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Networks of DNA and RecA Protein Are Intermediates in Homologous Pairing†

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ABSTRACT: Partial coating of single-stranded DNA by recA protein causes its aggregation, but conditions that promote complete coating inhibit independent aggregation of single strands and, instead, cause the mutually dependent conjunction of single- and double-stranded DNA in complexes that sediment at more than 10000 S. This coaggregation is independent of homology but otherwise shares key properties of homologous pairing of single strands with duplex DNA: both processes require ATP, $MgCl_2$, and stoichiometric amounts of recA protein; both are very sensitive to inhibition by salt and ADP. Coaggregates are closed domains that are intermediates in homologous pairing: they form faster than joint molecules, they include virtually all of the DNA in the reaction mixture, and they yield joint molecules nearly an order of magnitude faster than they exchange DNA molecules with the surrounding solution. The independent aggregation of single-stranded DNA differs in all respects except the requirement for Mg^{2+}, and its properties correlate instead with those associated with the renaturation of complementary single strands by recA protein.

Escherichia coli recA protein promotes two kinds of homologous pairing of DNA molecules in vitro, each of which may play a direct role in genetic recombination, namely, the renaturation of complementary single strands (Weinstock et al., 1979) and the formation of joint molecules from single-stranded or partially single-stranded DNA plus duplex DNA (Shibata et al., 1979; Radding, 1982). The latter of these two kinds of pairing reaction is an ordered process in which at least three sequential phases can be distinguished: (1) A slow *presynaptic phase* consists of the polymerization of recA protein on single-stranded DNA (Cox & Lehman, 1982; Dunn et al., 1982; Kahn & Radding, 1984; Radding, 1982; Flory et al., 1984a,b), a reaction that is hindered by secondary structure in the single strands (Muniyappa et al., 1984). (2) A rapid *synaptic* phase can be subdivided into two sequential steps, which are *conjunction*, the coming together of single-stranded DNA and duplex DNA that is mediated by recA protein independent of homology, and *homologous alignment*, which occurs at least in part by facilitated diffusion within the complex of DNA and protein that results from conjunction (Gonda & Radding, 1983). (3) A slow *postsynaptic phase* of strand exchange produces heteroduplex DNA and correspondingly displaces an old strand (DasGupta et al., 1980; Cox & Lehman, 1981; Kahn & Radding, 1984).

A few of the intermediates in this sequence of reactions have been isolated or identified. The product of the presynaptic reaction is a highly ordered nucleoprotein filament which is at least 1.5 times the length of duplex DNA and consists of repeating subunits of about 6 molecules of recA protein and 20 nucleotide residues (Flory et al., 1984a,b; Tsang et al., 1985). Homologous alignment involves a nascent three-stranded intermediate in which the incoming single strand can pair with its complement in the duplex DNA without the true interwinding of strands that exists in duplex DNA (Bianchi et al., 1983).

The experiments described in this paper concern additional putative intermediates in the two distinct pairing reactions that are promoted by recA protein: an intermediate that is associated with *conjunction*, the step between the formation of a presynaptic complex and homologous alignment in the formation of D loops, and an aggregated form of single-stranded

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Table I: Aggregation of ssDNA vs. Coaggregation of ssDNA and dsDNA by RecA Protein[a]

	$[MgCl_2]$ (mM)			DNA in reaction			% DNA in aggregates +ATP		% DNA in aggregates -ATP	
line	preincubation	final	SSB	M13 ss	ϕX174 ss	M13 ds	ss	ds	ss	ds
1	13	13	+	+		+	93	94	0	0
2	13	13	+		+	+	94	99	0	0
3	13	13	+	+			0		0	
4	13	13	+			+		0		0
5	13	13		+		+	86	72	95	0
6	13	13			+	+	91	27	95	0
7	13	13		+			84		94	
8	13	13				+		0		0
9	1	12		+		+	94	96	0	0
10	1	12			+	+	96	98	0	0
11	1	12		+			0		0	
12	1	12				+		0		0

[a] Single-stranded DNA (ssDNA) and recA protein were preincubated at the indicated concentrations of $MgCl_2$ for 10 min at 37 °C. When ATP or SSB was added, they were included in the preincubation. Subsequently, the concentration of $MgCl_2$ was adjusted to 12 or 13 mM, and 10 μM double-stranded (dsDNA) was added where indicated. Incubation was continued for 10 min more at 37 °C before aggregation was measured as described under Experimental Procedures. The final concentrations of recA protein, single-stranded DNA, and SSB, respectively, were 2.0, 2.8, and 0.28 μM. In the column headings, ss denotes single-stranded DNA and ds denotes double-stranded DNA.

DNA and recA protein that is probably an intermediate in the renaturation of complementary single strands.

Experimental Procedures

Enzymes. RecA protein was purified by the method of Shibata et al. (1981a). Single-strand binding protein (SSB) of *E. coli* was generously provided by Dr. John Chase from the Department of Molecular Biology, Albert Einstein College of Medicine, New York, NY. Restriction endonuclease *Stu*I and *Hin*cII were from New England Biolabs. Creatine phosphokinase (type I) was from Sigma.

Preparation of DNA Substrates. Circular single-stranded and duplex DNAs from phage M13 or phage ϕX174 were prepared as described previously (Cunningham et al., 1980). The supercoiled duplex DNAs from phage M13 and phage ϕX174 were linearized with the restriction endonucleases *Hin*cII and *Stu*I, respectively. Single-stranded DNA fragments were formed by heating single-stranded DNA from phage ϕX174 for 10 min in boiling water, which produces fragments with a mean length of 500–1000 nucleotide residues (Shibata et al., 1981a).

