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On the Mechanism of Pairing of Single- and Double-stranded DNA Molecules by the recA and Single-stranded DNA-binding Proteins of *Escherichia coli**

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The pairing of single- and double-stranded DNA molecules at homologous sequences promoted by recA and single-stranded DNA-binding proteins of *Escherichia coli* follows apparent first-order kinetics. The initial rate and first-order rate constant for the reaction are maximal at approximately 1 recA protein/3 and 1 single-stranded DNA-binding protein/8 nucleotides of single-stranded DNA. The initial rate increases with the concentration of duplex DNA; however, the rate constant is independent of duplex DNA concentration. Both the rate constant and extent of reaction increase linearly with increasing length of duplex DNA over the range 366 to 8623 base pairs. In contrast, the rate constant is independent of the size of the circular single-stranded DNA between 6,400 and 10,100 nucleotides. No significant effect on reaction rate is observed when a single-stranded DNA is paired with 477 base pairs of homologous duplex DNA joined to increasing lengths of heterologous DNA (627–2,367 base pairs). Similarly, heterologous T7 DNA has no effect on the rate of pairing. These findings support a mechanism in which a recA protein-single-stranded DNA complex interacts with the duplex DNA to produce an intermediate in which the two DNA molecules are aligned at homologous sequences. Conversion of the intermediate to a paranemic joint then occurs in a rate-determining unimolecular process.

The exchange of strands between a circular single-stranded (ss) DNA[1] molecule and its homologous duplex has been a useful model for the analysis *in vitro* of homologous recombination (Cox and Lehman, 1981a; Das Gupta *et al.*, 1980). This reaction, which is promoted by the recA and single-stranded DNA-binding proteins of *Escherichia coli*, is believed to occur in several steps. Initially, a recA-ssDNA complex is formed (Cox and Lehman, 1982; Kahn and Radding, 1984) which then interacts with dsDNA in an as yet unknown way to produce homologously paired structures. Two such structures have been observed and distinguished by their different stabilities and requirements for formation. *Paranemic joints,* which appear early in the reaction, can form at homologous internal sites along a DNA duplex and are unstable in the absence of protein or at elevated temperatures (Bianchi *et al.*, 1983; Riddles and Lehman, 1985a). *Plectonemic joints,* which are formed later in the reaction, are stable in the presence of deproteinizing agents or at elevated temperatures and require free duplex ends homologous to the ssDNA (Bianchi *et al.*, 1983; Riddles and Lehman, 1985a). Their order of appearance suggests that paranemic joints are intermediates in the formation of plectonemic joints, but this remains to be shown rigorously. *E. coli* SSB stabilizes the recA-ssDNA complex, affects the ssDNA-dependent ATPase activity of recA protein, and may bind the displaced single-strand generated during strand exchange (McEntee *et al.*, 1980; Cox and Lehman, 1982; Soltis and Lehman, 1983; Cox *et al.*, 1983b; West *et al.*, 1982). Whether SSB acts solely through its binding to ssDNA (Muniyappa *et al.*, 1984) or by direct interaction with recA protein in the recA-ssDNA complex, or both, is presently unclear.

We present here an analysis of the kinetics of homologous pairing between circular ss- and linear dsDNAs using binding to nitrocellulose filters as an assay (Riddles and Lehman, 1985a). We show that the reaction follows apparent first-order kinetics, a finding that is consistent with the formation of a ssDNA-recA-dsDNA complex as an intermediate. We have also examined the effect of DNA length and of heterologous sequences on the pairing rate. The results are interpreted in terms of a mechanism in which the DNA molecules are aligned at homologous sequences in this intermediate which is then converted to a paranemic structure in a rate-determining first-order reaction.

EXPERIMENTAL PROCEDURES

Materials—recA protein was purified from *E. coli* strain KM1842 by ATP elution from ssDNA cellulose (Cox *et al.*, 1981) or by the procedure of Griffith and Shores (1985) through the spermidine acetate precipitation step. Nalidixic acid was omitted in the latter case. *E. coli* SSB was a gift from D. Soltis, of this department. Restriction enzymes were purchased from New England Biolabs or Boehringer Mannheim, except *Eco*RI, which was a gift from P. Modrich, Duke University. Creatine phosphate, creatine phosphokinase (rabbit muscle, type I), and ATP were from Sigma. NACS52 resin was purchased from Bethesda Research Laboratories.

