Kinetics of Homologous Pairing Promoted by RecA Protein: Effects of Ends and Internal Sites in DNA†

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ABSTRACT: When recA protein was preincubated with single-stranded DNA in the presence of an ATP-regenerating system prior to the addition of homologous duplex DNA, a slow presynaptic step was eliminated, and the subsequent homologous pairing was revealed as a reaction whose rate exceeds by 1 or 2 orders of magnitude the calculated rate of spontaneous renaturation in 0.15 M NaCl at T_m −25 °C. The pairing reaction displayed saturation kinetics with respect to both single-stranded and double-stranded DNA, indicating the existence of a rate-limiting enzyme–substrate complex. The signal observed in the assay of the pairing reaction was due to pairing at free homologous ends of the duplex DNA, as well as pairing in the middle of the duplex molecule, away from a free end. The apparent rate of pairing of circular single strands with linear duplex DNA was equal to the sum of the rates of pairing at sites located at either end of the duplex DNA or at interior sites, but the apparent rates attributable to ends were greater, and nicks also stimulated the apparent rate.

Escherichia coli recA protein promotes the homologous pairing of double-stranded DNA with single-stranded or partially single-stranded molecules. The reaction proceeds in three phases: a *presynaptic phase*, during which recA protein polymerizes on single-stranded DNA, *synapsis*, which comprises the conjunction and homologous alignment of molecules, and a *postsynaptic* phase of strand exchange which slowly enlarges heteroduplex joints to thousands of base pairs in length [see Radding (1982) for a review].[1]

Synapsis is one of the most interesting aspects of homologous recombination and of the related reaction that is promoted by recA protein in vitro. On the basis of previous observations, we have developed the following view of synapsis as promoted by recA protein in vitro. In a formal sense, synapsis includes both the conjunction and homologous alignment of DNA molecules. RecA protein appears to promote both of these steps. The idea that it promotes conjunction came from the observation that single-stranded DNA stimulates the binding of duplex DNA by recA protein, in the presence either of ATP or of its nonhydrolyzable analogue adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS) (Shibata et al., 1979b; Radding et al., 1980). A series of additional observations have supported the concept that recA protein promotes the homologous alignment of a single strand and its duplex counterpart without regard to the existence or location of free ends and that when an end or a break exists in one strand of the nascent pair, recA protein causes strand exchange and the creation of heteroduplex joints that may be thousands of nucleotide residues in length [Bianchi et al., 1983; for a review, see Radding (1982)].

The small DNA phages are the source of a particularly useful pair of substrates, namely, linear duplex DNA and circular viral or plus strands. In this combination, the linear molecule presents three classes of sites at which pairing can occur. Pairing in the middle of the duplex molecule produces a noninterwound or paranemic joint, which is intrinsically unstable but which may serve as a step toward pairing at one of the ends. Pairing at the ends rapidly produces a joint in which the newly paired strands are interwound, resulting in displacement of a strand from the original duplex molecule (Wu et al., 1982; Bianchi et al., 1983). Because recA protein promotes strand exchange in a polar fashion (Kahn et al., 1981; Cox et al., 1981; West et al., 1981), the joints formed at the ends also differ in stability. When the 5′ end of the plus strand is displaced in the initial pairing reaction, subsequent strand exchange in the 5′ to 3′ direction enlarges and stabilizes the heteroduplex joint. By contrast, when the 3′ end of the plus strand is displaced in the initial pairing reaction, subsequent strand exchange decreases the size of the heteroduplex joint and causes it to dissociate (Wu et al., 1982).

Previous observations have shown that the kinetics of homologous pairing promoted by recA protein resemble enzyme kinetics rather than the second-order renaturation of complementary single strands (Radding et al., 1980, 1981). In this paper, we have investigated the kinetics of the reaction in detail, guided by improved understanding of its phases, of the adverse effect of ADP (Wu et al., 1982), and of the topological features discussed above.

Materials and Methods

Enzymes. RecA protein (Shibata et al., 1979a) was purified essentially as described previously. Restriction endonucleases were purchased from New England Biolabs. Pancreatic DNase I was purchased from Boehringer Mannheim. RecA protein concentration was measured by using a modified Lowry procedure (Geiger & Bessman, 1972) and by absorbance with $E^{1\%}_{280nm} = 4.53$.

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[1] In our terminology, *homologous pairing* and *pairing* are used interchangeably to describe the overall homology-dependent joining of molecules without signifying any particular step in the process or any particular extent of strand exchange. The term *conjunction* is used in its ordinary nontechnical sense to mean coming together, and therefore denotes a process that is necessarily independent of homology.

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Preparation of DNA. Circular single-stranded and circular duplex DNA from phages G4, M13, and M13G*ori*I were prepared as described previously (Cunningham et al., 1980, 1981; DasGupta et al., 1980). The preparations of circular single-stranded DNA contained less than 5% linear molecules as judged by electrophoresis in 1.8% agarose. The preparations of superhelical DNA usually contained less than 10% nicked molecules, as determined by the assay of Kuhnlein et al. (1976); preparations that had a greater percentage of nicked molecules were repurified in CsCl/ethidium bromide gradients. DNA concentration was measured by the absorbance at 260 nm using ϵ = 6800 for duplex DNA and ϵ = 8500 for single-stranded DNA. Unless otherwise stated, all concentrations of DNA are expressed in micromoles of nucleotide residues.

Supercoiled M13G*ori*I [^{3}H]DNA (<3% nicked) was nicked with pancreatic DNase I in reaction mixtures (70–75 μL) containing 50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) (pH 7.5), 5 mM $MgCl_2$, 15 nmol of duplex DNA, and 2–5 ng/mL pancreatic DNase I. After 20 min at room temperature, the DNase I was inactivated by heating to 70 °C for 10 min. Reactions in which 70% of the DNA was nicked had less than 5% linear molecules as judged by agarose gel electrophoresis.