Standard Reaction Conditions. Unless otherwise stated, the reaction mixtures contained 33 mM (Tris-HCl) (pH 7.5), 1.8 mM dithiothreitol, 1.3 mM ATP, and 88 μg of bovine serum albumin (BSA) per milliliter, 10 units of phosphocreatine kinase per milliliter, 3 mM phosphocreatine, and either 13 or 1 mM $MgCl_2$. The concentrations of single-stranded [^{32}P]DNA, double-stranded [^{3}H]DNA, recA protein, and SSB were as stated for each experiment. Concentrations of DNA are expressed in terms of moles of nucleotide residues. In experiments where SSB was present to promote the formation of presynaptic complexes of recA protein and single-stranded DNA, SSB was added to the reaction mixture within 30 s after recA protein. Reaction mixtures were usually incubated at 37 °C for 10 min.

Assay for Aggregation of DNA. After incubation for 10 min at 37 °C, a reaction mixture of 30 μL was centrifuged in a 0.5-mL Eppendorf tube at 15600*g* for 2 min at room temperature. Three sequential aliquots of 9 μL were taken from the supernatant. The remaining 3 μL of supernatant and the pellet were resuspended in 200 μL of distilled water. The amount of labeled DNA in each aliquot of the supernatant and in the resuspended material was measured and added to give the total DNA recovered. The amount of DNA in the total supernatant (30 μL) was estimated from the average counts of the first two aliquots of supernatant. In every experiment, the recovery of labeled DNA in the supernatant and pellet was 90% or greater, and residual counts on the wall of the tube were less than 10%. Except as noted, when we studied the coaggregation of single- and double-stranded DNA, we used heterologous combinations to avoid the formation of joint molecules.

Assay of Joint Molecules. The assay is based upon the retention of single-stranded DNA by nitrocellulose filters which allows one to detect the linkage of duplex DNA to homologous single-stranded DNA (Beattie et al., 1977). An aliquot of 5–10 μL from a reaction mixture was taken and diluted with 100 μL of 25 mM ethylenediaminetetraacetic acid (EDTA) (pH 9.0) on ice, followed immediately by 3 mL of ice-cold 1.5 M NaCl and 0.15 M sodium citrate. The sodium was then filtered through a nitrocellulose filter (Sartorius, type SM11306, 0.45 μm) and the filter was processed as described previously (Gonda & Radding, 1983).

Results

Aggregation of Single-Stranded DNA by RecA Protein. When recA protein was mixed with single-stranded DNA, virtually all of the DNA was found in aggregates that sedimented to the bottom of a tube after centrifugation at 15600*g* for as little as 1 min (see below). Aggregation, when measured in this way, required Mg^{2+} but did not require ATP (Table I, line 7). RecA protein did not cause the aggregation of duplex DNA when single-stranded DNA was absent (Table I, line 8, and see below). At 4–16 μM single-stranded DNA, one molecule of recA protein per 10–23 nucleotide residues was required for complete aggregation (Figure 1A).

E. coli single-strand binding protein (SSB) is a helix-destabilizing protein which, by destabilizing the secondary structure in single strands, promotes the complete coating of the latter by recA protein (Muniyappa et al., 1984). We found that SSB inhibited completely the aggregation of single-stranded DNA by recA protein (Table I, compare lines 3 and 7). SSB was able not only to inhibit the formation of aggregates but also to rapidly dissociate preexisting complexes. Aggregates formed in the absence of SSB were completely dispersed within 3 min after the addition of SSB (data not shown). Thus, presumably even interior sites in the aggregates were accessible to SSB, and aggregates are not dead-end by-products.

Elsewhere, we have shown directly that the binding of recA protein to single-stranded DNA can be favored by SSB or by reduction of the concentration of Mg^{2+} to 1 mM (Tsang et

