Kinetic Modeling of the RecA Protein Promoted Renaturation of Complementary DNA Strands†

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ABSTRACT: Quantitative agarose gel assays reveal that the recA protein promoted renaturation of complementary DNA strands (ϕX DNA) proceeds in two stages. The first stage results in the formation of unit-length duplex DNA as well as a distribution of other products ("initial products"). In the second stage, the initial products are converted to complex multipaired DNA structures ("network DNA"). In the presence of ATP, the initial products are formed within 2 min and are then rapidly converted to network DNA. In the absence of ATP, the initial products are formed nearly as fast as with ATP present, but they are converted to network DNA at a much lower rate. The time-dependent formation of initial products and network DNA from complementary single strands for both the ATP-stimulated and ATP-independent reactions can be modeled by using a simple two-step sequential kinetic scheme. This model indicates that the primary effect of ATP in the recA protein promoted renaturation reaction is not on the initial pairing step (which leads to the formation of initial products) but rather is to increase the rate at which subsequent pairing events can occur.

The recA protein of *Escherichia coli* is essential for homologous recombination and for the postreplicative repair of damage to DNA. The purified recA protein is a single-stranded DNA-dependent ATPase and will promote a variety of ATP-dependent DNA pairing reactions that presumably reflect in vivo functions. These reactions include the assimilation of linear single strands into duplex DNA (D-loop formation) and the reciprocal exchange of strands between linear duplex and homologous circular single-stranded DNA (Radding, 1982). The molecular mechanisms of these DNA pairing reactions are not known.

The renaturation of complementary DNA strands is the simplest DNA pairing activity associated with the recA protein (Weinstock et al., 1979). As such, it is an attractive model reaction for the analysis of the recA protein dependent alignment of homologous DNA sequences. In previous work, we examined the interaction of recA protein with (+) circular bacteriophage ϕX174 single-stranded DNA (ϕX ssDNA)[1] in the absence of its complementary partner (Bryant et al., 1985). We also carried out an analysis of the recA protein promoted renaturation of the linear complementary (+) and (−) strands of ϕX DNA (Bryant & Lehman, 1985). These studies established the following: (1) optimal renaturation occurs at a ratio of 1 recA monomer/30 nucleotides of ssDNA, a level of recA protein sufficient to cover about 15% of the ssDNA; (2) although recA protein promoted renaturation is stimulated by ATP, a significant reaction occurs in the absence of ATP; (3) the ATP-stimulated reaction follows apparent first-order kinetics, indicating that the reaction mechanism involves the formation of intermediate recA–ssDNA complexes prior to renaturation of the complementary strands. The experiments described in this paper were designed to further explore the mechanism of the recA protein promoted renaturation reaction.

Experimental Procedures

Materials

RecA protein was purified to homogeneity as described (Cox et al., 1981). RecA protein concentrations were calculated by using the extinction coefficient 0.59 A_{280} mg^{-1} mL (Craig & Roberts, 1981). S1 nuclease and calf thymus DNA were from Sigma. GF/C filters were from Whatman.

Unlabeled and ^{3}H-labeled (+) circular ϕX ssDNAs were prepared as previously described (Cox & Lehman, 1981). Unlabeled linear duplex ϕX DNA (form III ϕX DNA) was prepared by either *Pst*I or *Xho*I cleavage of circular duplex ϕX DNA as previously described (Cox & Lehman, 1981). ^{3}H-Labeled linear duplex ϕX DNA (*Pst*I cleaved) was prepared as previously described (Cox & Lehman, 1981). ^{32}P-Labeled linear duplex ϕX DNA (*Xho*I cleaved) was prepared by using the T4 polynucleotide kinase exchange reaction and [γ-^{32}P]ATP as described (Maniatis et al., 1982). Linear duplex ϕX DNA was denatured to the complementary single strands by heating at 100 °C for 3–5 min. Alternatively, in order to prepare the separated (+) and (−) linear strands, linear duplex ϕX DNA was denatured by addition of NaOH to a concentration of 0.1 M. The alkaline denaturation mixture was then loaded onto an agarose gel (0.8%) and subjected to electrophoresis in the presence of ethidium bromide (0.5 μg/mL). The separated strands were recovered from the gel by electroelution (Maniatis et al., 1982), and the identity of each strand was determined by its reactivity with (+) circular ϕX DNA in a recA protein promoted renaturation reaction. The slower migrating strand on agarose gels reacted with (+) circular ssDNA in the presence of recA protein to yield form II DNA and therefore was identified as the complementary (−) linear strand (see Figures 4 and 5). The faster migrating strand was unreactive with (+) circular ssDNA and was therefore identified as the (+) linear strand (gel not shown). DNA concentrations were calculated by using an A_{260} of 1 as equivalent to 36 μg/mL ssDNA and 50 μg/mL duplex DNA. All DNA concentrations are expressed as total nucleotides.

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[1] Abbreviations: ssDNA, single-stranded DNA; ϕX, bacteriophage ϕX174; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); SDS, sodium dodecyl sulfate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid.

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Methods

DNA Renaturation Assays. Renaturation reaction solutions contained 25 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, and 5% glycerol. Concentrations of DNA, recA protein, and nucleotide cofactor are given in the figure legends. All renaturation reactions were carried out at 25 °C.

S1 nuclease assays were carried out as previously described (Bryant & Lehman, 1985). The renaturation of (+) and (−) linear strands was measured by using denatured ^{3}H-labeled linear duplex ϕX DNA; the renaturation of (+) circular and (−) linear single strands was measured by using ^{3}H-labeled (+) circular ϕX ssDNA and unlabeled (−) linear ϕX ssDNA. We have previously shown that the recA protein promoted renaturation reaction results in the formation of final renaturation products that are 75% resistant to S1 nuclease digestion (Bryant & Lehman, 1985). The agarose gel assays described in this paper show that the formation of the 75% S1 nuclease resistant products corresponds to the complete disappearance of the complementary single strands (Figure 1a). Therefore, the measured S1 nuclease resistance of the DNA in renaturation reaction mixtures at various time points was normalized relative to a maximum possible nuclease resistance of 75% in order to express the percentage of the DNA that had undergone renaturation.

