DNA STRAND EXCHANGES

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I. INTRODUCTION AND BACKGROUND

The metabolism of DNA in the cell can be divided into three events: (1) replication, (2) transcription, and (3) recombination and the related repair events. Our understanding of homologous, or general, recombination lags far behind our perception of the molecular mechanisms of replication and transcription. This is because of some sharp differences in the natures of these reactions. For example, while replication and transcription are net synthetic events, in a DNA strand exchange, several thousand bases of DNA may be transferred from one DNA molecule into another with little or no synthesis of new DNA. Thus, the powerful assay, which measures the incorporation of radioactive nucleotides into acid insoluble material, offers little to aid the investigator of strand exchange. Structurally, the reactions are profoundly different. Whereas single monomers of RNA or DNA polymerase are capable of catalyzing the basic synthetic reactions, single monomers of RecA or UvsX protein are inactive. Instead, the active unit is a large protein-DNA filament constructed from the ordered arrangement of hundreds of protein monomers bound to the DNA (Figure 1). Possibly this assembly is stable — with the protein remaining bound to the DNA throughout the reaction — or the protein may undergo an active treadmilling as the point of exchange is driven forward. In either case, these reactions are very different from simple synthetic reactions and require assays that reflect the involvement of large structures.

A comprehensive review of all of the work on RecA protein alone is beyond the scope of this series. This review, therefore, focuses on DNA strand exchanges from a structural point of view, emphasizing the importance of the structure of the protein and DNA-protein complexes involved. Most of this work has been done on RecA protein. Newer studies with the bacteriophage T4 UvsX protein and the recl protein of *Ustilago* are brought in, where appropriate, to indicate similarities and possible functional differences.

This review begins with an overview of the assays that have been used to follow strand exchange. The remainder of this review is organized to parallel the step-by-step processes employed by recombination proteins to complete strand exchange. In vitro, before RecA protein even contacts DNA, it shows a propensity to form several types of aggregates. This may greatly influence how it subsequently binds to DNA and carries out its function. Once RecA or UvsX protein contacts DNA, it assembles into large filaments. We describe what is known about the ultrastructure of these DNA-protein filaments and follow that with a discussion of how the proteins accomplish this assembly. DNA strand exchanges are introduced by a discussion of the topology of pairing and by a comparison between paranemic and plectonemic joints. Strand exchange itself is broken into separate descriptions of the search for homology, synapsis, and the pairings between (1) linear single-stranded DNA (ssDNA) and supertwisted double-stranded DNA (dsDNA), and (2) circular ssDNA and linear dsDNA. Finally, we discuss and contrast several models of strand exchange.

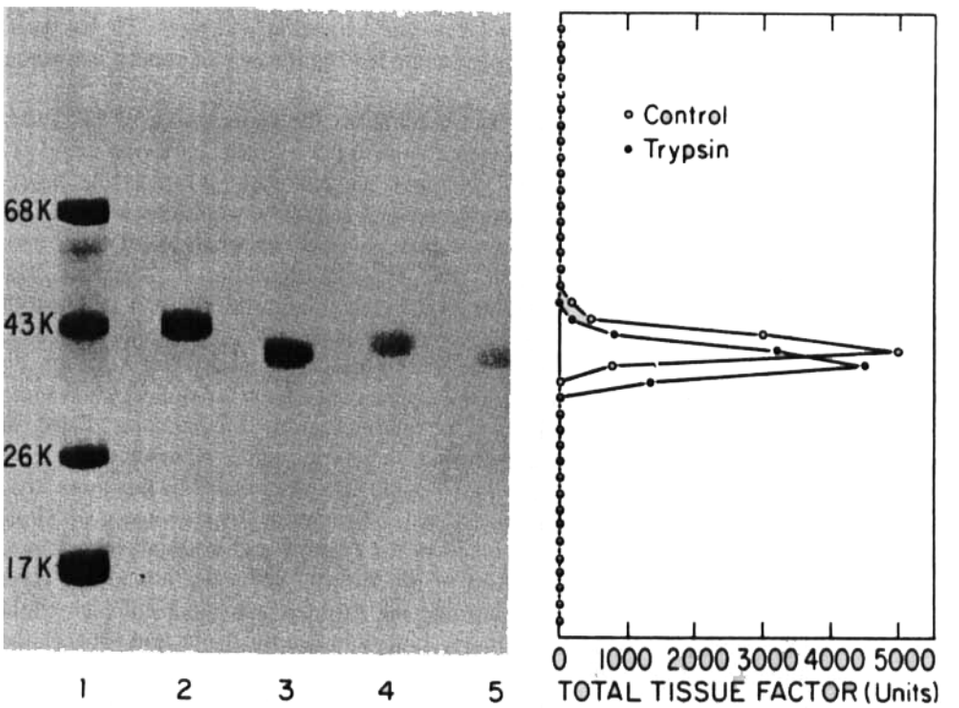

FIGURE 1. Bacteriophage T4 UvsX protein bound to a circular superhelical dsDNA. In this micrograph (see Reference 58), UvsX protein was complexed with an 11-kb supertwisted plasmid DNA. The complex was processed for visualization by mounting onto a glow-charged thin carbon film in a buffer containing spermidine, washing with distilled water and graded ethanol solutions, air drying, and then rotary shadowcasting with tungsten; it is shown in reverse contrast. Each repeat of the protein helix contains four turns of the DNA helix.

II. ASSAYS FOR STRAND EXCHANGE

Many assays are available for following the interaction of recombination enzymes with DNA and the subsequent catalysis of strand exchange. These may: (1) monitor the binding of proteins to the DNA, (2) follow the progress of the DNA strands through strand exchange, or (3) follow the rate of ATP hydrolysis, an enzymatic activity of the protein required for strand exchange. The complexity of the DNA-protein intermediates in the strand exchange reaction makes it difficult to determine the pathways of a reaction from a method such as nuclease protection or filter binding alone; a full understanding of the mechanism of strand exchanges requires information from both structural and biochemical assays.

To follow the binding of proteins to DNA during strand exchanges, nitrocellulose can be treated with alkali to prevent naked ssDNA from binding so that only protein-bound DNA will be measured (Table 1). Protection of ssDNA or dsDNA in a DNA-protein complex from nuclease or restriction enzyme attack has also been exploited by a number of investigators (Table 1).

Table 1
ASSAYS TO MEASURE BINDING OF PROTEINS TO DNA

Assay	Summary	Ref.
Nitrocellulose filter binding	Retention of radiolabeled DNA on filters which have been pretreated to bind only protein, not free DNA	1
Nuclease protection	Ability to protect DNA or oligonucleotides from nucleases	2—5
Electron microscopy	Direct visualization of proteins bound to DNA molecules	6, 7
Sucrose density gradient sedimentation	Alteration of migration of DNA through sucrose density gradients	8
Ethidium bromide (EtBr) blocking	Inability of EtBr to intercalate in DNA when protein is bound	9
Etheno-DNA fluorescence	Increase in fluorescence of ϵ-DNA when protein is bound	10, 11
Light scattering	Spectrophotometric monitoring of filament formation	12

Table 2
ASSAYS TO MEASURE PAIRING OR STRAND EXCHANGE

Assay	Summary	Ref.
Nitrocellulose filter binding	Radiolabeled dsDNA binds to filter only when complexed to ssDNA	13—15
Electron microscopy	Examine structure of intermediates in reaction	16—18
Gel electrophoesis	Follow altered migration of reactant DNAs	19
Nucleases:		
Nuclease S1 or endonuclease VII	Labeled dsDNA becomes sensitive and ssDNA becomes insensitive in reactions that create heteroduplex DNA	20, 21
Restriction enzymes	Only able to digest labeled ssDNA if it has paired to form heteroduplex DNA	3
Exonuclease I	Digests ssDNA from the 3′ end so no substrate is available unless reaction proceeds to a complete transfer of strands	22
Sucrose density gradient centrifugation	Separate products from reactants on gradient	23

Other physical approaches to the quantitation of protein binding to DNA have included sucrose sedimentation and optical techniques (Table 1). One powerful approach takes advantage of the fact that the fluorescence of etheno-derivatized DNA (ϵ-DNA) is increased by RecA protein binding.[10,11] This has been particularly informative when combined with assays that monitor the rate of ATP hydrolysis. Because the derivatization may break ssDNA, the state of the DNA must be monitored. Also, ϵ-DNA does not behave exactly the same as natural DNA; the stability of binding and the dissociation constant of RecA protein for ϵ-DNA have been shown to differ greatly from what is seen with natural DNA.[10,11] Electron microscopy (EM) offers the opportunity to examine single molecules and obtain number averages as contrasted to weight averages. The ability to visualize proteins bound along the DNA makes EM a powerful tool if the data are correlated with information from other sources.

Both gel electrophoresis and filter-binding assays have been used extensively to follow the progress of DNA strands through strand exchange. Filter binding to nitrocellulose is conducted under conditions in which only ssDNA binds to the filter (Table 2); radiolabeled dsDNA is retained only if it is linked to ssDNA. This has been possibly the single most useful assay of strand exchange. Riddles and Lehman[14] have extended this method to distinguish between paranemic and plectonemic joining. Joint complexes are bound to the nitrocellulose, then are rinsed with 5.2 *M* guanidium hydrochloride; only plectonemic joints remain bound. A caution in the use of this assay for crude systems is that contaminating nucleases which produce single-stranded regions on the dsDNA might generate spurious

results. Another assay takes advantage of the fact that exchange reactions involve the conversion of a ssDNA to a duplex by monitoring the resistance of the ssDNA to S1 nuclease. EM is particularly useful in demonstrating the presence of specific intermediates in exchanges and in distinguishing those which could only occur through a legitimate exchange from those which arise from artifactual pathways. The demonstrations by McEntee et al.[17] and Cunningham et al.[18] that RecA protein could produce D-loops in DNA, as seen by EM, initially provided the most convincing demonstration of the role of the RecA protein.

Although many of the assays described in Table 2 are suitable for studies of purified proteins, they may not be appropriate for the isolation of RecA-like proteins from crude cell lysates, unless they are coupled to other measurements. For example, take the simple assay in which strand transfer between a linear dsDNA and a circular ssDNA is followed by the appearance of a band on an agarose gel corresponding to relaxed circular dsDNA. This assay has been very useful in studies of purified RecA protein. But if it were used to isolate a RecA protein analog from crude cell lysates, one might instead purify: (1) a protein which would bind in an SDS-resistant manner to the ends of the linear dsDNA forming a relaxed dsDNA circle (such binding is observed with the protein described by Stillman and Bellet[24] in adenovirus and with the T4 phage topoisomerase;[25] (2) a helicase which would split the dsDNA allowing the (−) strand of the dsDNA to anneal to the circular (+) ssDNA, forming a relaxed dsDNA circle; (3) a highly processive exonuclease which might give the same result as (2); or (4) a modest exonuclease which would generate single-stranded tails on the linear ssDNA. The tails would anneal to the ssDNA circle generating a duplex circle with a ssDNA bush, a complex which migrates like a relaxed duplex circle. If, however, EM or other methods were used in parallel to demonstrate the presence of an intermediate diagnostic of true strand transfer, then the electrophoretic assay could be highly useful. We also note that purified UvsX protein yields a different product in this reaction (see Section VI.E).

Although the exact role of ATP hydrolysis in strand exchange reactions is not yet established, monitoring the DNA-dependent hydrolysis of ATP by RecA or UvsX protein provides a view into the progress of these reactions. It is known that RecA protein will hydrolyze (r,d)ATP, UTP, or CTP,[26,27] but only in reactions with (r,d)ATP will it catalyze recombination. ATPase activity can be inhibited by adding ADP, UTP, dTTP, or GTP, apparently because these molecules compete for a single nucleoside-binding site on the protein.[26,27] The nucleotide-binding site has been identified and shown to contain a single tyrosine residue which can be modified by ATP analogs.[28,29]

The hydrolysis of ATP by RecA protein is dependent on the binding of the protein to ssDNA or dsDNA. Thus RecA protein shows no ATPase activity with dsDNA at neutral pH, but does at pH 6.2 where it is known to bind dsDNA.[27] Some dsDNA-dependent ATPase activity can, however, be observed at pH 7.5, but only under conditions which promote partial melting of the duplex.[29a] Each of the major steps in RecA protein-catalyzed strand exchange — assembly onto ssDNA, synapsis, and strand transfer — has been shown to have different requirements for ATP and sensitivities to ADP. We describe in the following how this has made it possible to dissect and examine each step in the reactions. The UvsX protein is also a DNA-dependent ATPase and shares many properties with RecA protein in this regard. Notable differences are that UvsX protein displays no dsDNA-dependent ATPase activity under conditions where it readily binds dsDNA. In addition, UvsX protein not only hydrolyzes ATP to form ADP + Pi, but also yields as products AMP + PPi.[30,31]

III. STRUCTURE OF THE DNA-PROTEIN FILAMENTS

A. Structure of RecA Protein in the Absence of DNA

In vitro strand exchange reactions are catalyzed by the filaments formed when RecA protein binds to DNA. These filaments are likely to be equally important in vivo. In addition,

although the initial steps in the induction of the SOS response in *Escherichia coli* remain unclear, in vitro data suggest that ssDNA-RecA protein filaments greatly stimulate lexA repressor cleavage,[32] initiating the cascade of SOS events.

Unfortunately, many central questions relating to how RecA or UvsX proteins assemble onto DNA remain unresolved. One of the most central questions concerns the aggregation state of the proteins themselves in solution prior to assembly. Several older studies suggest that RecA protein may exist in one or more high molecular weight forms in the absence of DNA. For example, while the SSB protein is easily shown to exist in a tetrameric state in solution by sedimentation, gel filtration, and chemical cross-linking methods,[33,34] the same techniques have failed to elucidate the nature of RecA protein multimerization. McEntee et al.[8] found that RecA protein sedimented as a dimer at pH 6.2, but at higher pH values a broad range of sedimentation values has been observed. When RecA protein is filtered through Sephacryl S200 in a buffer containing 0.3 *M* ammonium sulfate, it elutes near the exclusion volume rather than at the position of a 40-kdalton protein.[35] Cotterill and Fersht[36] observed aggregates of monomers through hexamers following chemical cross linking with much of the protein not entering the gel. They also examined the light-scattering behavior of RecA protein under a variety of ionic conditions and found evidence for an assembly of RecA protein into higher molecular weight forms which was stimulated by adding magnesium and inhibited by adding ATP. The magnesium-induced aggregates were so large that they could be pelleted by low-speed centrifugation. There was no direct evidence from EM however to indicate what the structures were that formed upon addition of magnesium.

Early EM studies suggested that RecA protein could form short rods in the apparent absence of DNA. McEntee et al.[8] noted that the RecA protein seen in the background in preparations to which DNA and adenosine 5′-O-(3-thiotriphosphate) (ATPγS) had been added was in the form of short rods 10 to 20 nm in diameter. However, Register and Griffith[37] showed that such "self filaments" formed in the presence of ATPγS are actually likely to be complexes of RecA protein bound along poly(rA), which is polymerized from ADP by contaminating polynucleotide phosphorylase. Flory and Radding[7] showed that, on adding magnesium to RecA protein preparations, short rods formed very rapidly and they were disrupted on adding ATP. The rods had a range of lengths which, if there were 6 RecA monomers per 5.5 nm axial repeat,[6,38] would have contained from 20 to 200 or more RecA monomers. More recently, Williams and Spengler[39] confirmed the observation of Flory, reporting an 11 nm diameter for the RecA protein rods and a 7.5 nm helical pitch. Cotterill and Fersht[36] suggested that the large light-scattering signal they observed might be due to the formation of such rods, but the possibility remained that the signal was due to the formation of even larger structures. Takahashi et al.[40] point out that, if RecA protein is assumed to exist in solution as a monomer, estimates of the cooperativity value for its binding to DNA are irreconcilable with existing EM data. By their calculations, conflicting cooperativity values can only be resolved if RecA protein is assumed to exist as a multimer of about 30 RecA protein monomers.

Evidence that RecA protein could form large ordered structures was obtained when it was found that the addition of spermidine to RecA protein solutions caused them to become turbid and that, subsequently, the RecA protein could be pelleted at low speed.[41] EM examination revealed needle-like pseudo-crystals several micrometers long. The dependence of pseudo-crystal formation upon spermidine and other cations was examined and found to be strongly inhibited by chloride concentrations over 30 m*M* or by even lower concentrations of sulfate and phosphate ions. These dependencies showed a strong similarity to the findings of Cotterill and Fersht,[36] in light-scattering studies, implying that the signal they saw resulted from the formation of large pseudo-crystals and not from 10-nm rods.