Plasmid pWR1 (Fig. 1*A*) was constructed from pXF3 (Hanahan, 1983) and M13mp8 form I DNA (Messing, 1981) (gifts from Z. Livneh of this department) digested with *Pst*I and *Cla*I. The fragments were separated by agarose gel electrophoresis, and the 608-bp fragment from M13mp8 and the 2353-bp fragment from pXF3 were recovered by elution onto DEAE paper as described (Livneh, 1983). The fragments were ligated and transformed into *E. coli* strain HB101 using

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[1] The abbreviations used are: ssDNA, single-stranded DNA; SSB, *E. coli* single-stranded DNA-binding protein; dsDNA, double-stranded DNA; ATPγS, adenosine-5′-*O*-(3-thiotriphosphate); RF, replicative form; bp, base pair(s).

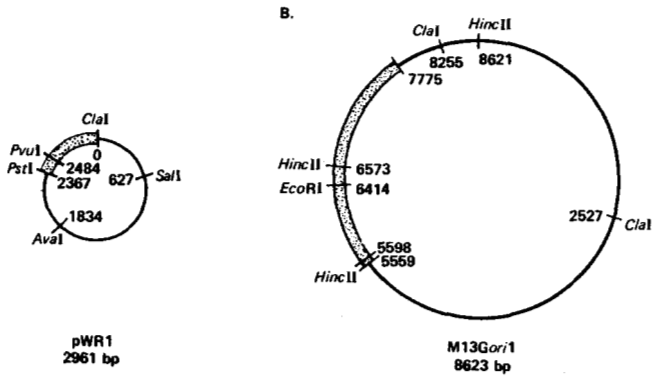

FIG. 1. *A*, structure of pWR1. *Stippled curve* is the *Pst*I/*Cla*I fragment of M13mp8 replacing the corresponding fragment of pXf3. Restriction sites are numbered as the first nucleotide of the recognition sequence. *B*, structure of phage M13*Gori*1 DNA. *Stippled region* is the G4 DNA inserted into M13. Numbering is clockwise in the 5′→3′ direction around the viral (+) strand.

standard techniques (Maniatis *et al.*, 1982). Tritium-labeled supercoiled pWR1 was prepared as follows: *E. coli* strain HB101 (Maniatis *et al.*, 1982) carrying pWR1 was grown at 37 °C to A_{600} = 0.1 in 100 ml of the medium described by Boyer and Roulland-Dussoix (1969), 2.5–5 mCi of [methyl-^{3}H]thymidine (ICN, 60–90 Ci/mmol) and 20 mg of uridine were added, and growth was continued until saturation (about 8 h). The cells were harvested, lysed, and the supercoiled plasmid DNA (specific activity = 100,000–300,000 cpm/μg) purified as described (Davis *et al.*, 1980). Tritium-labeled M13*Gori*1 RF I DNA (Fig. 1*B*) (Kaguni and Ray, 1979) was prepared by infecting *E. coli* strain K37, grown to A_{600} = 0.5 in M9CA medium (Maniatis *et al.*, 1982) with phage (multiplicity of infection = 20). Growth was continued for 10 min at 37 °C, at which time 5 mCi of [^{3}H]thymidine and 200 mg of uridine were added per liter of culture. The cells were harvested after 1.5 h, lysed, and the supercoiled phage DNA isolated as above. Single-stranded DNAs from bacteriophages M13 (wild type), M13*Gori*1, M13H*ori*1, and M13mp8 were gifts from D. Soltis or were prepared from CsCl-banded phage. The phage were incubated at 60–65 °C for 3–5 min in 2% sodium dodecyl sulfate, cooled to 37 °C, and proteinase K (EM Laboratories) (10 mg/ml in 10 mM Tris, pH 8.0, 10 mM $CaCl_2$, 1 mM EDTA, and 50% (v/v) glycerol) added to a final concentration of 200 μg/ml. The solution was kept for 30 min at 37 °C. More enzyme (100 μg/ml) was added, and the incubation continued for 30 min. NaCl was added to 0.1 M, the solution extracted 2–4 times with phenol-chloroform, and the DNA precipitated with ethanol or isopropanol. T7 DNA was prepared essentially as described by Richardson (1966) and Yamamoto *et al.* (1970).

Methods—Restriction endonuclease digestions were carried out under the conditions recommended by Maniatis *et al.* (1982), and restriction fragments were isolated from agarose gels by elution onto DE81 paper (Whatman) as described (Livneh, 1983; Dretzen *et al.*, 1981).

