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Reversibility of Strand Invasion Promoted by recA Protein and Its Inhibition by *Escherichia coli* Single-stranded DNA-binding Protein or Phage T4 Gene 32 Protein*

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When recA protein promotes homologous pairing and strand exchange involving circular single strands and linear duplex DNA, the protein first polymerizes on the single-stranded DNA to form a nucleoprotein filament which then binds naked duplex DNA to form nucleoprotein networks, the existence of which is independent of homology, but requires the continued presence of recA protein (Tsang, S. S., Chow, S. A., and Radding, C. M. (1985) *Biochemistry* 24, 3226–3232). Further experiments revealed that within a few minutes after the beginning of homologous pairing and strand exchange, these networks began to be replaced by a distinct set of networks with inverse properties: their formation depended upon homology, but they survived removal of recA protein by a variety of treatments. Formation of this second kind of network required that homology be present specifically at the end of the linear duplex molecule from which strand exchange begins. *Escherichia coli* single-stranded DNA-binding protein or phage T4 gene 32 protein largely suppressed the formation of this second population of networks by inactivating the newly formed heteroduplex DNA, which, however, could be reactivated when recA protein was dissociated by incubation at 0 °C. We interpret these observations as evidence of reinitiation of strand invasion when recA protein acts in the absence of auxiliary helix-destabilizing proteins. These observations indicate that the nature of the nucleoprotein products of strand exchange determines whether pairing and strand exchange are reversible or not, and they further suggest a new explanation for the way in which *E. coli* single-stranded DNA-binding protein and gene 32 protein accelerate the apparent forward rate of strand exchange promoted by recA protein, namely by suppressing initiation of the reverse reaction.

Prior to the formation of joint molecules, single-stranded DNA coated with recA protein forms large networks with naked duplex DNA, within which the processive search for homology is facilitated (Tsang *et al.*, 1985a; Chow and Radding, 1985; Gonda and Radding, 1986). The formation of such nucleoprotein networks is independent of homology and, under suitable conditions, includes virtually all of the DNA in the reaction mixture. We have suggested that networks form because recA protein has at least two binding sites for DNA, a site that binds single-stranded DNA relatively strongly, and a site that binds either duplex DNA or naked single strands relatively weakly. Consequently, the single-stranded nucleoprotein filament is polyvalent with respect to the binding of naked DNA molecules.

During strand exchange, the incoming single strand pairs in a directional fashion with the complementary strand of the duplex molecule to form heteroduplex DNA and simultaneously displaces the anticomplementary strand starting from the 5′ end of the latter. When the substrates are circular single-stranded DNA and full-length linear duplex DNA, the products at the completion of strand exchange are nicked circular heteroduplex DNA and a completely displaced linear strand derived from the parental duplex. By probing the DNA-recA protein complexes with nucleases during and after strand exchange, we found that the displaced strand from the parental duplex as well as the newly formed heteroduplex DNA are coated with recA protein (Chow *et al.*, 1986). Since our previous observations indicated that single strands fully coated with recA protein do not bind to one another and that network formation depends on the interaction of single-stranded and double-stranded DNA via binding to common molecules of recA protein, we expected that the nucleoprotein network would dissociate at the completion of strand exchange because, at that stage, both DNA products are coated with recA protein. Similarly, at the completion of strand exchange, when one strand of a labeled linear duplex molecule is incorporated into circular duplex DNA and the other labeled strand is fully displaced, we expected that only half of the total radioactivity would be retained by nitrocellulose filters at high concentrations of salt (see Fig. 1).

Observations that differed markedly from these expectations led us to explore further the products of strand exchange, as described here. These studies provide new information on the reversibility or nonreversibility of strand invasion and the role of SSB[1] in accelerating strand exchange.

EXPERIMENTAL PROCEDURES

Enzymes—Unlabeled recA protein was purified by the method of Shibata *et al.* (1981), and ^{35}S-labeled recA protein was prepared as described previously by Tsang *et al.* (1985b). The concentration of unlabeled or labeled recA protein was measured by absorbance using a value for $E^{1\%}_{277\,nm}$ (corrected for light scattering) of 6.33 (Tsang *et al.*,

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[1] The abbreviations used are: SSB, *E. coli* single-stranded DNA-binding protein; SDS sodium dodecyl sulfate.

1985b). *Escherichia coli* single-stranded DNA-binding protein was kindly provided by Dr. John Chase (Albert Einstein College of Medicine, New York). Gene 32 protein of phage T4 was a generous gift from Dr. Kenneth Williams (Yale University). Restriction endonucleases *Bam*HI, *Eco*RI, *Hinc*II, and *Hpa*I were purchased from International Biotechnologies, Inc. Creatine phosphokinase (type I) and S1 nuclease were obtained from Sigma. Proteinase K was purchased from EM Laboratories, Inc.

Preparation of DNA Substrates—Unlabeled circular single-stranded DNA and circular duplex [^{3}H]DNA from phages G4, M13, and M13*Gori*1 were prepared as described (Cunningham *et al.*, 1980, 1981; DasGupta *et al.*, 1980). Preparations of circular single-stranded DNA contained less than 5% linear molecules as determined by agarose gel electrophoresis. Preparations of circular duplex [^{3}H]DNA contained less than 5% nicked molecules as determined by agarose gel electrophoresis and by the nitrocellulose filter assay of Kuhnlein *et al.* (1976). All concentrations of DNA are expressed in moles of nucleotide residues.

Unless indicated otherwise, the supercoiled DNAs from phages G4 and M13 were linearized with the restriction endonucleases *Eco*RI and *Hinc*II, respectively. Linearization was performed under conditions specified by the supplier, and the completeness of cleavage was determined by agarose gel electrophoresis and by the assay of Kuhnlein *et al.* (1976).

Standard Reaction Conditions—Unless otherwise stated, the preincubation mixture contained a final concentration of 33 mM Tris-HCl (pH 7.5), 1.3 mM ATP, 6 mM creatine phosphate, 10 units/ml creatine phosphokinase, 88 μg/ml bovine serum albumin (nuclease-free, Bethesda Research Laboratories), 1.8 mM dithiothreitol, 1 mM $MgCl_2$, and 3 μM circular single-stranded DNA. The mixture was incubated for 2 min at 37 °C; recA protein was added at 2 μM, and the incubation was continued for another 10 min at 37 °C. Pairing and strand exchange were initiated by adding linear duplex [^{3}H]DNA at 6 μM and adjusting the final concentration of $MgCl_2$ to 13 mM. In reactions that included SSB, the preincubation mixture contained 13 mM $MgCl_2$ (instead of 1 mM), 2 μM recA protein, and 0.19 μM SSB. The mixture was incubated for 15 min at 37 °C prior to addition of linear duplex DNA at 6 μM to start the pairing and strand exchange.

Assay for Joint Molecules (the D-loop Assay)—This assay measures the retention by nitrocellulose filters of duplex DNA that has attached to single-stranded or partially single-stranded DNA (Beattie *et al.*, 1977). At the appropriate times, we took a 10-μl aliquot of the reaction mixture and placed it directly into a 12 × 75-mm culture tube containing 200 μl of 25 mM EDTA (pH 7.5) at 0 °C. This was followed 2 min later with 4.0 ml of 1.5 M NaCl, 0.15 M sodium citrate (pH 7.5), also at 0 °C. The solution was filtered through a nitrocellulose filter (Sartorius type SM11306, 0.45-μm pore size) that had been soaked in 1.5 M NaCl, 0.15 M sodium citrate (pH 7.5). The filters were then rinsed three times with 2 ml of 1.5 M NaCl, 0.15 M sodium citrate, dried under a heat lamp, and put into vials with 5 ml of Optifluor (Packard Instrument Co.). The radioactivity was determined in a scintillation counter.