Agarose gel assays were carried out as follows. Aliquots (30 μL) were removed from renaturation reaction mixtures at the indicated times and quenched as indicated in the appropriate figure legend. The samples were then subjected to electrophoresis on 0.8% agarose gels (containing 0.5 μg/mL ethidium bromide) using a Tris–acetate–EDTA buffer system (Maniatis et al., 1982). The gels were photographed under UV irradiation using Polaroid type 55 Pos/Neg film.

In the quantitative agarose gel assays, the photographic negatives were scanned by using a Quick Scan R&D densitometer (Helena Laboratories) coupled to an Epson LQ-1500 plotter with a Nelson Analytical 760 series interface. Peaks corresponding to single-stranded DNA, form III DNA, and network DNA were quantitated by comparison to appropriate standard curves. Single-stranded DNA, form III DNA, and network DNA standard curves were derived from densitometric analyses of gels containing various known amounts of the appropriate DNAs. The network DNA used in the network DNA standard curve was prepared from a known amount of single-stranded DNA using the ATP-stimulated renaturation reaction (15-min incubation to ensure the complete conversion of the single-stranded DNA to network DNA; see Figure 3); network DNA refers specifically to the renaturation products that do not migrate out of the wells of the agarose gels.

Results

Analysis of RecA Protein Promoted Renaturation by Agarose Gel Electrophoresis. In our previous studies, the recA protein promoted renaturation reaction was monitored by measuring the conversion of the S1 nuclease susceptible complementary (+) and (−) linear strands of *Pst*I-cleaved ϕX DNA to an S1 nuclease resistant duplex form (Bryant & Lehman, 1985). Although this assay allows the overall rate of renaturation to be determined, it provides only limited information about the nature of the renaturation reaction products or the possible occurrence of intermediates along the reaction pathway. We have therefore investigated the recA protein promoted renaturation of (+) and (−) linear ϕX single strands by agarose gel electrophoresis. In our agarose gel assays, we use densitometry to quantitate the time-dependent disappearance of the complementary ϕX single strands; renaturation time courses are then constructed by subtracting the amount of single-stranded DNA remaining at various time points from the amount of single-stranded DNA present at the beginning of the reaction. The renaturation reactions were carried out at 1 μM recA protein and 30 μM ϕX ssDNA [15 μM each of the (+) and (−) linear strands], conditions previously shown to be optimal for both the ATP-independent and ATP-stimulated renaturation reactions (Bryant & Lehman, 1985).

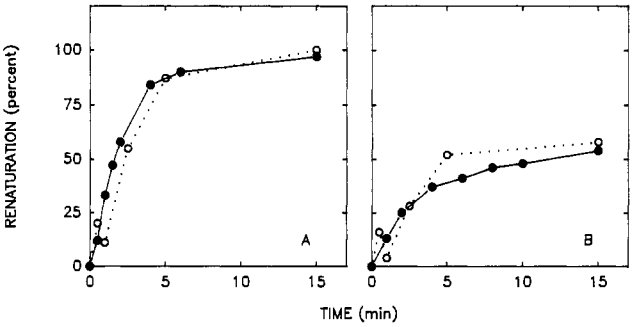


FIGURE 1: Comparison of the S1 nuclease and agarose gel assays of recA protein promoted renaturation. Reactions were carried out as described under Experimental Procedures. Reaction solutions contained 30 μM denatured ϕX DNA [15 μM each of the (+) and (−) linear strands], 1 μM recA protein, and either 500 μM ATP (panel A) or 0 μM ATP (panel B). RecA protein was added last to initiate the reactions. At the times indicated, aliquots were quenched with SDS (1%) and assayed by (●) the S1 nuclease assay or (○) the agarose gel assay. The points (●) represent the relative amount of denatured ϕX DNA that has been converted to a S1 nuclease resistant (duplex) form. The points (○) represent the relative amount of denatured ϕX DNA that has undergone renaturation; these values were obtained from agarose gel assays by subtracting the amount of single-stranded DNA remaining at each time point (as determined by densitometry) from the amount of single-stranded DNA present at the beginning of the reaction.

The time course of the ATP-stimulated renaturation reaction (500 μM ATP) is shown in Figure 1, panel A. The renaturation time course that was determined from the disappearance of complementary single strands in the agarose gel assay agrees well with the time course that was determined from the appearance of nuclease-resistant DNA in the S1 nuclease assay. Both assays indicate that the ATP-stimulated renaturation reaction follows apparent first-order kinetics with a half-time of about 2 min.

The time course of the ATP-independent renaturation reaction is shown in Figure 1, panel B. Again, the renaturation time course that was determined by using the agarose gel assay agrees well with the time course that was determined by using the S1 nuclease assay. In the absence of ATP, the renaturation reaction exhibits biphasic kinetics; the initial rate of renaturation is about 2-fold lower than that measured in the presence of ATP.

No renaturation was detected in the absence of recA protein (with or without ATP) by either the agarose gel assay or the S1 nuclease assay.

Time-Dependent Formation of Renaturation Reaction Products. The agarose gel assays described above measure renaturation as the disappearance of single-stranded ϕX DNA. The time-dependent formation of the products of the ATP-stimulated and ATP-independent renaturation reactions were also measured by using the agarose gel assay.

An agarose gel showing the time course of the ATP-stimulated renaturation reaction is presented in Figure 2A. The (+) and (−) linear strands are well-separated on the gel and decrease concurrently throughout the reaction time course. Initially, unit-length linear duplex ϕX DNA (form III DNA)