Digestion of duplex DNA by restriction endonucleases was performed by using the buffers recommended in Davis et al. (1980). Prior to doing the preparative reactions, we assayed the restriction endonucleases in analytical reactions of 5–10 μL to determine the least amount of enzyme needed to digest the DNA completely. We monitored the endonuclease reactions by agarose gel electrophoresis.

Full-Sized Linear Duplex M13 and M13GoriI DNAs. Supercoiled [^{3}H]DNA was digested with the appropriate enzyme. The reaction mixture was extracted with phenol, extracted several more times with ether, and dialyzed vs. 10 mM Tris-HCl (pH 7.5) and 1 mM ethylenediaminetetraacetic acid (EDTA).

Large AccI or AvaI Fragments of M13GoriI. Supercoiled M13G*ori*I [^{3}H]DNA was digested to completion with *Acc*I or *Ava*I restriction endonucleases. *Acc*I cuts the M13G*ori*I DNA into two pieces of 6490 and 2133 base pairs; *Ava*I cuts the DNA into two pieces of 7362 and 1261 base pairs. The DNA was loaded onto a 5–20% neutral sucrose gradient (in 1 M NaCl and 1 mM EDTA) and centrifuged for 18 h at 32 000 rpm in a Beckman SW41Ti rotor at 15 °C. Fractions containing the large *Acc*I and *Ava*I fragments were pooled and precipitated with ethanol. In the experiment shown in Figure 7, the full-length linear duplex M13 [^{3}H]DNA was also passed through a sucrose gradient, so as to control against the potential effects of the sucrose gradient preparation on the efficiency of subsequent pairing reactions. Pairing reactions with DNAs that had been passed through a sucrose gradient yielded the same rate as pairing reactions with DNAs that had not been so treated.

SphI and BstNI Mid-Sized Fragments. Supercoiled M13G*ori*I [^{3}H]DNA was digested to completion with *Bst*NI; NaCl was added to the reaction mixture to a final concentration of 150 mM, and digestion was continued with *Sph*I to yield three fragments. The DNA was electrophoresed through a 1.8% low melting point agarose gel (Bethesda Research Laboratories, gel electrophoresis grade) to separate the fragments. To find the DNA in the gel, we cut the marker lanes out of the gel and stained them with ethidium bromide to allow the visualization of the DNA bands under UV light. We made a notch in the gel at the position of the desired band and then set the marker lanes back into the preparative gel to guide the cutting of the untreated gel. We reisolated the DNA by melting appropriate slices of the gel in a 5-fold greater volume of 50 mM Tris-HCl (pH 7.5) and 1 mM EDTA, following which we extracted the solution with phenol in siliconized Corex tubes, as described in Maniatis et al. (1982). Pairing reactions with DNAs that had been passed through a low melting point agarose gel yielded the same rate as pairing reactions with DNAs that had not been so treated.

Reactions. Reactions were performed in 1.5-mL Eppendorf tubes set in a 37 °C water bath. All reaction mixtures contained 20 mM $MgCl_2$, 61 mM Tris-HCl (pH 7.5), 0.6 mM NaEDTA, 0.6 mM dithiothreitol, 6% glycerol, an ATP regenerating system (6 mM phosphocreatine and 10 units/mL creatine phosphokinase), and concentrations of recA protein and DNA as indicated. Except where noted, all reactions were set up as follows: Reaction mixtures lacking duplex DNA and ATP were incubated at 37 °C for 10 min to warm them; ATP was added, and incubation was continued for 20 min at 37 °C before duplex DNA was added to start the pairing reaction.

Nitrocellulose Filter Assay for Joint Molecules (D-Loop Assay). This assay measures the trapping by nitrocellulose filters of duplex DNA that has become partially single stranded (Beattie et al., 1977). It detects the attachment by any means of fully duplex DNA to single-stranded or partially single-stranded DNA. The assay used in the present experiments is a modification of previously published procedures (Beattie et al., 1977; Shibata et al., 1979a). At the appropriate times, we took a 20-μL aliquot of a reaction mixture, placed it directly into 200 μL of 25 mM EDTA (pH 9.4) at 0 °C, and rapidly mixed the solution. We immediately added 4.0 mL of 1.5 M NaCl and 0.15 M sodium citrate, also at 0 °C, and set the mixture on ice. We filtered the sample at approximately 4 mL/10 s through a nitrocellulose filter (Sartorius or Millipore, 0.45-μm pore size) which had been washed with 4 mL of NaCl and sodium citrate. We rinsed the tube twice with 4 mL of NaCl and citrate, filtering the rinses, and rinsed the filter and apparatus again with 4 mL of NaCl and sodium citrate. Some lots of filters caused a salt-dependent quenching of radioactivity; we found that we could eliminate the quenching by giving the filters an additional rinse with 5 mL of 0.3 M NaCl and 0.03 M sodium citrate before the filters were dried. We determined the total amount of radioactivity by spotting a 20-μL aliquot of the reaction mixture directly onto a dry filter. Filters were dried under a heat lamp and put into vials with Betafluor (National Diagnostics); radioactivity was measured in a liquid scintillation counter. As measured by this assay, 100% of radioactive single-stranded DNA was retained by the filters.

Measurement of Initial Rates. We sampled the pairing reactions at least at 10, 25, and 40 s after starting the reaction by the addition of duplex DNA. The time course of formation of joint molecules was usually linear for the first 60 s of the reaction. We computed the initial rate of pairing from the linear portion of the time course. For time courses which were not linear, but nevertheless yielded smooth, well-defined curves, we estimated the initial rate from the first two points sampled at 10 and 25 s. The reproducibility of rates was good in general. Nevertheless, for quantitative comparisons (e.g., Table I and Figure 5), we relied on rates measured side by side, within the same experiment.