Assay of DNA Networks or Coaggregates—The assay used here is identical to that described by Tsang *et al.* (1985a). The assay measures the formation of complexes of DNA that sediment at more than 10,000 S. To measure the formation of such complexes, we prepared a reaction mixture containing single-stranded DNA and recA protein as described under "Standard Reaction Conditions." At the specified times after the addition of duplex [^{3}H]DNA, a 30-μl aliquot of the reaction mixture was centrifuged in a 0.5-ml Eppendorf tube at 15,600 × *g* for 2 min at room temperature (Brinkmann Model 5414).

To measure the formation of DNA networks that are stable in the absence of recA protein, we used four different treatments to dissociate recA protein from DNA just prior to centrifugation: (i) addition of proteinase K and SDS to a final concentration of 300 μg/ml and 0.1%, respectively, and incubation at 37 °C for 5 min; (ii) incubation in an ice bath for 15 min; (iii) addition of 5 mM ADP and incubation at 37 °C for 2 min; and (iv) addition of 1% SDS and incubation in an ice bath for 6 min. By monitoring the disposition of ^{35}S-labeled recA protein, we showed that all of the above treatments completely removed the protein from DNA. These different treatments were used in order to ensure that the effects observed were due to the removal of recA protein and not a result of the treatment itself. Unless indicated otherwise, a 30-μl aliquot of the reaction mixture was centrifuged at 15,600 × *g* for 2 min at room temperature immediately after deproteinization. Following centrifugation, both for protein-dependent and -independent networks, three sequential 9-μl aliquots were taken from the supernatant fraction. We estimated the total radioactivity in 30 μl of supernatant from the average concentration of radioactivity in the first two fractions. The remaining 3 μl of supernatant and the pellet were suspended in 200 μl of distilled water, and the radioactivity was determined. A correction was made for radioactivity attributable to the 3 μl of supernatant that was not separated from the pellet. In every experiment, the recovery of labeled DNA in the supernatant and pellet was 95% or greater, and residual radioactivity on the wall of the tube was 5% or less.

Determination of the Extent of Strand Exchange—The extent of strand exchange was measured by determining the fraction of ^{3}H-labeled duplex DNA that became sensitive to S1 nuclease as a strand was displaced by the incoming circular single strand of DNA (Cox and Lehman, 1981; Wu *et al.*, 1982). At the appropriate times, a 10-μl aliquot of the reaction mixture was removed for the D-loop assay. Simultaneously, a 25-μl aliquot was taken and placed in a 1.5-ml Eppendorf tube containing 2.2 μl of 10% SDS. The samples were kept on ice for 15 min and then diluted into 363 μl of S1 buffer (0.5 M NaCl, 50 mM sodium acetate, and 1 mM zinc acetate (pH 4.6); heat-denatured calf thymus DNA was added to 30 μg/ml; and S1 nuclease was added to a final concentration of 2 units/ml. After incubation for 30 min at 37 °C, 10 μl of calf thymus DNA at 5 mg/ml was added as carrier, followed by 800 μl of cold 10% trichloroacetic acid. After 30 min on ice, samples were centrifuged at 15,600 × *g* (Brinkman Model 5414) for 10 min at 4 °C, and an aliquot of the supernatant was removed to determine acid-soluble counts. A control reaction, in which single-stranded DNA was omitted, was treated identically to determine the background of label rendered acid-soluble in the assay.

Since only one strand of the labeled duplex DNA can be displaced and degraded, the amount of ^{3}H-labeled duplex DNA that became sensitive to S1 nuclease was multiplied by a factor of 2 and then divided by the fraction of duplex molecules engaged in complexes with the single-stranded DNA by the amount of [^{3}H]DNA in joint molecules as determined by the D-loop assay to yield the extent of strand exchange.

RESULTS

Persistence of DNA Networks and Joint Molecules following Strand Exchange—When we paired circular single-stranded DNA with linear duplex DNA, contrary to the expectations outlined in Fig. 1 (see "Introduction"), we found that both networks and joint molecules remained at high levels, even 120 min after initiation of the pairing reaction (Fig. 2*A*). Under identical conditions, the extent of strand exchange, which was measured by the percent of labeled duplex DNA that became sensitive to digestion by S1 nuclease, had already reached a maximal level at 60 min. As expected, when the single-stranded DNA used was heterologous to the duplex DNA, there was no formation of joint molecules or strand exchange, but homology-independent networks formed rapidly, as previously described (Tsang *et al.*, 1985a). At later times, the level of homology-independent networks declined steadily (Fig. 2*B*). The drop probably reflects a gradual accumulation of ADP, which has been shown to dissociate recA protein from DNA and to dissolve nucleoprotein networks (Tsang *et al.*, 1985a; Chow *et al.*, 1986).

The persistence of networks and joint molecules shown in Fig. 2*A* revealed that strand exchange does not proceed in the idealized manner illustrated in Fig. 1. Since the levels of networks at later times were much higher when the two forms of DNA were homologous than when they were heterologous, these observations further indicated that homologous and heterologous networks differ.

Characterization of Networks Formed following Homologous Pairing and Strand Exchange—The persistence of networks during strand exchange (Fig. 2*A*) suggested that the stability of these networks, in contrast to the type formed by heterologous contacts (Fig. 2*B*), might be independent of recA protein. We examined the stability of networks in the absence of protein by treating the network with proteinase K and SDS (Fig. 3). As expected from previous experience, treatment with proteinase K and SDS had no effect on the yield of joint