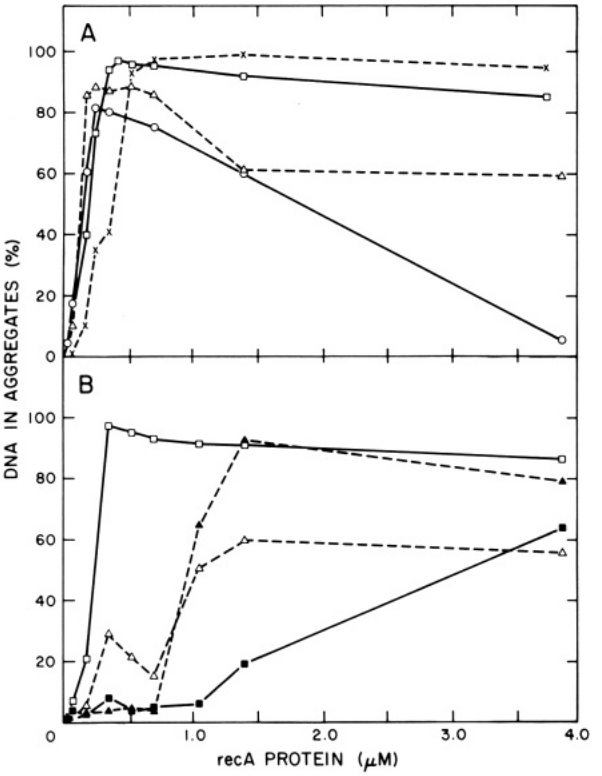

FIGURE 1: Aggregation and coaggregation vs. concentration of recA protein. (A) Aggregation of circular single-stranded ϕX174 DNA. Reactions were performed in 1 mM $MgCl_2$ under the standard conditions described under Experimental Procedures with circular single-stranded [^{32}P]DNA at 1 (○), 2 (△), 4 (□), or 16 μM (×). The reaction mixture was incubated for 10 min at 37 °C. (B) Coaggregation of circular single-stranded ϕX174 DNA and linear duplex M13 DNA. Circular single-stranded [^{32}P]DNA (4 μM) was preincubated with recA protein in a reaction mixture (see above) containing 1 (△, ▲) or 12 mM $MgCl_2$ (□, ■) for 10 min at 37 °C. Following the preincubation period, coaggregates were formed by adding 4 μM linear duplex [^{3}H]DNA and adjusting the final concentration of $MgCl_2$ to 13.3 mM. The reaction mixture was then incubated for an additional 5 min at 37 °C. Open symbols represent single-stranded DNA found in aggregates; filled symbols represent duplex DNA in aggregates.

al., 1985). However, the formation of joint molecules (Shibata et al., 1981b) and recA-dependent aggregation of single strands in the presence of ATP require a concentration of $MgCl_2$ in excess of 10 mM (Figure 2A). When we preincubated recA protein with single-stranded DNA in 1 mM $MgCl_2$ and then raised the concentration to 12 mM, no aggregation occurred (Table I, line 11). Thus, SSB itself is not specifically required to inhibit aggregation, but rather the saturation of single-stranded DNA with recA protein and the concomitant elimination of secondary structure appear to be responsible (Muniyappa et al., 1984). Consistent with this view is the observation that the addition of excess recA protein also inhibited the aggregation of single–stranded DNA (Figure 1A). The latter observation suggests in addition that aggregation does not result from the interaction of molecules of recA protein that are bound to different single strands. Aggregation was also inhibited when the single-stranded DNA was fragmented (see Figure 3b, below).

When single-stranded DNA was absent, recA protein did not cause significant aggregation of duplex DNA at ratios of protein to DNA varying from 1 per 29 base pairs to 1.4 per base pair (data not shown). Thus, aggregation of duplex DNA was provoked neither by low nor by high concentrations of recA protein.

Coaggregation of Single-Stranded DNA and Double-Stranded DNA. Whereas in the presence of SSB, recA protein caused the aggregation of neither single-stranded nor double-stranded DNA separately (Table I, lines 3 and 4), the two kinds of DNA, when mixed with recA protein and SSB, coaggregated in a reaction that was independent of homology (Table I, lines 1 and 2).

Similarly, when the independent aggregation of single strands was suppressed by a preincubation with recA protein in 1 mM $MgCl_2$, coaggregation occurred upon the addition of double-stranded DNA with additional Mg^{2+} to raise the

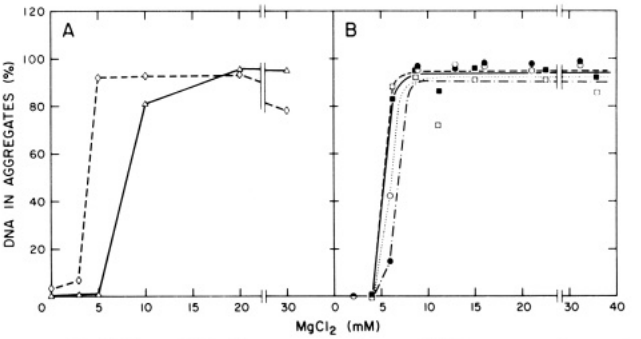

FIGURE 2: Effect of $MgCi_2$ concentration on DNA aggregation and coaggregation promoted by recA protein. (A) Aggregation. Reactions were performed with 3 μM single-stranded DNA from phage M13 and 2.1 μM recA protein in the presence (△) or absence (◇) of ATP. The concentrations of $MgCl_2$ in the reaction mixtures were varied as indicated. DNA aggregates were assayed after an incubation of 10 min at 37 °C. (B) Coaggregation. Presynaptic complexes were first formed at 1 mM $MgCl_2$ in the presence of ATP by preincubating recA protein and single-stranded DNA from phage ϕX174 for 10 min at 37 °C. The complexes containing 3 μM single-stranded DNA and 2.1 μM recA protein were reacted with 14 μM linear double-stranded DNA from phage M13 at various $MgCl_2$ concentrations. Aggregates of single-stranded DNA (○) and double-stranded DNA (●) were measured after 10 min at 37 °C. A similar experiment was done with presynaptic complexes formed in 3 mM $MgCl_2$ in the presence of 0.3 μM SSB protein. Coaggregation of single-stranded DNA (□) and double-stranded DNA (■) was determined.