Results

A Slow Step Attributable to the Presynaptic Association of RecA Protein with Single-Stranded DNA. Recent observations have shown that there is a presynaptic step in the pairing reaction that is very sensitive to the accumulation of

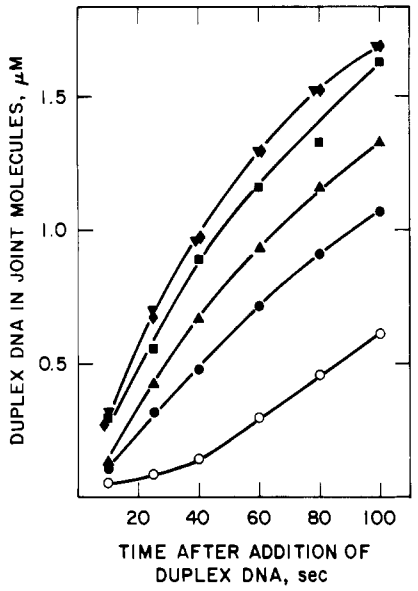

FIGURE 1: Preincubation of recA protein with single-stranded DNA accelerates the rate of formation of joint molecules. Reaction mixtures containing 2.0 μM circular single-stranded M13 DNA, 5.0 μM recA protein, 12 mM $MgCl_2$, 1.2 mM ATP, 14 mM Tris-HCl (pH 7.5), and an ATP-regenerating system were incubated at 37 °C for various lengths of time before addition of linear duplex M13 [^{3}H]DNA (prepared by cleavage of supercoiled duplex DNA with restriction endonuclease *Acc*I) to a final duplex DNA concentration of 4.58 μM. (○) Duplex DNA added after no preincubation; (●) 2 min of preincubation; (▲) 4 min; (■) 6 min; (▼) 12 min; (♦) 18 min.

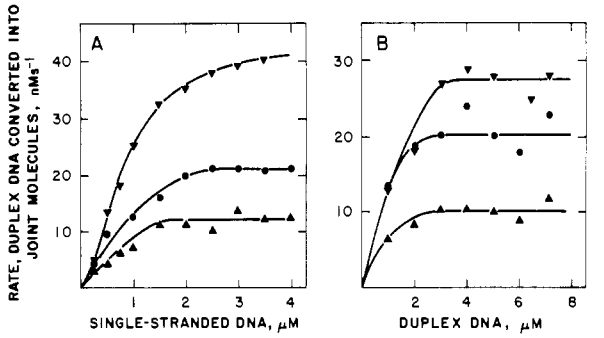

FIGURE 2: Pairing reaction displays saturation kinetics. Circular single-stranded DNA, recA protein, and ATP were preincubated in the presence of an ATP-regenerating system for 20 min at 37 °C prior to starting the pairing reaction by the addition of duplex DNA. The final concentration of recA protein was 10.0 μM. Circular single-stranded DNA was from the phage M13. (A) Initial rate vs. concentration of single-stranded DNA at three concentrations of duplex DNA. The linear duplex M13 [^{3}H]DNA was prepared by cleavage of supercoiled DNA with *Ava*I. Duplex DNA concentration: (▲) 2.5 μM; (●) 5.0 μM; (▼) 10.0 μM. (B) Initial rate vs. concentration of duplex DNA at three concentrations of single-stranded DNA. The linear duplex M13 [^{3}H]DNA was prepared by cleavage of supercoiled DNA with *Acc*I. Single-stranded circular DNA concentration: (▲) 0.33 μM; (●) 0.67 μM; (▼) 1.00 μM. The rates at 1 μM single-stranded DNA and 5 μM duplex DNA in panels A and B are different. We attribute this difference to sequence differences at the ends of the duplex DNA molecules made by cutting with *Acc*I and *Ava*I [see also Figure 5 of this paper and Muniyappa et al. (1984)].

ADP (Wu et al., 1982; Kahn & Radding, 1984). The use of an ATP-regenerating system, which can forestall the accumulation of ADP for an hour or more, permits one to examine the effect of incubating recA protein with single-stranded DNA prior to the addition of homologous duplex DNA. In the experiments described below, the reaction mixture that was preincubated at 37 °C included Mg^{2+}, ATP, phosphocreatine, and creatine phosphokinase, in addition to recA protein and single-stranded DNA. After preincubation, the pairing reaction was started by adding homologous double-stranded DNA.

When there was no preincubation, we observed a lag of approximately 30–40 s before any joint molecules were formed. Preincubation of the reaction mixture for several minutes eliminated the lag. Preincubation for longer times progressively increased the subsequent rates of formation of joint molecules. Under the conditions described in Figure 1, the maximal initial rate of formation of joint molecules was achieved after 12 min of preincubation. Preincubation eliminated the lag and increased the initial rate of the reaction only when ATP was present, preincubation of double-stranded DNA with recA protein and ATP had no affect on the lag, and preincubation of excess recA protein with heterologous single strands did not produce an activated form of free recA protein (Kahn & Radding, 1984). Flory et al. (1984) isolated a complex of recA protein and single-stranded DNA that forms joint molecules more rapidly than equivalent concentrations of the free components.

Preincubation of recA protein with single-stranded DNA and ATP also changed the amount of recA protein needed to reach the maximal rate (data not shown). When we did not preincubate recA protein and single-stranded DNA, we observed a maximal rate with slightly less than one monomer of recA protein per nucleotide residue of single-stranded DNA. After 20 min of preincubation, not only the rate was increased but also the reaction required almost 3 times as much recA protein to reach the new maximal rate. If free recA protein and single-stranded DNA exist in equilibrium with some presynaptic complex, as suggested by several studies (Cox et al., 1982, 1983; Kahn & Radding, 1984), the large excess of recA protein may be necessary to maximize the concentration of the presynaptic product. The requirement for a long preincubation of recA protein and single-stranded DNA suggests that there may also be a kinetic barrier to the formation of the presynaptic product. With or without a preincubation, the need for recA protein was still determined by the concentration of single-stranded DNA (data not shown).