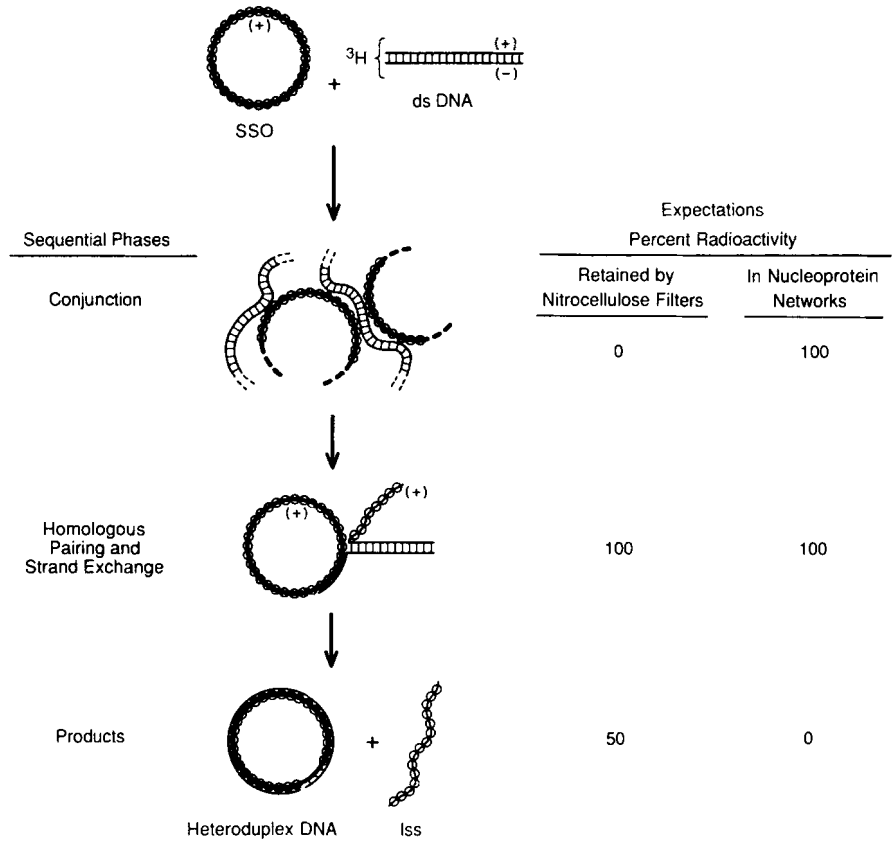

FIG. 1. **Expected values from assays that monitor the sequential phases of homologous pairing promoted by recA protein.** Unlabeled, circular single-stranded DNA coated with recA protein is reacted with ^{3}H-labeled duplex DNA. The DNA substrates are brought together independent of homology during the conjunction phase. The search for homology within the nucleoprotein network results in homologous pairing, followed by strand exchange. The products at the completion of strand exchange are a nicked, circular heteroduplex DNA and a displaced linear single strand (DasGupta *et al.*, 1980; Cox and Lehman, 1981). When the pairing reaction is monitored by the amount of radioactivity retained by nitrocellulose filters (see "Experimental Procedures"), retention should reach 100% during homologous pairing and strand exchange when every molecule of duplex DNA in the reaction mixture pairs with a single strand. At the completion of strand exchange, the radioactivity retained should fall to 50% because one strand of the labeled duplex is incorporated into the heteroduplex DNA product which is not retained by the filters. On the other hand, when the pairing reaction is monitored by the formation of nucleoprotein networks, we expect 100% of the labeled duplex to be included in the network during conjunction, homologous pairing, and strand exchange. However, at the completion of strand exchange, the network should be dissolved because both the heteroduplex DNA and the linear single strand are coated with recA protein (Tsang *et al.*, 1985a; Chow *et al.*, 1986). *ds DNA*, double-stranded DNA; *SSO*, circular (viral) single strands; *lss*, linear single strands.

molecules measured by the standard D-loop assay. Networks, however, showed a biphasic response to deproteinization: at early times, 0–20 min, networks were dissolved by treatment; whereas at later times, 60–120 min, they became resistant to treatment, *i.e.* independent of the further presence of recA protein (Fig. 3). Controls showed that untreated networks contained one monomer of recA protein/4.5 nucleotide residues of single-stranded DNA, whereas treated networks had no detectable recA protein (Fig. 3).

On the basis of the results shown in Figs. 2 and 3, we hypothesized that the formation of protein-independent networks is due to reinitiation of pairing and strand exchange by the partially displaced strands of joint molecules formed in the first round of pairing and strand exchange (Fig. 4). Therefore, unlike networks that result from heterologous contacts, *i.e.* networks formed by heterologous molecules or networks formed prior to homologous pairing, the networks formed after the initiation of homologous pairing and strand exchange would be stabilized by hydrogen bonding of complementary bases instead of by DNA-protein interactions. The postulated reinitiation would also explain the high percentage of retention of duplex DNA by nitrocellulose filters since it might slow the completion of strand exchange. Under the conditions of the D-loop assay, the retention of duplex DNA by a nitrocellulose filter depends solely upon its attachment to single-stranded DNA and is, therefore, not affected by the presence or absence of recA protein (see Fig. 3, for example). According to this hypothesis, the sensitivity of networks to proteinase K and SDS treatment during the early part of the reaction probably reflects the minor fraction of DNA in stable networks that are attributable to reinitiation of strand invasion at that early time. Furthermore, joints formed by reinvasion at early times might be short and therefore might spontaneously dissociate by branch migration upon removal of recA protein.

Further support for this hypothesis came from experiments in which we removed recA protein from the networks by incubating them at 0 °C (Fig. 5). Low temperature dissociates recA protein from duplex DNA (Shibata *et al.*, 1982) and, at the same time, decreases the rate of spontaneous branch

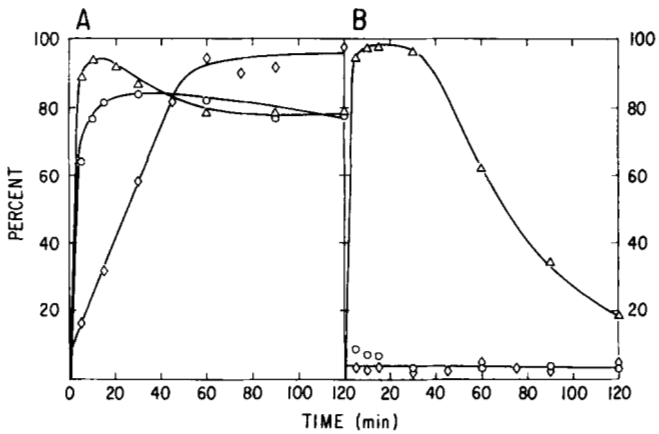

FIG. 2. **Persistence of nucleoprotein networks and joint molecules following strand exchange.** *A*, homologous substrates. Circular single-stranded DNA (3 μM) of phage G4 was preincubated with 2 μM recA protein in the presence of 1 mM $MgCl_2$ for 10 min at 37 °C. After preincubation, we initiated the pairing reaction by adding ^{3}H-labeled linear duplex DNA of phage G4 at a final concentration of 6 μM and bringing the concentration of $MgCl_2$ to 12 mM. The formation of networks (△) and joint molecules (○) and extent of strand exchange (◇) were determined as cited under "Experimental Procedures." *B*, heterologous substrates. Conditions were identical to those described above except that circular single-stranded DNA of phage M13 was used in the reaction with linear duplex G4 [^{3}H]DNA. Symbols have the same significance as for *A*.

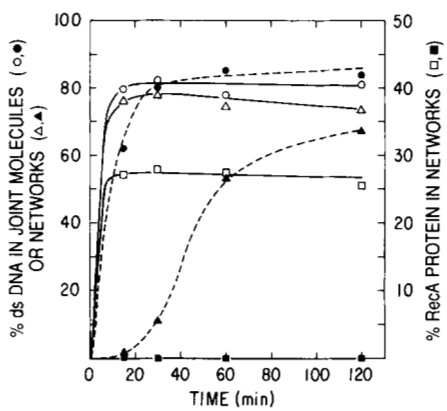

FIG. 3. **Effects of proteinase K and SDS treatment on the stability of networks and joint molecules.** Circular single-stranded DNA (3 μM) of phage G4 was paired with 6 μM linear duplex [^{3}H]DNA from the same phage in the presence of 2 μM ^{35}S-labeled recA protein. At various times after initiation of the reaction, aliquots of the reaction mixture were removed, and additions were made of proteinase K at a final concentration of 300 μg/ml and of SDS at 0.1% (●, ▲, ■). An equal volume of distilled water was added in control aliquots (○ △, □). The reaction mixture was incubated for another 5 min at 37 °C before the measurements were made of joint molecules (○, ●), duplex [^{3}H]DNA in networks (△, ▲) and ^{35}S-labeled recA protein in networks (□, ■). *ds DNA*, double-stranded DNA.

migration which might dissociate short joints (Radding *et al.*, 1977). When a reaction mixture containing homologous DNA substrates and recA protein was transferred to an ice bath following a 30-min incubation at 37 °C (Fig. 5*A*), recA protein gradually dissociated from the network and was not detectable after 15 min of cold treatment. However, the percent of duplex DNA in networks showed only a slight decline when compared to the reaction mixture that was maintained at 37 °C throughout. On the other hand, the network that formed in the presence of heterologous DNA substrates disintegrated in proportion to the amount of recA protein dissociated from the network (Fig. 5*B*).