Two recent studies have contributed to the investigation of this complex issue. Brenner et al.[41a] used a combination of light scattering and EM to examine the aggregation state of

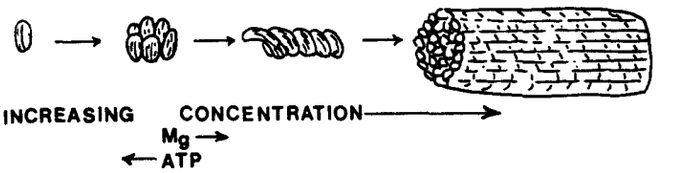

FIGURE 2. Assembly of ordered RecA protein aggregates. This diagram summarizes the work of recent studies which show that, with increasing protein concentration, RecA protein is driven from a state in which it is present as monomers or dimers to rings (most likely hexamers), then to short helical rods, and finally into very large side-by-side aggregates or microcrystals. This assembly is strongly promoted by the addition of magnesium, spermidine, or spermine and is partially inhibited by ATP. Under normal strand exchange conditions, RecA protein appears to exist primarily in rings and short rods (see text).

RecA protein in solution under normal strand exchange conditions. In a parallel study, Heuser and Griffith[41b] have used a new EM technique to approach the same problem. In this method, the RecA protein is adsorbed to a slurry of small mica flakes and then flash frozen by rapid contact with a copper block chilled in liquid helium as described by Heuser.[42] Freeze etching and shadowcasting at low temperature revealed very fine structure in the samples. The results of these studies are summarized in Figure 2. Four aggregation states of RecA protein were found: (1) small 5- to 6-nm particles, very likely RecA protein monomers and dimers; (2) RecA protein rings 14 nm in diameter containing 5 to 8 monomers (Figure 3); (3) helical rods 12 nm in diameter that were 20 to 200 nm in length and had a 5.5 nm helical repeat (Figure 3), and (4) very large aggregates micrometers in dimensions (Figure 4). These large objects were needle-like when allowed to form slowly (by the addition of 2 to 4 m*M* magnesium) but were more spherical when induced to form rapidly (with 12 m*M* magnesium) (Figure 4). The needles were identical to the aggregates observed to form upon the addition of spermidine to RecA protein.[41] Their structure strongly suggested that they were constructed by the side-by-side aggregation of the 12-nm rods. The 14-nm diameter of the rings was reasonably greater than that of the rods, indicating that the rods were not formed from a simple stacking of the rings one upon the other. However, were the rings to have a lockwasher structure and to coil as they added to a growing rod, this would account for the difference.

Parallel light-scattering data confirmed the EM observations. Upon addition of magnesium to a dilute RecA protein solution, a biphasic response was observed, which consisted of a rapid, but small, increase in the light-scattering signal followed by a much larger increase that required up to several hours to plateau, depending on the concentration of magnesium added (Figure 5). The addition of ATP rapidly eliminated this large signal, and it was also inhibited if chloride ions were present in the original solution above 30 m*M*.

These studies provide a picture of the aggregation state of RecA protein in which the protein is seen to exist in a series of larger and larger ordered structures driven by the propensity of the protein to arrange itself into helical filaments. It will be critical to determine which of these forms (monomers or dimers, rings, rods) are active in binding to ssDNA. Because the bulk of the RecA protein was found to be in rings and possibly rods under conditions normally used in strand exchange reactions (12 m*M* magnesium, 30 m*M* NaCl, 3 m*M* ATP), it is possible that one of these two forms could be the direct precusor of assembly onto ssDNA. However, if assembly occurs from the monomeric or dimeric state, then the larger aggregates could sequester the active form and thus affect the kinetics of assembly. Morrical and Cox[12] have shown that the formation of the very large needle-like pseudo-crystals lowers the binding of RecA protein to ssDNA, which implies that this form

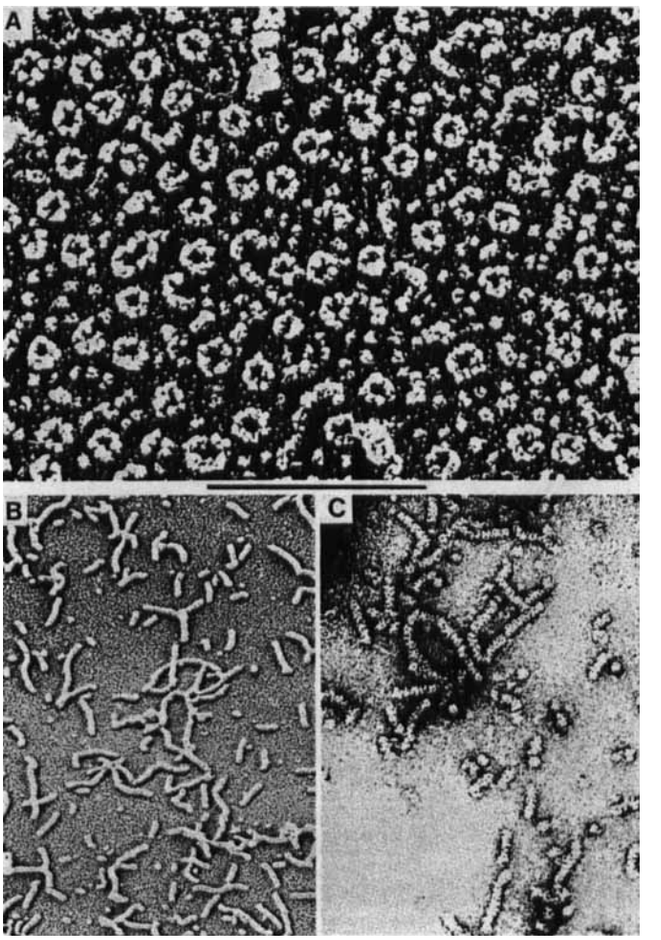

FIGURE 3. Visualization of RecA protein rings and rods. Preparation of RecA protein for visualization by ultrarapid freezing and freeze etching (at 300 μg/mℓ protein) showed that RecA protein exists primarily in rings (A) formed from about six smaller particles, some of which can be seen free on the background. Following addition of 12 m*M* $MgCl_2$ and 30 m*M* NaCl to a RecA protein preparation at the same concentration, many short 10-nm diameter rods can be visualized by either rotary shadowcasting (B) or negative staining (C). (Bars = 0.1 μm.) (Figure courtesy of Dr. John Heuser.)

is not an intermediate in DNA-protein filament formation. Clearly, it will be essential to understand much more about these aggregation states.

B. Structure of SSB Protein-ssDNA Complexes

Chrysogelos and Griffith[43] reported that, when SSB protein was added to M13 ssDNA at a ratio of 1 tetramer per 70 nucleotides, circular chains of nucleosome-like beads were observed by EM (Figure 6). Binding under these conditions did not appear to be highly cooperative as it had been reported to be under conditions of higher concentrations of SSB protein and low salt.[44] Digestion of the complexes with micrococcal nuclease revealed a chromatin-like ladder of ssDNA fragments beginning at 145 bases (Figure 7A). Combining these data with a count of the number of beads complexed to a given DNA molecule, the

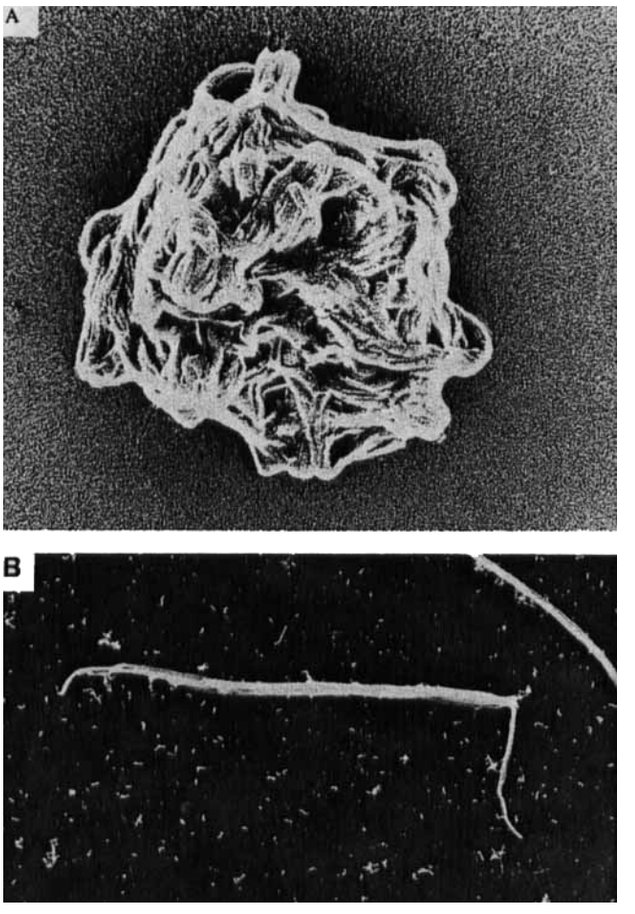

FIGURE 4. Visualization of large RecA protein aggregates. Very large RecA protein aggregates can be induced by the addition of magnesium to a RecA protein solution. Here, addition of 12 m*M* magnesium led to the rapid appearance of compact spherical particles (A), while addition of 3 m*M* magnesium and longer incubation generated needles (B). Prepared by mounting samples onto thin carbon films and rotary shadowcasting as in Figure 1. (Bar = 2 μm.)

investigators concluded that each bead contained 2 SSB protein tetramers and 145 bases and, at this stoichiometry, each bead was spaced by about 30 bases. When less SSB protein was added per nucleotide of ssDNA, SSB protein bound but did not disrupt the secondary structure of the M13 ssDNA circles.[45] When SSB protein, in low salt buffers, was added in increasing amounts to ssDNA, a transition from the beaded form to a "smooth-contoured" filament form occurred as the amount of SSB protein exceeded 1 tetramer per 35 nucleotides. The smooth-contoured form had a length twice that of the beaded form (Figure 6), and no breaks in the protein sheath were evident as there were in the beaded form.[45] When the same experiments were carried out in solutions containing 12 m*M* magnesium and 3 m*M* ATP, the beaded complexes were found at the same low stoichiometries, but the transition to the smooth, extended form appeared to be inhibited.

Using optical methods, Lohman and Overman[46] and Bujalowski and Lohman[47] examined the binding properties of SSB protein and found that SSB protein will bind ssDNA in several

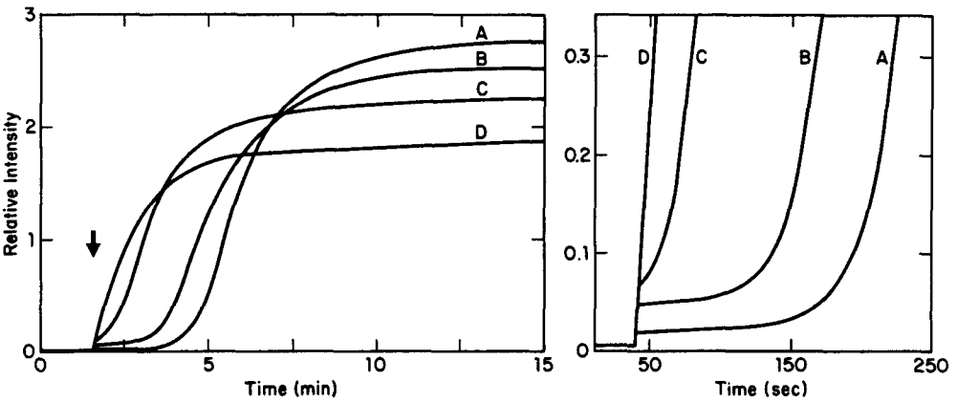

FIGURE 5. Light-scattering behavior of RecA protein. This figure illustrates the dependence of the light-scattering behavior of RecA protein on its starting aggregation state. In (A), RecA protein was diluted from a stock at 135 to 0.5 μM in a buffer containing 20 mM HEPES pH 7.5 and then $MgCl_2$ was added to 12 mM. By diluting the protein to a state likely composed primarily of monomers and dimers, a long lag is seen in which rods first form (low rise in light scattering) followed by the formation of large aggregates (large increase in the signal). If the RecA protein were diluted to 10 μM (B) or 20 μM (C) in the HEPES buffer and then diluted further to 0.5 μM in a buffer containing 12 mM $MgCl_2$, the lag would be reduced or no lag would be seen (D) if the protein were diluted to 0.5 from 135 μM directly into a buffer with 12 mM $MgCl_2$. (Figure courtesy of Dr. Stephen Brenner, the Dupont Company.)

modes which differ from each other in the number of nucleotides bound per tetramer of SSB protein, i.e., 35 ± 2, 40 ± 2, 56 ± 3, and 65 ± 3 nucleotides per tetramer. They observed that the relative population of each binding mode is determined by the concentration and valence of the cations present and that the higher site size modes are found in solutions of higher salt and valence. Furthermore, the "high-salt" or beaded mode of 65 bases per tetramer was found to possess only low-to-moderate cooperativity at equilibrium. They have used these observations[46,47] to present a detailed mathematical model of SSB protein binding in the beaded $(SSB)_{65}$ mode in which it is suggested that, when SSB protein binds to ssDNA under salt conditions which would be used in most strand exchange reactions, it would be present as a mixture of tetramers complexing 65 bases per tetramer and octamers binding 130 bases with only a low affinity between the adjacent units. This model agrees very well with the EM data and is further supported by earlier DNase I digestion patterns of ssDNA-SSB protein complexes that revealed fragments sizes near 40, 60, and 130 to 150 bases.[43] EM studies in the near future using the ultrarapid freezing method of Heuser,[42] which avoids artifactual associations of adjacent tetramers and octamers that might be created by fixation, should provide an important test of this model.

C. Structure of the RecA Protein-DNA Filaments

The best-characterized filament formed by the binding of RecA protein to DNA is that formed with dsDNA in the presence of ATPγS. Although the use of the analog is understood to be nonphysiological, it is possible that it may trap complexes in an intermediate stage of strand exchange. The great stability and remarkable ultrastructure of this filament has made it a popular topic of study, particularly by EM, and there is little argument among the several EM laboratories over its structure. In this filament, RecA protein is bound tightly and cooperatively. The filament is 10 nm in diameter, has a 7.5- to 9.5-nm repeat along the helical axis, and it can be calculated that there are 18 bp per repeat (Figures 8 and 9).[6,48-50] The observation that, as measured by EM, these filaments are 1.6 times longer than the DNA in them (Figure 8) suggested that the dsDNA must be unwound.[48,51] Measurement

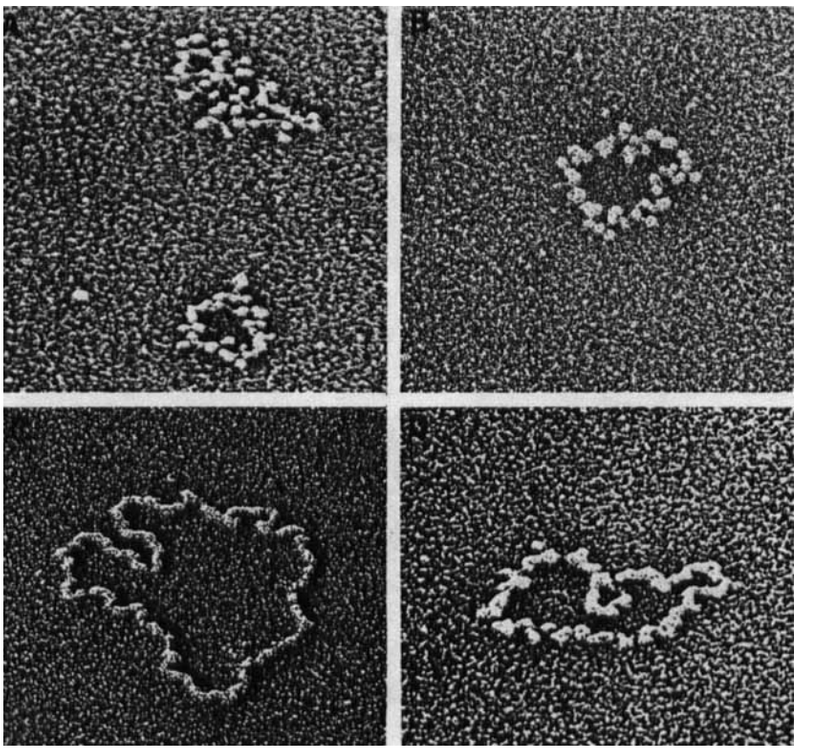

FIGURE 6. Visualization of SSB protein-ssDNA complexes. When SSB protein is incubated with ssDNA in 0.01 *M* Tris®, pH 7.5, 1 m*M* EDTA solutions at low stoichiometries (1 tetramer per 140 nucleotides), the DNA secondary strucure is not fully disrupted (A), but it is disrupted at 1 tetramer per 70 nucleotides (B) where open beaded loops consisting primarily of octamers bound to the ssDNA are observed. A distinct transition occurs upon increasing the SSB protein concentration to 1 tetramer per 35 nucleotides where extended, smooth-contoured filaments are found (C). Even at high stoichiometries (1 tetramer per 17 nucleotides) in a buffer containing 3 m*M* ATP and 12 m*M* $MgCl_2$, the beaded appearance is retained (D). Prepared for EM as in Figure 1.[44] (Bar = 0.25 μm.)

of the unwinding was carried out by Stasiak and DiCapua,[38] who derived a value of 15° of unwinding per base pair from the appearance of partially RecA protein-covered DNA loops, and also by the authors' laboratory,[51,52] which measured the amount of RecA protein coverage needed to relax a given number of supercoils to arrive at a value of 11.5 ± 2° per base pair. Examples from this study are in Figures 10 and 11. This value corresponds to one third of a complete unwinding of the two strands. Mass measurements using scanning transmission electron microscopy provided an estimate of six RecA protein monomers per helical repeat,[6] a value consistent with a sixfold symmetry of partially ordered RecA protein crystals.[53] An estimate of the stoichiometry from the authors' laboratory[51] was, it appears, low for unknown reasons.