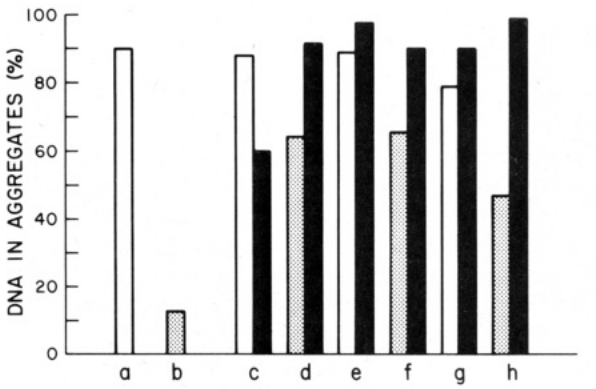

FIGURE 3: Coaggregation provoked by fragments of single-stranded DNA. In (a–d), the concentration of $MgCl_2$ was 13 mM: (a) circular single-stranded ϕX174 DNA (open bar); (b) fragments of single-stranded ϕX174 DNA (stippled bar); (c) circular single-stranded ϕX174 DNA plus linear duplex M13 DNA (filled bar); (d) fragments of single-stranded ϕX174 DNA plus linear duplex M13 DNA. In (e) and (f), single-stranded DNA was preincubated with recA protein in 1 mM $MgCl_2$, and the concentration was raised to 13 mM when duplex DNA was added: (e) DNA as in (c); (f) DNA as in (d). In (g) and (h), 0.27 μM SSB was included and the $MgCl_2$ concentration was 13 mM: (g) DNA as in (c); (h) DNA as in (d). All mixtures contained 1.9 μM recA protein. The final concentrations of DNA in the indicated combinations were 3 μM circular single-stranded ϕX174 [^{32}P]DNA or fragments thereof and 5.9 μM linear double-stranded M13 [^{3}H]DNA. Presynaptic complexes were formed by preincubation of recA protein with intact or fragmented single-stranded DNA for 10 min at 37 °C at the indicated concentrations of $MgCl_2$ and in the presence of ATP and an ATP-regenerating system (see Experimental Procedures).

concentration of the latter to 12 mM (Table I, lines 9–12). Under these conditions, coaggregation was maximal at a ratio of 1 molecule of recA protein per 2.9 nucleotide residues of single-stranded DNA (Figure 1B), which is the ratio required to saturate single-stranded DNA with recA protein (Tsang et al., 1985). However, when the concentration of $MgCl_2$ was 12 mM during preincubation, single-stranded DNA aggregated independently at a ratio of 1 molecule of recA protein per 11 nucleotide residues, but coaggregation of duplex DNA required much higher concentrations of recA protein (Figure 1B). Thus, the independent aggregation of single strands can interfere with the incorporation of duplex molecules into coaggregates, and increasing concentrations of recA protein reduce aggregation but favor coaggregation.

Coaggregation also occurred when the single-stranded DNA was fragmented (Figure 3d,f,h), another condition under which the independent aggregation of single strands was suppressed (Figure 3b). Fragments of single-stranded DNA provoked coaggregation whether preincubation with recA protein was in 1 mM (Figure 3f) or 13 mM $MgCl_2$ (Figure 3d), or whether SSB was present (Figure 3h) or not (Figure 3c–f).

The conditions described above that suppress aggregation but promote coaggregation are conditions that favor the complete coating of single-stranded DNA by recA protein: preincubation of single strands with recA protein in 1 mM Mg^{2+} and high concentrations of recA protein, addition of SSB, and fragmentation of single-stranded DNA [see Muniyappa et al. (1984)].

Aggregation and Coaggregation Have Different Properties. Apart from a similar requirement for Mg^{2+} (see Figure 2), aggregation and coaggregation differ significantly in requirements and properties, including the requirement for different amounts of recA protein already described (Figure 1).

Coaggregation requires ATP (Table I, lines 1 and 2) whereas aggregation does not (Figure 2A; Table I, line 7). When we omitted SSB, in order to compare aggregation and coaggregation directly under the same conditions (Table I, lines 5–8), we observed the aggregation of single strands whether or not ATP was present (Table I, line 7). When double-stranded DNA was also present under these conditions, it coaggregated with the single-stranded DNA only in the presence of ATP (Table I, lines 5 and 6). Similarly, when aggregation of single-stranded DNA was inhibited by preincubation of recA protein and single strands in 1 mM $MgCl_2$ (see above), coaggregation occurred only in the presence of ATP (Table I, lines 9–12).

We examined the effect of salt on coaggregation under two of the conditions that suppress aggregation, namely, the presence of SSB or the preincubation of single strands with recA protein in 1 mM $MgCl_2$ (Figure 4A,B); we also examined aggregation and coaggregation under the condition that permits both to occur, namely, in 13 mM $MgCl_2$ without SSB or any preincubation (Figure 4C,D).

When aggregation of single-stranded DNA was suppressed, coaggregation was very sensitive to the concentration of NaCl: 50 mM salt completely inhibited coaggregation (Figure 4A,B). By contrast, 50 mM NaCl had little effect on the aggregation of single strands in the presence of ATP, and complete inhibition required 150–200 mM NaCl (Figure 4D). In another experiment, we incubated single-stranded DNA with recA protein in 13 mM $MgCl_2$, which causes incomplete coating of the single strands by recA protein and aggregates the DNA as described above. To these aggregates, we added duplex DNA and various amounts of NaCl. Concentrations of NaCl