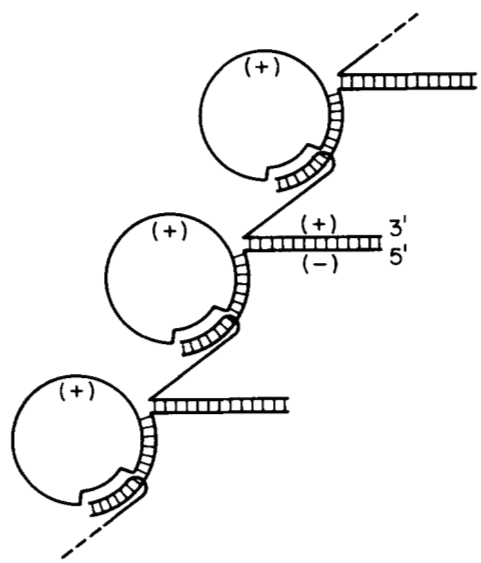

FIG. 4. **A model to rationalize the formation of a distinct second population of networks, the formation of which required homology but whose existence did not require the continued presence of recA protein.** The 5′ end of the partially displaced plus strand invades the heteroduplex region of another joint molecule and starts a second round of pairing and strand exchange. The cycling process of strand exchange and reinitiation produces a network of interlaced molecules which is stabilized by hydrogen bonding of complementary bases. The formation of such networks requires that the circular single strand share homology with the duplex DNA only at the near end of the duplex molecule with respect to strand exchange. (+) denotes the plus or viral strand; (−) denotes the minus complementary strand.

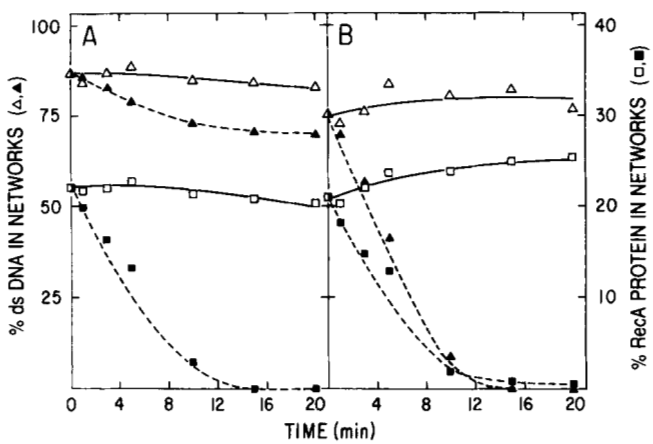

FIG. 5. **Cold treatment dissociates recA protein from DNA but does not dissolve homologous networks.** *A*, homologous substrates. Circular single-stranded DNA (3 μM) of phage G4 was reacted at 37 °C with 6 μM linear duplex [^{3}H]DNA from the same phage in the presence of 2 μM ^{35}S-labeled recA protein. Thirty minutes after initiation of the reaction, an aliquot of the reaction mixture was put into an ice bath (▲, ■), whereas an equal aliquot of the reaction mixture was maintained at 37 °C (△, □). The time of switch of temperature is time 0 in this plot. The percent of duplex DNA (△, ▲) and the amount of recA protein (□, ■) in networks were measured at various times after the temperature adjustment. *B*, heterologous substrates. Conditions were identical to those described for *A* except that circular single-stranded DNA of phage M13 was used to react with linear duplex [^{3}H]DNA of phage G4. *Symbols* have the same significance as for *A*. *ds DNA*, double-stranded DNA.

Time Course of Formation of Protein-independent Networks—At various times after the pairing reaction started, we subjected different aliquots of the reaction mixture to cold treatment or to ADP, which is yet another way to remove

recA protein from DNA (Cox *et al.*, 1983b; Menetski & Kowalczykowski, 1985; Tsang *et al.*, 1985a). The percent of duplex DNA in joint molecules was not significantly affected by either method of removing recA protein as compared to the untreated control (Fig. 6, *upper*). On the other hand, the removal of protein by these treatments applied 1 min after the start of homologous pairing decreased the fraction of duplex DNA in networks by 4-fold (Fig. 6, *lower*). However, by 5 min, networks began to become resistant to treatments that removed recA protein.

Thus, as early as 1–5 min after the initiation of homologous pairing, some event or events begin to produce networks whose stability no longer depends upon the further presence of recA protein. By about 30 min, most of the DNA was still in networks, but all of it was converted into the type that is stable in the absence of recA protein.

Basis for the Formation of Networks That Are Stable after Removal of recA Protein—To test the hypothesis that the protein-independent networks result from the reinitiation of strand invasion (Fig. 4), we took advantage of previous observations which showed that homologous pairing can take place whether homology is restricted to either end or to the middle of the duplex molecule, whereas the propagation of strand exchange requires that the 5′ end of the plus or noncomplementary strand be located in a region of homology (Wu *et al.*, 1982). Therefore, we measured the formation of protein-independent networks when homology was restricted to different parts of the duplex DNA.

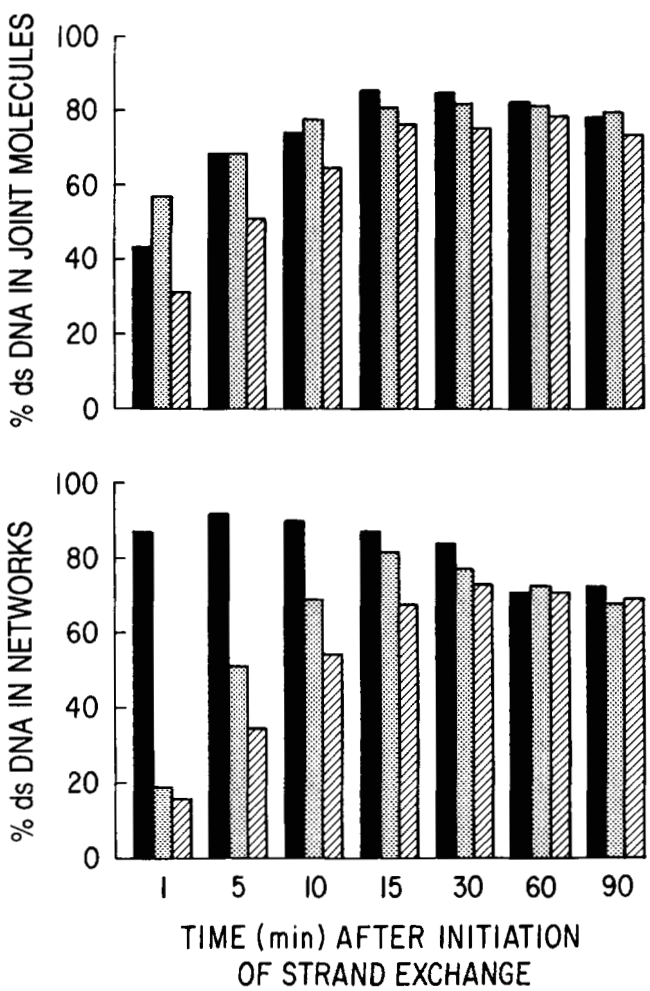

FIG. 6. **Time course of the formation of protein-independent networks.** The pairing reaction was initiated by adding 6 μM linear duplex [^{3}H]DNA of phage G4 to an incubation mixture containing 3 μM circular single-stranded DNA of phage G4 and 2 μM recA protein. At the indicated times after the addition of duplex DNA, three aliquots of the reaction mixture were removed. Each aliquot was split for assay of joint molecules and networks. *Solid bars* represent the aliquots which were assayed immediately without further treatment to serve as controls. *Stippled bars* represent the aliquots that were transferred to a 0 °C ice bath for 15 min prior to the assays. *Cross-hatched bars* represent the aliquots to which 5 mM ADP was added, followed by incubation for another 2 min before the assay. *ds DNA*, double-stranded DNA.