Assay for D-loops—This assay detects both paranemic and plectonemic joints, as defined previously (Riddles and Lehman, 1985a). In the standard reaction, recA protein (1 monomer/3.0 nucleotides of ssDNA) was incubated with 866 pM (molecules of DNA) of circular ssDNA for 5 min at 37 °C in 25 mM Tris·HCl, pH 7.5, 10 mM $MgCl_2$, 5% glycerol (v/v), and 1 mM dithiothreitol. SSB (1 monomer/8.1 nucleotides of ssDNA) and ATP (1.0 mM) were added and the incubation continued for 5 min. An ATP regeneration system, consisting of 4 mM creatine phosphate and 0.02 mg/ml creatine phosphokinase, was added to some reactions (see figure legends) immediately before addition of ATP. ^{3}H-Labeled linear dsDNA (866 pM in DNA molecules) was then added to initiate the reaction. Unless otherwise indicated, equal numbers of ss- and dsDNA molecules were always present. Aliquots were removed and treated as described (Riddles and Lehman, 1985a), except that the initial quenching was in 2 M NaCl, 22 mM EDTA at 0 °C. The diluted aliquots were poured through nitrocellulose filters (Millipore, type HAWP, 0.45-μm pore size), dried, and their radioactivity determined by scintillation counting. An undiluted aliquot from each reaction mixture was dried directly onto a filter to give the total radioactivity in the aliquot. No reaction (<2%) was detectable in the absence of homologous sequences. Variations from the standard conditions are given in relevant figure legends and under "Results."

Reactions were followed to completion and first-order rate constants (k) calculated either from the slope of plots of log $\left(\frac{cpm_\infty - cpm_t}{cpm_\infty}\right)$ *versus* time, where cpm_∞ is the radioactivity bound to the filter at the completion of the reaction (end-point) and cpm_t is the radioactivity bound at time t, or by fitting the data directly to Equation 1

$$cpm_t = cpm_\infty (1 - e^{-kt}) \quad (1)$$

using a modified version of the nonlinear least squares FORTRAN program of Cleland (1969).

RESULTS

Kinetics of D-loop Formation—Typical time courses for the appearance of ^{3}H-labeled structures that bind nitrocellulose filters (D-loops) are shown in Fig. 2*A*, with first order plots of the data in Fig. 2*B*. An experiment in which the reaction components (*i.e.* recA protein, SSB, ssDNA, and dsDNA) were diluted 2-fold gave essentially the same first-order rate constant (1.01 min^{-1}) as the undiluted reaction (0.86 min^{-1}, Figs. 1*B* and 6*A*), suggesting that the process is true first-order. The extent of reaction was 70–80% of the total dsDNA present. In a second experiment, rate constants of 0.75 and 0.80 min^{-1} were obtained for undiluted and 2-fold diluted reactions, respectively (data not shown).

Figs. 3 and 4 show the results of experiments in which the concentrations of recA and SSB proteins were varied, with

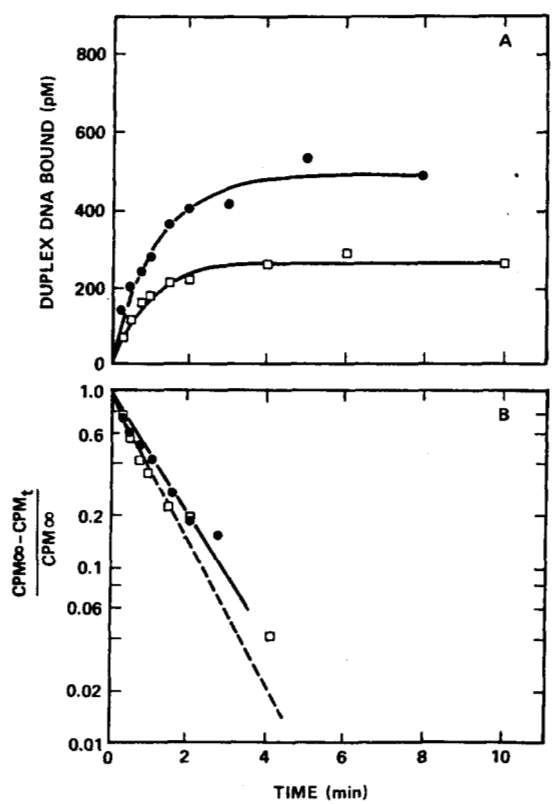

FIG. 2. **Time course of appearance of D-loops.** Reaction conditions were as in the standard assay with: 866 pM M13*Gori*1 ssDNA and 684 pM M13*Gori*1 dsDNA (linearized with *Eco*RI) (●); 433 pM ssDNA and 353 pM dsDNA (□). *A*, smooth curves are calculated from computer fits of the data to Equation 1. Calculated values of the rate constants are 0.86 min^{-1} (●) and 1.01 min^{-1} (□). *B*, semi-log plot of data shown in *A*. Lines were calculated from the computer-fitted parameters. *Solid line* corresponds to *filled circles* and *dashed line* to *open squares*.

