology (Fig. 1) and should, therefore, produce ends that are homologous to the circular ssDNA. As shown in Fig. 7,

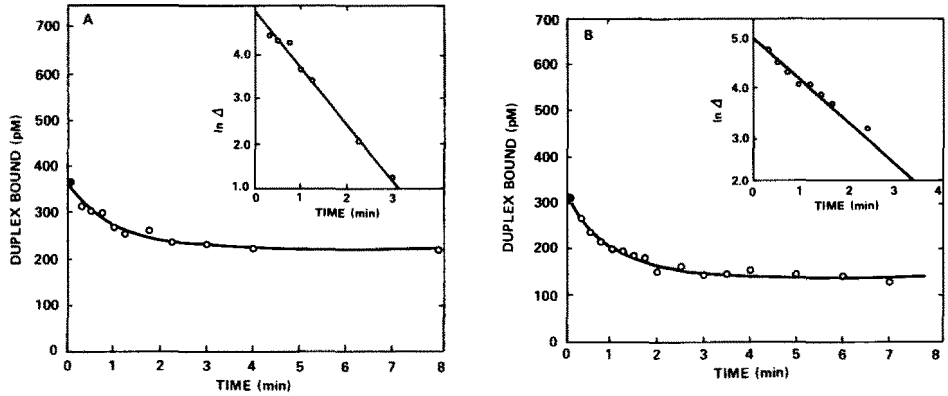

FIG. 5. **Stability of D-loops.** *A*, D-loops were formed between M13*mp*8 ssDNA and M13*mp*8 duplex DNA (Fig. 3*A*); *B*, D-loops formed between M13G*ori*1 ssDNA plus M13G*ori*1 duplex DNA (Fig. 3*B*). Reactions were performed as described under "Methods." A sample of each reaction was mixed with 5.2 M GHCl, 22 mM EDTA, 0.1 M Tris-HCl (pH 7.5) at 56 °C at 3.5 min (*A*) or 5 min (*B*); thereafter, aliquots were withdrawn and diluted into 1 ml of ice-cold 2 M NaCl, 0.15 M sodium citrate at the times indicated. Binding to nitrocellulose filters was determined as described under "Methods." The *insets* show a logarithmic plot from which a first-order rate constant was determined, where Δ = (duplex bound at completion − duplex bound at any time). The zero time point determined from this plot is shown (●).

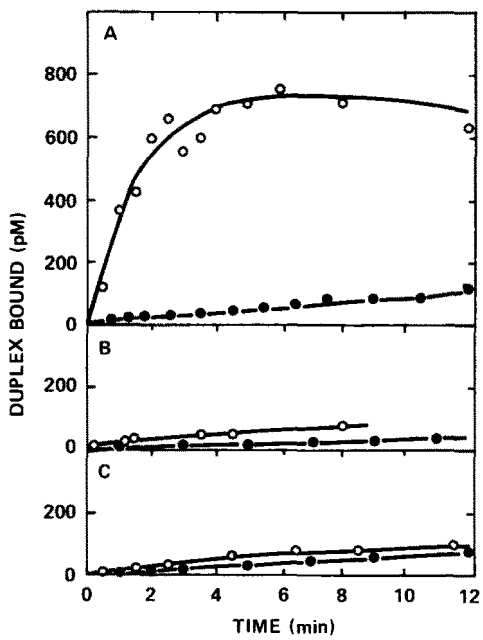

FIG. 6. **Reaction between M13 wild-type ssDNA and M13G*ori*1 duplex DNA linearized with *Xho*I (see Fig. 1).** Reactions were performed as described under "Methods." *A*, standard reaction conditions; *B*, standard reaction conditions with SSB omitted; *D*, M13*mp*8 ssDNA and M13*mp*8 duplex DNA linearized with *Pst*I; SSB omitted. Treatments were with 2 M NaCl, 0 °C for 10 min (○) or 5.2 M GHCl, 32 °C for 10 min (●) which determine paranemic and plectonemic joints, respectively.

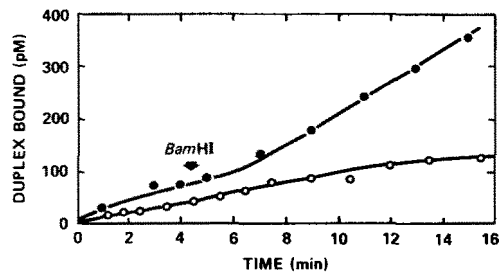

FIG. 7. **Effect of treatment with *Bam*HI on reaction between M13 wild-type ss and M13G*ori*1 duplex DNA linearized with *Xho*I (see Fig. 1).** Reactions were formed as described under "Methods", and the products were treated with GHCl at 32 °C for 10 min. *Bam*HI restriction endonuclease (12 units) was added to the reaction as indicated (●); no *Bam*HI added (○).

there was an increase in plectonemic joint formation upon addition of *Bam*HI. However, it is not known whether the site of endonucleolytic cleavage is within the paranemic joint or adjacent to it.

During the course of treatment of samples withdrawn from the reaction there was little if any change in the products, either paranemic or plectonemic. For example, there was no decrease in the level of paranemic joints during 20 min at 0 °C in 2 M NaCl. Similarly, a sample treated with 5.2 M GHCl for 10 min at 0 °C and then diluted into 2 M NaCl, 0.15 M citrate showed a similar stability (data not shown).