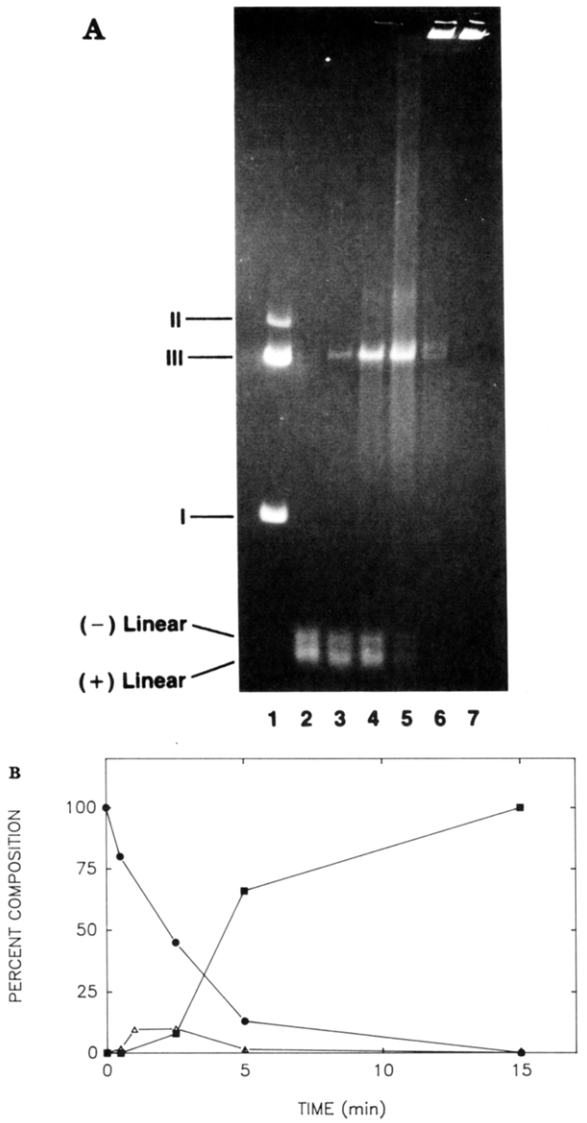


FIGURE 2: Agarose gel assay of the ATP-stimulated renaturation reaction. Reactions were carried out as described under Experimental Procedures. Reaction solutions contained 30 μM denatured linear ϕX DNA [15 μM each of the (+) and (−) linear strands], 1 μM recA protein, and 500 μM ATP. RecA protein was added last to initiate the reaction. At the indicated times, aliquots were quenched with SDS (1%) and then subjected to agarose gel electrophoresis. (A) Lane 1 contains form I, form II, and form III ϕX DNA markers; lanes 2–7 are 0-, 0.5-, 1.0-, 2.5-, 5-, and 15-min reaction time points. (B) A photographic negative of the gel shown in (A) was analyzed by densitometry, and the relative amounts of single-stranded DNA (●), form III DNA (△), and network DNA (■) are plotted as a function of reaction time.

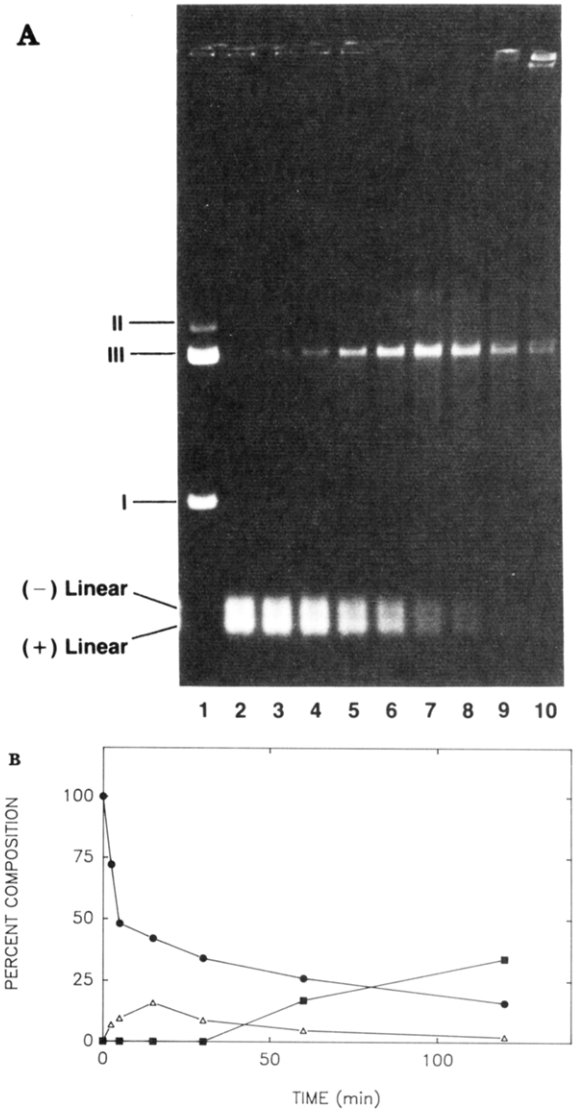


FIGURE 3: Agarose gel assay of the ATP-independent renaturation reaction. Reactions were carried out as described under Experimental Procedures. Reaction solutions contained 30 μM denatured linear ϕX DNA [15 μM each of the (+) and (−) linear strands], 1 μM recA protein, and no ATP. RecA protein was added last to initiate the reaction. At the indicated times, aliquots were quenched with SDS (1%) and then subjected to agarose gel electrophoresis. (A) Lane 1 contains form I, form II, and form III ϕX DNA markers; lanes 2–10 are 0-, 0.5-, 1.0-, 2.5-, 5-, 15-, 30-, 60-, and 120-min reaction time points. (B) A photographic negative of the gel shown in (A) was analyzed by densitometry, and the relative amounts of single-stranded DNA (●), form III DNA (△), and network DNA (■) are plotted as a function of reaction time.

as well as a distribution of other renaturation products (which appear as a smear on the gel) are formed; these products will be referred to as "initial products". After 5 min, these initial products are completely converted to a form that does not migrate from the gel well. We have previously shown that these final products correspond to complex networks of multipaired DNA molecules (Bryant & Lehman, 1985); these products will be referred to as "network DNA". The relative amounts of single-stranded DNA, form III DNA, and network DNA at each time point were determined by densitometry and are plotted as a function of reaction time in Figure 2B.

An agarose gel showing the time course of the ATP-independent renaturation reaction is presented in Figure 3A. Again, the (+) and (−) linear strands decrease concurrently throughout the reaction as form III DNA and a distribution of other initial products are formed. In contrast with the ATP-stimulated reaction, these initial products are only slowly converted to network DNA; network DNA is detectable only after 60 min, and even after 120 min, a significant amount of initial products remains. The relative amounts of single-stranded DNA, form III DNA, and network DNA in the ATP-independent reaction are plotted as a function of reaction time in Figure 3B.