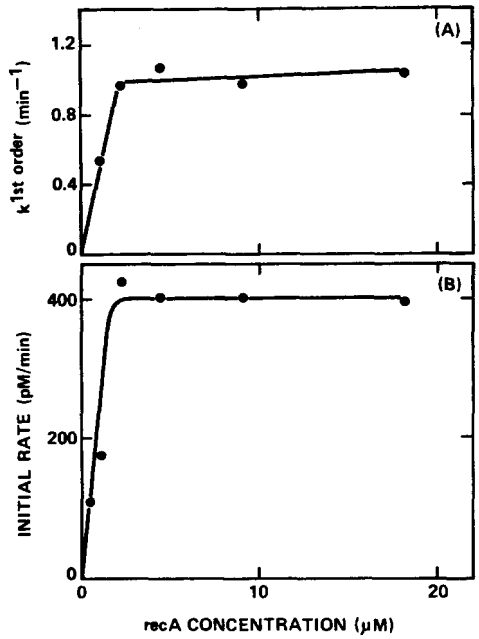

FIG. 3. **Effect of recA protein concentration on the first order rate constant (*A*) and initial rate (*B*) of D-loop formation.** Circular M13G*ori*1 ssDNA was paired with *Eco*RI-linearized M13G*ori*1 dsDNA, using standard assay conditions but varying recA protein concentration. A first-order rate constant was not calculated for the experiment at the lowest recA protein concentration, because the extent of reaction was less than 20% of the total dsDNA present (all other runs went to 63–93%).

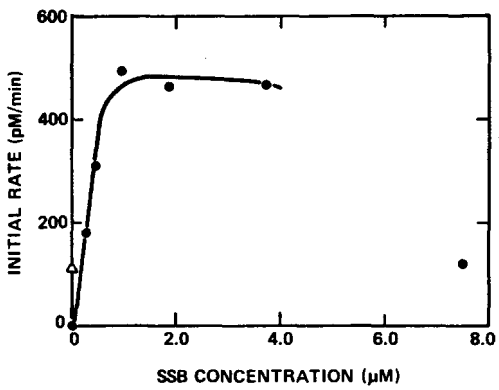

FIG. 4. **Dependence of initial rate of D-loop formation on SSB concentration.** M13G*ori*1 ssDNA was paired with *Eco*RI-linearized M13G*ori*1 dsDNA in the standard assay (●). In one experiment to which no SSB was added, ssDNA was preincubated for 20 min at 37 °C with 2.5 recA protein/nucleotide, ATP was added, and after 5 more min the reaction initiated by adding labeled dsDNA (△).

the concentrations of the remaining components kept constant. The rate constants showed the same concentration dependence as the initial rates. Extents of reaction were about 80% of completion in the concentration-independent plateau, but were somewhat less in nonoptimal reaction conditions. The rate saturated at about 1 recA protein/3 and 1 SSB/8 nucleotides of ssDNA, similar to previous findings for recA protein-promoted DNA strand exchange carried out under the same conditions (Soltis and Lehman, 1984), although somewhat different from results obtained using other conditions or preincubation protocols (Cox and Lehman, 1982; Cox *et al.*, 1983a). Essentially no reaction was observed in the absence of SSB, but an increase in the concentration of recA protein and longer preincubation periods allowed some reaction to occur. However, this reaction was significantly slower than that in the presence of SSB and proceeded to only about 35% of completion. The dependence on ATP concentration (data not shown) was sigmoidal, as has been observed in recA protein-catalyzed ATP hydrolysis (Weinstock *et al.*, 1981), with essentially no reaction at ATP concentrations below 20 μM and a maximal rate over the range 0.1–1.0 mM. Higher ATP concentrations led to substantial inhibition, apparently due to chelation of Mg^{2+} by ATP. Thus, no reaction was observed with both ATP and Mg^{2+} at 10 mM, but the rate constant at 10 mM ATP and 16 mM Mg^{2+} (0.20 min^{-1}) was essentially identical to that at 4 mM ATP and 10 mM Mg^{2+} (0.22 min^{-1}). No D-loop formation was observed at 30 mM Mg^{2+} in the absence of ATP, conditions in which recA protein-dependent DNA renaturation can occur (Bryant and Lehman, 1985).