RecA protein will bind to dsDNA in the presence of magnesium and ATP, but only at pH values below 6.5.[8] The binding appears to be cooperative but much less stable than that observed with ATPγS, although once the protein has bound, it can remain bound, despite changes in salt and pH.[8] RecA protein has recently been reported to be associated with dsDNA following a cycle of recombination with the dsDNA or following association with a single-stranded region (see Section VI.D).

RecA protein binds readily to ssDNA; several different filament forms have been observed,

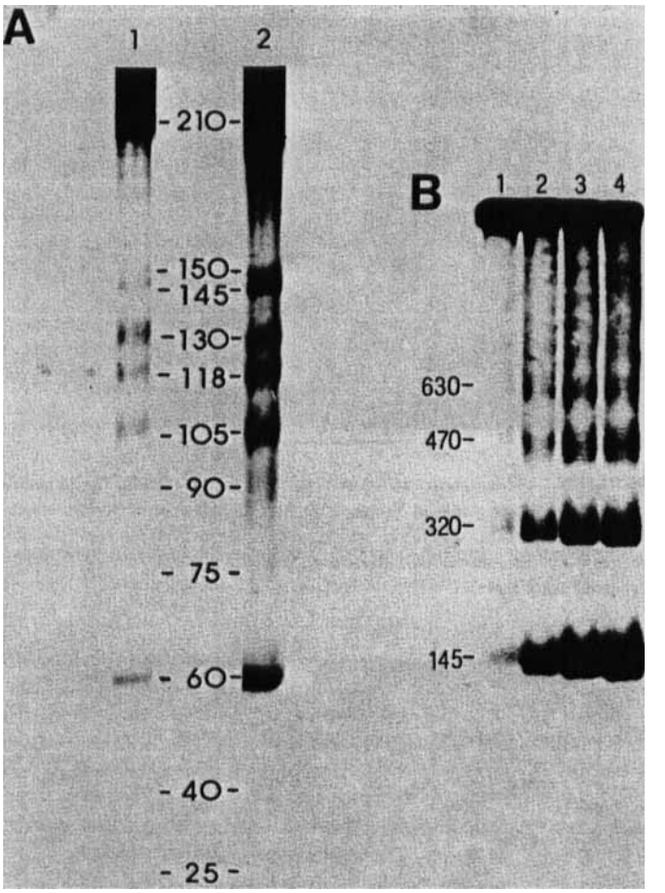

FIGURE 7. Nuclease digestions of SSB protein-ssDNA complexes. As described in Reference 42, digestion of SSB protein-ssDNA complexes with DNase 1 (A) leads to a pattern of ssDNA fragments displayed on acrylamide gels with particularly prominent bands in the regions of 40, 60, 130, and 130 to 150 bases. These likely correspond to the protection of the ssDNA by different arrangements of tetramers and octamers as described by Lohman and colleagues.[46,47] When the complexes are treated with a limited amount of micrococcal nuclease (B), higher order nucleosome-like ladders of fragment sizes are observed.

depending on the cofactors used and the route of assembly. When RecA protein is mixed with M13 ssDNA in low salt buffers, it will disrupt the secondary structure of the DNA without the aid of the SSB protein.[7,48] This binding produces a smooth-contoured filament 10 nm in diameter, with a barely detectable 5.5-nm repeat as seen by EM.[39,48] The filaments form rapidly, are stable to prolonged incubation, and measure 1.8 Å/nucleotide.

RecA protein will bind very tightly to ssDNA if magnesium and ATPγS are present as cofactors. These filaments resemble those formed on dsDNA under the same conditions in that the protein is very tightly bound to the ssDNA, a distinct helical repeat of 8 to 9 nm is seen by EM, and the ssDNA is extended to 4 Å/nucleotide.[39,49] RecA protein protects the sugar/phosphate backbone of oligonucleotides from nucleases, but the bases are left unobstructed, which would presumably function to make the bases available for pairing with complementary bases during strand exchange.[5]

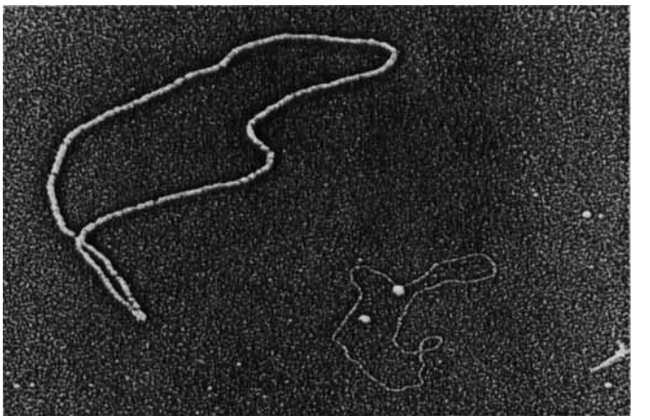

FIGURE 8. Visualization of RecA protein bound to dsDNA. The binding of RecA protein to a nicked circular dsDNA in the presence of ATPγS leads to the formation of a stiff helical nucleoprotein filament measuring 1.6 times the length of the protein-free DNA (one molecule of each shown). Sample prepared as in Figure 1.[47] (Bar = 0.5 μm.)

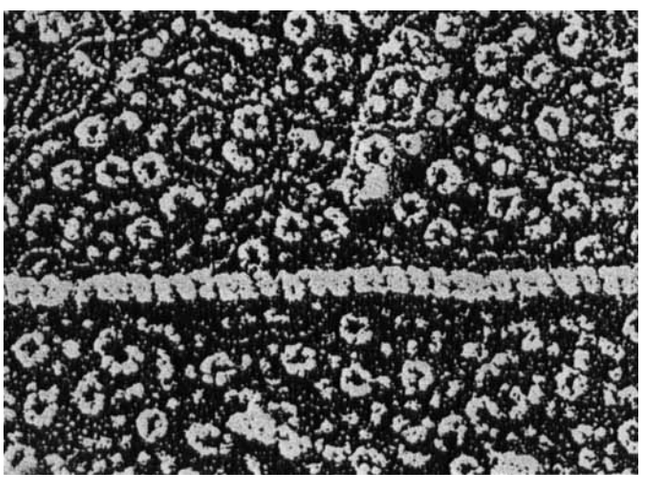

FIGURE 9. Visualization of the helical structure of RecA protein-dsDNA filaments. When RecA protein-dsDNA complexes formed in the presence of ATPγS are prepared for EM by ultrarapid freezing and freeze etching,[41] the very regular 9-nm helical repeat is clearly visible and there is a suggestion of a deep crack along the filament length. Free RecA protein rings are visible on the background. (Figure courtesy of Dr. John Heuser.)

Finally, as described in the following, under strand exchange conditions (12 m*M* magnesium, 3 m*M* ATP), RecA protein can be assembled onto ssDNA through a three-step pathway promoted by SSB protein.[45,54] These filaments are 10 nm in diameter, the RecA protein is bound in a highly cooperative manner, and the filaments are stable to challenge with 0.3 *M* NaCl, as long as the ADP levels remain low. In this laboratory, the length of such filaments is found to depend on the manner in which they are prepared for EM. If the filaments are fixed with formaldehyde and glutaraldehyde and then mounted onto carbon

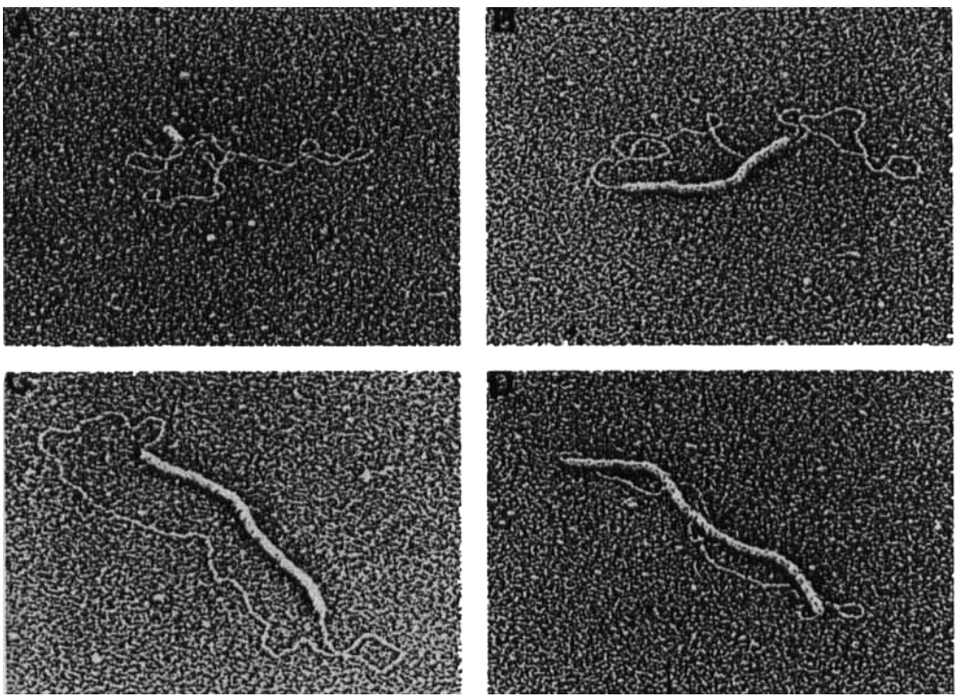

FIGURE 10. Progressive unwinding of dsDNA by RecA protein. When RecA protein binds to dsDNA in the presence of ATPγS, the DNA is unwound by 11.5°/bp.[50] Here, progressive stages of the unwinding and resulting relaxation of the natural superhelicity of a plasmid DNA (A to D) are illustrated by the synchronous binding and polymerization of single RecA protein tracts on the DNA. Quantitation is described in Figure 11.[50] Prepared for EM as in Figure 1.

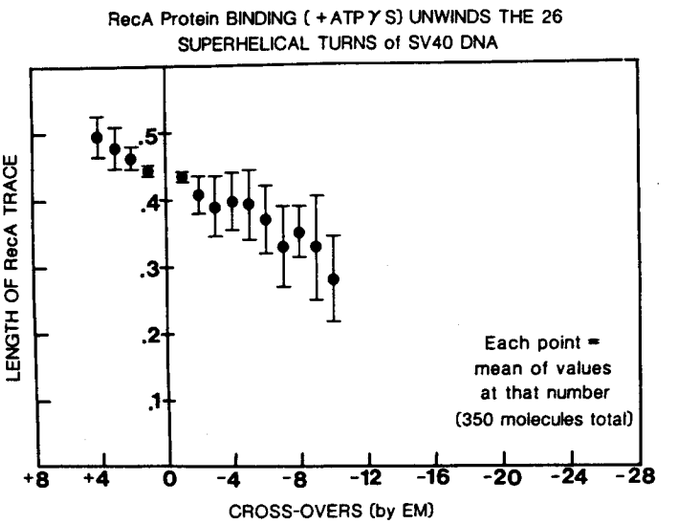

FIGURE 11. Quantitation of the unwinding of dsDNA by RecA protein. When the lengths of RecA protein tracts on molecules such as those shown in Figure 10 are measured and the number of DNA crossovers on the same molecules are scored, a linear relation is obtained relating the length of the RecA protein tracts to the number of supertwists remaining. Such analysis led to the determination that this interaction unwinds dsDNA by 11.5°/bp.[50]

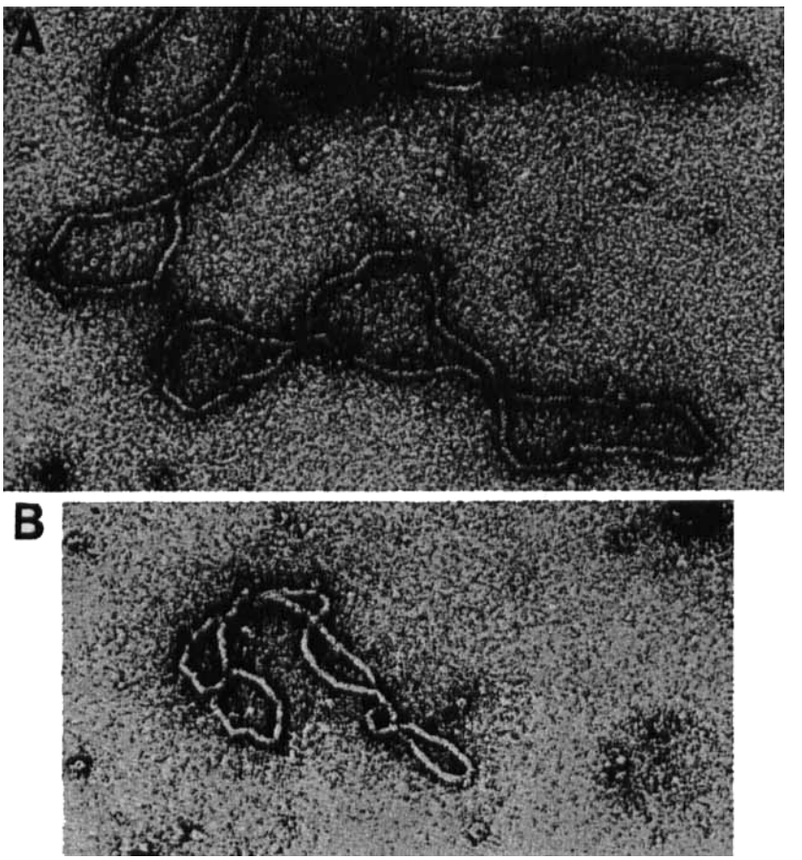

FIGURE 12. Visualization of RecA protein bound to ssDNA. When RecA protein is assembled onto ssDNA in the presence of SSB protein, 12 m*M* magnesium, and 3 m*M* ATP, open nucleoprotein filament loops can be observed which appear to undergo a cooperative change from a smooth-contoured form with a length equivalent to 2.6 Å/nucleotide (B) to a form with 4.8 Å/nucleotide and a 9-nm helical striation (A). Here, the two forms were photographed from the same EM grid following negative staining of unfixed complexes. (Bar = 1 μm.)

supports in the presence of spermidine, they have a length of 2.6 Å/nucleotide. If the same (unfixed) filaments are mounted onto carbon films in 70 m*M* KCl, some filaments measure 2.6 Å/nucleotide, while others measure 4 Å/nucleotide; the latter show a distinct 8-nm helical repeat (Figure 12). If the (fixed) filaments are spread on a solution of 10% formamide, they also measure 4 Å/nucleotide. A value of 4 Å/nucleotide has been reported by Flory and Radding[7] using other EM methods.[7] Thus these filaments must be remarkably extendable, even after fixation, and the extension must be highly cooperative.

Stasiak and Egelman[50] have used image-enhancement technology on electron micrographs of negatively stained RecA protein-DNA complexes to elucidate details of their structure. The model they propose depicts the RecA protein filament as a spring-like helix, capable of extending or compressing, yielding a highly flexible overall structure. Their images show RecA protein reacting with ssDNA to produce two types of complexes. In the absence of any nucleotide cofactor, the complex is "closed" and displays a 64-Å pitch. In the presence of ATP or ATPγS, RecA protein forms a "open" complex on ssDNA with a helical pitch of 95 Å. Their studies also yield a model for RecA protein-dsDNA interactions.[50,55] RecA

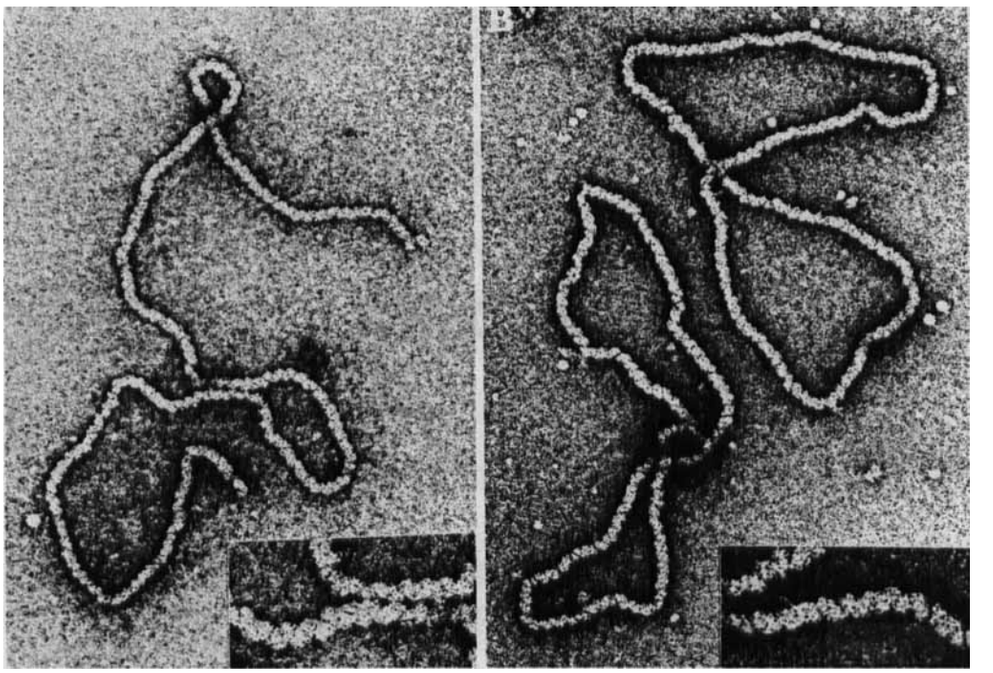

FIGURE 13. Visualization of UvsX protein bound to ss- and dsDNA. The binding of UvsX protein to linear M13mp7 dsDNA (A) and to circular M13mp7 ssDNA (B) followed by negative staining with 1% uranyl acetate reveals the same 12- to 14-nm filament diameter and 12-nm helical repeat in both filaments.[58] (Bar = 0.2 μm [0.1 μm for insets].)

protein forms the same type of spring-like helix on dsDNA, with a pitch varying from 90 to 100 Å. Regions of the filament with a greater pitch show a correlating decrease in filament diameter, which further supports the model of a spring-like construction. Egelman and Stasiak[55] also show that RecA protein-dsDNA filaments which align themselves side by side show perturbation in the usual helical structure. Image enhancement reveals bridges of RecA protein which link the adjacent filaments. The studies produce an image of the filament as a flexible, bendable spring, capable of structural alterations to allow for some sort of interaction with other RecA protein filaments.

