Homologous pairing of single-stranded circular DNAs catalyzed by recA protein

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ABSTRACT

RecA protein catalyzes annealing between pairs of circular single-stranded DNA molecules containing complementary sequences varying in length from 3550 nucleotides to 181 nucleotides. The reaction requires ATP and catalytic amounts of recA protein. Molecules containing large complementary inserts are annealed by recA protein to form large multimeric aggregates that migrate slowly in agarose gels. In contrast the products formed from circular molecules containing short complementary regions are principally dimeric structures. We have used electron microscopy, thermal denaturation and kinetic studies to analyze these reaction products. Our results indicate that recA protein catalyzes multiple nucleation events between complementary DNA sequences in the absence of a free end and when these sequences are flanked by extensive noncomplementary regions.

INTRODUCTION

The recA protein of *Escherichia coli* is necessary for homologous recombination. The properties and enzymatic activities of purified recA protein suggest that this enzyme is responsible for the formation and extension of the heteroduplex joint which is proposed to be an essential early intermediate in genetic exchange between parental duplexes (1). *In vitro*, recA protein, alone or in combination with single-stranded DNA binding protein (SSB) promotes formation of heteroduplex regions between homologous duplex and single-stranded DNA (2,3).

Our laboratory has been interested in the mechanism by which recA protein pairs homologous DNA segments. We have chosen to investigate the simplest pairing reaction catalyzed by recA protein, the annealing of complementary single-stranded DNAs. Unlike reactions involving duplex substrates, the annealing of single-stranded molecules requires only catalytic amounts of recA protein (4 and unpublished results). In this report we describe experiments involving single-stranded circular DNA molecules containing limited complementary regions. We have used these DNA substrates to investigate the role of ends and the effects of heterologous DNA on the annealing reaction. Our

results demonstrate that free ends are not required for formation of duplex joints between these DNAs. Moreover, recA protein catalyzes annealing of complementary regions 181 nucleotides long when these sequences are flanked by more than 6 kb of noncomplementary DNA.

MATERIAL AND METHODS

The recA protein was purified from strain KM1842 as described (5). Viral DNA substrates were prepared by phenol extraction of CsCl purified viruses described (6). The different DNA substrates used in the annealing experiments are described in Table I.

DNA Annealing by recA Protein.

Annealing reactions were performed in 50-100 μ l and contained 20 mM Tris-HCl (pH 7.5), 20 mM KCl, 5 mM MgCl₂, 1 mM dithiothreitol and 1 mM ATP. Equal molar amounts of the complementary DNAs (1.25-2.6 nmol) were added and incubated with recA protein at a molar ratio of 1:25 or 1:60 (recA protein: total nucleotides) at 37°C for the indicated time, terminated by the addition of 50 mM EDTA and 0.5% sodium dodecyl sulfate and analyzed by electrophoresis through agarose gels (0.7%) using a Tris-acetate electrophoresis buffer (7). Gels were stained with 1 μ g/ml ethidium bromide for 1 hr and photographed. Densitometry was performed using a Helena Quikscan densitometer.

Electron Microscopy of Annealed Molecules.

A portion of the reaction mixture was spread for electron microscopy using the method of Kleinschmidt (8). The spreading solution contained 50% formamide, 0.1 M Tris-HCl 10 mM EDTA (pH 8.5), cytochrome C 50 μ g/ml). The hyphophase consisted of 0.1 M Tris-HCl, 10 mM EDTA (pH 8.5) and 17-20% formamide. Grids were stained in uranyl acetate, dehydrated in 90% ethanol, rotary shadowed with platinum-palladium and examined and photographed using a Zeiss EM109 transmission electron microscope. Molecules were measured using a Hewlett-Packard 9825A calculator with a 9864A Digitizer.

RESULTS

Annealing of Circular DNAs by recA Protein.