Effect of Increasing Size of ss- and dsDNA on D-loop Formation—The first-order rate constants for the pairing of a duplex *Cla*I fragment of M13G*ori*1 (2895 bp) (see Fig. 1*B*) with circular ssDNAs of increasing size under standard assay conditions are given in Table I. The constants for reaction with M13 wild type (6407 nucleotides), M13G*ori*1 (8623 nucleotides), and M13H*ori*1 (10,100 nucleotides) ssDNAs were essentially independent of size. In contrast, when the size of the ssDNA was kept constant and the length of the homologous dsDNA varied, the rate constants and initial rates (not shown) exhibited a linear dependence on the length of the dsDNA (Table I and Fig. 5). In this experiment, the reaction with the full-length M13G*ori*1 dsDNA always reached about 80% of completion, but the extents with the smaller duplex fragments were lower (39% with the 366-bp fragment). Low

TABLE I

Rate constants for pairing of ss- and dsDNAs by recA protein

Reactions were performed under standard assay conditions (see text) except as noted below.

dsDNA	Size	ssDNA	Size	$k^{1st\ order}$
	bp		*nucleotides*	min^{-1}
M13G*ori*1				
*Cla*I fragment	2,895	M13	6,407	0.50 (0.05)[a]
*Cla*I fragment	2,895	M13G*ori*1	8,623	0.62 (0.07)
*Cla*I fragment	2,895	M13H*ori*1	10,100	0.56 (0.05)
M13G*ori*1[b]				
*Cla*I/*Hin*cII fragment	366	M13G*ori*1	8,623	0.14 (0.016)
*Cla*I/*Hin*cII fragment	975			0.25 (0.018)
*Cla*I/*Hin*cII fragment	2,529			0.48 (0.040)
Linearized with *Eco*RI	8,623			1.67 (0.18)
pWR1[c]				
*Pvu*I/*Cla*I fragment	477	M13mp8	7,229	0.070 (0.009)
*Pvu*I/*Sal*I fragment	1,104			0.073 (0.006)
*Pvu*I/*Ava*I fragment	2,311			0.066 (0.008)
*Pvu*I/*Pst*I fragment	2,844			0.064 (0.006)

[a] Values in parentheses are standard errors calculated by the least-squares fitting program.

[b] Reaction conditions were as described under "Experimental Procedures" with 1 recA protein/1.6 nucleotides of ssDNA and an ATP regeneration system; see Fig. 1*B* for origin of *Cla*I/*Hin*cII dsDNA fragments.

[c] Reaction conditions were as in Fig. 7; see Fig. 1*A* for origin of pWR1 dsDNA fragments.

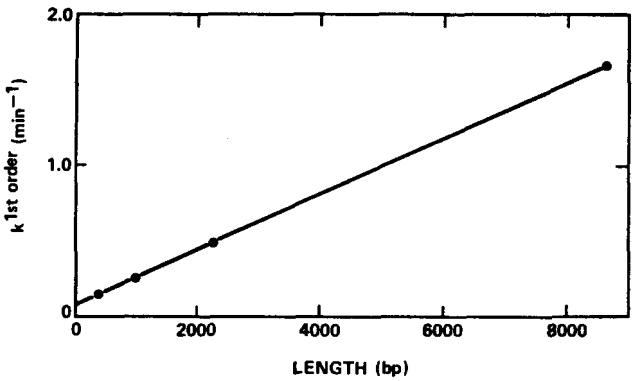

FIG. 5. **Effect of length of dsDNA on first-order rate constant for D-loop formation.** The rate of pairing between ssDNA (M13*Gori*1) and dsDNA fragments from *Cla*I/*Hin*cII-digested (366, 975 and 2529 bp) and *Eco*RI-linearized (8623 bp) M13*Gori*1 was measured in the standard assay with 4.0 mM ATP, and first-order rate constants were calculated by fitting to Equation 1. Average values of duplicate determinations are plotted.

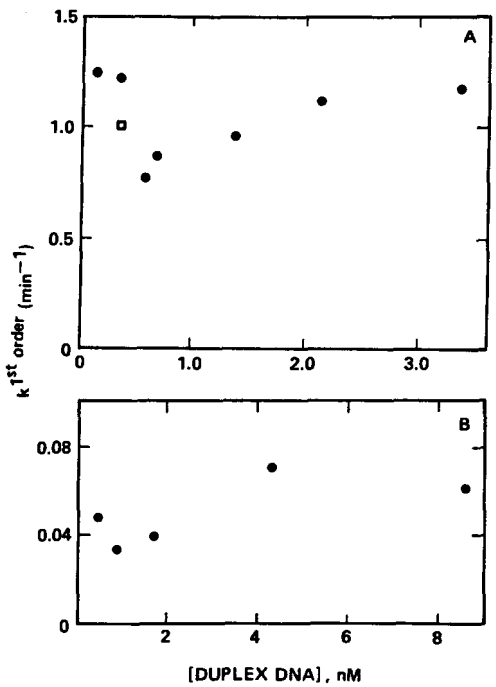

FIG. 6. **Effect of varying dsDNA concentration on the apparent first-order rate constant for D-loop formation.** *A*, M13*Gori*1 dsDNA linearized with *Eco*RI was paired with M13*Gori*1 ssDNA in the standard assay with the concentration of dsDNA molecules as shown (●). Standard errors calculated by the least-squares program were about 10% the magnitude of the rate constants. In one experiment, the concentration of ssDNA was halved to 433 pM (molecules of DNA), with recA protein and SSB present in the same ratios as in the standard assay (1 recA protein/3 nucleotides and 1 SSB/8 nucleotides) (□). *B*, the 477 bp *ClaI*/*PvuI* fragment of pWR1 was paired with M13mp8 ssDNA in the standard assay.