These studies show that RecA protein binds readily to ssDNA in the presence of ATP, but the protein cycles off much more quickly than it would in the absence of any nucleoside cofactor.[2] ADP not only prevents binding, but also disrupts filaments which have already been formed by lowering RecA protein's intrinsic affinity for ssDNA.[56] Once ADP/ATP ratios reach a level of 0.6 to 1.5, the filaments dissociate.[57] Because RecA protein is a ssDNA-dependent ATPase, reactions with ATP will lead to an accumulation of ADP, which, in turn, will stop the progress of the reaction unless an ATP regeneration system is employed.

D. Structure of the UvsX Protein-DNA Filaments

In contrast to the variety of RecA protein-DNA filaments described, the binding of UvsX protein to DNA appears to be less complex. UvsX protein binds tightly to both ssDNA and dsDNA and appears to form the same helical structure with both.[58] As with RecA protein, the binding appears to be highly cooperative and to involve a rare nucleation event followed by a rapid polymerization of additional protein. The filaments formed by UvsX protein are 14 nm in diameter, compared with 10 nm for the RecA protein filaments, and a helical repeat of 12 nm can be easily observed (Figure 13). No striking effect of ATP analogs in

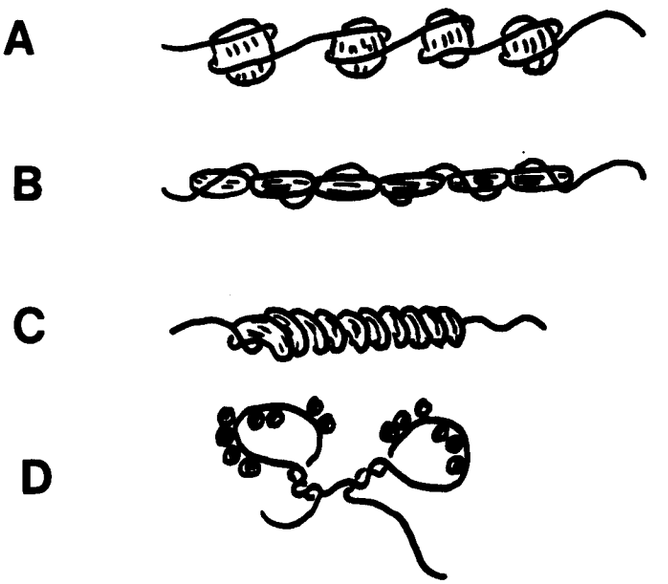

FIGURE 14. Four binding modes of single-stranded binding proteins. Four different ways in which ssDNA-binding proteins can interact with ssDNA are (A) beaded or nucleosomal with low cooperativity; (B) highly cooperative and smoothly covering the ssDNA; (C) highly cooperative with a helical structure; and (D) cooperative or not but unable to disrupt the secondary structure of the ssDNA. SSB protein can be found in (A) or (B) forms, T4 gene 32 protein in (B), RecA and UvsX proteins in (C), and UvrD shows mode (D) binding (see text).

promoting a tight DNA-protein complex has yet been reported.[58] However, in parallel to the binding of RecA protein to ssDNA, two UvsX protein-ssDNA filaments are observed, one with a length factor of 1.8 Å/nucleotide when binding is carried out in a 0.01 *M* Tris®, 1 m*M* EDTA solution, and the other, with a length factor of 2.6 Å/nucleotide when the solution contained 0.01 *M* Tris®, 5 to 12 m*M* $MgCl_2$, and 5 m*M* ATP. Stoichiometric measurements indicate that the helical repeat unit for the filaments containing dsDNA contains exactly 4 turns of the dsDNA helix and (4 × 10.5 bp) and 12 *UvsX* protein monomers. The filament formed with ssDNA (in the presence of magnesium and ATP) contains 12 protein monomers and 27 bases of ssDNA per repeat.[58]

E. Comment: Different Single-Strand DNA-Binding Proteins May Interact with DNA in at Least Four Different Modes

From the results summarized previously, it is useful to note that there are at least four different modes of binding between ssDNA-binding proteins and ssDNA that can be envisioned (Figure 14). The first is exemplified by the binding of the T4 gene 32 protein to ssDNA. Here, the highly cooperative binding melts the secondary structure in the DNA and there is a strong interaction between adjacent protein units in a nonpolar fashion (see review in Reference 59). This creates a smooth protein sheath along the DNA which shields it from interactions with other proteins. SSB protein shows such binding under very low salt and high protein to DNA stoichiometries.[43,45-47] A second mode of interaction is seen in the binding of SSB protein to ssDNA under higher salt and strand exchange conditions in which the secondary structure of natural ssDNA is also removed but where adjacent binding units show much less affinity for each other. The result, in this case, is a distribution of SSB

protein tetramers and octamers bound along the ssDNA often separated by protein-free DNA linkers.[43,45-47] Such an ordering could provide a means of removing the secondary structure of the DNA and arranging it into an ordered state, while providing access to the DNA by other proteins (e.g., RecA protein). The importance of such a binding mode is demonstrated by SSB protein, which stimulates RecA protein nucleation on DNA when it is in the beaded mode but inhibits nucleation when it assumes the "smooth-contoured", low salt mode.[45] A third mode of binding, which has not been well studied, appears to be exhibited by the UvrD protein.[45a] The EM observations indicate that it binds ssDNA but not with an affinity high enough to remove the secondary structure. Finally, a fourth binding mode is exhibited by the RecA and UvsX proteins. Here, the affinity of single monomers for DNA is less than that of SSB protein tetramers. Nonetheless, RecA protein is able to displace SSB protein from ssDNA. It does so through a highly cooperative polar assembly in which the protein monomers are interlocked into a regular helical protein sheath. This sheath shields the ssDNA so that once SSB protein is displaced and the RecA protein sheath has covered that DNA segment the SSB protein is no longer able to gain access to the DNA. In discussing the binding of these proteins with DNA and with one another and in considering models of SSB-RecA protein interactions, the inherently different properties of these four binding modes must be appreciated.

IV. ASSEMBLY OF RecA AND UvsX PROTEIN-DNA COMPLEXES

A. Assembly of RecA Protein onto ssDNA

The assembly of RecA protein onto ssDNA is the first step in the initiation of RecA protein-directed DNA strand exchange and may be an early step in the induction of the SOS response. It is often referred to as presynapsis and, in kinetic studies, appears as a lag time after the DNA is mixed with the proteins, but before recombination can be detected.[60,61] This assembly has been examined in numerous studies and appears to proceed through a complex multistep pathway that is strongly affected by the concentrations and the orders of addition of ATP, SSB and RecA proteins, cations, and salts.

The relationship between the RecA and SSB proteins is complex. At low concentrations, SSB protein promotes RecA protein's binding to ssDNA and enhances the rate of subsequent strand exchange.[1,45,61] However, at higher concentrations, SSB protein prevents nucleation of RecA protein on DNA.[45] Mutant gene products of the SSB protein which bind more tightly to DNA than their wild-type form impair RecA protein activity.[62] Several studies seem to indicate that SSB protein interacts directly with RecA protein to promote its assembly onto ssDNA. In the presence of SSB protein, RecA protein binds more stably to ssDNA.[20] More ssDNA-RecA protein filaments are formed, and the structure of the filaments is reported to be more extended than that of the filaments formed in the absence of SSB protein.[63] The binding of SSB protein to ssDNA can be studied without disrupting the filaments by monitoring the decrease in light absorbance of tryptophan residues in the protein. Results of such studies have been taken to argue that SSB protein remains bound to ssDNA throughout RecA protein filament assembly and strand exchange.[64] However, when RecA protein-ssDNA filaments assembled in the presence of SSB protein have been isolated, little evidence of interaction between RecA and SSB proteins is found. Tsang et al.[65] showed that the amount of RecA protein bound to an isolated filament is inversely proportional to the amount of SSB bound, which indicates that the two proteins bind competitively. As shown in the authors' laboratory, RecA protein-ssDNA filaments assembled onto circular ssDNA in the presence of radiolabeled SSB protein contained no significant radiolabel following isolation on sucrose density velocity gradients (Figures 15 and 16). Other studies, which have followed the binding stability of one protein in the presence of the other, offer verifying evidence that the two proteins compete for binding sites on the DNA.[66,67] Furthermore, the enhance-

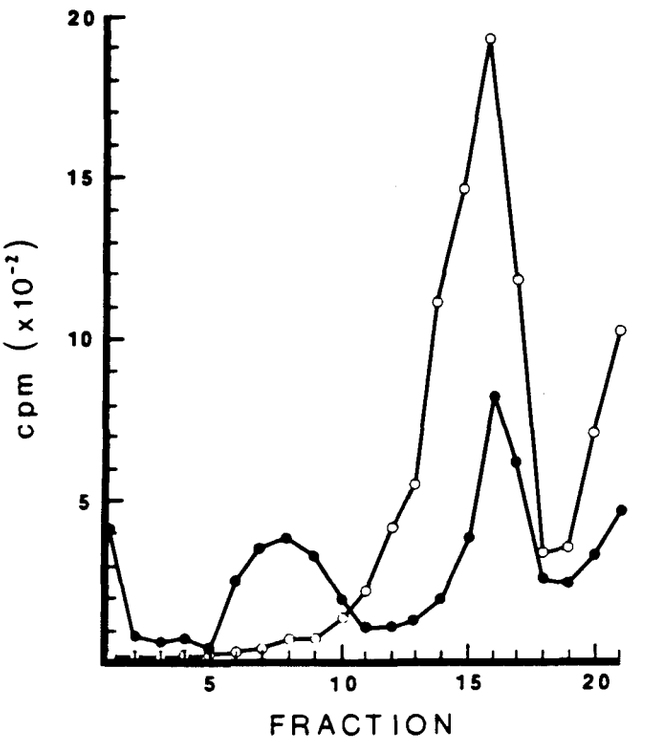

FIGURE 15. Sedimentation analysis of RecA protein-ssDNA complexes. When RecA protein is assembled onto ssDNA in the pathway described in Figures 17 and 18, but with 2 m*M* ATP, the nucleation step is depressed, and it is possible to achieve a nearly equal mixture of fully RecA protein-covered and fully SSB protein-covered complexes after 30 min of incubation. Here such a mixture was formed with either the ssDNA labeled (●) or the SSB protein labeled (○). Following incubation and fixation, the complexes were sedimented on 5 to 20% sucrose gradients. The two gradients are shown here superimposed. The profiles show very little SSB protein remaining in the fractions corresponding to the faster sedimenting RecA protein-ssDNA complexes, and this amount could be accounted for by contaminating SSB protein-ssDNA loops as seen by EM. EMs of gradient fractions 8 and 16 are shown in Figure 16.

ment and stablization of RecA protein binding to ssDNA in the presence of SSB protein can be imitated in its absence by lowering the concentration of Mg^{2+}, increasing the temperature of the reaction, or assembling RecA protein onto DNA molecules devoid of secondary structure.[68,69] This suggests that the SSB protein serves only to break up the secondary structure of the ssDNA; thus it eliminates sequence-specific "cold spots"[68] that blocked complete polymerization of RecA protein along the ssDNA.

An indirect argument for the absence of SSB protein in the fully assembled filaments is that the EM studies have revealed a highly regular helical substructure that is very similar to that created by the binding of RecA protein alone to dsDNA (in the presence of ATPγS). Given this fact, it is difficult to draw a model of the RecA protein-ssDNA filament incorporating 75,000-dalton SSB protein tetramers (the minimal SSB protein unit) or octamers in such a regular, tightly organized protein helix. A rational conciliation for the apparently conflicting results is that, as observed in the authors' laboratory, even after extended assembly times some of the M13 ssDNA circles were always found to remain fully complexed with

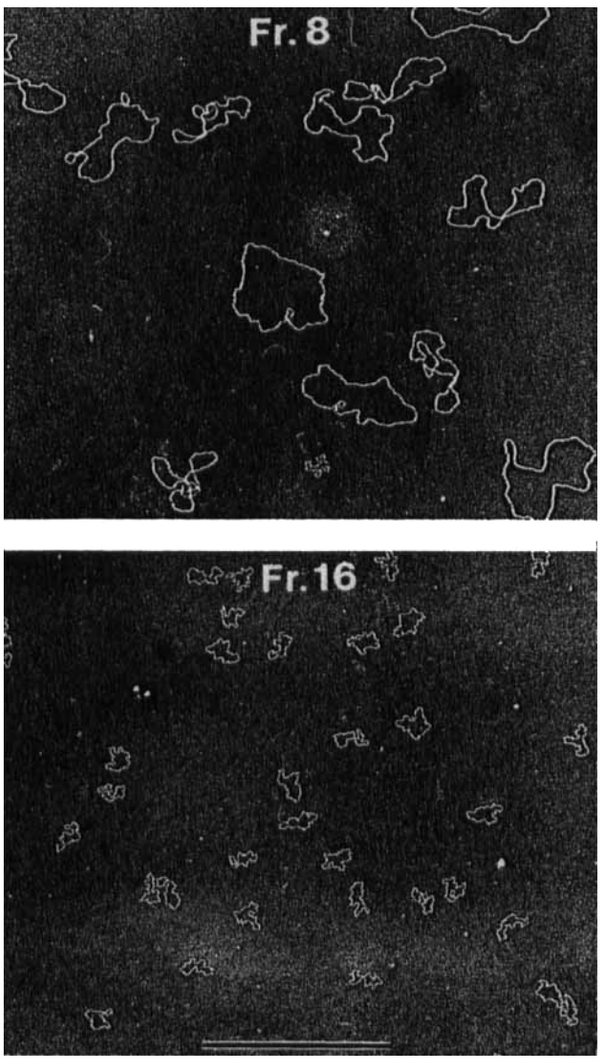

FIGURE 16. Visualization of separated SSB and RecA protein-ssDNA complexes. Fractions 8 and 16 from the sedimentations in Figure 15 were prepared for EM as described in Figure 1. A single smaller SSB protein-ssDNA complex can be seen among the longer fully assembled RecA protein-ssDNA complexes in Fr. 8. (Bar = 1 μm.)

SSB protein and this fraction could be as high as 50% depending on the initial conditions (Figures 17 and 18). Analysis of such a sample for composition might erroneously lead to the conclusion that all complexes contain a mixture of SSB and RecA proteins, rather than there being a mixture of two different complexes.