Pairing Reaction Displays Saturation Kinetics. To achieve a maximal initial rate, we preincubated recA protein with single-stranded DNA and an ATP-regenerating system, usually for 20 min. By titrating various amounts of single-stranded DNA with recA protein, we determined that 8–10 μM recA protein saturates all of the reactions described here. We started the pairing reaction by adding duplex DNA and measured the synthesis of joint molecules during the first minute or two (see Figure 1 for sample time courses). We estimated initial rates from the time course during the first minute. The maximal initial rates were very fast compared with the thermal renaturation of DNA at comparable concentrations (see below).

As indicated by earlier experiments (Radding et al., 1981), when recA protein was in excess and the concentration of duplex DNA was held constant at one of several different levels, the rate of the reaction saturated with regard to single-stranded DNA (Figure 2A). Saturation occurred at a ratio of about one molecule of circular single-stranded DNA per molecule of linear duplex DNA (Figure 2A). The rate of the reaction also saturated with regard to duplex DNA when single-stranded DNA was held constant at one of several different levels. In this case, saturation occurred at a ratio that varied from one to four molecules of duplex DNA per circular single strand (Figure 2B). The variation seemed to depend at least in part on the choice of duplex DNA. The initial rate achieved at saturation, V_{max}, was proportional to

Table I: Initial Rates of Pairing at the Three Sites on the Duplex DNA Are Additive[a]

set	duplex DNA	single-stranded DNA	sites of pairing	initial rate	
1	(b) (0.23)	M13 (0.23)	5′(+) end + middle	1.07	1.71
	(b) (0.23)	G4 (0.23)	3′(+) end	0.64	
	(b) (0.23)	M13*Gori*I (0.23)	all sites	1.72	
2	(c) (0.31)	M13 (0.47)	3′(+) end + middle	2.07	3.12
	(c) (0.31)	G4 (0.47)	5′(end)	1.05	
	(c) (0.31)	M13*Gori*I (0.47)	all sites	3.12	
3	(d) (0.23)	M13 (0.23)	middle	0.43	1.57
	(d) (0.23)	G4 (0.23)	3′(+) end + 5′(+) end	1.14	
	(d) (0.23)	M13*Gori*I (0.23)	all sites	1.52	

[a] The numbers in parentheses are the concentrations of DNA in nanomoles of DNA molecules per liter. Initial rates are expressed as picomoles of DNA molecules converted into joint molecules per liter per second. Letters in the column labeled duplex DNA refer to the substrates shown in Figure 3. In the three sets of reactions shown, circular single-stranded DNA, recA protein, and ATP were incubated in the presence of an ATP-regenerating system for 20 min at 37 °C prior to starting the pairing reaction by the addition of duplex DNA. RecA protein was in excess.

the concentration of the species that was held constant during the titration, whether that species was single- or double-stranded DNA (Figure 2).

The well-behaved saturation kinetics resemble classical Michaelis–Menten kinetics and suggest an enzyme-like mechanism in which there is an intermediate enzyme–substrate complex whose conversion to products limits the rate of the reaction.

Contribution to the Overall Rate of Pairing by the Sequence in the Middle vs. Sequences at the Ends of Duplex DNA. Homologous pairing of circular single strands with linear duplex DNA can occur at three classes of site: middles, 5′(+) ends, and 3′(+) ends (see the introduction). Since our assay for joint molecules measures only the attachment of duplex DNA to single-stranded DNA, any paired structure of sufficient stability might contribute to the measurement. Because of the differing stabilities of the three types of joints in solution, and the possible differences in the efficiency of their retention by filters in our assays, we cannot measure the actual amount of joint molecules of different types. However, we can determine the contribution that each type of pairing makes to the *signal* we observe with the nitrocellulose filter assay. Understanding the relative contributions to the assay made by the different types of pairing is important in the interpretation of kinetic experiments. To do this, we used chimeric DNA, which we could cleave in various ways to limit homology to particular regions of the molecule (Figure 3).

From the chimeric phage M13*Gori*1, made by Kaguni & Ray (1979), we prepared duplex fragments that contained mostly M13 DNA sequences with a small block of G4 DNA sequences at the 3′(+) end (large *Acc*I fragment or large *Ava*I fragment, Figure 3). The maximal rate at which these duplexes formed joint molecules with single-stranded circular M13 DNA was $^3/_5$ to $^2/_3$ of the rate observed when duplex M13 DNA paired with single-stranded circular M13 DNA, whether duplex DNA or single-stranded DNA was in excess (first set of Table I, and data not shown). The large *Acc*I fragment and *Ava*I fragment of M13*Gori*I and the linear derivative of M13 DNA are nearly identical in size (see Figure 3a,b,e). The principal differences between these substrates are the presence of heterologous sequences at the 3′(+) end of the chimeric duplex DNA, preventing pairing at this end.