In the presence of Mg⁺⁺ and ATP, *E. coli* recA protein efficiently catalyzes renaturation of heat-denatured phage λ or P22 DNA (4 and Figure 1 lanes 1 and 2). This reaction, proceeds rapidly under conditions where DNA is in molar excess relative to recA protein (25:1). ATP binding and hydrolysis are required for renaturation of the single-stranded molecules (Figure 1 lanes 4 and 5).

The products of the reaction of recA protein with linear viral DNAs are

Figure 1. Agarose gel electrophoresis of DNA complexes annealed by recA protein. The indicated DNAs were incubated with recA protein as described in Materials and Methods. Reactions were terminated by addition of 50 mM EDTA and 1% SDS and samples were electrophoresis in 0.7% agarose gels lane 1, complete reaction, heat denatured P22 DNA; lane 2, complete reaction, heat denatured λ DNA; lane 3, $-\text{Mg}^{++}$, heat denatured P22 DNA; lane 4, $-\text{ATP}$, heat denatured P22 DNA; lane 5, $-\text{ATP}$, heat denatured λ DNA; lane 6, $-\text{recA}$ protein, heat denatured λ DNA; lane 7, $-\text{recA}$ protein, M13oriC26/M13oriC81 DNA; lane 8, $-\text{ATP}$, M13oriC26/M13oriC81 DNA; lane 9, complete reaction, M13oriC26/M13oriC81 DNA; lane 10, $-\text{M13oriC81}$ DNA, $+\text{M13oriC26}$ DNA; lane 11, $-\text{M13oriC26}$ DNA, $+\text{M13oriC81}$ DNA; lane 12, $-\text{recA}$, M13oriC26/M13oriC81 DNA; lane 13, complete reaction, M13oriC26/M13oriC81 DNA; lane 14, $-\text{ATP}$, M13oriC26/M13oriC81 DNA; lane 15, $-\text{ATP}$, $+\text{UTP}$, M13oriC26/M13oriC81 DNA; lane 16, $-\text{ATP}$, $+\text{ATP}\gamma\text{S}$, M13oriC26/M13oriC81 DNA.

DNA aggregates held together by short base-paired regions (4). The large size and branched character of these aggregates retard their mobility through agarose gels. In order to study more completely the details of this reaction we have examined the ability of recA protein to anneal circular DNA molecules containing limited regions of sequence homology. A series of recombinant single-stranded M13 viruses have been constructed by Ray and coworkers (9,10), containing separated complementary sequences that are derived from the replication origin regions of the *E. coli* chromosome or from phage ϕX174 . These complementary DNA sequences are nested within more than 6 kb of noncomplementary viral plus strand DNA. The DNAs chosen for this work contain complementary inserts as large as 3550 bases (M13oriC81/M13oriC26) or as short as 181 nucleotides (M13 ϕX80 /M13 ϕX81) (see Table I).

Incubation of recA protein with the M13oriC81/M13oriC26 DNAs resulted in the formation of large DNA complexes that migrated slowly into agarose gels

Table I. DNA substrates used in recA protein catalyzed annealing reactions

DNA	source of insert DNA	length of complementary	ref
		insert (nucleotides)	
M13oriC81	XhoI fragment	3550	9
M13oriC26	of <i>E. coli</i> ori plasmid pJS5	3550	
M13ØX50 ^a	HaeII fragment	1565	10
M13ØX60	of ØX174 RF	1565	
M13ØX80 ^b	AluI-HinfI	181	10
M13ØX81	fragment of ØX174 RF	181	

^a during propagation of the M13ØX50 phage a variant containing a small deletion within the insert fragment was produced and outgrew the parental phage.

^b These phages are derived from the M13bla61 cloning vector containing the β -lactamase gene from Tn3

(Figure 1 lanes 9 and 13), similar to the result obtained with denatured linear DNAs. The formation of these complexes required recA protein, Mg^{++} and ATP (Figure 1 lanes 8 and 14). Incubation of only M13oriC81 DNA or M13oriC26 DNA with recA protein and ATP did not lead to the formation of slowly migrating DNA complexes (Figure 1 lanes 10 and 11) demonstrating the requirement for complementary sequences within the DNAs for aggregate formation.