The assembly pathway of RecA protein onto ssDNA in the presence of SSB protein, derived from work in the authors' laboratory, is illustrated in Figure 17. When RecA and SSB proteins are incubated together at 37°C in a buffer containing 12 m*M* magnesium and 3 m*M* ATP and a natural ssDNA is added to this mixture, SSB protein binds rapidly to the ssDNA, disrupting its secondary structure, providing a beaded nucleoprotein template for RecA protein assembly. When this assembly is followed by EM (Figure 18), samples taken

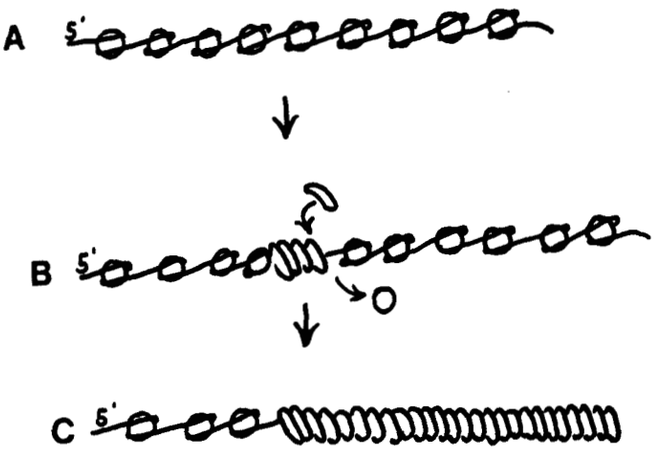

FIGURE 17. Assembly pathway of RecA protein onto ssDNA. When SSB and RecA proteins are incubated together in a buffer containing 12 m*M* magnesium and 3 m*M* ATP and then ssDNA is added, a three-step assembly of RecA protein onto ssDNA can be observed.[44] In the first step (A), SSB protein rapidly binds and disrupts the secondary structure. Next in the rate limiting step (B), RecA protein nucleates on this template most likely in the protein-free segments between the SSB protein octamers. In the third step (C), additional RecA protein polymerizes along the ssDNA 5′ to 3′, with the concomitant displacement of the SSB protein.

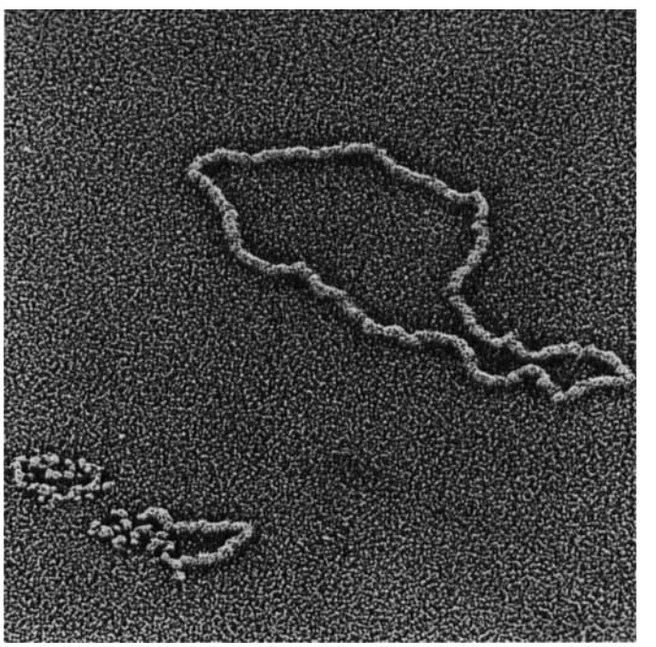

FIGURE 18. Visualization of the stages of RecA protein assembly onto ssDNA. In this micrograph, RecA protein assembly was initiated on circular M13mp7 ssDNA as described in Figure 17 and in Reference 44. All three stages of assembly are visible. Sample was prepared for EM as in Figure 1. (Bar = 0.5 μm.)

shortly after addition of the ssDNA appear indistinguishable from those formed in the absence of RecA protein. With continued incubation, short tracts of a 10-nm diameter filament structure begin to appear on the SSB protein-ssDNA complexes, usually one per complex, and these tracts grow in length at the expense of the beaded form. Eventually, 10-nm diameter filament loops are observed, which contain no trace of the beaded structure. Thus, it is convenient to consider this assembly in three separate steps: (1) rapid assembly of SSB protein onto the ssDNA, (2) nucleation of RecA protein on this template, and (3) polymerization of additional RecA protein with the concurrent release of the SSB protein.

The first step, SSB protein binding (described previously), occurs rapidly and can result in either a beaded or smooth-contoured structure, depending on salt and stoichiometric conditions.[45-47] We have shown that the two modes differ in their promotion of the second step, RecA protein nucleation.[45] Examining the ability of RecA protein to nucleate on different SSB protein-ssDNA templates, it was found that the rate of nucleation was highly dependent on the initial ratio of SSB protein monomers to nucleotides of ssDNA such that RecA protein assembly was optimally stimulated by relatively low amounts of SSB protein and was inhibited at higher amounts.[45] The optimal stoichiometry was that needed to disrupt the secondary structure of the ssDNA and form a repeating chain of beads, but also leave protein-free segments between each SSB protein tetramer or octamer. At higher stoichiometries, the beads were more closely packed, and the rate of nucleation was greatly depressed. This suggests that nucleation of the helical RecA protein filament on ssDNA occurs by RecA protein binding directly to the ssDNA in the segments separating the SSB protein units and that specific SSB-RecA protein interactions may not be important. This explains how SSB protein could work, at different concentrations, either to increase or to compete with RecA protein binding.

The third step, RecA protein polymerization and release of SSB protein, appears, as seen by EM, to occur relatively rapidly once nucleation has occurred.[45,54] During the third step, the ratio of free SSB protein to nucleotides of protein-free ssDNA must increase greatly. Nonetheless, it appears that fully RecA protein-covered circles can be formed and that full coverage of M13 ssDNA is accomplished within 5 min after the nucleation of RecA protein. In a recent study,[54a] we measured the *net* rate of RecA protein polymerization onto ssDNA. The rate was found to be 900 RecA protein monomers per minute when SSB protein was present at 1 protein monomer per 36 nucleotides and decreased by 66% when the SSB protein concentration was increased to 1 monomer per 9 nucleotides.

B. RecA Protein Assembly onto ssDNA is Polar

Visualization of RecA protein assembled onto linear ssDNA revealed that one end of each linear filament contained a short tract that remained bound by SSB protein even after extended assembly times[54] (Figure 19). The explanation offered was that RecA protein assembly onto linear molecules began with a random nucleation on the linear SSB protein-covered ssDNA followed by RecA protein assembly in only one direction (5′ to 3′ or 3′ to 5′). Polar RecA protein assembly would produce filaments with mixed tracts unless RecA protein nucleation occurred at the exact end of the molecule. This hypothesis was confirmed by showing that, when assembly occurred on duplex DNA having long ssDNA tails with 5′ ends, SSB protein tracts were found at the free (5′) end of the ssDNA tail. When the tails had 3′ ends, the SSB protein tracts were internal and the RecA protein tracts were at the free end. Thus assembly was shown to be polar and to grow in the 5′ to 3′ direction (Figure 20).[54] This was confirmed by Cassuto and Howard-Flanders,[70] who showed that, when RecA protein was assembled onto dsDNA which contained ssDNA gaps, it was able to cover the ssDNA and then continue covering the dsDNA for a short distance in the region outside the gap. However, the protein only bound to dsDNA which was 3′ to the ssDNA gap.

Egelman and Stasiak's image enhancement work reveals that RecA protein filaments are

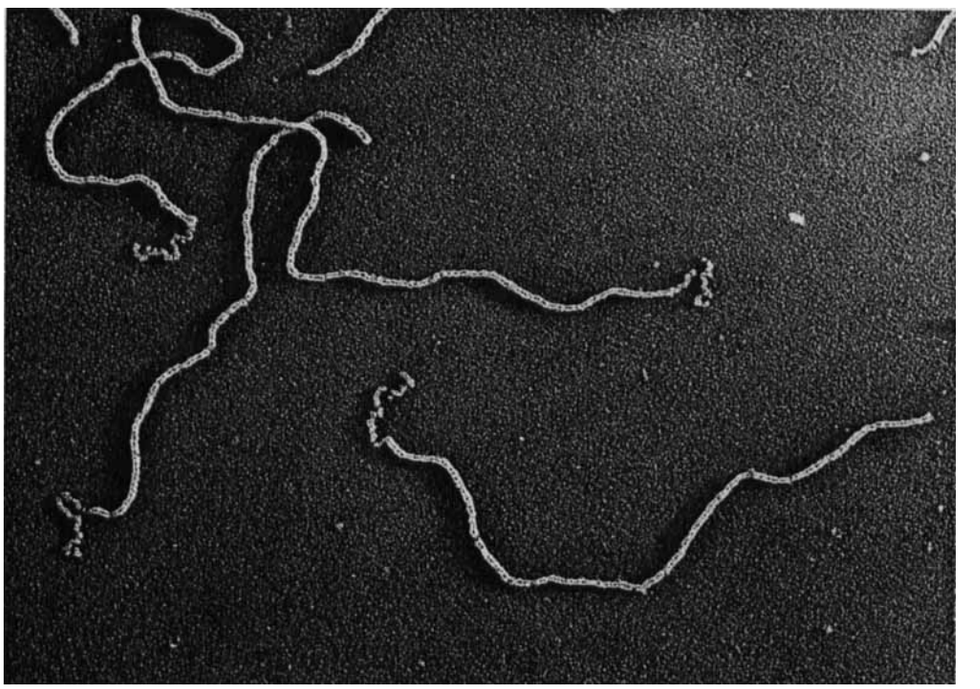

FIGURE 19. RecA protein assembled onto linear ssDNA. The assembly of RecA protein onto ssDNA via the SSB protein pathway (see Figure 17) yields linear RecA protein-ssDNA filaments, each with a single-beaded SSB protein-covered tract at one, and only one, end. Samples prepared for EM as described in Figure 1. (Bar = 1 μm.)

constructed with units that have a visible structural polarity.[55] Further evidence for an absolute polarity in the RecA protein-ssDNA filaments formed by the SSB protein assembly pathway can be found in the phenomenon of end-to-end joining of linear filaments reported by Register and Griffith.[71] It was found that, when filaments were formed with linear ssDNA, they would join end to end, but only if a 5′ end could join to a 3′ end (filaments would not join 3′ to 3′ or 5′ to 5′). These observations are important to models of treadmilling or dynamic filaments (see following) because it argues that the two ends are structurally different — otherwise one filament should show no preference for joining with one end over the other of another filament.

C. Assembly of UvsX Protein onto DNA

The UvsX protein shows both similarities and differences with RecA protein in its assembly onto ssDNA. As seen by EM, its binding involves a nucleation step followed by a highly cooperative polymerization.[58] Whether this polymerization occurs at one or both ends of the filament and whether it grows 5′ to 3′ or 3′ to 5′ has not been shown. An important difference is that, under conditions in which UvsX protein will promote synapsis (20 m*M* HEPES, pH 7.5, 5 to 12 m*M* magnesium, 3 to 5 m*M* ATP, and 0 to 150 m*M* KCl), it will disrupt the secondary structure of natural ssDNA and form the protein-DNA filaments without the assistance of the T4 single-strand-binding protein, gene 32 protein. Indeed, Griffith and Formosa[58] observed that the two proteins compete for ssDNA-binding sites, producing tracts that appeared to be covered by one or the other protein (Figure 21). This was unexpected in light of earlier findings that the UvsX protein would bind to a gene 32 protein affinity column.[72]

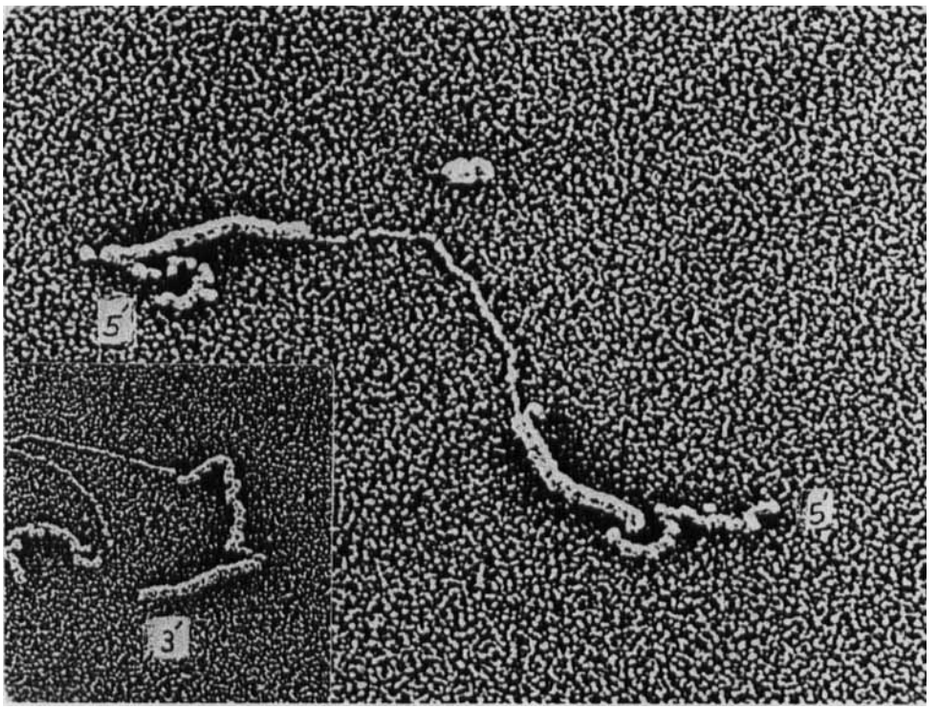

FIGURE 20. Determination of the polarity of RecA protein assembly. When RecA protein was assembled onto ssDNA by the pathway described in Figure 17 using linear duplex DNAs having single-stranded tails with 5′ or 3′ termini, the orientation of the SSB protein and RecA protein tracts could only be explained if, following nucleation, the RecA protein tract had grown 5′ to 3′ along the ssDNA. Here, two such molecules are shown and the termini indicated.[53]

V. TOPOLOGY OF PAIRING AND DISCOVERY OF PARANEMIC JOINING

A. Topology of Synaptic Joints

The pairing of a single strand of DNA with a homologous duplex can be drawn in several ways, two of which have been shown to be catalyzed by RecA protein. In the classic plectonemic D-loop (Figure 22A), the in-coming strand forms Watson-Crick base pairs with the complementary strand of the duplex and displaces the strand of the same sequence. This invasion and displacement requires a free homologous DNA end, and a necessary result of the separation of the two strands of the duplex is its complete unwinding. If the dsDNA is a covalently closed circle, this unwinding will relieve the natural supercoiling of the dsDNA. The Watson-Crick base pairing between the two DNAs renders the association stable to deproteinization. A similar plectonemic pairing when one strand of the duplex is displaced occurs in the pairing of a homologous co-linear ssDNA and dsDNA molecules (Figure 22B). The dsDNA is also unwound, but these turns are absorbed by a swiveling of the free dsDNA ends.

Pairing of two DNAs could also occur in ways that might not be stable to deproteinization. In Figure 22D, a ssDNA circle is shown paired with a dsDNA at a site of homology, but in such a way that the three strands are not topologically interwound. Such a pairing would not require a free end and is termed paranemic. Evidence for such a joining is described in the following. In this diagram (Figure 22D), the two strands of the dsDNA are separated over the length of the joint. Such a separation should be detected by the removal of supertwists

FIGURE 21. Visualization of T4 gene 32 and UvsX proteins bound to ssDNA. When T4 gene 32 and UvsX proteins were mixed with ssDNA in equal mass amounts, regions of the ssDNA appeared to be covered by one or the other but not a comixture of the two proteins.[58] Sample prepared for EM as described in Figure 1. (Bar = 0.25 μm.)

if the dsDNA partner is a natural covalently closed circle. The presence of free ends does not preclude paranemic joining, and paranemic joining may represent a major interaction between any DNA substrate pairs which can undergo net strand exchange. The way in which paranemic joining may promote plectonemic interactions on one hand, or may sequester the substrates in nonproductive complexes on the other, remains a central question to be resolved before the kinetics of pairing reactions can be fully understood.

A third and formal way in which three DNA strands might interact is illustrated in Figure 22C. A linear ssDNA is shown wrapped about a linear dsDNA with all three strands wrapping in the same direction. Such a joint* would be expected to be labile to deproteinization like the joints shown in Figure 22D, but would not be expected to produce a net unwinding of the dsDNA partner. Three-stranded wrapping might occur if the DNA ends were not homologous, a situation Bianchi et al. have termed buried homology.[13] However, such a substrate pair could also form paranemic joints like those in Figure 22D. Whether three-stranded wrapping is catalyzed by RecA or UvsX protein is not known.

* Working definition of joints: for the purpose of this review, the criteria of Riddles and Lehman[14] are used to distinguish paranemic joining from plectonemic joining. Plectonemic joints involve a net topological interwinding of the two DNAs with Watson-Crick base pairing. They are stable to deproteinization, require a free homologous DNA end, and bind to nitrocellulose through the ssDNA. Paranemic joints also require homology, require protein for stability, and bind to nitrocellulose through ssDNA or protein, but are released from the filter with 5.2 *M* guanidine hydrochloride.