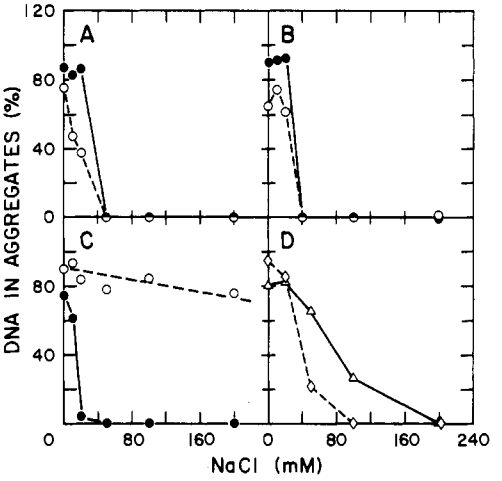

FIGURE 4: Differential inhibition of aggregation and coaggregation by NaCl. (A) Presynaptic complexes were first formed at 13 mM $MgCl_2$ under the standard conditions in the presence of SSB protein. Then, the complex containing 3 μM single-stranded DNA, 2.1 μM recA protein, and 0.3 μM SSB protein was reacted with 3 μM linear double-stranded DNA from phage M13 at various concentrations of NaCl. Single-stranded DNA (○) and double-stranded DNA (●) in aggregates were measured after 10 min at 37 °C. (B) Experiment from (A) repeated with presynaptic complexes formed at 1 mM $MgCl_2$ in the absence of SSB protein. (C) Experiment from (A) repeated with presynaptic complexes formed at 13 mM $MgCl_2$ in the absence of SSB protein. (D) Aggregation of single-stranded DNA in 13 mM $MgCl_2$. Experiments were performed with 3 μM single-stranded DNA from phage M13 and 2.1 μM recA protein in the presence (△) or absence (◇) of ATP. (No SSB was present in either case.) DNA aggregates were assayed after a 10-min incubation at 37 °C.

up to 200 mM failed to dissolve the preformed aggregates of single-stranded DNA, but as little as 20 mM NaCl prevented the duplex DNA from coaggregating (Figure 4C).

To examine the effect of ADP on aggregation vs. coaggregation, we omitted the ATP-regenerating system and preincubated single strands with recA protein for 2–3 min in 13 mM $MgCl_2$. During that time, less than 10% of the ATP was hydrolyzed, but when duplex DNA was added, none of it was detectable in aggregates whereas 90% of single-stranded DNA aggregated. Aggregation of single-stranded DNA was not reversed by the subsequent addition of amounts of ADP as great as 50% of the total adenine nucleotide (data not shown). In the presence of an ATP-regenerating system, 80% or more of single-stranded and double-stranded DNAs aggregated.

Coaggregates Are Intermediates in Homologous Pairing. In the experiments described above, we assayed the formation of aggregates by centrifugation for 2 min. To examine the time course of formation of aggregates, we determined the centrifugation time required to recover all of the complexes in the pellet. One minute sufficed for maximal sedimentation of complexes, and 20 s brought down 80% of the maximal amount (data not shown). Therefore, we were able to use an assay that took only 20 s and found that the initial rate of coaggregation was at least 4 times greater than the initial rate of formation of joint molecules (Figure 5B). The time course of coaggregation was similar whether the single-stranded DNA and duplex DNA were heterologous or homologous (Figure 5B). Coaggregates also formed faster than joint molecules in the absence of SSB (data not shown). This experiment shows that the formation of coaggregates reflects a set of interactions of presynaptic complexes with duplex DNA that are fast enough to be intermediate steps in homologous pairing.

All of the DNA in coaggregates, consisting of more than 95% of double-stranded DNA and more than 85% of single-

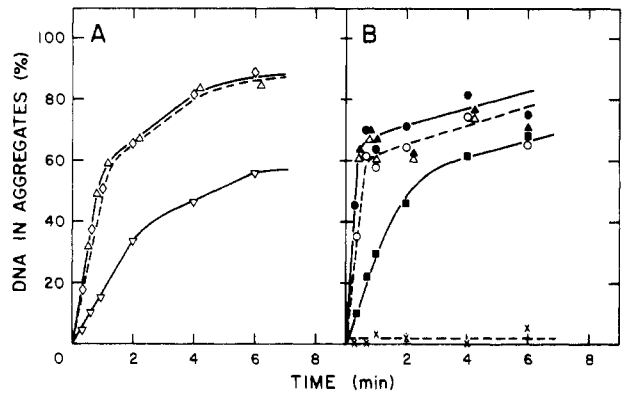

FIGURE 5: Time courses of aggregation, coaggregation, and formation of joint molecules. (A) Aggregation of single-stranded DNA: A reaction mixture containing single-stranded DNA from phage ϕX174, recA protein, and 13 mM $MgCl_2$ was incubated for various times at 37 °C. Aggregates were assayed by centrifugation for 20 s: (∇) 3 μM DNA and 2.1 μM recA protein; (Δ) 10 μM DNA with 2.1 μM recA protein; ($\diamond$) 10 μM DNA with 4.2 μM recA protein. (B) Coaggregation vs. the formation of joint molecules. Reaction mixtures containing 3 μM single-stranded DNA were incubated with 2.1 μM recA protein and 0.3 μM SSB protein for 10 min at 37 °C. Then, 2 μL of 120 μM double-stranded DNA and 2 μL of 26 mM $MgCl_2$ were added to a 30-μL aliquot of the reaction mixture. Coaggregation of single-stranded ϕX174 DNA (○) and duplex M13 DNA (●); coaggregation of single-stranded M13 DNA (Δ) and duplex M13 DNA ($\blacktriangle$); aggregation of double-stranded M13 DNA in the absence of any single strands (×); formation of joint molecules from single-stranded and duplex M13 DNA as determined by the D-loop assay (see Experimental Procedures) (■). The assay for coaggregation was performed by centrifugation for 20 s.