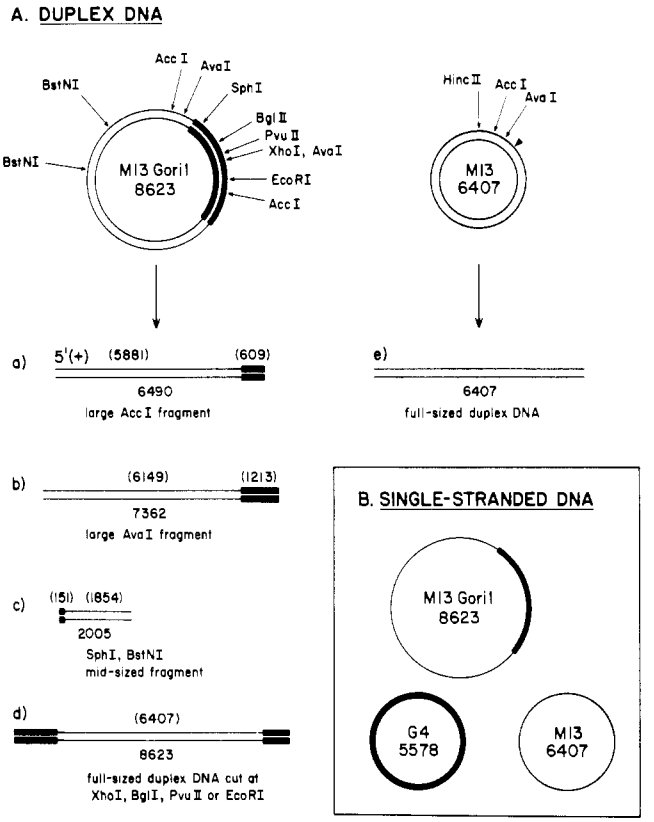

FIGURE 3: DNA substrates. (A) Maps of the single-stranded DNA phages used and the duplex DNA substrates derived from them by restriction endonuclease cleavage of the double-stranded replicative forms. The phage M13*Gori*I was made by Kaguni & Ray (1979) from the phage M13 by the insertion of a 2216 base pair *Hae*II fragment of the phage G4 into a *Hae*II site of M13 [indicated by an inverted triangle (▼) on the M13 map]. Thin lines denote M13 sequences; thick lines denote G4 sequences. The linear duplex DNAs are oriented so that the 5′ end of the (+) strand is to the left. Chimeric fragments were isolated by gel electrophoresis or sucrose gradient centrifugation as described under Materials and Methods. (a) The 6490 base pair fragment of M13*Gori*I formed by cleavage with *Acc*I; (b) the 7362 base pair fragment of M13*Gori*I formed by cleavage with *Ava*I; (c) the 2005 base pair fragment of M13*Gori*I formed by cleavage with *Bst*NI and *Sph*I; (d) full-length linear M13*Gori*I DNA formed by cleavage with *Bgl*I, *Pvu*II, *Xho*I, or *Eco*RI, all of which cut within the G4 region of the DNA; (e) full-length linear M13 DNA formed by cleavage with *Hinc*II, *Ava*I, or *Acc*I. The numbers in parentheses represent the length in base pairs of the region of DNA over which they appear; the numbers without parentheses represent the total length in base pairs of the DNA molecule. (B) The circular single-stranded DNA molecules used. Numbers represent the total length of bases. Thin lines, M13 sequences; thick lines, G4 sequences.

To explore the contribution to the apparent rate made by pairing in the middle of the duplex molecule, we compared the rate of pairing of single-stranded circular M13 DNA with duplex M13 DNA vs. duplex M13*Gori*I DNA cleaved at its *Xho*I site in the G4 region (Figure 3). In the latter combination, the M13 sequences are flanked by heterologous G4 sequences, so that homology is present only in the middle of the molecule. When homologous sequences were so buried, the reaction continued to show saturation kinetics, and the measured rate was 8 times less than that observed for the totally homologous combination (Figure 4). The total length of homologous M13 DNA was identical in the two cases.

The relative contributions of middles and ends to the apparent rate depend at least in part upon the nature of the ends. With all other features controlled, the observed rates of pairing attributable to buried sequences were reproducible from one experiment to another (Figure 5A). However when duplex M13*Gori*I molecules were prepared by cleavage with different

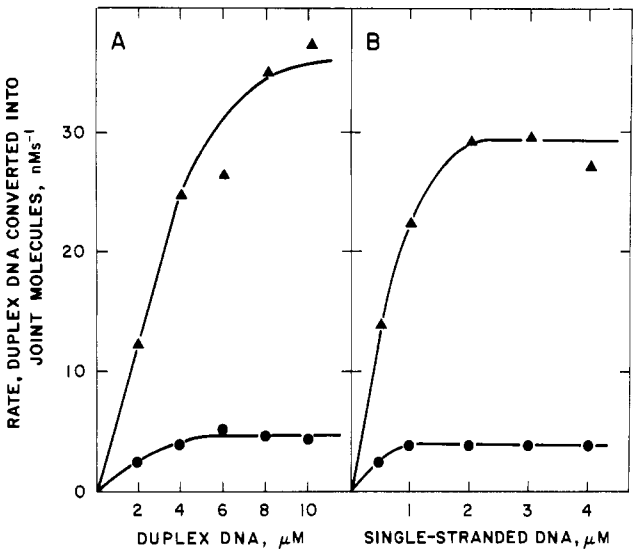

FIGURE 4: Initial rate of pairing in the absence of a homologous end ("buried homology"). Circular single-stranded DNA, recA protein, and ATP were incubated in the presence of an ATP-regenerating system for 20 min at 37 °C prior to starting the pairing reaction by the addition of duplex DNA. The final concentration of recA protein was 8.3 μM. Duplex DNA: (▲) linear duplex M13 [^{3}H]DNA prepared from supercoiled DNA by cleavage with *Acc*I (Figure 3e); (●) linear duplex M13*Gori*I [^{3}H]DNA prepared from supercoiled DNA by cleavage with *Xho*I (buried homology, Figure 3d). (A) Initial rate of pairing vs. the concentration of duplex DNA. Circular single-stranded DNA was from the phage M13 and was present at a final concentration of 1.0 μM. (B) Initial rate of pairing vs. the concentration of single-stranded DNA. Reaction mixtures contained a final concentration of 5.0 μM duplex DNA. Circular single-stranded DNA was from the phage M13.