In the initial stage of both the ATP-stimulated and ATP-independent renaturation reactions (i.e., before network DNA appears), form III DNA accounts for about 20–30% of the single-stranded DNA that has undergone renaturation (Figures 2B and 3B). The remainder of the renatured single strands must therefore be accounted for by the distribution of other renaturation products that appears simultaneously with the form III DNA during this time period.

Inactivity of Form III DNA in RecA Protein Promoted

Network Formation. The gel assays described in the previous section show that both the ATP-stimulated and the ATP-independent renaturation reactions lead to the formation of form III DNA as the major initial renaturation product and that this form III DNA is subsequently converted to network DNA. The following experiments were performed in order to determine whether the recA protein can directly convert form III DNA to network DNA.

First, form III ϕX DNA (30 μM) was incubated with recA protein (1 μM) both in the absence and in the presence of ATP (500 μM), and the mixtures were analyzed at various times by agarose gel electrophoresis. No change in the form III DNA was apparent after 60 min (plus ATP) or 120 min (minus ATP) of incubation (gels not shown). This indicates that recA protein cannot directly convert free form III ϕX DNA into network DNA during the time course of the renaturation reaction.

Next, form III ϕX DNA (15 μM) was incubated with recA protein (1 μM) in the presence of purified (−) linear ϕX ssDNA (15 μM). No change in either the form III DNA or the (−) linear ssDNA was apparent after 15 min (plus ATP) or 120 min (minus ATP) of incubation (gels not shown). This indicates that recA protein cannot directly combine free form III DNA with single-stranded DNA to form network DNA during the time course of the renaturation reaction.

In a third experiment, ϕX ssDNA [15 μM each of the (+) and (−) linear strands] was incubated with recA protein (1 μM) in the presence of a small amount (1.5 μM) of ^{32}P-labeled form III ϕX DNA. Agarose gel assays showed that the single-stranded DNA was largely converted to network DNA, both in the presence of ATP (60-min incubation) and in the absence of ATP (120-min incubation). However, an autoradiogram of the gels showed that all of the radiolabel remained as form III DNA; no label was detected in network DNA (gels not shown). This indicates that recA protein cannot convert free form III DNA to network DNA during an ongoing renaturation reaction.

RecA Protein Inactivation/Reactivation Experiments. The experiments described in the previous section demonstrate that recA protein cannot convert free form III DNA into network DNA under the conditions of our renaturation reactions. This indicates that free form III DNA is not an intermediate in the recA protein promoted renaturation reaction and that the form III DNA that is observed in our agarose gel assays is derived from some sort of recA–form III DNA complex (since our renaturation time points were obtained by first quenching aliquots of the reaction solutions with EDTA/SDS to inactivate the recA protein, it is possible that the renaturation products that are observed in our agarose gel assays are not fully formed until after the recA protein is removed). Alternatively, it is conceivable that the form III DNA that is produced during the renaturation reaction does exist free in solution but has local imperfections or single-stranded regions (that are not present in our form III DNA standard) that allow the recA protein to rebind and carry out subsequent network formation.

In order to distinguish between these possibilities, we carried out a series of experiments in which we inactivated the recA protein during an ongoing renaturation reaction by adding sufficient EDTA to complex all of the Mg^{2+} in the reaction solution (we have previously shown that the binding of recA protein to DNA requires Mg^{2+}, as measured by nitrocellulose filter binding, and that the addition of EDTA will cause the dissociation of any recA–DNA complexes). We then reactivated the DNA binding activity of the recA protein by adding additional Mg^{2+} to the reaction solution. If free form III DNA is a viable intermediate in the renaturation reaction, then the inactivation and reactivation of the DNA binding activity of the recA protein should have no effect on the conversion of the form III DNA to network DNA. If, on the other hand, a recA–form III DNA complex is an obligatory intermediate in the renaturation reaction, then the dissociation of these complexes will result in free form III DNA that cannot be converted to network DNA, even after the addition of more Mg^{2+}. The results of these studies are presented in Figure 4 and are described below.