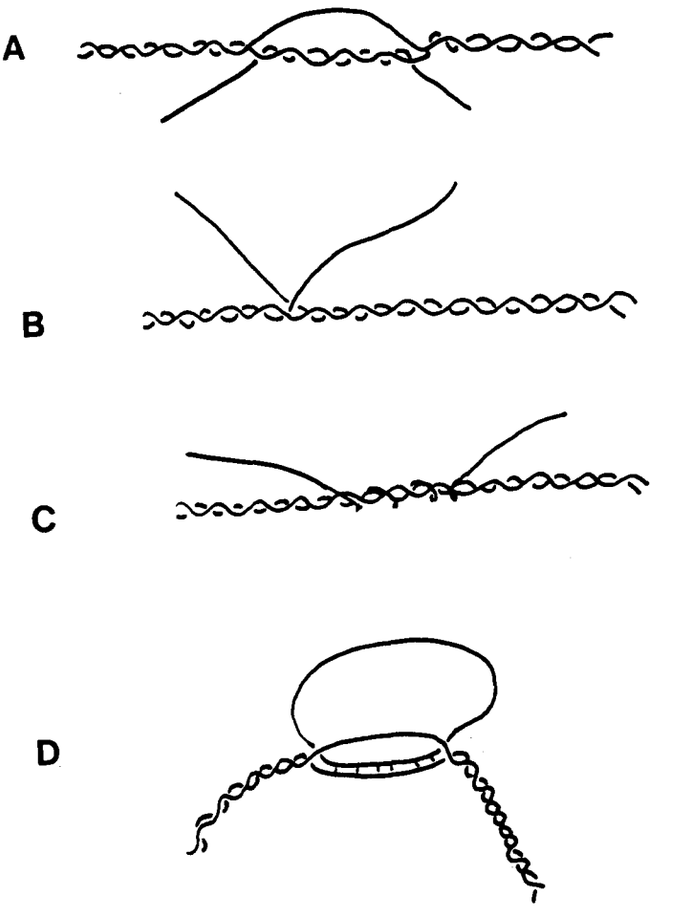

FIGURE 22. Structure of joints formed between ssDNA and dsDNA. (A) In the classic D-loop, a single strand invades a duplex in a region of homology displacing the strand of the same sequence. (B) Similar joint formed at the end of a dsDNA. Both (A) and (B) are plectonemic. (C) Illustration of a putative three-stranded wrapping in a region of homology. (D) Illustration of a paranemic joint between a circular ssDNA and a dsDNA at a region of homology. Here, the three strands interact while retaining a linking number of zero. The DNA is drawn with a duplex-like and single-stranded segment, but it is not yet known how the three strands are arranged in the RecA protein filament.

B. Discovery of Paranemic Joining

The first experimental support for paranemic joining came from EM observations by DasGupta et al.[73] of rare RecA protein-dependent pairings of circular phage G4 dsDNA with circular M13 ssDNA containing a short insert of G4 sequences. Cassuto et al.[74] found that RecA protein could form synapses using dsDNAs whose ends were cross-linked, and therefore unable to unwind, but that these joints were less stable to heat than joints formed with regular dsDNA. Cunningham et al.[75] presented additional evidence of nonplectonemic DNA synapsis by showing that RecA protein and topoisomerase 1 could act on superhelical DNA and homologous circular ssDNA to topologically link the two DNAs. Bianchi et al.[13] demonstrated that paranemic joining occurred at a high frequency and estimated the size of the joints to be approximately 500 bp. Riddles and Lehman[14] have developed a filter-binding

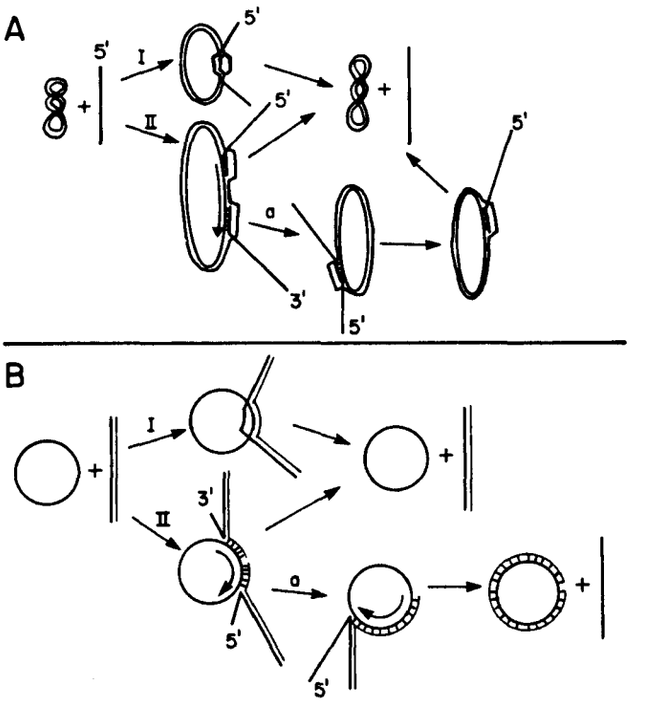

FIGURE 23. Schematic illustration of two DNA pairings. (A) In the pairing of a superhelical dsDNA and a linear ssDNA, the DNAs can pair either paranemically (top path) or plectonemically (bottom path), the latter leading to a "rolling D-loop" reaction. (B) In the pairing of a circular ssDNA and a linear dsDNA, pairing can also be paranemic (top) or plectonemic (bottom). The product of this reaction is a circular dsDNA and linear ssDNA.

assay which distinguishes paranemic joints from plectonemic joints by their relative lability to certain protein denaturants (5.2 *M* GuHCl). Julin et al.[76] used this technique to follow the kinetics of paranemic joining. The observation that paranemic joints form rapidly[76] and that cutting the dsDNA in paranemic synapses to yield homologous ends leads to plectonemic joint formation[14] suggests that these structures may serve as precursors to plectonemic joints in vivo.

A description of the ultrastructure of paranemic joints was presented by Christiansen and Griffith.[16] In that study, EM was used to visualize the joining of supertwisted M13 dsDNA and either circular M13mp7 ssDNA or linear M13mp7 ssDNA cleaved in the lac insert. This cleavage placed 400 bases of non-M13 sequences at the ends of the ssDNA, blocking strand invasion of the dsDNA. With either set of substrates, RecA protein catalyzed the formation of paranemic joints, which were labile to deproteinization, in regions of shared homology. In these joints, the dsDNA entered the RecA protein-ssDNA filament over a length of 360 ± 80 bp, without visibly altering its ultrastructure, and then exited the filament (Figure 23). Although the dsDNA in the complexes appeared topologically relaxed, deproteinization released dsDNA with 40 supertwists, indicating that the dsDNA was unwound by 34°/bp in the paranemic joint. When a chimeric dsDNA with 60 supertwists, containing M13 and *E. coli* sequences, was joined to the linear M13mp7 ssDNA-RecA protein filaments, the joints were proportionately larger but the unwinding per base pair was the same. When supertwisted M13 dsDNA was paired with circular M13 ssDNA, joints identical to those

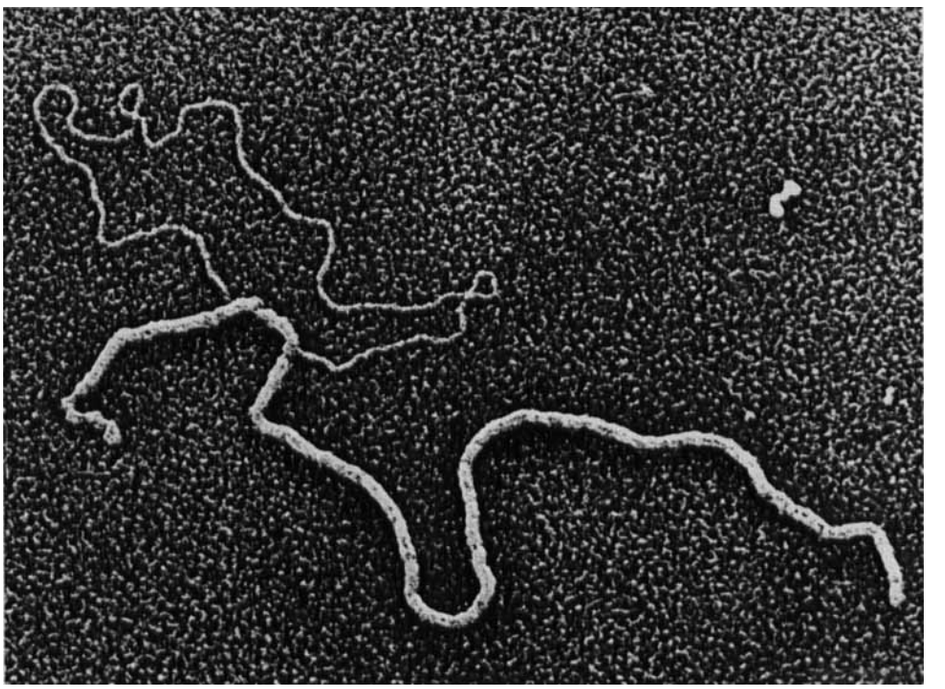

FIGURE 24. Paranemically joined ss- and dsDNA. When filaments of RecA protein assembled onto linear M13mp7 ssDNA were incubated with circular M13 dsDNA containing about 40 superhelical turns, the 2 DNAs were found joined over a distance of 350 bp and the dsDNA appeared relaxed.[16] Such joints were paranemic because the ssDNA contained segments at its ends which were not homologous with the dsDNA and the joints were labile to deproteinization. Samples were prepared for EM as described in Figure 1. (Bar = 1 μm.)

observed with the ssDNA having nonhomologous ends were observed and both the dsDNA and the RecA protein-complexed ssDNA appeared as relaxed circles that were not tightly coiled about each other. The fact that the frequency of joint formation, the ultrastructure, and the degree of unwinding of the dsDNA was exactly the same for all joints observed in both template pairs would argue against the three-strand wrapped structure as playing any major role in the joining of these DNAs.

In order to learn what functions paranemic joining may play in vivo, numerous questions need to be answered. It is known that paranemic joints are catalyzed rapidly by RecA protein,[14,73,76] but it is not known what the lifetime of a joint is at a single site, how dependent paranemic joining is on DNA sequence, whether paranemic joints move along the DNA once they form at a site, and whether they move in one or both directions relative to the polarity of the ssDNA.

Kmiec and Holloman[77] have presented evidence for the presence of left-handed (Z-DNA) DNA in paranemic joints catalyzed by the rec1 protein of *Ustilago*. In the course of paranemic joining, the DNA was transiently able to bind antisera raised to left-handed DNA. They showed that in pairings where only paranemic joining could occur, reactivity to the anti-Z-DNA antisera was observed, while no reactivity was observed when the pairings were carried out with DNA substrates that could pair plectonemically.

Christiansen and Griffith[16] demonstrated that the dsDNA segment in the joint was fully melted and, furthermore, that the two DNA partners did not counterwrap about each other outside the joint (Figure 24). One possible way this could occur would be if the joint contained a duplex-like segment and a single-stranded segment, as it does in a plectonemic D-loop.

If so, the duplex segment would have to contain an equal number of left- and right-handed turns so that the linking number would remain zero. Evidence for DNA that appears duplex-like by EM, yet contains both left- and right-handed segments, can be found in the studies of form V DNA in which (+) and (−) complementary circular ssDNAs are allowed to anneal one with the other.[78] It is possible, however, that, in the paranemic joint, all three DNA strands are bound in separate linear groves in the protein filament, a result that is equally consistent with these observations.

VI. JOINT FORMATION AND STRAND EXCHANGE

A. Search for Homology and the Role of Protein-DNA Aggregates

The ability of RecA protein to unwind dsDNA (see discussion under Section III.C) has often been postulated to be a function which would expose the dsDNA bases and promote a search for homology. Presumably, once the dsDNA bases are open for scanning, a search for homology can begin, but the mechanism by which these proteins direct a successful search for homology remains perhaps the least understood aspect of these reactions. By EM, no simple associations of RecA protein-covered ssDNA and nonhomologous dsDNA have been reported. This would argue against the formation of a presynaptic structure (possibly similar to paranemic joints) that would form regardless of homology and then scan one DNA across the other.

A common observation with both RecA and UvsX proteins has been the appearance of large DNA-protein aggregates containing both ssDNA and dsDNA.[79,80] These aggregates are observed immediately following the mixture of the homologous DNA substrates and, with time, the aggregates appear to resolve into individual units of protein-covered ssDNA joined to a dsDNA in either a paranemic or plectonemic fashion. Tsang et al.[81] proposed that these aggregates are a requisite step in the search for homology, providing a local high concentration of RecA protein. They form quickly with dsDNA and either homologous or heterologous ssDNA, and their stability is dependent on the length of the dsDNA. ssDNA added to the reaction subsequent to the formation of the aggregates is excluded from homologous pairing.[82]

Two laboratories have shown that by increasing the length of the dsDNA in a pairing reaction both the rate and extent of the reaction are increased.[76,82,83] Gonda and Radding[82] have found that long stretches of heterologous dsDNA promote pairing. When the heterologous dsDNA flanks short regions of homologous ssDNA, the rate of pairing is faster than if the short homologous pieces are alone, or if the heterologous and homologous pieces are separate.[82] These data support a model in which conjoined molecules move progressively along each other until they reach a region of homology. However, more recent studies by Gonda and Radding[83] conflict with such a model for a one-dimensional sliding mechanism. This work[83] shows that long stretches of dsDNA, whether homologous or heterologous and whether linked to or separated from short homologous dsDNAs, promote the rate of strand exchange over what is seen with short dsDNAs. The investigators correlate this phenomenon with an increase in the formation and stability of RecA protein-DNA networks. (Figure 25 shows an example from such a network from this laboratory.) Thus, Gonda and Radding postulate that long dsDNAs allow for the formation of stable coaggregates within which three-dimensional diffusion of reactant DNAs is promoted. These conclusions are partially in agreement with work by Julin et al.,[76] who find that increasing lengths of dsDNA increases the rate and extent of pairing, but, according to their data, this is true only for homologous dsDNAs. The addition of heterologous dsDNAs, regardless of their size, appears to have no effect on the rate of pairing. Despite the conflicting data of these studies, it seems possible that a search for homology can be conducted within DNA-protein aggregates.

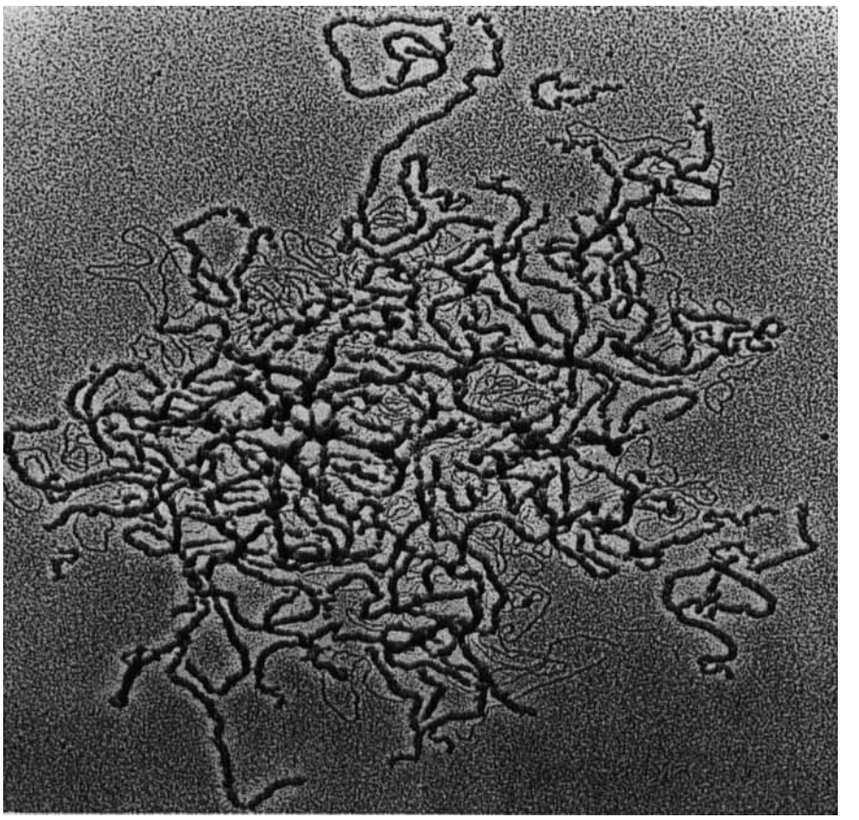

FIGURE 25. RecA protein-DNA aggregates formed early in synapsis. When linear RecA protein-ssDNA filaments are paired with fully homologous supertwisted dsDNA, very early in the incubations much of the DNA is often observed to be in large aggregates, as shown here. Sample prepared for EM as described in Figure 1.

B. Synapsis

Joint formation and synapsis are terms that have been used interchangeably. The terms describe the stage between filament assembly and strand exchange: the ssDNA-RecA protein filament finds a homologous region on a dsDNA and aligns the two DNAs in a ternary complex. Wu et al.[21] distinguished this phase from the subsequent strand exchange by the following criteria: synapsis is rapid, nonpolar, and sensitive to low levels of ADP, whereas strand exchange is slower, directional, and less ADP-sensitive. With the discovery of paranemic joining, models for synapsis now incorporate much historically conflicting data.

Evidence indicated that complete strand exchange could not occur without the presence of a free, homologous end, and yet some kind of localized interwinding occurred in its absence.[81,84,85] Several laboratories demonstrated that filaments formed with RecA protein and ATPγS (instead of ATP) could form joints with homologous dsDNA, but ATP hydrolysis was necessary for productive strand exchange.[86-88] Riddles and Lehman[89] concluded that, while paranemic joints can be formed with ATPγS, plectonemic joint formation requires ATP hydrolysis.