When the duplex DNA was completely homologous to the circular single-stranded DNA or when both ends of the duplex DNA were homologous, joint molecules formed efficiently, and more than 40% of the duplex DNA was found in protein-independent networks (Fig. 7*B*). When the ends of the duplex DNA were heterologous, joint molecules formed somewhat less efficiently, as seen before (Bianchi *et al.*, 1983); but virtually no protein-independent networks were formed, indicating that at least one homologous end is required for the formation of networks (Fig. 7*B*).

The data in Fig. 7*C* show that homology is required specifically at the proximal end of duplex DNA, with respect to the polarity of strand exchange. A 2-kilobase fragment of chimeric DNA (Fig. 7*A*, *substrate c*) was reacted either with G4 single-stranded DNA which was homologous to the proximal end or with M13 DNA which was homologous to the distal end. G4 single-stranded DNA formed joint molecules and protein-independent networks. M13 DNA formed stable joint molecules somewhat less efficiently, consistent with the relatively small size of the duplex DNA and the unfavorable polarity of strand exchange (Gonda and Radding, 1986; Wu *et al.*, 1982); but M13 DNA did not give rise to any protein-independent networks.

Suppression by SSB or T4 Gene 32 Protein of Networks That Are Independent of recA Protein—The addition of SSB before the start of homologous pairing and strand exchange changed strikingly the time course of events (compare Fig. 8, *B* with *A*). The percent of labeled duplex DNA in joint molecules and in total networks started to decrease after 15 min and reached a minimum about 60 min after the start of the pairing reaction. The quantitative changes agreed with the idealized expectations diagramed in Fig. 1: the level of retention of radioactivity by nitrocellulose filters fell nearly to 50%, and total networks dissipated completely. In addition, virtually all of the networks formed at any time in the presence of SSB were sensitive to deproteinization by treatment with SDS (Fig. 8*B*). Thus, SSB not only inhibited the persistence of networks, but specifically eliminated the formation of those networks whose stability does not depend upon the continued presence of recA protein.

In these experiments, single-stranded DNA was preincubated with recA protein in 1 mM Mg^{2+} and, 1 min later, SSB or buffer was added prior to the addition of duplex DNA. Similar results were obtained when SSB was added along with duplex DNA or when the single strands were preincubated in 13 mM Mg^{2+} in the presence of recA protein and SSB (data not shown). Consistent with earlier observations (Kahn and Radding, 1984; Tsang *et al.*, 1985a), SSB had little or no effect on the rate of formation and yield of joint molecules and networks, but stimulated the rate of strand exchange about 2-fold (Fig. 8).

In addition, we found that gene 32 protein of phage T4 had the same action as SSB in eliminating the persistence of both joint molecules and networks at the completion of strand exchange. The time course of formation of joint molecules and networks in the presence of gene 32 protein (data not

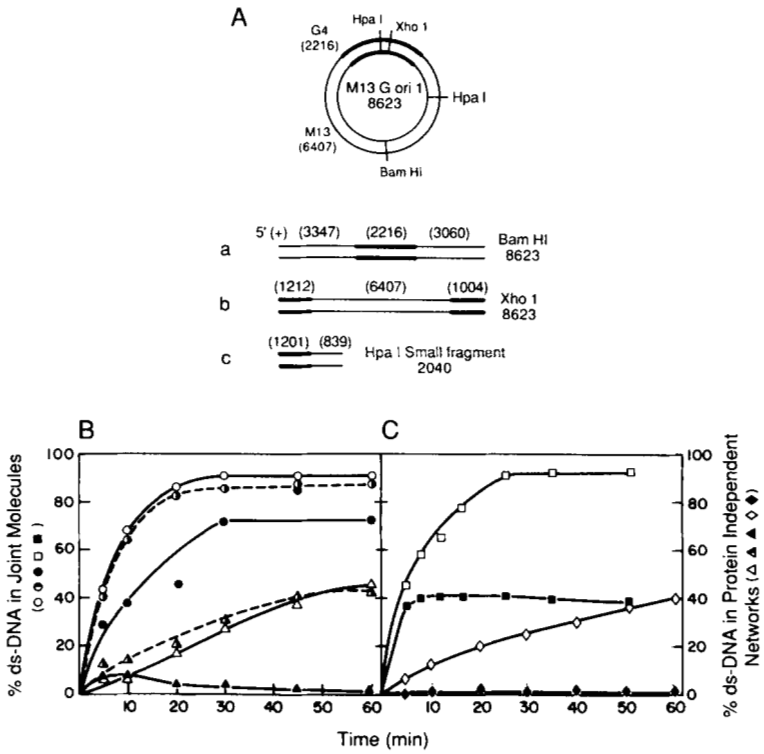

FIG. 7. The formation of protein-independent networks requires homology at the end of linear duplex DNA from which strand exchange begins. *A*, maps of duplex DNA substrates derived from chimeric phage M13G*ori*1 by digestion with restriction endonucleases. *Thin lines* denote M13 sequences, and *thick lines* denote G4 sequences. The 5′ terminus of the plus strand in each molecule is represented by the *left end* of the *upper line*. The *numbers* without parentheses represent the total length in base pairs of the DNA molecule; *numbers* in *parentheses* represent the length in base pairs of the region by which it appears. *B* and *C*, formation of joint molecules and protein-independent networks when homology was restricted to various portions of the duplex DNA. Circular single-stranded DNA was preincubated for 15 min at 37 °C with an excess of recA protein and 1.2 mM $MgCl_2$. After preincubation, pairing was initiated by the addition of duplex DNA and adjustment of the final concentration of $MgCl_2$ to 12 mM. At the times indicated, 10-μl aliquots were taken to measure the formation of joint molecules (*circles*), and 30-μl aliquots were taken to assay protein-independent networks (*triangles*). For the latter assay, 3 μl of 10% SDS was added to each 30-μl aliquot of the reaction mixture, and the sample was kept on ice for 6 min before centrifugation at 15,000 × *g* for 5 min at 4 °C. ○ and △, *substrate a* and circular single-stranded M13 DNA (both ends homologous). The reaction mixture contained 1 μM recA protein, 2.0 μM single-stranded DNA, and 5.4 μM *substrate a*, the *Bam*HI derivative of duplex M13G*ori*1 DNA. ◑, and ◭, *substrate a* and circular single-stranded M13G*ori*1 DNA (complete homology). The reaction mixture contained 1 μM recA protein, 2 μM circular single-stranded M13G*ori*1 DNA, and 4 μM *substrate a*. ● and ▲, *substrate b* and single-stranded circular M13 DNA (homology only in the middle of the duplex molecule). The reaction mixture contained 1.2 μM recA protein, 2.4 μM circular single-stranded M13 DNA and 5.4 μM *substrate b*, the *Xho*I derivative of duplex M13G*ori*1 DNA. □ and ◇, *substrate c* and circular single-stranded G4 DNA (homology only at the proximal end of the duplex molecule). The mixture contained 2.1 μM recA protein, 4.2 μM circular single-stranded G4 DNA, and 3 μM *substrate c*, the small *Hpa*I fragment derived from duplex M13G*ori*1 DNA. ■ and ◆, *substrate c* and circular single-stranded M13 DNA (homology only at the distal end of duplex DNA). The mixture contained 2.4 μM recA protein, 4.8 μM M13 circular single-stranded DNA, and 3 μM *substrate c*. *ds-DNA*, double-stranded DNA.

shown) was indistinguishable from that seen in the presence of SSB, as exemplified in Fig. 8*B*. Except for the helix-destabilizing property and the high affinity for single-stranded DNA, the physical characteristics and modes of binding of SSB and gene 32 protein are very different from one another (Kowalczykowski *et al.*, 1981; Bujalowski and Lohman, 1986). This implies that the mechanism by which SSB and gene 32 protein suppress protein-independent networks is mainly related to their helix-destabilizing action.