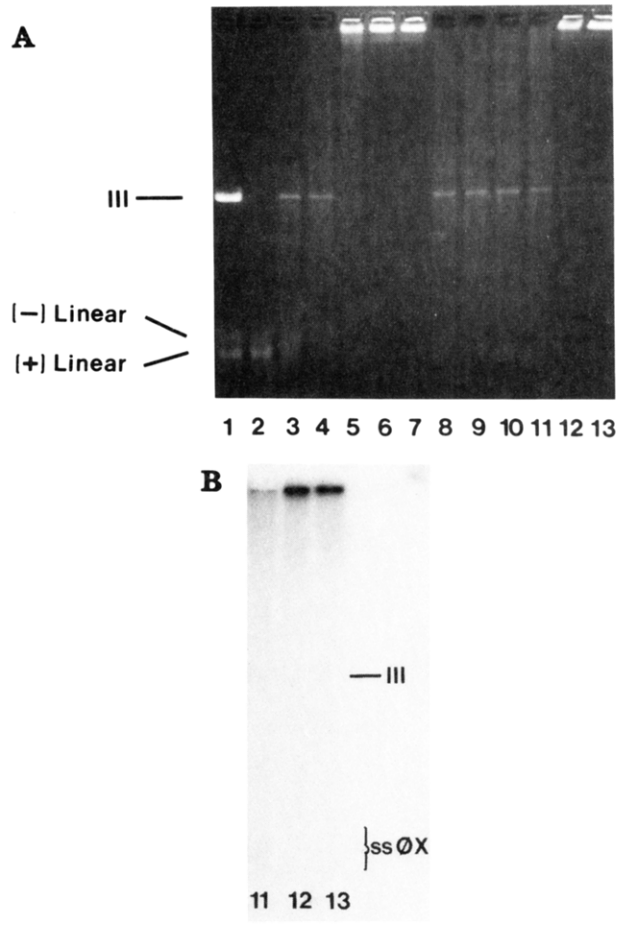


FIGURE 4: Inactivation/reactivation of recA protein during an ongoing renaturation reaction. (A) Reactions were carried out as described under Experimental Procedures. Reaction solutions contained 30 μM denatured linear ϕX DNA [15 μM each of the (+) and (−) linear strands, *Xho*I cleaved] and 1 μM recA protein; recA protein was added last to initiate the reactions. At the indicated times, aliquots were quenched with EDTA (25 mM) and subjected to agarose gel electrophoresis; lane 1 contains ss ϕX DNA and form III ϕX DNA standards; lanes 2–4 are 0-, 5-, and 15-min time points following the addition of recA protein. After the initial 15-min incubation, the reaction solution was divided into three portions, and the indicated additions were made: lanes 5–7 are 5-, 15-, and 45-min time points following the addition of 500 μM ATP; lanes 8–10 are 5-, 15-, and 45-min time points following the addition of 10 mM EDTA and 500 μM ATP; lanes 11–13 are 5-, 15-, and 45-min time points following the addition of 10 mM EDTA, 500 μM ATP, 1.5 μM ^{32}P-labeled denatured linear ϕX DNA, and 10 mM $MgCl_2$, in that order. (B) Autoradiograph of lanes 11–13. Aliquots corresponding to the time points shown in lanes 11–13 in (A) were electrophoresed on a thin (0.4-cm) agarose gel. An autoradiograph was then prepared by drying the gel onto Whatman 3MM chromatography paper followed by exposure to Kodak XAR-5 film.

In the first reaction, ϕX ssDNA [15 μM each of the (+) and (−) linear strands] was incubated with recA protein (1 μM). After 15 min, ATP (500 μM) was added, and the incubation was continued for an additional 45 min. The

agarose gel assay shows that some unreacted single-stranded DNA as well as a substantial amount of form III DNA (but no network DNA) is present after the initial 15-min incubation (Figure 4A, lanes 2–4) and that the addition of ATP at this point results in the rapid and complete conversion of both the unreacted single-stranded DNA and the form III DNA to network DNA (Figure 4A, lanes 5–7).

In the second reaction, ϕX ssDNA [15 μM each of the (+) and (−) linear strands] was incubated with recA protein (1 μM). After 15 min, EDTA (10 mM) was added to complex all of the Mg^{2+} present in the reaction mixture (10 mM). ATP (500 μM) was then added, and the incubation was continued for an additional 45 min. The agarose gel assay shows that the addition of EDTA completely prevents any further renaturation reaction (Figure 4A, lanes 8–10).

In the third reaction, ϕX ssDNA [15 μM each of the (+) and (−) linear strands] was incubated with recA protein (1 μM). After 15 min, EDTA (10 mM) was added to stop the reaction. ATP (500 μM), $MgCl_2$ (10 mM), and a small amount of radiolabeled ϕX ssDNA (0.75 μM each strand) were added, and the incubation was continued for an additional 45 min. The agarose gel assay of this reaction shows that this sequence results in the complete disappearance of the single-strand DNA and the formation of network DNA (Figure 4A, lanes 11–13). Thus, the addition of Mg^{2+} to the EDTA-quenched reaction results in the reactivation of the renaturation activity of the recA protein. Unlike in the first reaction, however, a substantial amount of form III DNA persists and is not converted to network DNA (Figure 4A, lanes 11–13). An autoradiogram of this gel showed that the radiolabeled ssDNA (which was added after the EDTA quench) had been completely converted to network DNA; no radiolabel was detected in the form III DNA band (Figure 4B). This result shows that the dissociated/reassociated recA protein can efficiently convert the unreacted ssDNA to network DNA but that the form III DNA that was formed prior to the dissociation step remains unreactive toward further conversion to network DNA.

Analogous results were obtained in a second series of experiments in which only Mg^{2+} (no ATP) was added to the EDTA-quenched renaturation reaction mixtures; the unreacted ssDNA was completely converted to network DNA by the reactivated recA protein, but the form III DNA formed prior to the inactivation step remained unreactive toward further conversion to network DNA (gels not shown).

These results indicate that both the ATP-independent and the ATP-stimulated conversion of form III DNA to network DNA requires a continuing association between the form III DNA and recA protein and that free form III DNA is not a viable intermediate in either reaction. However, these experiments cannot completely rule out the possibility that imperfect form III like DNA is an intermediate in the renaturation reaction, if these imperfections are dissipated in the absence of Mg^{2+}.

Renaturation of (+) *Circular and* (−) *Linear* ϕ*X Single Strands.* The recA protein promoted renaturation of (+) circular ϕX ssDNA and (−) linear ϕX ssDNA was also examined. The conditions employed were those previously determined to be optimal for the renaturation of (+) and (−) linear strands. The DNA concentration was 30 μM [15 μM each for the (+) circular and (−) linear strands], and the recA protein concentration was 1 μM, giving the optimal ratio of 1 recA monomer/30 nucleotides of ssDNA (Bryant & Lehman, 1985). Reactions were initiated by adding recA protein to a mixture of the (+) circular and (−) linear strands and were carried out in the presence and absence of ATP. The renaturation reactions were monitored by using the S1 nuclease assay (Figure 5).

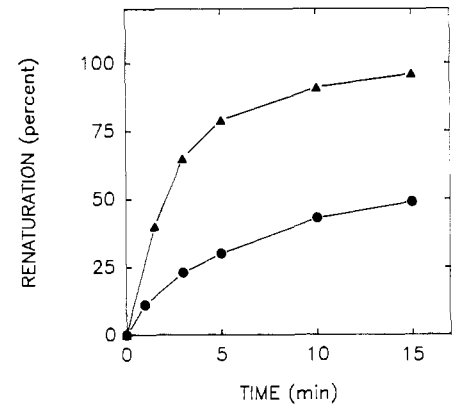


FIGURE 5: Renaturation of (+) circular and (−) linear ϕX single strands by the recA protein. Reactions were carried out as described under Experimental Procedures. Reaction solutions contained 15 μM ^{3}H-labeled (+) circular ϕX ssDNA, 15 μM (−) linear ϕX ssDNA, 1 μM recA protein, and either 500 μM ATP (▲) or 0 μM ATP (●). All components except recA protein were preincubated for 5 min before addition of the recA protein. At the indicated times, aliquots were quenched with SDS and analyzed by using the S1 nuclease assay.