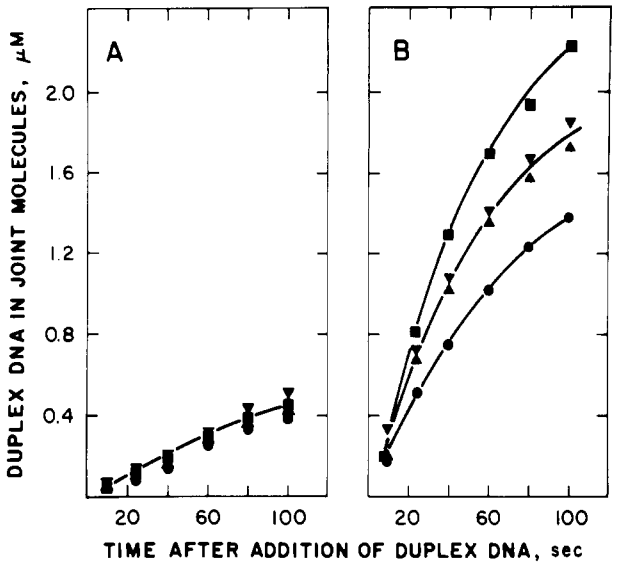

FIGURE 5: Initial rates vs. different homologous ends. Duplex DNA was prepared by cleavage of supercoiled M13*Gori*I [^{3}H]DNA at one of four sites which bisect the G4 region of the DNA: *Xho*I (●); *Bgl*I (▲); *Eco*RI (▼), *Pvu*II (■). Circular single-stranded DNA, recA protein, and ATP were incubated in the presence of an ATP-regenerating system for 20 min at 37 °C prior to starting the pairing reaction by the addition of duplex DNA. The final concentration of recA protein was 8.0 μM; the final concentration of duplex DNA was 4.0 μM. (A) Circular single-stranded DNA was from the phage M13 and was present at 1.5 μM; with this DNA, pairing could only occur in the middle of the chimeric duplex DNA molecules. (B) Circular single-stranded DNA was from the phage M13*Gori*I and was present at 2.0 μM; with this DNA, pairing could occur anywhere on the duplex DNA molecules, including at the ends.

restriction enzymes in the G4 region, the rates of pairing with M13*Gori*I single strands varied by as much as 2-fold (Figure 5B; see Discussion).

To limit homology to various portions of the duplex DNA, we used derivatives b, c, and d of the chimeric M13*Gori*I DNA (Figure 3) and paired each with M13, G4, and M13*Gori*I in separate reactions. For any given derivative (sets 1, 2, and 3 in Table I), the rate of pairing with single-stranded DNA that was homologous to the entire duplex was the sum of rates attributable to different parts of the double-stranded molecule. These observations suggest that pairing occurs at all possible sites on the duplex DNA molecule and that the reaction of a duplex molecule with a totally homologous circular single strand can be described as the sum of the pairing reactions at the 3′(+) end, the 5′(+) end, and the middle (see Discussion).

We used the observed additivity of rates to deduce the apparent rate of pairing at ends vs. middles for the experiments discussed above (Figures 4 and 5, Table I). To make the calculation, we assumed that early in the reaction the two ends of a duplex molecule would react at the same rate. The assumption is supported by the data of Wu et al. (1982) which show that in the presence of an ATP-regenerating system, reactions at the 3′(+) end and the 5′(+) end of a duplex DNA molecule occur with very nearly the same efficiency. Thus, knowing any two of the following parameters, we could calculate the others: the rate of pairing at a 3′(+) end or a 5′(+) end (e), or at both ends (2e); the rate of pairing in the middle of the duplex DNA, away from an end (m); the rate of pairing at an end and in the middle (e + m); the rate of pairing of a totally homologous combination of substrates, in which pairing can take place at all sites (2e + m). Our analysis shows that the apparent rate of the reaction at an end can be from 1 to 5 times that of the apparent rate in the middle of a duplex molecule. Ends tend to give a greater signal than middles, sometimes by a large factor (see Discussion for a consideration of the possible variables involved).

Rate of the Reaction Is Directly Related to the Number of Free Ends. Since two homologous ends in the duplex molecule produced a faster rate of pairing than one homologous end, we asked if more ends, in the form of nicks, could further increase the rate of pairing. Supercoiled duplex M13*Gori*I DNA was nicked to varying extents with pancreatic DNase, the average amount of nicking not exceeding one nick per duplex molecule. The circular molecules were then cleaved with *Xho*I. As the nicking in the population of duplex molecules increased, the rate of pairing of this DNA with single-stranded circular M13*Gori*I DNA also increased (Figure 6). Each nick produces two free ends. On a random basis, half of the nicks should be in the plus strand. If the two ends of each nick in the plus strands behave independently, then on the average each nick produces one free end per plus strand. On the basis of these assumptions, the increase in rate per free end was about 17–20 nM s^{-1}, which is comparable to the best rates calculated for reaction at the end of a duplex molecule at similar concentrations of DNA (Figure 5B).

We showed above that the overall rate of pairing of duplex DNA with a homologous circular single strand can be described as the sum of the rates of pairing at the different sites on the duplex. Introduction of a nick in the middle of the molecule appears to have created a more efficient pairing site, and therefore increased the overall rate of pairing, which is congruent with the observation that, per nucleotide residue, ends make a larger contribution than middles to the overall rate (Figures 4 and 5, Table I). Consistent with earlier topological observations (DasGupta et al., 1980), these data show, moreover, that a nick is roughly equivalent to the actual

Table II: Comparison of Initial Rates of RecA-Promoted Homologous Pairing with the Rate of Thermal Renaturation of DNA[a]

duplex DNA concn (μM)	single-stranded DNA concn (μM)	initial rate of recA-promoted pairing (nM s^{-1})	corresponding initial rate of thermal renaturation (nM s^{-1})	ratio of rates
1.0	0.33	6.7	0.056	120
1.0	0.67	13.5	0.113	120
1.0	1.00	12.9	0.169	76
2.0	1.00	21.6	0.337	64
5.0	0.50	29.2	0.422	69

[a] For the first three entries, the rates of pairing promoted by recA protein were taken from Figure 2B; the rates for the last two entries are from data not shown. We calculated the initial rates of thermal renaturation (column 4), in 0.15 M NaCl at T_m −25 °C, for complementary single strands of the same length and complexity as the substrates used in the recA reactions, according to Wetmur & Davidson (1968).