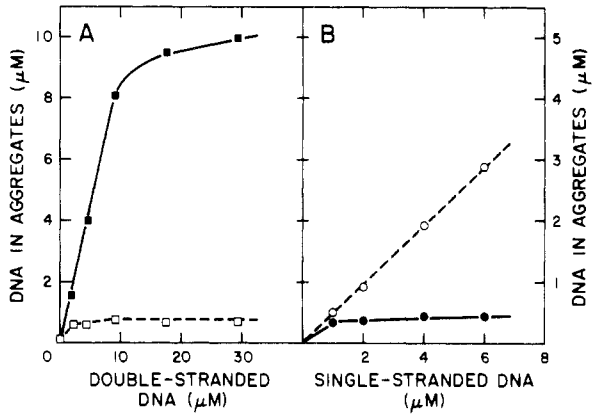

FIGURE 6: Ratios of single- and double-stranded DNA in coaggregates. (A) Titration of presynaptic complexes with double-stranded DNA. Single-stranded DNA from phage ϕX174 at 1 μM was incubated at 37 °C for 10 min with 1.4 μM recA protein, 0.1 μM SSB protein, and 13 mM $MgCl_2$. Then linear double-stranded DNA from phage M13 was added at various concentrations. The amounts of single-stranded DNA (□) and double-stranded DNA (■) in aggregates were assayed after 10 min. The final concentration of single-stranded DNA was 0.88 μM. (B) Presynaptic complexes were formed by incubating 8 μM single-stranded DNA from ϕX174 with 3.9 μM recA protein, 0.55 μM SSB protein, and 13 mM $MgCl_2$ at 37 °C for 10 min. Double-stranded M13 DNA at a final concentration of 0.5 μM was titrated with aliquots from the reaction mixture of concentrated presynaptic complexes. Reactions were continued for 10 min before single-stranded DNA (○) and double-stranded DNA (●) in aggregates were assayed. The concentrations plotted on the ordinates represent the micromoles of DNA per liter of reaction mixture that was present in the large complexes detected by the aggregation assay.

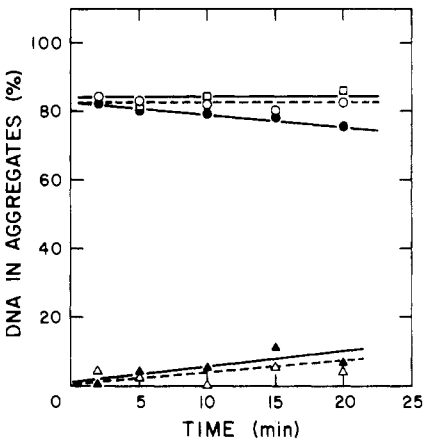

FIGURE 7: Lack of exchange of DNA in coaggregates. Presynaptic complexes were formed from 0.7 μM recA protein, 1 μM single-stranded DNA from phage ϕX174, 0.1 μM SSB protein, and 13 mM $MgCl_2$ in a total volume of 225 μL. After 10 min at 37 °C, 1 μL of 240 mM $MgCl_2$ and 15 μL of 240 μM ^{3}H-labeled linear double-stranded DNA from phage M13 were added. To aliquots of 32 μL, taken 10 min later, we added 2 μL of unlabeled linear double-stranded DNA from phage M13 (○) or phage ϕX174 (●) to make the final concentration of unlabeled DNA 13 μM. For the control, we added 2 μL of 10 mM Tris–0.1 mM EDTA (□) to each aliquot. Incubation was continued for various times, and DNA aggregates were assayed. In another set of experiments, unlabeled double-stranded DNA from phage M13 was added first to the reaction mixture to react with the presynaptic complexes. Again 32-μL aliquots were taken, and ^{3}H-labeled double-stranded DNA from phage M13 (Δ) or ϕX174 ($\blacktriangle$) was added finally at the same concentrations described above. In all experiments, the amount of single-stranded [^{32}P]DNA in aggregates was more than 90% (data not shown).

stranded DNA, was in the pellet after centrifugation for 1 min at 16000g in an Eppendorf Model 5414 centrifuge. According to this observation, most of the DNA was included in particles whose sedimentation coefficient exceeds 10^4 S. Using ^{35}S-labeled recA protein, we found that the coaggregates contain 1 molecule of recA protein per 3.6–5.0 nucleotide residues of single-stranded DNA, which agrees well with the stoichiometric binding ratio determined by other methods (Tsang et al., 1985). To characterize the large particles further, we held constant the amounts of single-stranded DNA, recA protein, and SSB while we added increasing amounts of duplex DNA. At saturation, there were about 10 molecules of duplex DNA per molecule of single-stranded DNA (Figure 6A). The complementary experiment, saturation of complexes with single-stranded DNA, is complicated by the need to vary simultaneously the amount of single strands and recA protein, as well as to optimize the concentration of SSB. In an experiment done simply by taking various amounts of presynaptic complex from a concentrated stock, we failed to saturate duplex DNA even when there were 10 molecules of single-stranded DNA per duplex molecule (Figure 6B). Thus, coaggregates can accommodate wide variations in the relative amounts of single- and double-stranded DNA.

The saturation of coaggregates by duplex DNA provided the means to assess the turnover of duplex DNA in the complexes. We preincubated 1 μM circular single-stranded ϕX174 DNA with 0.7 μM recA protein and 0.1 μM SSB following which we added 15 μM linear duplex M13 [^{3}H]DNA, a saturating concentration. Ten minutes later, we added an equal amount of unlabeled M13 duplex DNA and assayed the amount of labeled DNA in coaggregates for the next 25 min. Over this period, there was no measurable release of labeled duplex M13 DNA from the coaggregates (Figure 7). In a complementary experiment, coaggregates formed from single-stranded ϕX174 DNA and excess unlabeled duplex M13 DNA were titrated with labeled duplex M13 DNA. Little or no labeled M13 DNA entered the preformed coaggregates (Figure 7). These experiments show that coaggregates that are saturated with respect to heterologous double-stranded DNA are stable entities that do not readily exchange duplex