RecA protein promoted the rapid renaturation of (+) circular and (−) linear strands in the presence of ATP (500 μM). Renaturation also occurred in the absence of ATP, but at an initial rate that was 2–3-fold lower than measured in its presence (Figure 5). No renaturation was detected in either case in the absence of recA protein. These reaction time courses are similar to those determined for the recA protein promoted renaturation of (+) and (−) linear strands (see Figure 1).

The products of these renaturation reactions were analyzed by agarose gel electrophoresis. After 15 min, the ATP-independent reaction yielded nicked circular duplex DNA (form II) as the major renaturation product (gels not shown). The formation of form II DNA in this reaction is analogous to the formation of form III DNA in the renaturation of (+) and (−) linear strands. This result supports our conclusion that the prominent band that appears in the early time points of our various renaturation reactions is indeed unit-length duplex DNA and not some other DNA structure that happens to comigrate with unit-length DNA.

Discussion

Quantitative agarose gel assays reveal that the recA protein promoted renaturation reaction proceeds in two stages. The first stage results in the formation of unit-length duplex DNA as well as a distribution of other renaturation products; these products will be referred to, collectively, as "initial products". In the second stage, the initial products are converted to complex multipaired DNA structures that do not migrate out of the gel wells; these final products will be referred to as "network DNA".

In order to analyze the kinetics for the formation of initial products and network DNA, the data in Figures 3 and 2 were replotted to show the time-dependent formation of the total population of initial products (Figures 6A and 7A). In these replots, all renaturation products, except network DNA, were considered to be initial products; the curves showing the relative amounts of initial products were constructed by subtracting the amount of single-stranded DNA and network DNA at each time point (as determined by densitometry) from the total amount of DNA.

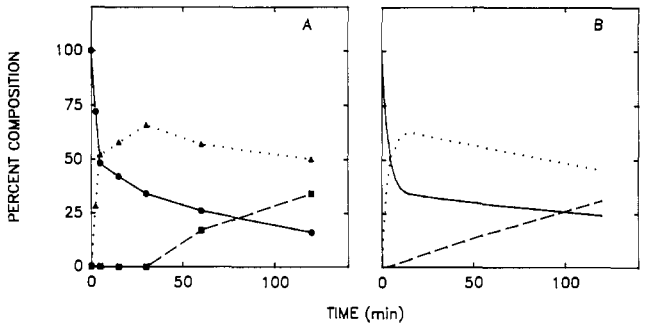


FIGURE 6: Simulation of the ATP-independent renaturation reaction using Scheme I. (A) The experimental curves showing relative amounts of single-stranded DNA (●) and network DNA (■) as a function of time were obtained directly from the agarose gel data in Figure 3. The curve showing the relative amounts of initial products (▲) was calculated by subtracting the amount of single-stranded DNA and network DNA at each time point from the total amount of DNA. (B) The simulated curves showing the relative amounts of single-stranded DNA (—), initial products (···), and network DNA (– –) as a function of time were calculated from Scheme I by using the parameters $k_1 = 3.3 \times 10^{-3}$ s^{-1}, $k_{-1} = 1.7 \times 10^{-3}$ s^{-1}, and $k_2 = 8 \times 10^{-5}$ s^{-1}. The general equations for the two-step sequential reaction are given in Moore and Pearson (1981).

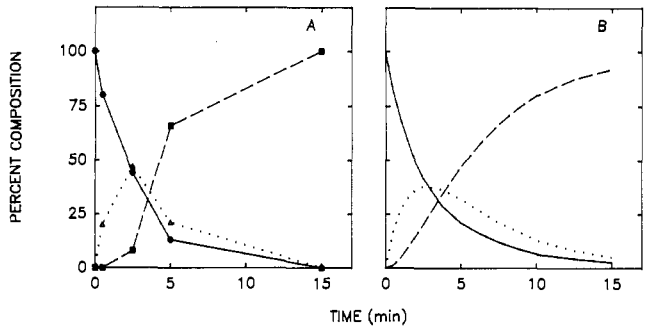


FIGURE 7: Simulation of the ATP-stimulated renaturation reaction using Scheme I. (A) The experimental curves showing the relative amounts of single-stranded DNA (●) and network DNA (■) as a function of time were obtained directly from the agarose gel data in Figure 2. The curve showing the relative amounts of initial products (▲) was calculated by subtracting the amount of single-stranded DNA and network DNA at each time point from the total amount of DNA. (B) The simulated curves showing the relative amounts of single-stranded DNA (—), initial products (···), and network DNA (– –) as a function of time were calculated from Scheme I by using the parameters $k_1 = 6.6 \times 10^{-3}$ s^{-1}, $k_{-1} = 1.7 \times 10^{-3}$ s^{-1}, and $k_2 = 5 \times 10^{-3}$ s^{-1}. The general equations for the two-step sequential reaction are given in Moore and Pearson (1981).

We point out that our definitions of initial products and network DNA are operational definitions that we have introduced to facilitate the kinetic analysis. In fact, many of the initial product molecules may be more complex than simple linear duplex form III molecules and may actually be networklike in structure. However, the appearance and disappearance of the form III products parallel the appearance and disappearance of the total population of initial products (compare Figure 2B with Figure 7A, Figure 3B with Figure 6A). This suggests that the form III products and the other initial products are formed in a common step and that the entire population of initial products behaves as a single kinetic species in the subsequent conversion to network DNA. The successful modeling of both the ATP-stimulated and the ATP-independent renaturation reactions by the simple two-step sequential reaction scheme discussed below supports this interpretation.

The time courses for both the ATP-independent and the ATP-stimulated renaturation reaction can be modeled with the two-step sequential kinetic mechanism shown in Scheme I. In Scheme I, ss represents the (+) and (−) single strands, I represents the total population of initial products (including form III DNA), and N represents the network DNA.