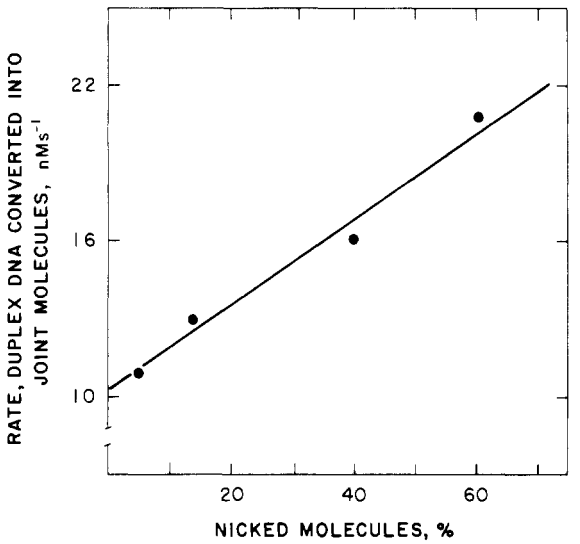

FIGURE 6: Initial rates vs. the fraction of nicked duplex molecules. Supercoiled M13*Gori*I [^{3}H]DNA was nicked to various extents with pancreatic DNase and then linearized with *Xho*I. Circular single-stranded DNA, recA protein, and ATP were incubated in the presence of an ATP-regenerating system for 20 min at 37 °C prior to the starting of the pairing reaction by the addition of duplex DNA. The final concentration of recA protein was 10.0 μM. The final duplex DNA concentration was 5.0 μM; single-stranded circular DNA from the phage M13*Gori*I was present at 2.5 μM. Percent nicked molecules is the percent of duplex DNA molecules with at least one nick in them as determined by the assay of Kuhnlein et al. (1976), before the circular DNA was cut with *Xho*I.

terminus of a duplex molecule in favoring the formation of joint molecules. We conclude that when other factors are held constant, the rate of the overall reaction is directly related to the number of homologous free ends. Since joints formed in the middle of a molecule are less stable (Bianchi et al., 1983), we presume that nicks and other ends influence the net rate of the reaction by producing more stable intermediates.

Rapidity of Homologous Pairing Promoted by RecA Protein. A practical appreciation of the rapidity of the pairing reaction promoted by recA protein can be had from Figure 5B. In the case of the best substrate, about half of the duplex DNA was incorporated into joint molecules in 80 s. To obtain a more quantitative standard for comparison, we calculated the rate at which a plus strand of the same size and complexity would reanneal with its complement at T_m −25 °C in 0.15 M NaCl, a calculation made possible by the classic study of Wetmur & Davidson (1968). The assay used to measure the formation of joint molecules by recA protein detects the incorporation of an entire duplex molecule as soon as a sufficiently stable joint is made. In thermal renaturation, nucleation is the rate-limiting step (Wetmur & Davidson, 1968). Renaturation kinetics measure the rate of appearance of nucleotide residues in complete duplex molecules. Accordingly, we compared directly the conversion of duplex DNA into joint molecules by recA protein with the production of duplex DNA by thermal renaturation, both expressed in moles of nucleotide residues in duplex DNA per liter per second (Table II).

From various experiments, we selected data which represent rates observed below saturation for various ratios of single-stranded DNA to duplex DNA. Consistent with our findings on the processive mechanism of homologous pairing promoted by recA protein (Gonda & Radding, 1983), its rate of reaction compares most favorably with calculated reannealing rates at low concentrations of DNA (Table II). In the range of DNA concentrations shown in Table II, recA protein paired single strands with their complements in duplex DNA 1 or 2 orders of magnitude faster than two complementary single strands can reanneal at T_m −25 °C in 0.15 M NaCl.

Discussion

In these experiments, we have used conditions that optimize the measurement of rates of formation of joint molecules, namely, the use of an ATP-regenerating system and preincubation of single strands with recA protein to complete the initial slow polymerization of protein on single-stranded DNA (Kahn & Radding, 1984; Flory et al., 1984; Figure 1 this paper). Using these conditions, we have explored the kinetics of homologous pairing and the features of DNA molecules that affect the apparent rate of the reaction.

The kinetics of homologous pairing promoted by recA protein show two outstanding characteristics. First, the reaction displays well-behaved, enzyme-like saturation kinetics, indicating the existence of a ternary complex of single-stranded DNA, duplex DNA, and recA protein whose conversion to product limits the apparent rate of the reaction. Second, the pairing is extremely fast: recA protein at 37 °C causes a single strand to find its complement in duplex DNA even faster than comparable single strands can reanneal at T_m −25 °C in 0.15 M NaCl. These observations underscore the uniqueness of the pairing reaction promoted by recA protein and indicate a reaction mechanism that is much more complex than the simple renaturation of complementary strands.

We have formulated many of the observations on recA-promoted pairing as indicated in Figure 7. The presynaptic phase (a) is represented as the stoichiometric association of recA protein with single-stranded DNA (S) to produce a presynaptic complex (SA_n). This association is slow and under some conditions can be rate limiting (Figure 1). Presynaptic complexes have been isolated and shown to be active in forming joint molecules (Flory et al., 1984; Tsang et al., unpublished results). The saturation kinetics indicate that there are ternary complexes which contain recA protein, single-stranded DNA, and duplex DNA. At saturation, the conversion of such a complex to product limits the rate of the reaction, much as a classical enzyme–substrate complex does (Figure 2). In a formal sense, synapsis comprises both the conjunction (b) and homologous alignment (c_1, and/or c_2) of DNA molecules (Figure 7). On a statistical basis, the initial conjunction of

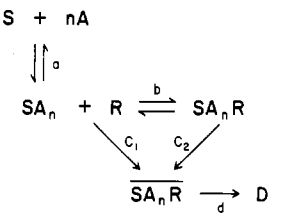

FIGURE 7: Formulation of homologous pairing and the formation of joint molecules promoted by *Escherichia coli* recA protein. S = single-stranded DNA; A = recA protein; R = linear duplex DNA (replicative form III); D = joint molecules (D loops); SA_nR = ternary complexes in which the single-stranded DNA and duplex DNA are not homologously aligned; $\overline{SA_nR}$ = ternary complexes in which homologous sequences are aligned via a nascent heteroduplex joint. (a) The presynaptic polymerization of recA protein on single-stranded DNA; (b) conjunction of single-stranded and double-stranded DNA without homologous alignment; (c_1) conjunction and simultaneous homologous alignment; (c_2) homologous alignment of previously conjoined molecules; (d) formation of a stable heteroduplex joint. A purely reiterative pathway is described by steps b plus c_1 and a purely processive pathway by steps b plus c_2.