The simplest explanation for these observations is that SSB inhibits reinitiation of pairing and allows strand exchange resulting from the first round of pairing to proceed to completion. This conclusion is supported by comparing the assays shown in Fig. 8 with electrophoretic analysis of the reaction in the presence and absence of SSB (Fig. 9). In a pairing reaction involving circular single strands and linear duplex DNA, the products at the completion of one round of strand exchange should be linear single strands and nicked circular heteroduplex DNA (see Fig. 1). Assuming a rate of strand exchange of 2 base pairs/s (Kahn and Radding, 1984), we would expect complete strand exchange involving M13 DNA to require approximately 50 min. However, in the presence of recA protein alone, strand exchange produced only a small amount of nicked circular DNA even 80 min after the start of the reaction. By contrast, in the presence of SSB, but in an otherwise identical reaction, a large fraction of DNA molecules migrated on the gel as nicked circular DNA between 20 and 40 min of the reaction (Fig. 9). In the absence of SSB, DNA products that did not enter the gel accumulated; whereas in the presence of SSB, such products declined. The electrophoretic observations, which confirm earlier reports (Cox and Lehman, 1981; Bianchi and Radding, 1983), correlate the suppression of recA protein-independent networks with the

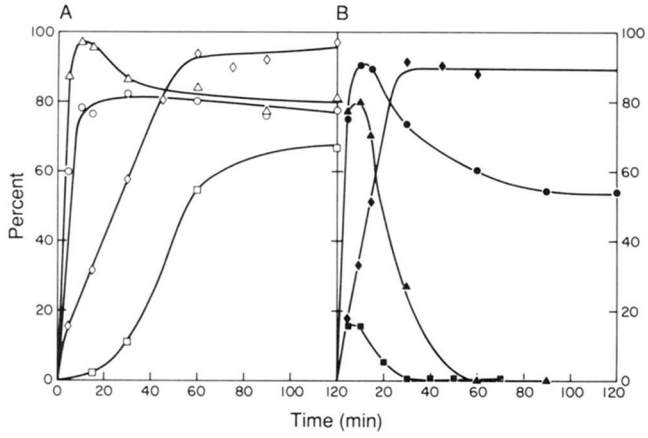

FIG. 8. **Addition of SSB suppressed the persistence of joint molecules and inhibited the formation of the distinct second population of networks whose existence did not require the continued presence of recA protein.** Circular single-stranded G4 DNA (3 μM) was added to a preincubation mixture containing 1 mM $MgCl_2$ and 1.3 mM ATP. recA protein (2 μM) was added to the mixture after it was warmed to 37 °C for 2 min. Buffer (*A*) or 0.19 μM SSB (*B*) was added 1 min after the recA protein. The reaction mixture was incubated for an additional 10 min at 37 °C before the addition of 6 μM linear duplex G4 [^{3}H]DNA and 12 mM $MgCl_2$ to start the pairing reaction. △ and ▲, percent of duplex DNA in networks; ○ and ●, percent of duplex DNA in joint molecules; ◇ and ◆, extent of strand exchange; □ and ■, percent of duplex DNA in protein-independent networks, assayed as described for Fig. 7.

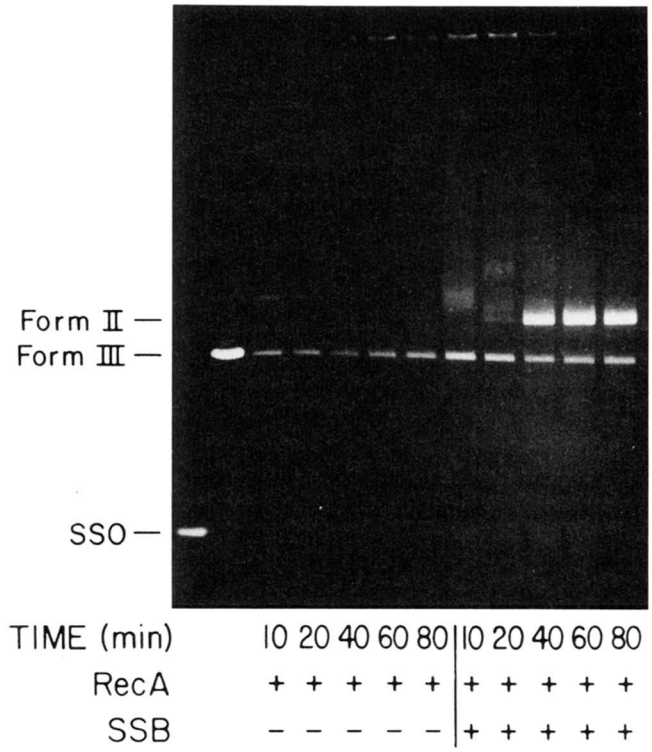

FIG. 9. **Correlation of efficient completion of strand exchange with the suppression by SSB of networks whose persistence is independent of recA protein.** Circular single-stranded DNA of M13 (8 μM) was preincubated with 6 μM recA protein in a reaction mixture containing 1.2 mM $MgCl_2$ for 15 min at 37 °C. Homologous pairing and strand exchange were initiated by the addition of 16 μM linear duplex M13 [^{3}H]DNA and adjustment of the final concentration of $MgCl_2$ to 12 mM. In a separate reaction which included SSB, circular single-stranded DNA (8 μM) was preincubated with 6 μM recA protein and 0.5 μM SSB in the presence of 12 mM $MgCl_2$ for 15 min at 37 °C prior to the addition of 16 μM linear duplex M13 [^{3}H]DNA. At various times after the start of the pairing reaction, 30-μl aliquots of the reaction mixture were taken and mixed with 3 μl of 10% SDS and 4 μl of 0.2 M EDTA. After 20 min on ice, the aliquots were treated with proteinase K (50 μg/ml) for 30 min at 37 °C and then adjusted to 10% (v/v) glycerol, 0.1% (w/v) bromphenol blue, and 0.1% (w/v) xylene cyanol prior to electrophoresis (5 V/cm) in a 0.8% agarose gel at 4 °C for 12 h. *SSO*, circular M13 (viral) single strands; *Form III*, linear M13 duplex DNA; *Form II*, circular M13 duplex DNA containing a nick in one strand.

efficient completion of strand exchange in the presence of SSB.