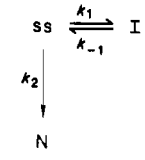
Scheme I

$$\text{ss} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{I} \xrightarrow{k_2} \text{N}$$

The time course for the ATP-independent renaturation reaction (Figure 6A) can be modeled with Scheme I and the parameters $k_1 = 3.3 \times 10^{-3}$ s^{-1}, $k_{-1} = 1.7 \times 10^{-3}$ s^{-1}, and $k_2 = 8 \times 10^{-5}$ s^{-1} (Figure 6B). The ATP-stimulated renaturation reaction time course (Figure 7A) can be modeled with Scheme I and the parameters $k_1 = 6.6 \times 10^{-3}$ s^{-1}, $k_{-1} = 1.7 \times 10^{-3}$ s^{-1}, and $k_2 = 5 \times 10^{-3}$ s^{-1} (Figure 7B).[2] The relative values for k_1 indicate that the formation of initial products is only about 2-fold faster in the presence of ATP than in its absence. The relative values for k_2, on the other hand, indicate that the conversion of initial products to network DNA is 65-fold faster in the presence of ATP than in the absence. Thus, the primary effect of ATP in the recA protein promoted renaturation reaction is not on the formation of initial products but rather is to increase the rate of formation of network DNA.

The modeling of the recA protein promoted renaturation reactions as a two-step sequential reaction is undoubtedly an oversimplification; the initial products are probably not converted to network DNA in a single step. Even so, the fit of our experimental data to the theoretical curves predicted by the two-step model is quite reasonable. The only significant discrepancy between the experimental and theoretical curves is in the formation of network DNA in the ATP-independent reaction; the experimental curve has a more pronounced lag than is accounted for by the two-step model. This discrepancy is probably due to the limitations of the two-step model. If the conversion of initial products to network DNA occurs in more than one step, and if the products formed in the intermediate steps migrate from the gel wells, then our experimental measurements of network DNA (defined strictly as the products that do not migrate from the gel well) will underestimate the amount of products formed in steps subsequent to the formation of initial products. This would result in a longer lag in the early portion of the experimental time course for the formation of network DNA than is predicted by the two-step model.

Scheme II



A second model that we considered in analyzing our kinetic data is shown in Scheme II. In this model, the initial products and network DNA are formed from ssDNA in competing pathways rather than on a sequential pathway. If the formation of initial products is reversible and the formation of network DNA is irreversible, then this second model can account for the transient appearance of initial products during the renaturation reaction. However, this second model predicts that the rate of formation of network DNA will be at a maximum at the beginning of the reaction (when the con-

[2] The value $k_{-1} = 1.7 \times 10^{-3}$ s^{-1} was used to simulate both the ATP-independent and the ATP-stimulated reaction. This value is essential for the ATP-independent reaction simulation because it determines the shape of the biphasic curve. The ATP-stimulated reaction, however, can be satisfactorily simulated by using only the k_1 and k_2 steps (i.e., by setting $k_{-1} = 0$).

centration of ssDNA is at a maximum) and will then decrease as some of the ssDNA is converted to initial products in the competing pathway. This prediction is inconsistent with our experimental data which show that network DNA is formed after a pronounced kinetic lag in both the ATP-independent and ATP-stimulated reaction. These kinetic lags are more consistent with the sequential model shown in Scheme I which predicts that the rate of formation of network DNA will initially be low and then will increase as initial product precursors accumulate after the first kinetic step.

We emphasize that the kinetic model shown in Scheme I is simply a device for quantitatively analyzing the rates of formation of initial products and network DNA. We have deliberately omitted recA protein from the kinetic formulation; since our renaturation data points were obtained by first quenching aliquots of the reaction solutions with EDTA/SDS, it is conceivable that the renaturation products that are observed in our agarose gel assays are not formed as such until after the recA protein is removed. Nevertheless, the results of our kinetic results can be related to possible molecular mechanisms for the recA protein promoted renaturation reaction.

Scheme I depicts an equilibrium between the initial products and the single strands in a step preceding the conversion of the initial products to network DNA. This implies that the initial products can break down (i.e., denature) to regenerate the (+) and (−) single strands. This may not be the correct physical interpretation of the data. An alternate possibility is that the recA protein first binds to the (+) and (−) single strands and then forms a complex (in a rapid step) that serves to bring the DNA strands together into a higher effective concentration. If the single strands are randomly held by recA protein in these complexes, then some of the strands may be in position to renature with complementary strands to form initial products while others may not be. In this alternate interpretation, the relative values of k_1 and k_{-1} that were used to model the agarose gel data according to the general two-step sequential reaction scheme would reflect the renaturation efficiency of the single strands held within the initial recA protein–ssDNA complexes rather than rate constants for an actual renaturation/denaturation process.

We found that recA protein is unable to convert purified form III DNA (with or without complementary single strands) directly to network DNA under renaturation reaction conditions. This indicates that free form III DNA is not an intermediate in the recA protein promoted renaturation reaction and that the form III DNA that is observed in our renaturation assays is derived from some sort of "recA–form III DNA" complex. Furthermore, when an ongoing renaturation reaction is treated with EDTA to dissociate recA–DNA complexes, the free form III DNA that is generated is not converted to network DNA by the recA protein when Mg^{2+} is added back to the reaction solution. These results indicate that the association of recA protein with complementary single strands leads to the formation of a recA–form III DNA complex and that a continuing association between the recA protein and the form III DNA is required in order for the form III DNA to be further converted to network DNA.

This mechanistic analysis raises the important question of how the recA protein physically brings two DNA strands together so that pairing can occur. One possibility is that DNA strands are brought together directly by means of two DNA binding sites on the recA protein monomer. A second possibility is that DNA strands are brought together by protein–protein interactions between recA protein molecules bound to different DNA strands. We previously suggested that recA protein may have two DNA binding sites on the basis of our studies of the binding of recA protein to ϕX ssDNA (Bryant et al., 1985). In the absence of a nucleotide cofactor, each recA monomer protects approximately four nucleotides of ssDNA from nuclease digestion, and a similar value is obtained in the presence of ATP (plus an ATP regeneration system). In the presence of ATPγS (a nonhydrolyzable analogue of ATP), however, the stoichiometry of nuclease protection increases by exactly 2-fold, to one recA monomer per eight nucleotides of ssDNA. A plausible explanation for this observation is that the binding of ATPγS traps recA protein in a conformation in which it is bound tightly to two strands of ssDNA. Indirect support for this idea was obtained by electron microscopy, which showed that addition of ATPγS to ssDNA in the presence of subsaturating concentrations of recA protein resulted in extensive cross-linking of the recA–ssDNA complexes into large networks (Bryant et al., 1985).