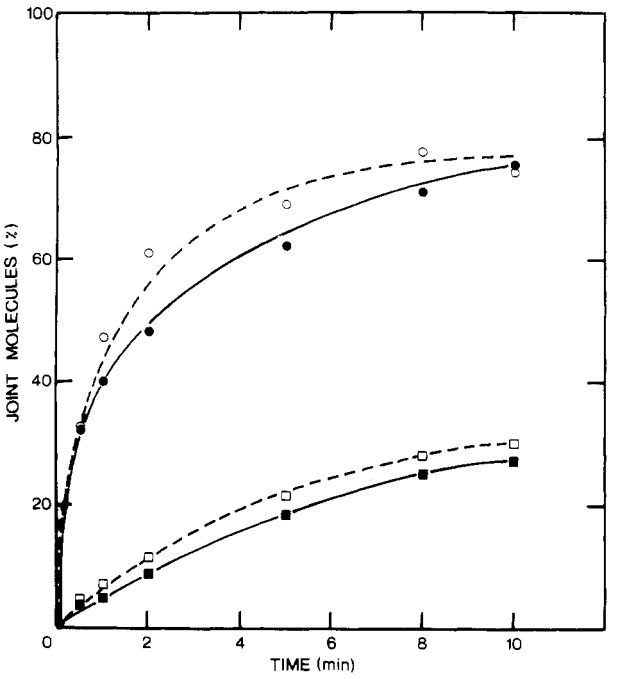

FIGURE 8: Formation of joint molecules by mixtures of homologous or heterologous coaggregates. Presynaptic complexes were formed in four separate tubes from 4 μM circular single-stranded DNA, 2.8 μM recA protein, and 1 mM $MgCl_2$ by incubation at 37 °C for 10 min. Coaggregates were then formed in each tube by raising the concentration of $MgCl_2$ to 10 mM and adding linear double-stranded DNA. Homologous coaggregates, which were made in two separate tubes and mixed 30 s later, contained a final concentration of 2 μM ϕX174 single-stranded DNA plus 4 μM ϕX174 double-stranded [^{32}P]DNA (○) and 2 μM M13 single-stranded DNA plus 4 μM M13 double-stranded [^{3}H]DNA (●). Heterologous coaggregates, handled in the same way, contained a final concentration of 2 μM ϕX174 single-stranded DNA plus 4 μM M13 double-stranded [^{3}H]DNA (■) and 2 μM M13 single-stranded DNA plus 4 μM ϕX174 double-stranded [^{32}P]DNA (□). The time course of formation of joint molecules at 37 °C was measured by the D-loop assay following the mixing of the homologous or heterologous coaggregates.

molecules with the surrounding solution.

In the same set of experiments, we investigated exchange when the challenging duplex DNA was homologous to the single-stranded ϕX174 DNA in the saturated coaggregates. The concentrations and procedures were the same as described above for heterologous DNA. Qualitatively, the results were similar whether the challenging DNA was homologous or heterologous; however, there were small quantitative differences: in 20 min, unlabeled ϕX174 duplex DNA chased about 10% of the labeled M13 duplex DNA out of the complexes (Figure 7). Little labeled challenging DNA was incorporated into coaggregates, and thus the measurement was imprecise because contaminating supernatant would make a significant contribution to label in the pelleted coaggregates (Figure 7). These observations do not exclude a low level of interaction between DNA sequestered in coaggregates and DNA in the surrounding solution.

To test directly whether coaggregates are intermediates in homologous pairing, we compared the formation of joint molecules by mixtures of homologous coaggregates vs. mixtures of heterologous coaggregates. To do so, we made presynaptic complexes by preincubating single-stranded DNA with recA protein in 1 mM $MgCl_2$ at 37 °C, added duplex DNA for 30 s in the presence of 10 mM $MgCl_2$, and then mixed coaggregates made in separate tubes. Homologous and heterologous combinations of M13 and ϕX174 DNA were used to make coaggregates. Double-stranded M13 DNA was labeled with ^{3}H and double-stranded ϕX174 DNA with ^{32}P.

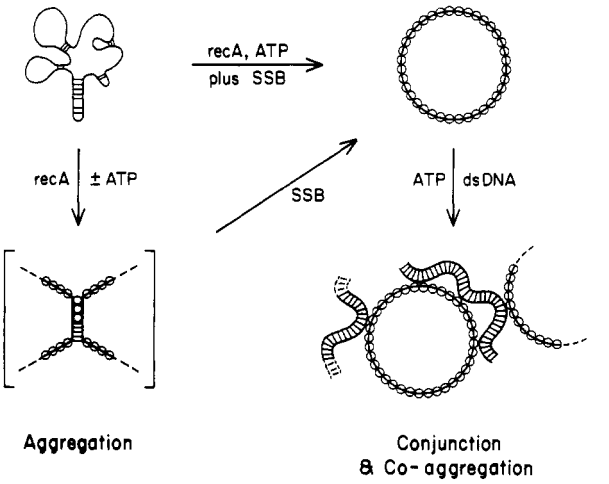

FIGURE 9: Diagram of the distinction between aggregation and coaggregation. Aggregation of single-stranded DNA by recA protein does not require ATP but is caused by conditions that impede complete coating of single-stranded DNA by recA protein. Aggregation may be attributable to the binding of a second single strand to the site that is normally filled by duplex DNA and/or the pairing of incidental short complementary sequences. Aggregation is relatively insensitive to inhibition by NaCl and ADP. Factors that reduce secondary structure in single-stranded DNA, including SSB, low concentrations of $MgCl_2$, fragmentation of the single strands, and excess recA protein, cause recA protein and single strands, in the presence of ATP, to form saturated presynaptic complexes that do not aggregate. The addition of duplex DNA, however, results in the mutually dependent coaggregation of the two forms of DNA as a result of their conjunction at multiple sites via recA protein. Like the formation of joint molecules, coaggregation is very sensitive to inhibition by NaCl and ADP. Conjunction, which is independent of homology, occurs rapidly and sequesters all of the DNA in large complexes within which homologous pairing occurs.