extents (20–50%) were usually observed in reactions with short DNAs using several different recA protein samples prepared in different ways. A number of experiments were performed in attempts to understand the low extents of reaction. D-loops formed with the 366-bp M13*Gori*1 fragment were stable at 0 °C in the high salt solution used to quench the reaction, and low extents were observed with DNAs from several different plasmid preparations, regardless of the length of time since isolation of the DNA. Another set of restriction fragments (the 366-, 975-, and 3071-bp *Hin*cII/*Cla*I fragments of M13*Gori*1) were isolated by a procedure different from that described under "Experimental Procedures," *i.e.* electrophoresis in low-melting agarose (Maniatis *et al.*, 1982) followed by chromatography on NACS52 resin. Reaction extents were again 50–60%, and the same linear dependence of rate constant on size of dsDNA was observed. Inclusion of an ATP regeneration system or addition of more ATP, ssDNA, recA protein, SSB mixture, or ^{3}H-labeled dsDNA after the reaction had stopped elicited no further reaction. Extents were generally greater (~50%) in reactions containing an excess (5-fold) of ssDNA, recA protein, and SSB over dsDNA than when both DNAs were present in equimolar amounts (~25–30%), suggesting that the reaction may go to some equilibrium level, but this result was not always reproducible. Extents were also greater at 1 mM ATP (50–70%) than at 4 mM (25–30%). As discussed below, the variability of extent of reaction with the small DNA fragments makes a detailed interpretation of kinetic data from these experiments difficult.

The concentration of dsDNA was varied in the two experiments shown in Fig. 6. Full-length linear M13*Gori*1 dsDNA (8623 bp) was paired with the corresponding ssDNA circle in Fig. 6*A*, while in Fig. 6*B* the 477-bp *ClaI*/*PvuI* fragment of pWR1 (see Fig. 1*A*) was paired with M13mp8 single strands. All time courses were fit to Equation 1 (see "Experimental Procedures") and first-order rate constants determined. As shown in Fig. 6*A*, the rate constants for the reaction with full length M13*Gori*1 dsDNA were independent of dsDNA concentration. The extents of reaction were 70–95% of the dsDNA reacted when ssDNA was in excess and were greater than 1 dsDNA reacted per ssDNA circle at excess dsDNA. Thus, at the highest dsDNA concentration, the amount of DNA bound to the filter at the maximal extent of reaction represented 1.6 D-loops/ssDNA circle. One explanation for this finding is that two duplexes can react with one circle, as has been observed previously by electron microscopic examination of the products of strand exchange (Cox and Lehman, 1981b), although in those experiments only a relatively small percentage of "double D-loops" was observed. The initial rate increased with increasing dsDNA concentration and did not reach complete saturation even at the highest concentrations used. There was, however, significant curvature, and saturation might have been observed at still higher concentrations. The rate constants for the reaction with the 477-bp DNA (Fig. 6*B*) were also independent of concentration; in this case, the extent increased with increasing concentration of dsDNA to a maximum of about 40–45% at a 5–10-fold excess of ds- over ssDNA. The initial rate also increased with increasing dsDNA concentration, reaching a maximum at a 5-fold excess.

Effect of Heterologous Sequences on Rate of D-loop Formation—The effect of heterologous sequences within the dsDNA on the rate of D-loop formation was tested by pairing M13mp8 ssDNA with restriction fragments of pWR1 (see Fig. 1*A*) containing 477 bp homologous to M13mp8, joined to varying lengths of heterologous DNA. As shown in Table I and Fig. 7, the heterologous DNA had little or no effect on the rate constant. Similarly, the initial rates in this experiment showed no significant dependence on the length of the heterologous DNA segment. This result contrasts with that reported by Gonda and Radding (1983), in which the addition of heterologous tails to a 151-bp dsDNA fragment increased the initial rate of pairing, giving a linear relation between tail length and initial rate.