The agarose gel results obtained with the circular DNA substrates were remarkably similar to those obtained with heat denatured linear λ or P22 DNAs and raised the possibility that complexes formed in the reaction might be due to annealing of linear M13oriC DNA contaminating the circular viral DNA preparations. In order to test this possibility, we examined the substrate and product DNAs by electron microscopy. Direct examination of the unreacted M13oriC DNA preparations indicated that the level of contamination with linear M13oriC fragments was approximately 21% (41 of 192 molecules). Following incubation with recA protein and ATP, two types of DNA complexes could be detected by electron microscopy, those in which at least one of the reacting strands was linear, and those in which only circular DNA molecules were evident (Figure 2, panels A through D). We estimate that approximately 70% of the complexes contained only circular molecules, a value close to the expected result. These results directly demonstrate that recA protein can anneal complementary regions within single-stranded DNA molecules that do not have free ends. Since recA protein lacks topoisomerase activity, these complexes must

Figure 2. Structure of DNA complexes annealed by recA protein. Circular DNAs (M13oriC26/M13oriC81) were incubated with recA protein as described in Materials and Methods and the products examined by electron microscopy. Molecules A)-D) were produced in reactions containing 500 μ M ATP. Molecules E) and F) were from reactions containing 500 μ M UTP.

be held together by joints composed of nonintertwined single-strands rather than the plectonemic coiling of normal duplex structures (11).

Structure of the aggregates formed from circular DNAs.

Most of the DNA complexes formed were composed of three or more DNA chains as determined by measurements of contour length using M13 viral single-stranded DNA as a standard. More than 90% of the aggregates were large aggregated structures that were too complex to allow us to identify the number or nature of the joints formed between the annealed molecules. We attempted to limit the size of these reaction products by shortening the time of incubation, reducing the DNA concentration or lowering the ATP or recA protein concentration. However, none of these modifications significantly altered the size or complexity of the products as judged by electron microscopy. We did note that substituting UTP for ATP produced complexes composed of fewer circular molecules (Figure 2, panels E and F). UTP, like ATP, is hydrolyzed by recA protein and supports the annealing of linear DNA's (4,12).

A series of 'simple' DNA aggregates formed by annealing M13oriC81 and M13oriC26 DNAs is shown in Figure 2. Measurements of these structures suggest that they are composed of fewer than four circular DNA molecules. We conclude that these structures form as a result of recA protein action and are not an artefact of preparing DNAs for electron microscopy because omitting recA protein or ATP from the reaction prevents formation of these aggregates (data not shown).

In the M13oriC81 and M13oriC26 DNAs, the complementary inserts comprises approximately one third of the length of the entire recombinant molecule. In many of the aggregates it was observed that the DNA chains interacted along the entire length of the molecule i.e. branch junctions, bubbles and extended duplex regions could be detected all along the contour of the DNAs (see Figure 2, A and D).

The Effect of Insert Size on Complex Formation.

By using M13 derivatives containing different size inserts, we were able to investigate the effects of altering the length of the complementary region on annealing. The reaction of recA protein and ATP with M13 ϕ X50/M13 ϕ X60 DNA containing a complementary region of approximately 1565 nucleotides, produced large DNA complexes as determined by electrophoresis in agarose gels (see Figure 4). We next tested a pair of substrates containing only 181 nucleotides of complementary DNA sequence, M13 ϕ X80/M13 ϕ X81 DNAs. The reaction of these molecules with recA protein and ATP resulted in the formation of products that migrated as discreet band in agarose gels (Figure 4). Electron

Figure 3. Dimeric products of annealed circular M13ΦX80 and M13ΦX81 DNAs catalyzed by recA protein. Incubations were performed as described in Materials and Methods and the reaction products were examined by electron microscopy. In A) and C) unreacted molecules can be seen.

microscopic analysis of the reaction mix indicated that structures composed of two circular molecules were the principal product (Figure 3). Unlike the reactions involving large inserts which converted all of the substrate circles into DNA complexes, only 50% of the M13ΦX80/M13ΦX81 DNAs were annealed. Addition of more ATP or recA protein did not alter the extent of the reaction.