molecules must produce a ternary complex in which the molecules are not homologously aligned (SA_nR). We have observed that such unaligned complexes can be detected directly (Radding et al., 1980; Tsang et al., unpublished observations). Homologous alignment may occur by two paths: In a purely reiterative pathway, unaligned ternary complexes (SA_nR) result from unfruitful collisions; the DNA molecules in such a complex would have to escape back into solution to try to pair again, and only those molecules that eventually collide in register (path c_1, leading to $\overline{SA_nR}$) would go on to form a joint molecule (D). Alternatively, in a processive pathway (c_2), unaligned ternary complexes (SA_nR) would be true intermediates in which some relative motion of the component DNA molecules would produce aligned complexes containing nascent heteroduplex joints ($\overline{SA_nR}$). The concept of the nascent heteroduplex joint was postulated previously to explain certain experimental observations (Wu et al., 1982). By definition, nascent heteroduplex joints can form anywhere in the molecule, at ends or away from ends. Paranemic joints form away from ends, and since they do not involve true intertwining of strands, they may be more closely related to nascent heteroduplex joints than are plectonemic joints. Once synapsis has been achieved, recA protein catalyzes the exchange of strands at a free homologous end to form a classical heteroduplex joint between the two molecules (Figure 7d). According to the observed saturation kinetics, the rate-limiting step is d if the reaction is purely reiterative, whereas the limiting step may be either c_2 or d if the reaction is processive. Evidence for a processive mechanism of searching for homology has been presented elsewhere (Gonda & Radding, 1983).

The ability to measure rates with reasonable precision and the kinetic characterization just described enabled us to explore features of DNA molecules that affect the apparent rate of the reaction. Previously we have shown that recA protein can pair a circular single strand with nonsuperhelical duplex DNA even when the homologous sequences in the duplex DNA are flanked by long regions of heterology (Wu et al., 1983; Bianchi et al., 1983). In such a case, the joint holding the molecules together is a noninterwound or paranemic joint which is less stable than a joint with interwound strands, viz., a plectonemic joint (Bianchi et al., 1983). Paranemic joints make a contribution to the signal detected by our D-loop assay (Figures 4 and 5, Table I). The size of the signal coming from the pairing at interior sites in a DNA molecule, relative to the signal from pairing at a free end, depends in part on the conditions of the assay [Bianchi et al. (1983) and unpublished observations] and in part of the efficacy of a given end (Figure 5). To an extent not yet determined, the signal also depends on the size and/or the sequence of the region of buried homology (Bianchi et al., 1983). In this study, we found that linear duplex molecules produced by cleavage of circular DNA by different restriction enzymes produced substrates of varying efficacy. A similar observation has been reported by Cox et al. (1983). In the experiment shown in Figure 5, pairing at sites in the middle of linear molecules with different ends was equally efficient for all four preparations of linear duplex DNA, which excludes extrinsic factors as being responsible for the observed variations in reactivity of the ends. In the experiment shown in Figure 5, the best rate was observed by using duplex DNA cleaved with *Pvu*II, which has a blunt-ended duplex molecule. Intermediate rates were observed by using duplex DNA cut with *Eco*RI (which has a four-base 5′ single-stranded overhang) and *Bgl*I (which has a three-base 3′ single-stranded overhang). The worst rate was seen with duplex DNA cut with *Xho*I (which also has a four-base 5′ single-stranded overhang). Therefore, the efficacy of an end does not correlate in a simple way with the physical structure of the end. Further work has been done which indicates that a major source of the variation observed with different ends is the presence or absence of strong secondary structure in the single-stranded DNA at a site corresponding to the end of the duplex DNA (Muniyappa et al., 1984).

Ends and nicks clearly affect the apparent or net rate of the pairing reaction: a molecule with two homologous ends reacts faster than one with only one homologous end, and nicks increase the rate of the reaction to the same degree as a good duplex end. Any studies of the kinetics of the recA reaction must take account of these effects.

Another observation is significant with regard to the interpretation of kinetic data, namely, that pairing at ends and pairing in the middle of the molecule make additive contributions to the net rate (Table I). On the one hand, this observation simplifies interpretation and indicates that the pairing seen when both ends of a duplex molecule are heterologous occurs at the same frequency in the middle of fully homologous molecules. On the other hand, this observation leads to the surprising conclusion that homologous alignment at sites away from ends does not accelerate the *apparent* rate of reaction at an end in the same molecule. Either homologous alignments at sites separated by even short distances are independent, which seems unlikely, or there is a step following alignment at an end that limits the rate of the reaction. Since the latter may be true, the present observation does not exclude the possibility that pairing anywhere in the molecule produces an intermediate which is on the pathway to forming a stable joint molecule.

Among the substrates that we used to check the additivity of rates was a 2005 base pair M13G*ori*I chimeric duplex fragment, which had 151 base pairs of G4 DNA at its 5′(+) end and 1854 base pairs of M13 DNA at its 3′(+) end. With this duplex fragment, we still observed that the rates attributable to different parts of the duplex molecule were additive (Table I). This is a remarkable observation, since one of the pairs of substrates shared only 151 base pairs of homologous sequences (Figure 3c, Table I). From the additivity of rates, we infer that even when homology was limited to a short sequence at one end, pairing occurred as rapidly as it occurred at the same end in a wholly homologous molecule. Elsewhere, however, we have shown that 151 base pairs of homology approaches the minimum length required to support pairing

at the end of a molecule at a constant rate (Gonda & Radding, 1983). Therefore, this substrate, or ones like it, with homology limited to one end, may be ideal for further kinetic studies. The study of homologous pairing limited to a single minimum site would avoid the complexity caused by the superposition of multiple strong and weak signals from molecules that share extensive homology.

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