In addition to SSB, the suppression of networks that survive deproteinization required excess recA protein. On the basis of a stoichiometric binding ratio of 1 molecule of recA protein/3.6 nucleotide residues of single-stranded DNA (Tsang *et al.*, 1985b), complete coating of 3 μM single-stranded DNA should have required no more than 0.9 μM recA protein. We found, however, that in the presence of SSB and 0.9 μM recA protein, both networks and joint molecules remained at high levels unless the concentration of recA protein was increased to 2 μM (data not shown). The initial rates of formation of networks and joint molecules were similar at both concentrations of recA protein, indicating that the differences were related to late events such as reinitiation rather than the initial round of pairing. We observed further that in the absence of SSB, even a 10-fold excess of recA protein was insufficient to suppress the formation of protein-independent networks. These observations are consistent with the reinitiation model (Fig. 4), if we assume that full coating of some intermediate with protein is required to inhibit reinitiation. Evidence in support of this assumption is presented below.

Heteroduplex DNA Formed in the Presence of SSB Is Inactive for Subsequent Pairing with Single-stranded DNA— Since SSB suppresses the phenomena that we attribute to reinitiation of strand exchange (Fig. 4), we reasoned that in its presence, one of the products of exchange, either the displaced single strand or the heteroduplex DNA, might be inactivated. We did the following experiments to examine the ability of both products to participate in a second round of exchange.

A standard reaction containing circular single strands and linear duplex DNA was performed in the presence of recA protein and SSB (Fig. 10). Sixty minutes after the start of the reaction, at which time strand exchange was completed and homologous networks totally disappeared (see Fig. 8*B*), we put additional linear duplex DNA into the reaction mixture. The addition of fresh duplex DNA caused a sharp rise in the formation of both networks and joint molecules (Fig. 10). The results show that in the presence of SSB, the displaced single strand formed at the end of recA-promoted strand exchange is capable of pairing with fresh exogenous homologous duplex DNA.

A similar approach was used to study the pairing ability of the heteroduplex DNA formed in the presence of SSB. Sixty minutes after the start of the pairing reaction, we added circular single-stranded DNA that had been preincubated with recA protein (Fig. 11). No increase in the formation of joint molecules or networks was observed after the addition, which indicates that the heteroduplex DNA formed at the completion of strand exchange in the presence of SSB is incapable of pairing with homologous single-stranded DNA.

Shibata *et al.* (1982) showed that following the formation of D-loops by single-stranded fragments and superhelical DNA, the latter was inactivated by extensive association of recA protein, but could be reactivated by cold treatment, which causes the dissociation of recA protein (see Fig. 5 in this paper). We have previously reported that in the absence

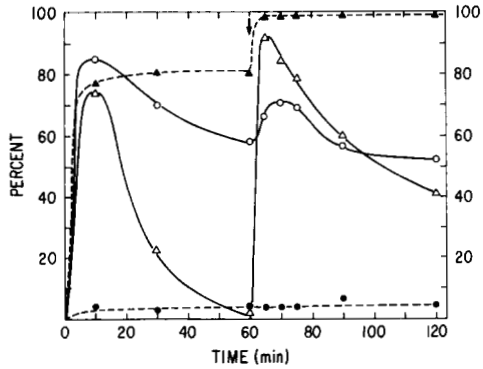

FIG. 10. **Strand displaced by exchange in the presence of SSB is capable of pairing with newly added duplex DNA.** Circular single-stranded DNA (3 μM) of phage G4 (○, △) or phage M13 (●, ▲) was preincubated at 37 °C with 13 mM $MgCl_2$ in the presence of 0.19 μM SSB for 5 min before the addition of 2 μM recA protein. Fifteen minutes later, the pairing reaction was initiated by adding 6 μM linear duplex [^{3}H]DNA of phage G4. Sixty minutes after the start of the reaction (indicated by the *arrow*), another 6 μM linear duplex [^{3}H]DNA of phage G4 was added to the reaction mixture, and the reaction was continued for an additional 60 min. △ and ▲, duplex DNA in networks; ○ and ●, duplex DNA in joint molecules. The percentage values, both before and after addition of extra [^{3}H]DNA at 60 min, are based on total counts/minute present in either phase of the reaction.

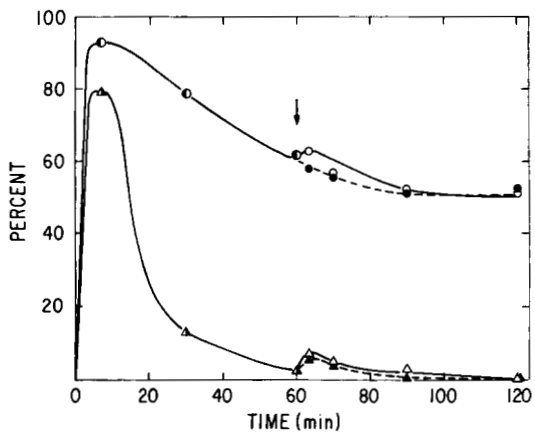

FIG. 11. **Nascent heteroduplex DNA formed by strand exchange in the presence of SSB is incapable of pairing with newly added single-stranded DNA.** Circular single-stranded DNA (3 μM) of phage G4 was preincubated at 37 °C with 13 mM $MgCl_2$ and 0.19 μM SSB for 5 min before the addition of 2 μM recA protein. The preincubation mixture was incubated for an additional 15 min, and the reaction was initiated by adding 6 μM linear duplex [^{3}H]DNA of phage G4. Sixty minutes after the start of the reaction (indicated by the *arrow*), 3 μM circular single-stranded DNA of phage G4 (△, ○) or phage M13 (▲, ●) was added. △ and ▲, duplex DNA in networks; ○ and ●, duplex DNA retained by nitrocellulose filters in the D-loop assay.

of SSB, heteroduplex DNA formed by circular single strands and linear duplex DNA is extensively coated by recA protein (Chow *et al.*, 1986). Pugh and Cox (1987) reported that in the presence of SSB, recA protein remains stably associated with heteroduplex DNA for at least 30 min after strand exchange is complete. To test whether such coating by recA protein, in the presence of SSB, is responsible for inactivation of the heteroduplex DNA described above (Fig. 11), we chilled the inactive reaction products at 60 min after the start of a pairing reaction. We observed that when the temperature was returned to 37 °C after incubation for 15 min at 0 °C, a new cycle of network formation and pairing took place (Fig. 12).

Under our standard reaction conditions at 37 °C, recA

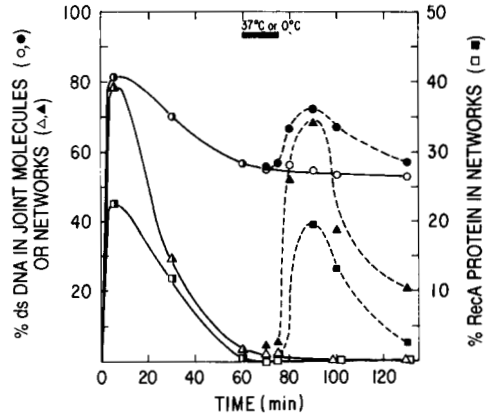

FIG. 12. **Cold treatment reactivates nascent heteroduplex DNA formed by strand exchange in the presence of SSB.** The pairing reaction was initiated by adding 6 μM linear duplex [^{3}H]DNA of phage G4 to an incubation mixture containing 3 μM circular single-stranded G4 DNA, 0.19 μM SSB, and 2 μM ^{35}S-labeled recA protein. Sixty minutes after the start of the reaction, the reaction mixture was divided into two aliquots. One aliquot (▲, ●, ■) was put into a 0 °C ice bath for 15 min (indicated by the *thick bar*) before returning to 37 °C for the remainder of the reaction. The other aliquot (△, ○, □) was incubated at 37 °C throughout the entire experiment. △ and ▲, percent of duplex DNA in networks; ○ and ●, percent of duplex DNA retained by nitrocellulose filters in the D-loop assay; □ and ■, percent of ^{35}S-labeled recA protein in networks. *ds DNA*, double-stranded DNA.

protein binds to single-stranded DNA, but not to double-stranded DNA (Shibata *et al.*, 1979; McEntee *et al.*, 1981; Stasiak *et al.*, 1984; Chow *et al.*, 1986). Hence, we interpret the reactivation described above to result from dissociation of recA protein from both single-stranded DNA and nascent heteroduplex DNA at 0 °C, followed by specific reassociation of recA protein with single-stranded DNA when the reaction mixture was incubated again at 37 °C.