Riddles and Lehman[14] suggest that plectonemic joint formation is the product of two first-order reactions. In the first reaction, RecA protein pairs two homologous DNAs in a paranemic joint, and in the second reaction, this is converted to a plectonemic joint. The authors

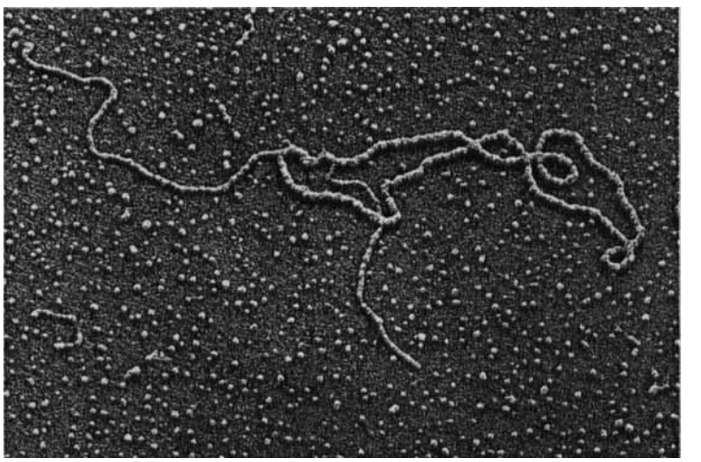

FIGURE 26. Plectonemic D-loop joint. When linear ssDNA and homologous supertwisted dsDNA are incubated with RecA and SSB proteins and then deproteinized, plectonemic D-loops are observed if the DNA is spread onto the surface of a 10% formamide solution for EM. Here, the linear ssDNA can be seen invading the dsDNA which appears relaxed, forming the D-loop. (Bar = 1 μm.)

offer two pathways for this conversion: (1) paranemic joints dissociate and then reform until they (randomly) reform at an end (or nick or gap); or (2) the joint extends until it reaches a free end. One could imagine a third possibility in which the paranemic synapse travels along the length of the DNAs (similar to the "rolling D-loop" described in the following) until it encounters an end.

C. Joint Formation between Linear ssDNA and Homologous dsDNA

In the pairing of a linear ssDNA and a homologous supertwisted dsDNA, joints form readily, but branch migration is blocked by the circularity of the dsDNA. This reaction is relevant in vivo, as it mimics initiation of pairing between a displaced linear ssDNA and supertwisted dsDNA which could occur at both replication forks and *chi* sites (see Reference 90 for a review). Pairing can be paranemic (Figure 23A — path I), in which the DNA ends are not involved and the DNAs are not topologically interwound, or plectonemic (Figure 23A — path II), in which the two DNAs are interwound, beginning at a DNA end.

Studies of the plectonemic pathway have generally used filter-binding methods following deproteinization of the DNAs. This work has shown that (1) due to the energy of supercoiling, plectonemic D-loops form more rapidly and to a greater extent than with other DNA pairs;[15] (2) plectonemic D-loop formation is reversible;[91] (3) the dsDNA in the joint is unwound[92,93] with the average D-loop length being proportional to the superhelical density of the dsDNA;[16] and (4) plectonemic D-loops formed with this DNA pair are more stable than those formed between other combinations of DNAs.[94]

Electron microscopic visualization of plectonemic D-loops was first made by McEntee et al.[17] and Shibata et al.[15] An example from the authors' laboratory is shown in Figure 26, where linear M13 ssDNA and supertwisted M13 dsDNA were incubated together for 5 min at 37°C in the presence of RecA and SSB proteins, then were deproteinized and surface spread in 10% formamide. As observed by others, the length of the dsDNA segment in the D-loop corresponds to the number of helical turns whose melting just relieves the natural supertwists of the dsDNA. This amounts to about 450 bp for a dsDNA with 40 supertwists. In these experiments, the D-loops were found at all distances from the ends of the linear

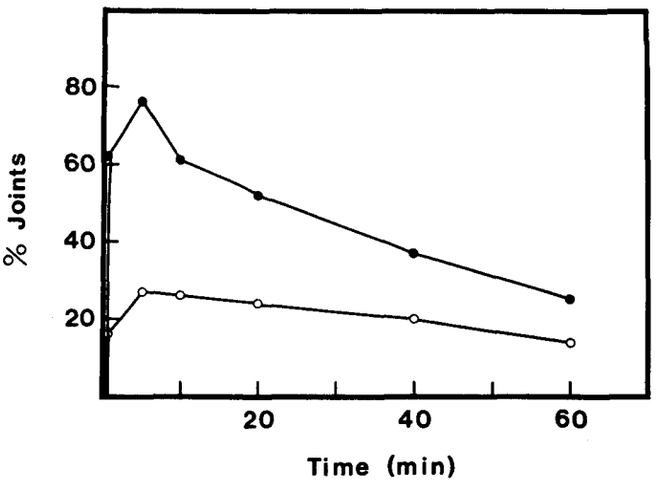

FIGURE 27. Kinetics of the joining of homologous linear ssDNA and supertwisted dsDNA. When linear M13mp7 ssDNA and M13mp7 supertwisted dsDNA were incubated together in the presence of RecA and SSB proteins, the total fraction of dsDNA present in joined complexes (plectonemic and paranemic) could be counted by fixing the samples and scoring the percentage of the dsDNA joined to ssDNA filaments after preparation for EM as described in Figure 1 (●). When parallel aliquots were deproteinized and surface spread as in Figure 26, only the fraction of molecules that were plectonemically joined remained (○). This indicates that roughly half of the joints were plectonemic and half were paranemic throughout the incubation. The dsDNA component was limiting in the incubations.

ssDNA. This might have resulted from joints located at the ends of the ssDNA which then branch migrated inwards following deproteinization. Alternatively, the joints might have been driven around the circular dsDNA in a strand exchange-like reaction exiting the dsDNA when the 3′ end of the ssDNA end was reached. Examination of the ultrastructure of the complexes with RecA protein fixed in place supported the second model.

Visualization of the RecA protein-DNA complexes engaged in these reactions has been recently carried out by Register et al.[95] Following the joining of linear ssDNA and homologous supertwisted dsDNA by RecA protein, the samples were treated with formaldehyde and glutaraldehyde to fix the proteins in place and examined directly by EM. The appearance of all the joint molecules was the same even though it was found that 50% were paranemic and 50% were plectonemic (Figure 27). While nearly 100% of the dsDNA could be found in joints after 5 to 10 min incubations, only 50% appeared as D-loops following deproteinization. In these joints, the dsDNA molecule appeared relaxed and entered a 10-nm diameter RecA protein-M13mp7 ssDNA filament over a short distance and then exited the filament without apparently altering the ultrastructure of the filament. The length of the joint was close to the length of dsDNA which, when melted, would just relieve the natural supertwisting of the dsDNA. Thus, like paranemic joints, the overall structure of the joint was dominated by the RecA protein-ssDNA filament. The finding of many joints at all distances from the ends of the linear ssDNA-RecA protein filament argued that RecA protein must be able to drive the movement of the plectonemic joints along the dsDNA in a "rolling D-loop" reaction.[95]

A similar study has been conducted with UvsX protein.[96] UvsX protein shows the same ability to pair DNAs either plectonemically or paranemically at any point along the length of the DNAs, but unlike the joints formed by RecA protein, paranemic joints formed by

UvsX protein are visibly distinguishable from plectonemic joints. The structural difference lies in the topological state of the dsDNA; UvsX protein is less able to unwind the dsDNA in paranemic joints, so that in joints formed with linear ssDNA and supertwisted dsDNA the supercoils of the dsDNA are only slightly relaxed. In plectonemic joints, however, the dsDNA relaxes concurrently with heteroduplex formation.

D. Kinetics of the D-Loop Cycle

The kinetics of the D-loop cycle have been studied in detail and have been found to be sensitive to alterations in both the DNA and protein concentrations. While excess RecA protein results in a lower yield of D-loops,[97] limiting the amount of RecA protein reduces the level of joint dissociation and promotes the appearance of a second round of joint formation (without subsequent dissociation).[91] Decreases in the ATP concentration result in kinetics very similar to those obtained when RecA protein concentrations are limiting.[91] Finally, in the presence of excess dsDNA, greater concentrations of RecA protein are required to observe the same level of joint dissociation obtained when the dsDNA and ssDNA are equimolar.[97]

The most plausible explanation for these observations is that either the RecA protein-ssDNA filaments or the dsDNA is inactivated during pairing. As discussed previously, many factors can change the assembly and stability of the RecA protein filaments. However, the changes in kinetics observed following variation of the dsDNA concentration[97] and the presence of dsDNA-dependent ATPase activity following dissociation of the joints[98] argue that dsDNA may be inactivated during the D-loop cycle. Shibata and colleagues[97] have concluded that the dsDNA is inactivated following one round of joining with ssDNA. They proposed that this results from the unwinding of the dsDNA by RecA protein which remains bound following dissociation of the ssDNA. These conclusions were based on three observations: (1) excess RecA protein inhibited the D-loop cycle, unless the concentration of dsDNA was increased proportionally;[97] (2) a restimulation of the joint cycle was observed when fresh dsDNA, but not fresh ssDNA or RecA protein, was added to the reaction following dissociation of a majority of the joints;[97] and (3) when aliquots of dsDNA from both before and after a D-loop cycle were treated with topoisomerase then electrophoresed on an agarose gel, the two DNAs were observed to migrate differently. This difference could be explained if the dsDNA which had been through a joint cycle was partially unwound.[99]

Studies in the authors' laboratory have verified that the initial dsDNA in the mixture is unable to undergo a second round of joining with the linear ssDNA-RecA protein filaments. When the dsDNA in the post D-loop reaction was examined by EM, the number of supertwists was found to be less than one half that of the original DNA, and short tracts of RecA protein were observed bound along the DNA. While these studies show that RecA protein binds to and unwinds dsDNA following the D-loop cycle, it has not been shown whether the bound RecA protein originates from what was bound to the ssDNA or from the free pool of protein. It is interesting to consider the possibility that a means exists in vivo for marking the DNA which has recently undergone recombination.

These observations relate directly to recent work of Shaner and Radding[100] and Shaner et al.,[101] who have demonstrated that if RecA protein binds to a single-stranded region linked to a duplex segment then the RecA protein will polymerize into the duplex region, covering it in a manner which protects the dsDNA from restriction endonucleases. Thus, contrary to the earlier conclusion that RecA protein will not bind dsDNA under strand-exchange conditions, it appears that RecA protein can be placed onto a dsDNA segment either through recombination with a ssDNA or by migration from a RecA protein-bound ssDNA segment.

E. Strand Transfer between Circular ssDNA and Linear dsDNA

Figure 23B illustrates the pathways of this pairing reaction which can be paranemic (path

I) or plectonemic (path II). Wu et al.[21] showed that when a circular (+) strand ssDNA is used plectonemic joints can form at either the 5′ or 3′ end of the (−) strand of the duplex DNA. Once the joint has formed, the length of the heteroduplex is extended by unidirectional branch migration.[23,102,103] Branch migration moves 5′ to 3′ relative to the ssDNA, so pairings at the 3′ ends are productive, while those at the 5′ end are not. Cox and Lehman[87] showed that continuous hydrolysis of ATP is necessary for RecA protein to catalyze branch migration. The extension of heteroduplex DNA was measured by following an increase in resistance to S1 nuclease by radiolabeled ssDNA. When ATPγS was added to the reaction, heteroduplex extension halted immediately. Contrasting results have been seen when branch migration was catalyzed by UvsX protein.[103a] In this study, heteroduplex extension was monitored by the ability of the branch to reach and pass a restriction site. When ATPγS was added to the reaction, branch migration actually accelerated briefly in a portion of the complexes, but then ceased altogether. These results form the basis for a model in which the polarity of assembly of UvsX protein onto DNA fuels branch migration. According to this model, addition of ATPγS would allow the UvsX protein to extend rapidly along the DNA, simultaneously pushing forward branch migration, but then would lock the protein in place so that branch migration would halt when the protein could no longer be recycled.

Kowalczykowski et al.[104] have also proposed such a "polar polymerization" model for RecA protein-promoted branch migration based on the nature of the duplex DNA-dependent ATPase activity of the RecA protein. By this model, polymerization of RecA protein along dsDNA works to transiently melt the duplex, moving the ssDNA forward into the dsDNA and extending the heteroduplex joint. In the *uvsX* system, this role may be filled by the *dda* helicase.[105] The dda protein binds specifically to gene 32 protein. It has been shown to specifically increase the rate of branch migration by UvsX protein. Other helicases will not substitute, and the dda protein is ineffective when used in conjunction with RecA protein.

The structure of the DNA intermediates and products of a plectonemic pairing and strand transfer between circular ssDNA and linear dsDNA have been examined by Kahn et al.[102] and Cox and Lehman.[103] Using surface spreading EM, the deproteinized DNA substrates were seen as circular ssDNA and linear dsDNA, the products were relaxed dsDNA circles and linear ssDNA strands, and the intermediates appeared as circles which were partially single stranded and partially double stranded. At one ssDNA/dsDNA junction were two linear arms, one single stranded, the other duplex. These structures are shown schematically in Figure 23B. Figure 28A is a micrograph from studies in the authors' laboratory.[95] We have observed that, although the maximum number of molecules involved in strand transfer was found after 10- to 15-min incubations, there appeared to be a great asynchrony in the reactions.

The structure of the DNA-protein intermediates in these pairings have been examined by Flory and Radding,[7] Stasiak and Egelman,[106] and the authors' laboratory.[95] However, the studies by Flory were done prior to the appreciation of the structure of paranemic joints. In studies in the authors' laboratory,[95] roughly 50% of the joined complexes visualized after the proteins had been fixed in place had the structure of an unbroken RecA protein-ssDNA filament with two dsDNA tails emerging from it (Figure 28B). This topology defined these joints as being paranemic and measurement indicated that they could contain as much as 1500 bp of dsDNA.

The joined complexes involving molecules undergoing a plectonemic strand transfer might have been expected to show a simple structure as illustrated in Figure 23B, where the ssDNA in the circle would be RecA protein covered, the displaced ssDNA would be covered by either SSB or RecA protein, and the dsDNA segments would be protein free. Such structures were seldom observed. Instead, highly variable complexes (Figures 29 and 30) in which only one (and sometimes no) dsDNA tail was attached to a circular RecA protein-ssDNA filament were found. Most striking were partially RecA protein-covered circles with no tails

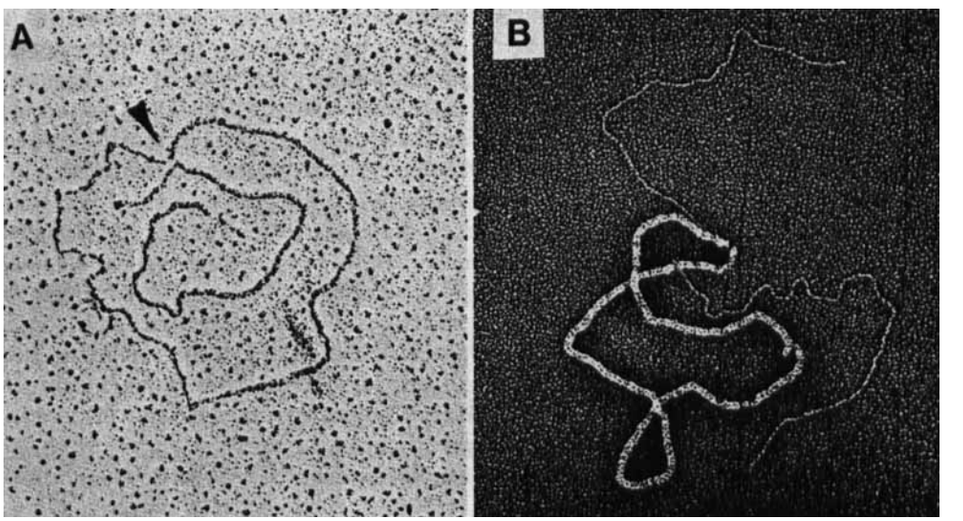

FIGURE 28. Visualization of the products of the joining of linear dsDNA and circular ssDNA. (A) The deproteinized product of the partial strand transfer between a linear dsDNA and a circular ssDNA promoted by RecA protein. Prepared for EM by surface spreading onto a 10% formamide solution following deproteinization. Arrowhead indicates heteroduplex joint. (B) A paranemic joint formed between the DNAs described in (A). Here the sample was fixed following incubation and mounted for EM as described in Figure 1 without deproteinization. (Bar = 1 μm.)