The initial rate of formation was nearly 10 times greater, and the final yield of joint molecules was 2.5 times greater in a mixture of homologous coaggregates than in a mixture of heterologous coaggregates (Figure 8). This experiment shows that coaggregates are domains whose exchange with the surrounding medium is far too slow and incomplete to account for the efficient formation of joint molecules in a few minutes via DNA and recA protein that may be outside of coaggregates. Since coaggregates form faster than joint molecules (Figure 5B) and include virtually all of the DNA in the reaction mixture, they must be intermediates in the overall reaction.

Discussion

Two distinctly different phenomena appear in the guise of DNA aggregation caused by recA protein: The aggregation of single-stranded DNA, and the mutually dependent coaggregation of single- and double-stranded DNA (Figure 9). The first of these phenomena is related to residual secondary structure in single-stranded DNA. Aggregation is abolished by factors that reduce secondary structure, such as the presence of helix-destabilizing proteins, preincubation of single strands with recA protein in low concentrations of Mg^{2+}, or fragmentation of single-stranded DNA [see Muniyappa et al. (1984)]. Aggregation is also reduced by excess recA protein, an observation that can be rationalized in the same way, and one which indicates further that aggregation of single strands is not attributable to protein–protein interactions. Consistent with that view are the observations that aggregation is relatively resistant to salt and to the accumulation of ADP which removes recA protein from single strands. There are several correlations between the conditions that are optimal for aggregation and those that are optimal for the renaturation of

complementary single strands by recA protein: Both aggregation and renaturation are optimal at subsaturating amounts of recA protein and are inhibited by helix-destabilizing proteins; neither has an absolute requirement for ATP (Weinstock et al., 1979; McEntee et al., 1980; Bryant et al., 1984). McEntee et al. (1980) suggested that SSB inhibits renaturation by preventing the binding of two single-stranded molecules to recA protein. The present observations provide direct evidence for that view. Other data indicate that SSB prevents recA protein from promoting renaturation by destabilizing secondary structure in single strands and consequently causing their complete coating by recA protein (Muniyappa et al., 1984; Tsang et al., 1985). In a completely coated or saturated complex, the single strand presumably occupies some preferred binding site for single-stranded DNA, and there is no extra naked single-stranded DNA to occupy a second site that normally is filled by duplex DNA. Bryant et al. (1984) have reported that the kinetics of renaturation are first order rather than second order, an observation that is consistent with the hypothesis that the aggregates of single-stranded DNA are intermediates in the pairing of complementary single strands.

The mutually dependent coaggregation of single strands and duplex DNA is a different process, which is favored by conditions that promote the complete coating of single strands by recA protein and their concomitant unfolding. Each single strand of DNA that is coated by recA protein has many binding sites for duplex DNA, and consequently, these coated strands cause large networks to form (Figure 9). RecA protein does not similarly bind to multiple molecules of double-stranded DNA in the absence of single strands, which suggests that the protein lacks more than one site for the binding of duplex DNA.

Kinetic observations predicted the occurrence of large complexes of single-stranded DNA and heterologous duplex DNA held together by many weak interactions (Gonda & Radding, 1983). The coaggregates described here appear to be such intermediates: they sediment at greater than 10^4 S and are readily dissolved by salt and low concentrations of ADP that remove recA protein from the DNA.

The observations described here show that coaggregates are intermediates in homologous pairing. Both coaggregation and homologous pairing require ATP, Mg^{2+}, and stoichiometric amounts of recA protein; both are inhibited by similar concentrations of salt and ADP; and correspondingly, both are favored by conditions that remove secondary structure from single-stranded DNA, which promotes their complete coating by recA protein. More directly, the data show that coaggregates are domains that form faster than joint molecules, that include most of the DNA in the reaction mixture, and that yield joint molecules nearly an order of magnitude faster than they exchange with one another or with free molecules in the surrounding solution. Thus, one cannot account for the rapid homologous pairing of all of the DNA by supposing that it proceeds via molecules that are outside of coaggregates, but rather, such three-dimensional networks must be intermediates directly on the reaction pathway.

From studies on the role of aggregation in catenation of DNA by topoisomerases, Krasnow & Cozzarelli (1982) concluded that "aggregates are dynamic structures accessible to enzymes and are a model for compacted DNA in vivo". The observations reported here support that concept. Aggregates of single-stranded DNA and recA protein are readily accessible to SSB, which dissociates such aggregates, and more strikingly, the large coaggregates of single- and double-stranded DNA made by recA protein facilitate the search for homology [this paper and Gonda & Radding (1983)]. In the case of recA protein, we would suppose that such functional networks are held together by many loose contacts none of which lasts very long, but which together retain the DNA and recA protein in a limited volume and thereby facilitate many trial pairings. The dynamism of this mechanism and the consequent speed and efficiency with which recA protein promotes homologous pairing seem remarkable. In vivo, the conditions for a similar process may be imagined to occur in phage crosses, bacterial conjugation, bacterial transformation, and synaptonemal complex formation, whenever pools of DNA molecules or multiple segments thereof are engaged in searching.

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