DISCUSSION

These experiments show that when recA protein pairs circular single strands with linear duplex DNA, the population of DNA networks changes from one that is held together by recA protein, but requires no homologous interactions, to one that is held together by homologous interactions, but no longer requires recA protein. This second qualitatively distinct population of networks starts to form within minutes of the start of the reaction and requires specifically that homology be present in the duplex DNA at the end where strand displacement begins. The formation of networks that link joint molecules together correlates with a low yield of the expected final DNA products of the reaction, namely nicked circular heteroduplex DNA and linear single strands.

These observations can all be rationalized by the interpretation that homology-dependent networks result from reinvasion of heteroduplex DNA by the 5′ end of the strands displaced from the original duplex molecules (Fig. 4). SSB and gene 32 protein suppress the formation of homology-dependent networks. This inhibition correlates with faster strand exchange and efficient production of final products. But more significant with regard to the mechanism of formation of homology-dependent networks is the observation that their suppression by SSB is accompanied by the production of inactive heteroduplex molecules that cannot participate in a second round of strand invasion and strand exchange.

The argument that homology-dependent networks result from reinitiation of strand invasion is further supported by

the observation that removal of recA protein from inactive heteroduplex DNA produced in the presence of SSB reactivates the heteroduplex DNA for a second round of pairing with the very same linear single strands displaced in the first round. Thus, we infer that SSB blocks the formation of homology-dependent networks specifically by helping recA protein itself to inactivate one of the substrates required for reinitiation.

To recapitulate, the observations made in the absence of SSB show that the formation of protein-independent networks specifically requires homology at the end of the duplex molecule from which strand displacement begins. No protein-independent networks form when homologous pairing occurs without strand displacement. The observations made in the presence of SSB show that reinitiation of strand exchange is blocked by inactivation of the heteroduplex product by bound protein, whereas the displaced single strand is capable of reinitiating strand exchange.

The failure of heteroduplex DNA coated with recA protein to pair with displaced single strands that were also coated with recA protein supports an earlier suggestion that 2 molecules of DNA that are completely coated with recA protein cannot pair (Tsang *et al.*, 1985a). This conclusion applies equally to a single strand plus a duplex molecule, as described here, or to a pair of complementary single strands (Bryant and Lehman, 1985).

The inactivation of heteroduplex that we observed and the reactivation that resulted from chilling are similar to observations made by Shibata *et al.* (1982) on the inactivation and reactivation of superhelical DNA following the formation of joint molecules by pairing with short single-stranded fragments. The observations, however, differed in one respect, namely that inactivation of non-superhelical DNA in our experiments required the presence of SSB, whereas in the experiments of Shibata *et al.*, the inactivation of closed circular duplex DNA did not require SSB. Roman and Kowalczykowski (1986) have also observed that starting from a circular single strand and a duplex molecule that was 42 base pairs shorter, recA protein, in the presence of SSB, promoted an apparently irreversible reaction as judged by gel electrophoresis. The difference may reflect a property of superhelical DNA.

The present results are also consistent with previous observations on the association of recA protein with heteroduplex DNA following strand exchange. Chow *et al.* (1986) observed that although recA protein largely remained attached to the incoming single strand after the latter was incorporated into heteroduplex DNA, the proximal end of the heteroduplex region tended to lose recA protein. Such an uncoated heteroduplex region could provide the target for reinvasion by a displaced strand coated with recA protein. On the other hand, in studies done in the presence of SSB, Pugh and Cox (1987) observed stable association of recA protein with the heteroduplex product of strand exchange, an observation that is in obvious agreement with the finding that in the presence of SSB, the heteroduplex product is resistant to reinvasion.

The literature on strand exchange promoted by recA protein contains divergent interpretations of the timing of strand displacement and the intermediates involved. On the basis of electron microscopic observations of fixed molecules and a model of strand exchange, Stasiak *et al.* (1984) proposed that a three-stranded intermediate persists until late in the reaction. The findings described here, on the other hand, suggest that in solution, at least the proximal end of the noncomplementary strand of the recipient duplex molecule is displaced, coated with recA protein, and active in homologous pairing within a few minutes of the onset of the reaction.

The precise way in which SSB suppresses reinitiation is not yet clear. Surprisingly, SSB appears in these experiments to favor the coating of heteroduplex DNA by recA protein, but further work is required to determine the location of recA protein and SSB on inactivated heteroduplex DNA. These observations, however, bear one striking similarity to earlier observations on the effects of SSB on the interaction of recA protein with single-stranded DNA, namely that specific protein-protein interactions are not involved since gene 32 protein, which is genetically unrelated, suppresses reinitiation just as well as SSB (Shibata *et al.*, 1980; Egner *et al.*, 1987).

The effects of SSB on the recombination activities of recA protein are multiple and complex, including effects on the formation and stability of nucleoprotein filaments and a 2–5-fold stimulation in the rate of strand exchange. By removing secondary structure from single-stranded DNA, SSB favors the binding of recA protein (Kowalczykowski *et al.*, 1987; Kowalczykowski and Krupp, 1987; Morrical *et al.*, 1986; Muniyappa *et al.*, 1984; Tsang *et al.*, 1985b). The nucleoprotein filament formed on single-stranded DNA by recA protein in the presence of SSB is more stable (Cox and Lehman, 1982; Cox *et al.*, 1983a) and contains SSB.[2] However, when formed in the absence of SSB under conditions that minimize secondary structure in single strands, recA nucleoprotein filaments were as stable (Kowalczykowski *et al.*, 1987) and nearly as active with respect to the initial rate of homologous pairing as those made in the presence of SSB (Tsang *et al.*, 1985b).

SSB also stimulates the rate of strand exchange, a distinct final phase of the recombination reaction promoted by recA protein. Cox *et al.* (1983a) attributed the stimulatory effect of SSB on strand exchange to stabilization of the recA filament. Kahn and Radding (1984) suggested that SSB might play another distinct role in strand exchange since optimization of the conditions for the formation of recA nucleoprotein filaments did not accelerate strand exchange, whereas addition of SSB did so. Our experiments indeed suggest additional explanations for the effect of SSB on strand exchange, namely that by inhibiting reinitiation, SSB blocks the actual reversal of strand exchange and thus enhances the apparent forward rate. Furthermore, by suppressing the formation of intermolecular connections, SSB may alleviate topological or steric barriers to the propagation of strand exchange.

From the beginning of studies on the pairing and strand exchange activities of recA protein, a recurring quandary has been posed by the apparent lack of a reverse reaction, namely the reinvasion of heteroduplex DNA by the displaced strand. The experiments described here suggest that the strand exchange reaction does indeed reverse itself when recA protein acts alone; whereas in the presence of SSB, the nature of the nucleoprotein filament that remains on heteroduplex DNA blocks that reversal.

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