DISCUSSION

Our principal finding is that recA protein together with SSB is able to support the pairing of ssDNA with linear duplex anywhere along its length. This conclusion confirms the previous observations of Das Gupta and Radding (11). However, there are a number of significant features of this pairing reaction that have not previously been described.

First, we have been able to resolve D-loops into two components, paranemic and plectonemic joints, based on the difference in their stability. The nitrocellulose filter-binding assay that we have used should detect both structures because they contain significant amounts of ssDNA. In addition, paranemic joints require some protein for their stabilization, and these structures may bind through protein-nitrocellulose interactions as well. It is evident, however, that the plectonemic joints bind only through their ssDNA component because treatment with GHCl at 32 °C deproteinizes the ssDNA completely, and duplex DNA is stable under these conditions (T_m = 71 °C). The minimum amount of ssDNA required to ensure binding of the plectonemic joint to nitrocellulose filters is not known, and it is possible that a plectonemic joint that has branch migrated to within a few base pairs of the end might not bind at all. Furthermore, plectonemic joints that consist of only a few base pairs are probably not stable under any conditions, since they would dissociate instantly by branch migration.

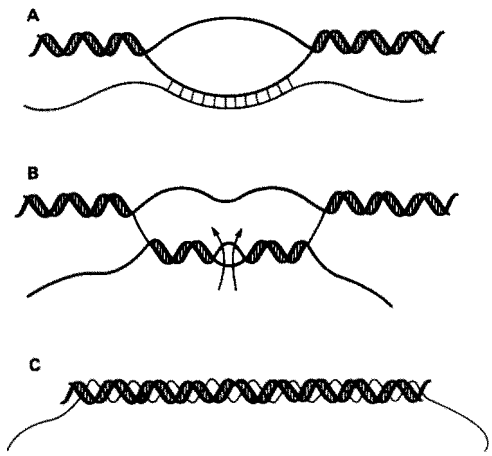

FIG. 8. **Possible structures of paranemic joints.** See "Discussion."

There are at least three ways in which a circular ssDNA might pair with a linear duplex, the ends of which remain intact (Fig. 8). First, the linear duplex may be unwound and the circular ssDNA paired with the looped out (now ss) DNA in a side-by-side manner (Fig. 8*A*). Second, the circular ssDNA can produce Watson-Crick helices of opposing handedness that are oriented in opposite directions (12) (Fig. 8*B*). Third, the duplex is not unwound and instead produces a triple-stranded helix, analogous to that proposed by Howard-Flanders *et al.* (19) (Fig. 8*C*). The experiments presented here cannot distinguish decisively between these three alternatives. However, the weight of evidence argues that the duplex DNA is unwound before pairing occurs (20). In this regard, Kmiec and Holloman have concluded that the rec1 protein from *Ustilago maydis* promotes formation of paranemic joints containing Z-DNA with unwinding of the duplex (21).

Secondly, we have also been able to show kinetically that paranemic structures form before plectonemic joints in a single reaction. An analysis of the reaction between M13*mp*8 ss- and duplex DNAs shows a good fit to a scheme in which there are two consecutive first-order reactions.

$$\text{Complex} \xrightarrow{k_1} \text{paranemic joint} \xrightarrow{k_2} \text{plectonemic joint}$$

where $k_1 = 0.7\ \text{min}^{-1}$ and $k_2 = 0.6\ \text{min}^{-1}$.[3] There are two simple mechanisms by which paranemic joints may be converted to plectonemic joints. First, paranemic joints can dissociate unless they are formed at an end and, therefore, yield plectonemic joints. Secondly, paranemic joints can be extended until an end (nick or gap) is reached, and then the free strand intertwines to form a plectonemic joint. Bianchi *et al.* (12) have estimated the number of base pairs in a paranemic joint to be about 500. Although both mechanisms may not occur exclusively, this value indicates that extensive unwinding of the duplex has occurred and, therefore, supports the second alternative. In either case, paranemic joints have a determining role in the complete pairing reaction and would certainly align regions of homology, whether or not they are directly paired.

Finally, we have found that at saturating concentrations of recA protein, SSB at 1 monomer/8.0 nucleotides can stimulate the formation of paranemic joints approximately 10-fold. This stimulation was observed in a reaction where plectonemic joints could not form. A similar result was obtained when free homologous ends were available. The simplest interpretation of these findings is that the formation of plectonemic joints depends on paranemic joints and that SSB promotes the formation of the latter. A plausible role for SSB would be to bind to the displaced strand ((+)-strand of the duplex DNA) at the paranemic joint, thus preventing reannealing of the duplex. Clearly, SSB would favor unwinding of the duplex DNA, presumably promoted by the recA protein. In the four-strand exchange reactions described by West *et al.* (22), SSB did not stimulate the pairing reaction and in fact had a slight inhibitory effect. In this case the equivalent strand (that binds SSB in the three-strand exchange) would have a complementary DNA with which to pair; consequently there would be no need for SSB.

[3] P. W. Riddles and I. R. Lehman, unpublished observation.

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