Heterologous dsDNA not linked covalently to the DNA reactant also had no influence on the rate of pairing. Thus, addition of 80 μM (nucleotides) of T7 dsDNA had virtually

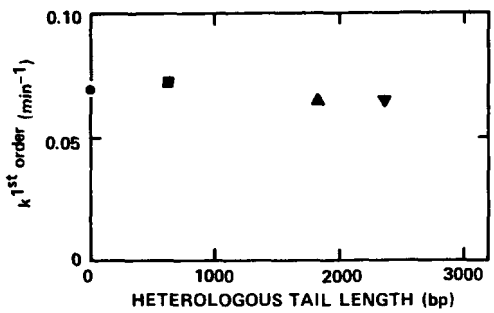

FIG. 7. **Effect of heterologous tail length on the first-order rate constant for D-loop formation.** M13mp8 ssDNA was paired with duplex restriction fragments of pWR1: *Pvu*I/*Cla*I fragment (477 bp, completely homologous, ●); *Pvu*I/*Sal*I fragment (477 + 627 bp heterologous tail, ■); *Pvu*I/*Ava*I fragment (477 + 1834 bp heterologous tail, ▲); *Pvu*I/*Pst*I fragment (477 + 2367 bp heterologous tail, ▼). The standard reaction conditions were used, except 1 recA protein/1.8 nucleotides of ssDNA and the ATP-regenerating system were included. Average values of duplicate determinations are plotted. Standard errors were 10–20%.

no effect on the pairing rate of M13G*ori*1 ssDNA (7.5 μM nucleotides) with the 3071-bp fragment (2.7 μM nucleotides) ($k^{1st\ order}$ = 0.17 min^{-1} without T7 DNA, and k = 0.15 min^{-1} in the presence of T7 DNA) and with the 366-bp fragment (0.32 μM nucleotides) (k = 0.083 min^{-1} without T7 DNA and k = 0.082 with added T7 DNA).

DISCUSSION

The formation of D-loops is a complex reaction involving ss- and dsDNA molecules, recA protein, and SSB. We had shown previously that recA protein binds tightly to ssDNA in the presence of SSB and ATP and does not exchange readily between ssDNA molecules (Cox and Lehman, 1982). The data of Fig. 3 support the conclusion that recA protein and ssDNA form a tight complex, which is then the active species in the pairing reaction. The first order rate constant and initial rate of pairing are maximal at a recA protein:nucleotide ratio of about 1:3, the same binding stoichiometry observed in direct assays of recA-ssDNA binding in the absence of SSB (Bryant *et al.*, 1985). Additional recA protein above this optimal level had no effect on the reaction rate, indicating that recA protein is bound tightly in the complex. The effect of SSB on the reaction rate reached saturation at a protein:nucleotide ratio of about 1:8, although at very high SSB levels the rate and extent were somewhat reduced, perhaps due to competition by the two proteins for binding to the DNA. Finally, the preincubation protocol used here allows the reaction to proceed without a lag (see Fig. 2), indicating that the complex forms during the preincubation period (Kahn and Radding, 1984; Riddles and Lehman, 1985b).

Any mechanism proposed for the formation of paranemic joints must account for the following experimental observations. (i) The reaction follows apparent first-order kinetics; (ii) both the initial rate and the first-order rate constant increase with increasing length of homologous sequences shared by the reacting DNA molecules; (iii) no rate enhancement is seen when either the ss- or dsDNA is made longer by the addition of DNA that is not homologous to the other reactant; (iv) heterologous DNA has no inhibitory effect; and (v) the rate constants are independent of dsDNA concentration.

The kinetics of paranemic joint formation may be treated as a two-component reaction in which a recA-ssDNA complex pairs with homologous duplex. Nevertheless, the time course and dilution experiment of Fig. 2 show that the reaction follows first-order kinetics. The fact that the reaction rate depends on both the dsDNA and recA-ssDNA concentrations (data not shown, and Gonda *et al.*, 1985) indicates a mechanism in which an intermediate ssDNA-recA-dsDNA complex is formed and converted to a D-loop in a rate-determining first-order process (Reaction 1).

$$\text{ssDNA-recA} + \text{dsDNA} \overset{K}{\rightleftharpoons} \text{ssDNA-recA-dsDNA complex} \xrightarrow{k} \text{D-loop}$$

The kinetics of such a mechanism will be first order if the equilibrium for complex formation lies well to the right, that is if most of the added DNA substrates form this relatively stable complex rapidly and the reaction rate is that at which the complex forms a D-loop with first order rate constant k. The reaction should become second order at very low DNA concentrations, but apparently this did not occur in the concentration range used. Moreover, deviations from first order behavior are expected to occur late in the reaction after depletion of a large percentage of the reactant; however, the precision of the filter-binding assay is not sufficient to detect accurately such deviations.