The notion of two DNA binding sites is also consistent with our studies of the mechanism of transfer of recA protein from one ϕX ssDNA molecule to another (Bryant et al., 1985). Transfer is relatively slow in the absence of nucleotide cofactors ($t_{0.5}$ = 30 min) but is stimulated by ATP ($t_{0.5}$ = 3 min). In both cases, transfer appears to be a cooperative process in which many recA monomers, possibly in the form of clusters, are transferred from one ssDNA molecule to another in a single event (Bryant & Lehman, 1986). Cooperative transfer may proceed by the intermediate formation of a complex between a recA–ssDNA complex and a second ssDNA molecule, followed by transfer of the recA protein from the first to the second strand. Kowalczykowski and co-workers have also suggested that the transfer of recA protein from one single strand to another may proceed via formation of a ssDNA–recA–ssDNA intermediate (Menetski & Kowalczykowski, 1987).

Tsang et al. (1985) have reported that incubation of recA protein with ssDNA results in the formation of recA protein–ssDNA aggregates that are large enough to be sedimented out of solution by low-speed centrifugation. Like the renaturation reaction, aggregation occurred optimally at subsaturating levels of recA protein (relative to the ssDNA) and was strongly inhibited by saturating levels of recA protein. These observations suggest that aggregation is due to ssDNA–recA–ssDNA interactions rather than recA–recA interactions. Furthermore, aggregation occurred equally well in the presence or absence of ATP. Thus, it seems likely that the aggregates reported by Tsang et al. correspond to DNA pairing intermediates on the recA protein promoted renaturation reaction pathway.

The results discussed above are consistent with the following tentative model for the recA protein promoted renaturation reaction. RecA protein first binds to ssDNA to form recA–ssDNA complexes. The binding is cooperative, and the reactive species in the renaturation reaction are ssDNA molecules that are only partially covered with clusters of recA protein (Bryant & Lehman, 1985). The next step may be the binding of a second DNA strand to form a transiently two-stranded complex. The formation of two-stranded complexes would increase the effective concentration of DNA and would serve to bring some fraction of complementary DNA sequences into homologous proximity. This would lead to the formation of form III DNA and other initial renaturation products in an initial pairing step that would be largely ATP-independent; this step would correspond to the k_1 step in Scheme I.

The molecular mechanism for the conversion of initial products to network DNA is not clear. It seems likely, however, that network DNA is built up from initial products by a series of additional pairing steps; these additional pairing steps would correspond to the k_2 step in Scheme I. If this is the case, our kinetic analysis suggests that ATP may increase the rate at which these additional pairing steps can occur. We have previously shown that ATP increases the rate at which recA protein moves from one DNA strand to another and it may be that this leads to an increase in the rate at which additional DNA pairing steps can occur. A more complete description of the mechanism for the formation of network DNA, and of the role of ATP in the recA protein promoted renaturation reaction, will require an analysis of the structural organization of the network DNA renaturation products.

The ATP-independent recA protein promoted renaturation of (+) circular ssDNA with (−) linear ssDNA described here is of additional interest because it leads to the formation of RFII molecules as the primary initial renaturation product. This reaction thus has an obvious relationship to the extensively studied three-strand exchange reaction involving circular ssDNA and homologous linear duplex DNA (Radding, 1982; Cox & Lehman, 1987). Howard-Flanders and co-workers have proposed that the three-strand exchange reaction proceeds by the formation of a recA protein filament on the ssDNA followed by assimilation of the duplex DNA into the filament via a second DNA binding site (Howard-Flanders et al., 1984). This somehow leads to an exchange of strands, resulting in the generation of form II molecules and displaced linear (+) strands. Unlike the renaturation reaction, the three-strand exchange reaction has an absolute requirement for ATP, presumably to drive the polar displacement of the (+) linear strand (Cox & Lehman, 1981; DasGupta et al., 1980). Since the renaturation reaction requires only DNA pairing whereas the three-strand exchange reaction requires both DNA pairing and strand displacement, a detailed comparison of these two reactions should help clarify the role of ATP hydrolysis in recA protein promoted DNA pairing reactions.

Registry No. ATP, 56-65-5.

References

Bryant, F. R., & Lehman, I. R. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 297–301.

Bryant, F. R., & Lehman, I. R. (1986) *J. Biol. Chem. 261*, 12988–12993.

Bryant, F. R., Taylor, A. R., & Lehman, I. R. (1985) *J. Biol. Chem. 260*, 1196–1202.

Cox, M. M., & Lehman, I. R. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 3433–3437.

Cox, M. M., & Lehman, I. R. (1987) *Annu. Rev. Biochem. 56*, 229–262.

Cox, M. M., McEntee, K., & Lehman, I. R. (1981) *J. Biol. Chem. 256*, 4676–4678.

Craig, N. L., & Roberts, J. W. (1981) *J. Biol. Chem. 256*, 8039–8044.

DasGupta, C., Shibita, T., Cunningham, R. P., & Radding, C. M. (1980) *Cell 22*, 437–446.

Howard-Flanders, P., West, S. C., & Stasiak, A. (1984) *Nature 309*, 215–220.

Kowalczykowski, S. C., Clow, J., & Krupp, R. A. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 3127–3131.

Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning*, Cold Spring Harbor, Laboratory, Cold Spring Harbor, NY.

Menetski, J. P., & Kowalczykowski, S. C. (1987) *J. Biol. Chem. 262*, 2085–2092.

Moore, J. W., & Pearson, R. G. (1981) *Kinetics and Mechanism: A Study of Homogeneous Chemical Reactions*, 3rd ed., Wiley-Interscience, New York.

Pugh, F. B., & Cox, M. M. (1987) *J. Biol. Chem. 262*, 1326–1336.

Radding, C. M. (1982) *Annu. Rev. Genet. 16*, 405–437.

Tsang, S. S., Chow, S. A., & Radding, C. M. (1985) *Biochemistry 24*, 3226–3232.

Weinstock, G. M., McEntee, K., & Lehman, I. R. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 126–130.