Rate of Formation of DNA Complexes.

We examined the relative rate of complex formation for the M13ΦX50/M13ΦX60 and M13ΦX80/M13ΦX81 DNA pairs. The kinetics of these reactions are

Figure 4. Agarose gel electrophoresis of DNA complexes formed by recA protein catalyzed annealing of M13φX50/M13φX60 and M13φX80/M13φX81 circular molecules. Annealing reactions were performed as described in Materials and Methods and at the indicated times samples were analyzed by agarose gel electrophoresis. S, substrate DNA, P₁, primary product; P₂, minor product.

shown in Figure 4. Early in the reaction of the M13φX50/M13φX60 DNAs, there is the appearance of a species migrating at the position expected of a dimer complex as well as a slower migrating species composed of (three) or more molecules. Complexes appear at the top of the agarose gel as early as 15 min at 37°C. Although the production of dimer structures preceeds the appearance of the very large complexes, these data alone do not permit us to conclude that these dimers are intermediates in the pathway to multimeric complex formation.

The formation of dimers using M13φX80/M13φX81 DNAs is also apparent by 15 min and reaches its maximum value by 60 min. Densitometry of these gel lanes was performed in order to quantitate the relative rates of these annealing reactions. As shown in Figure 5, annealing between DNAs containing the 181 bp insert proceeded at approximately one third the rate of annealing of molecules containing inserts almost ten times as large.

Figure 5. Kinetics of recA protein catalyzed annealing of circular DNAs containing long (1565 nucleotides) or short (181 nucleotides) complementary sequences. Densitometry of gel lanes in Figure 4 was performed and the relative amount of DNA in the indicated species was plotted. S, substrate, P₁ and P₂ are the products identified in Figure 4. In A) the substrate DNAs were M13ØX80/M13ØX81 (181 nucleotides) in B) M13ØX50/M13ØX60 (1565 nucleotides).

Stability of Joints Formed Between Circular Molecules.

The thermal stabilities of the multimeric and dimer complexes were determined by incubating the products of the annealing reaction at different temperatures and analyzing the results by agarose gel electrophoresis. These results, shown in Figure 6, indicate that the dimeric complexes are stable to elevated temperatures up to 65°C. The melting of the joints and dissociation of these dimers occur over a narrow temperature range (65-70°C) indicating that these joints are likely stabilized by accurate base pairing between the DNA strands.

A more complicated pattern is seen in the melting properties of the multimeric circular complexes (Figure 6B). These complexes are stable up to

Figure 6. Thermal denaturation of DNA complexes annealed by recA protein. DNA complexes were prepared as described in Materials and Methods. The annealing reactions were stopped by addition of EDTA (to 25 mM) or by proteinase K digestion of recA protein (200 µg/ml, 60 min). Samples were incubated for 10 min at the indicated temperature and electrophoresed in a 0.7% agarose gel. A) Annealed complexes of M13φx80/M13φx81 DNAs; B) Annealed complexes of M13φx50/M13φx60 DNAs.

65°C, after which there is an increase in the amount of DNA migrating at the position expected for dimeric circles. At higher temperatures, more DNA is formed which migrates at the position of a dimer. When the complexes are heated to 80–85°C, they dissociate to monomeric circles. This result indi-

cates that there is heterogeneity in the structure of the joints formed from DNA circles containing larger complementary inserts.

DISCUSSION

RecA protein promotes pairing of a variety of homologous DNA substrates. The most extensively studied of these reactions involves pairing of DNA molecules that are single-stranded or partially single-stranded with a duplex homology (13,14). An unusual feature of this class of reactions is a requirement for levels of recA protein which are stoichiometric with the single-stranded substrate. Radding and co-workers (15,16) have demonstrated that an early and presumably obligatory step is the formation of a presynaptic complex in which recA protein is polymerized on the single-stranded component. This presynaptic complex forms in the presence of ATP and is required for interaction with duplex molecules and their processive alignment.