We can consider possible structures for the ssDNA-recA-dsDNA intermediate. The DNAs could be held together nonspecifically by the protein, the rate-determining step being the first-order conversion of the complex to a form in which the DNAs are aligned homologously in a configuration that is stable to, and thus detected by, the conditions of the nitrocellulose filter-binding assay. Alternatively, the DNAs in the complex might already be aligned at homologous sites, the rate-determining step being conversion to a stable paranemic joint. Formation of this *unstable* intermediate complex could occur by either a random collision or by a facilitated diffusion mechanism (Berg *et al.*, 1981); these are kinetically equivalent in experiments in which only stably paired structures are scored.

The first-order rate constant for pairing shows a linear increase with size of the dsDNA in experiments where the dsDNA is completely homologous to the ssDNA (Fig. 5). As noted earlier, the reactions with small DNAs can be fit to a first-order rate equation, but the extents of reaction are lower than those with the full-length M13G*ori*1 dsDNA. The variable extents make precise interpretation of the rates and rate constants difficult since first-order reactions that proceed to different extents can have the same first-order rate constant but different initial rates. As mentioned previously, the initial rates of these reactions also increase linearly with dsDNA length. It is thus clear, these qualifications notwithstanding, that the reaction is faster with the longer DNAs.

No rate enhancement was observed when the size of the DNA was increased by adding heterologous sequences, either to the dsDNA (Fig. 7) or to the ssDNA (Table I). This result suggests that the rate enhancement shown in Fig. 5 cannot be explained by a greater number of nonspecific heterologous contacts forming between the recA·ssDNA complex and the longer dsDNAs. The fact that a large excess of T7 DNA did not inhibit the reaction suggests that such heterologous ternary complexes are relatively weakly bound and, therefore, short-lived. Heterologous ternary complexes have been observed in the presence of ATPγS (Shibata *et al.*, 1979; McEntee *et al.*, 1980) which causes recA protein to bind very tightly to DNA (Bryant *et al.*, 1985). They presumably form and dissociate rapidly on the time scale of these experiments due to random collisions between the DNA molecules, in a step preceding the rate-determining step.

These results support a mechanism in which the rate-determining step is conversion of an unstable homologously aligned recA·ssDNA·dsDNA complex to a structure that is stable to the high salt quenching conditions used in the nitrocellulose filter-binding assay, *i.e.* a paranemic joint. The detailed structure of this complex is unknown, but it could involve base pairing between the ssDNA and short regions of unwound duplex at internal sites along the dsDNA.

Neither attached heterologous sequences nor additional homologous sites introduced by increasing the concentration of dsDNA affect the pairing rate constant. Thus, the additional homologous interactions available in a longer dsDNA molecule, perhaps at distant sites, must enable the complex to be converted more readily to the stably paired structure. This mechanism differs from that proposed by Gonda and Radding (1983) in that location of homologous sites must be fast relative to conversion to a paranemic joint and is, therefore, not rate limiting. Thus, we are unable to discern whether facilitated diffusion plays a significant role in the process by which the search for homology proceeds.

The lack of an effect of attached heterologous DNA also differs from the findings reported by Gonda and Radding (1983), in which such heterologous sequences increased the initial pairing rate, although they report that heterologous DNA not covalently attached had no effect, a result in agreement with our findings. Gonda and Radding used a DNA containing only 151 bp homologous to the ssDNA *versus* 477 bp in the experiments reported here, and their reaction conditions were somewhat different than ours, especially in the use of large excesses of recA protein and the absence of SSB. In view of these differences the two experiments may not be fully comparable. The reaction rates which we measure in our standard assay conditions with full-length M13G*ori*1 dsDNA are comparable to those reported by Gonda *et al.* (1985) using the same DNA substrates (see our Figs. 3 and 4 and Table I in Gonda *et al.*, 1985), but our rates with 366- or 477-bp dsDNAs are somewhat less than that reported for a dsDNA of only 151 bp (Gonda and Radding, 1983). Furthermore, we have found that reactions performed under the experimental conditions described by Gonda and Radding (2.5 recA protein/nucleotide, SSB omitted, 1.2 mM ATP, 20 mM Mg^{2+}, and an ATP regeneration system; buffer, preincubation, and quenching conditions also identical to those of Gonda and Radding (1983)) had very low rates (2.4–4.6 pM/min *versus* 20–30 pM/min in our standard assay conditions), and the reactions did not reach completion (12–16% after 50 min). The initial rates were difficult to measure precisely and were lowest with the 1104-bp fragment (2.4 pM/min) and increased with the 2311- and 2844-bp fragments (3.0 and 4.5 pM/min, respectively). The rate was also higher with the 477-bp DNA lacking heterologous sequences (4.6 pM/min). The two groups have used recA protein and DNA substrates purified by different methods, but it is unclear whether this can explain the discrepancy.

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