RecA protein also promotes annealing of single-stranded DNA molecules to form duplex DNA (4). This annealing (or renaturation) reaction differs in three ways from those reactions involving a duplex substrate. First, annealing of complementary single-stranded molecules requires catalytic amounts of recA protein. Second, the annealing reaction proceeds nonprocessively (K. McEntee, unpublished results). Third, the annealing reaction is strongly inhibited by single-stranded DNA binding protein (SSB) (K. McEntee, unpublished results). Taken together these differences suggest that the enzymatic annealing of single-stranded DNAs by recA protein is mechanistically distinct from reactions involving duplex pairing events. In order to elucidate the relationship between these reactions and to provide a detailed characterization of enzymatic annealing of single-strands we have performed experiments to examine the requirements for molecular ends and the effects of heterology on this reactions.

RecA protein catalyzes formation of DNA aggregates from single-stranded circular DNAs containing large complementary inserts. The circular molecules in these complexes are joined by short base-paired regions. We infer that these joints are not normal duplex structures since the circular DNA substrates are topologically constrained and recA protein lacks any topoisomerase activity. Thus, these joints in which the single strands are not intertwined are different from the plectonemic coiling in normal duplex DNA. In this regard, the annealing of complementary circular molecules by recA protein differs from the annealing of linear DNA strands. Because of the topological constraints, there can be no net change in the linking number, α , in the over-

all reaction. For the unreacted single-stranded circles, $\alpha=0$, and therefore $\alpha=0$ for the aggregates. That is to say, for every right-handed turn introduced into a joint, there must be a compensating turn of opposite handedness introduced into the molecule. Because of the structural complexity of the products, we have been unable to investigate the topological details of the interactions.

Limiting the size of the complementary regions to 181 nucleotides alters the products of the annealing reaction considerably and the predominant species is a dimeric structure. Unlike the reaction between large insert containing DNAs in which recA protein joins nearly all of the circular molecules into large aggregates, the annealing of M130X80 and M130X81 DNAs reaches a limit of 50% of the molecules paired. This value is not changed by addition of more recA protein or ATP and likely represents an equilibrium between formation and dissociation of the joints catalyzed by recA protein.

Thermal stability experiments indicate that the joints formed between the M130X80 and M130X81 substrates containing 181 nucleotides of complementary sequence are dissociated at temperatures above 65°C. The sharpness of the transition suggests that these joints are homogeneous in character. However, the joints formed between the M130X50 and M130X60 molecules are characterized by a multistep melting profile indicating heterogeneity in the paired regions. At present we do not know the nature of this heterogeneity.

A kinetic analysis of complex formation indicates that the rate of recA protein catalyzed annealing of circular molecules does not increase in proportion to the length fraction of the complementary region. Thus by increasing the size of the complementary region almost 9-fold, the rate of annealing increases only three-fold. (It should be noted that the overall length of the molecules in these two cases is very nearly equal and therefore excluded volume effects on annealing are comparable.) More detailed kinetic studies are in progress in order to determine the mechanism of recA protein catalyzed annealing.

This and an earlier report (4) demonstrate that recA protein catalyzes pairing of single-stranded DNA molecules that contain complementary regions. Here we show that the annealing proceeds in the absence of free ends in the molecules and when the complementary regions are flanked by noncomplementary sequences. Although topological constraints presumably limit the size of the 'duplex' joints formed between DNAs, these joints are stable enough to survive electrophoresis in agarose gels, and spreading in 50% formamide for electron microscopy. Of course, joints that do not survive these treatments would not

be detected in these experiments.

The formation of highly branched aggregates with linear as well as circular DNA substrates argues that nucleation (the formation of a small base-paired region between aligned DNA strands) is efficiently catalyzed by recA protein, but that 'zippering' of these strands is a relatively slow process under these reaction conditions. This result contrasts with what is observed in spontaneous renaturation, where nucleation is rate limiting and extension of these duplex regions is rapid (17).

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