(Figure 30B). Here the RecA protein-covered segment must have contained all three DNA strands as did the region which appeared like protein-free duplex DNA. In many of the intermediates, the region over which all three DNA strands were held in a RecA protein filament contained as much as 2000 bp of dsDNA. Analysis of the structures observed[95] led to the model shown in Figure 31. In this model, three events — uptake of the dsDNA into the circular RecA protein-ssDNA loop, cooperative depolymerization from the region behind the point of exchange, and release of the end of the strand displaced from the newly formed duplex — occur independently of each other. This would explain the structure of the intermediates observed and the asynchrony in the reactions. The general model and EM observations are in agreement with models proposed by Stasiak and Egelman.[106]

These results suggest a three-step pathway of pairing and exchange as described by Register et al.:[95] (1) assembly of RecA protein onto the ssDNA and pairing with the homologous dsDNA; (2) envelopment of the two DNAs into the RecA protein filament and exchange of strands; and (3) dissociation of the RecA protein and resolution of the products. If this three-step pathway is found to be typical of the RecA and UvsX protein-driven reactions, then it argues for a very different mechanism from what could be explained by simple models, particularly those which involve treadmilling.

When the same DNA templates are paired in a reaction catalyzed by UvsX protein, a different product is observed. Instead of releasing nicked duplex circles and linear ssDNAs, UvsX protein integrates the DNAs into large networks.[30,103a] These networks are resistant to deproteinization and are maintained by heteroduplex base-pairing. Such networks arise as a result of multiple-strand exchange events between a single DNA strand and multiple partners, each of which could also have multiple partners. It has been suggested[103a] that RecA protein does not form these same networks because the DNA that has been involved in a pairing reaction remains coated with the RecA protein, making it unable to participate in further reactions.

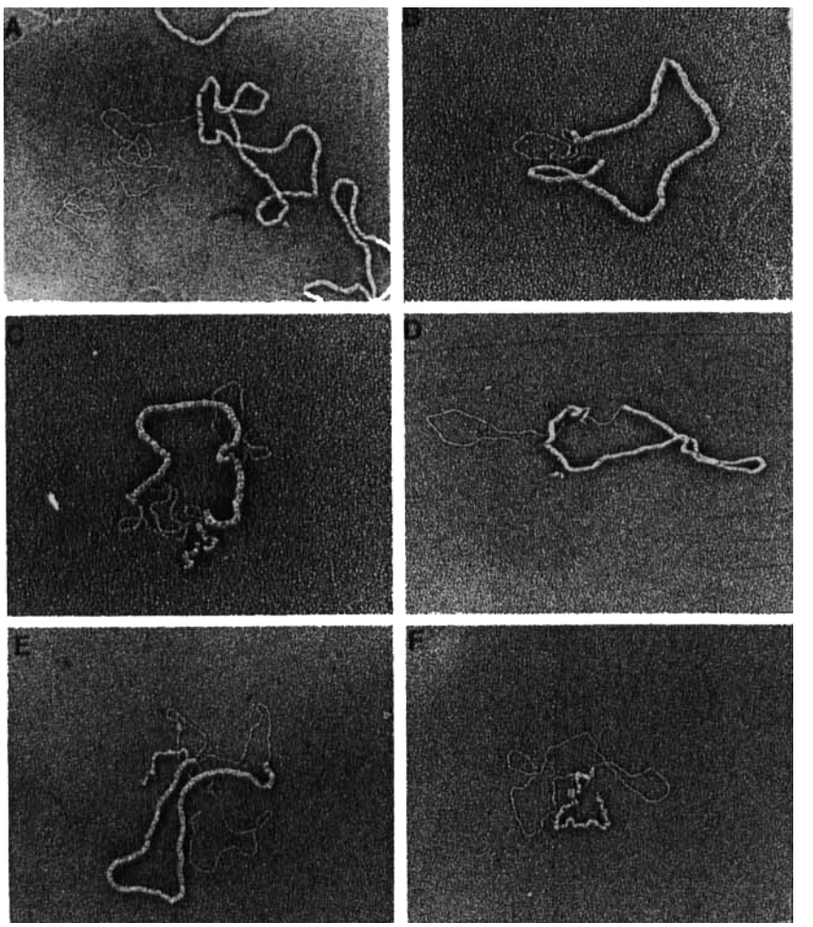

FIGURE 29. Visualization of intermediates in strand transfer reactions between linear dsDNA and circular ssDNA. When circular M13mp7 ssDNA was enveloped into filaments formed by RecA protein in the presence of SSB protein and then linear M13mp7 dsDNA was added, intermediates in the ensuing strand transfer (A to F) could be observed if the samples were fixed and processed for EM as described in Figure 1. A model is presented in Figure 31. (Bar = 1 μm.)

VII. ABILITY OF DNA STRAND EXCHANGES TO PASS OVER MISMATCHES AND BLOCKS OF NONHOMOLOGY

Experiments have been conducted to determine whether strand exchange reactions catalyzed by RecA protein will pass over single-base mismatches and short regions of nonhomology and whether they can incorporate large insertions or deletions into heteroduplex DNA. Such a reaction has been hypothesized in models of gene conversion. DasGupta and Radding[107] showed that exchanges will not occur between ΦX174 DNA and G4 DNA, which are 70% homologous; however, if one of the reactant molecules is supertwisted, exchange between these templates is possible.[108] RecA protein will readily catalyze heteroduplex formation between M13 and fd phage DNAs, which are 97% homologous.[22,107] Strand exchange between these two phage DNAs proceeded well up to a region which contained only one in ten homologous bases. Throughout the rest of the pairing, however, RecA protein was able to pair every possible combination of mismatches.

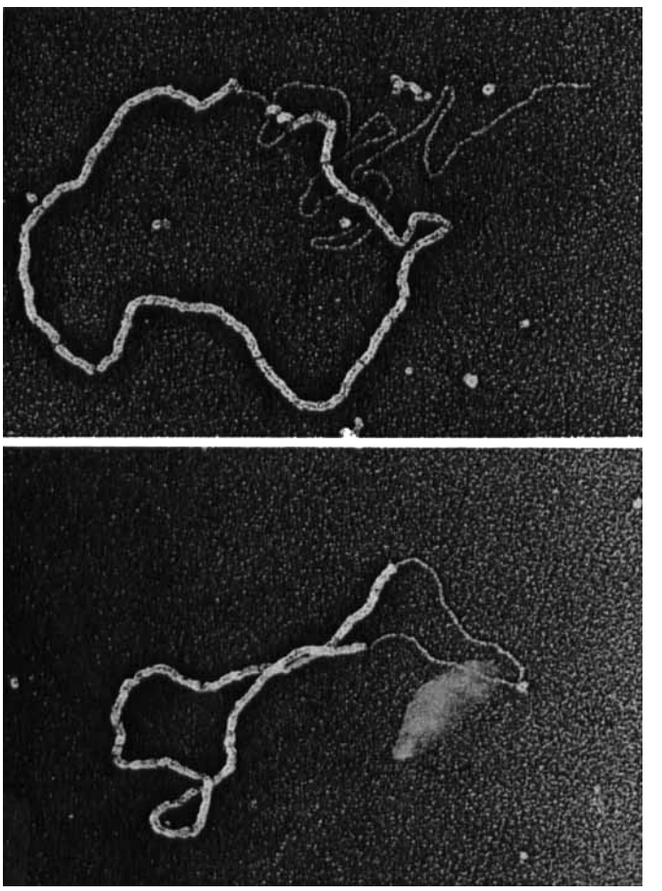

FIGURE 30. Intermediates in strand transfer. Two examples at higher magnification from the reactions described in the preceeding figure. (Bar = 1 μm.)

Initially, it was concluded that RecA protein could not catalyze strand exchange between DNAs if one included a large insertion.[107] However, it has since been shown that, in the presence of SSB protein and an ATP-regenerating system, RecA protein can pair circular ssDNA and linear dsDNA, whether an insertion was made in the ssDNA or dsDNA.[22] Increasing the length of the insertion decreased the amount of recombination, and insertions in the dsDNA were more sensitive to this length requirement than those in the ssDNA. However, the ability to pass through an insertion in the dsDNA at all demonstrates than RecA protein is able to unwind dsDNA and recognize homology far in advance of the point of strand exchange. The molecular mechanism by which this occurs is unknown.

VIII. MODELS OF STRAND EXCHANGE

It is worth comparing several models of the mechanism of DNA strand exchange. In the stable filament model, once RecA or UvsX protein has assembled onto a ssDNA molecule, it remains bound throughout the processes of pairing with a dsDNA and strand transfer and is released only after passage of the heteroduplex joint. In the RecA protein-catalyzed pairing of a linear ssDNA with a homologous supertwisted dsDNA, a stable RecA protein-ssDNA

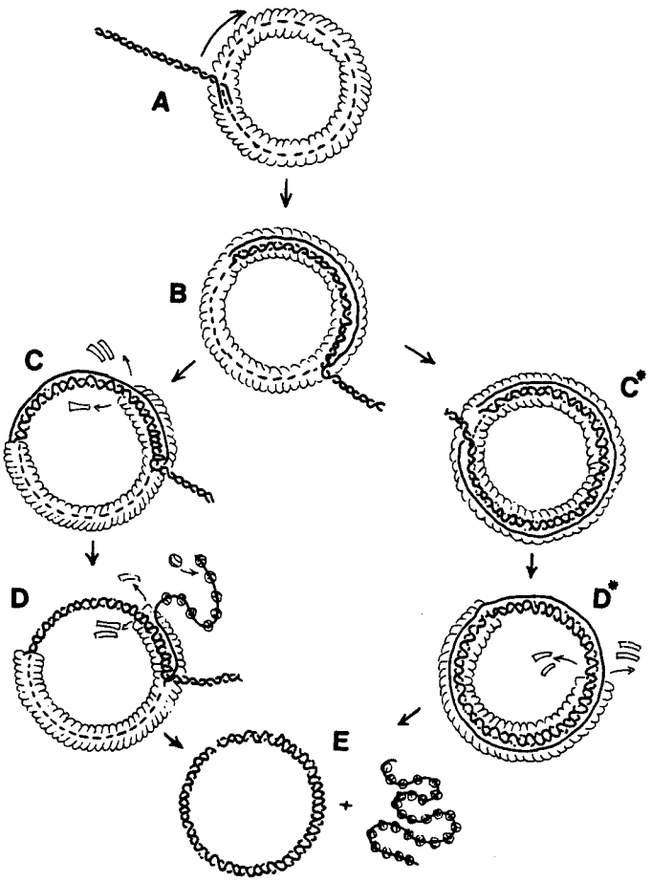

FIGURE 31. Model of the steps in strand transfer between a circular ssDNA and a linear dsDNA as promoted by RecA protein. As observed by Register et al.,[95] the large variety of structures observed by EM (see Figures 29 and 30) when the intermediates in a strand exchange between linear dsDNA and circular ssDNA are fixed and examined directly by EM can be explained on the basis of the diagram shown here. In (A), synapsis at the end of the dsDNA has just occurred, and in (B) the dsDNA has been enveloped into the circular RecA protein-ssDNA filament over a considerable distance, with the heteroduplex joint being shown near the site of exit of the dsDNA tail. The displaced strand remains within the filament. In (C*), this process continues until most of the dsDNA lies within the filament. In (D*), RecA protein has begun depolymerizing from the site of synapsis moving in the direction of exchange, and the displaced strand remains associated with the newly formed duplex, as is the case in (C), but here the RecA protein began depolymerization at an earlier point. In (D), the displaced strand has been released from its association with the newly formed duplex and is rapidly complexed with SSB protein. In (E), the final products are indicated. If we assume that the times when depolymerization of the RecA protein begins and the displaced strand loses its association with the newly formed duplex DNA are unrelated to the extent of strand transfer, then the large variety of intermediate structures observed by EM can be explained by this model. Although the dsDNA is shown exiting the filament at the site of the heteroduplex junction, it is possible that a paranemic joining precedes the heteroduplex joint melting the dsDNA.

filament would form and synapsis would occur at the 5′ end of the RecA protein-ssDNA filament, which would then proceed through a round of exchange in the rolling D-loop reaction described previously. In an exchange between a linear dsDNA and a circular ssDNA molecule, a stable circular RecA protein-ssDNA filament would form and the RecA protein would remain bound to the ssDNA as the exchange moves around the ssDNA circle, being released only after the heteroduplex joint had passed and the dissociation of the products had begun.

In the stable filament model, strand exchange is not driven by the entry of new RecA (or UvsX) protein from the pool of free protein. Energy could be supplied by ATP hydrolyzed within the assembled filament. Whether or not the RecA protein remains bound to the DNAs after passing of the point of exchange is not consequential to the model.

Support for this model includes the strong propensity of RecA and UvsX proteins to form highly ordered, helical filaments on ssDNA. Furthermore, Brenner et al.[109] have shown that ATP hydrolysis occurs throughout the length of RecA protein-DNA filaments. Finally, in the exchanges between circular ssDNA and linear dsDNA described previously, the linear dsDNA was observed to be taken up into the circular ssDNA-RecA protein filament; exchange occurred within the filament and only subsequently was the protein released from the products.

Arguments against the concept of an exchange driven by a stable filament are that, while the entire ssDNA may be covered by RecA or UvsX proteins in these DNA constructs in vitro, in the cell the regions of the exchanging DNAs that are single stranded may be small and may force a rapid turnover of the protein as the exchange moves. The model also does not explain how pairing can occur between a linear ssDNA and a supertwisted homologous dsDNA since, as seen by EM, the active (5′) end of the linear ssDNA is almost always complexed by SSB protein.

In the treadmilling model, based on analogy with tubulin and actin studies, strand exchange is driven by the cyclic assembly and disassembly of RecA or UvsX protein at the heteroduplex joint. The ATP-bound protein assembles onto the ssDNA at the heteroduplex joint then disassembles in an ADP-bound form at the end of the short protein filament, which encompasses the heteroduplex DNA. Assembly of the ATP-bound form may help in unwinding the dsDNA. By this model, in the pairing of a linear ssDNA and a homologous supertwisted dsDNA, the RecA protein would rapidly assemble and disassemble from the ssDNA as the D-loop is driven around the dsDNA circle. Support for the treadmilling model includes studies that show that ADP stimulates RecA protein dissociation from DNA, the requirement for ATP hydrolysis in strand exchange, and the polar growth of the protein assembly on ssDNA. Arguments against the treadmilling model are found in the EM observations of Stasiak and Egelman[106] and the authors' laboratory[95] in which both DNA substrates were seen enveloped in the RecA protein filament over several thousand base pairs during the exchange. Furthermore, the observation of Brenner et al.[109] that ATP hydrolysis occurs throughout the length of the RecA protein-DNA filaments argues against ATP hydrolysis being limited to a single site (the point of exchange) on the filament.

In a dynamic filament model, the stability of the RecA (or UvsX) protein-ssDNA filaments would depend on their length and form (circular, linear, or single-stranded gaps in duplex DNA). In this model, once nucleation on a ssDNA occurred, the protein would assemble in the 5′ to 3′ direction but would also disassemble 5′ to 3′ but at a slower rate linked to the buildup of ADP. If the ssDNA was circular, then the front of the growing filament would eventually overtake and join the back, stopping the disassembly process. Turnover of protein from the filament into the pool of free protein would then be very low so long as the ADP levels remain depressed. Assembly onto linear ssDNA however would produce a different result. Assembly would stop at the 3′ end of the ssDNA, while disassembly from the back (5′ end), followed by rebinding of SSB protein, would continue. This, in general, would not result in complete removal of the RecA protein because reinitiation of RecA

protein tracts and 5′-to-3′ polymerization to the current point of disassembly would produce a dynamic assembly-disassembly process on the ssDNA. With a long ssDNA molecule, this would result in a linear filament with a relatively stable complement of RecA protein toward the 3′ end, but near the 5′ end, there would be waves of RecA protein disassembly, SSB protein binding, and RecA protein renucleation and reassembly. As the length of the ssDNA became longer, it would approach the stable filament situation, and as it became shorter, most of the DNA would be involved in the turnover process. In this model, the tracts of SSB protein seen on the 5′ ends of the linear filaments might reflect the fractional length of the filament engaged in this turnover process. The length of the SSB protein tracts would also be expected to depend on the ATP/ADP levels. The turnover of RecA protein from single-strand gaps in duplex DNA might, by this model, be low if the association of the RecA protein with the 3′ terminus of the gap inhibited its release.

The dynamic filament model encompasses the results of Stasiak and Egelman[106] and the authors' laboratory,[95] which describe the appearance of the intermediates in exchanges between circular ssDNA and linear dsDNA. Furthermore, it could explain how the 5′ ends of the linear RecA protein-ssDNA filaments initiate pairing and exchange since it would be assumed that the 5′ ends would be covered by RecA protein a certain fraction of the time during the cyclic assembly-disassembly process.

The great differences in these models serve to illustrate major questions that remain unanswered. Work is needed that will show whether the UvsX protein-catalyzed exchanges resemble or differ from those catalyzed by RecA protein. Experiments employing labeled proteins or other methods that will directly measure the turnover of protein from the filaments during exchange must be carried out. Perhaps the greatest need is a determination of the role of ATP hydrolysis in protein assembly and strand exchange. Until these questions are answered, these models can only serve as guideposts for future experiments and not definitive explanations for the events.

IX. CONCLUSIONS

The work of many laboratories on RecA and other recombinational scaffolding proteins has begun to reveal the properties of a new class of DNA-binding proteins which function in a markedly different way from the protein complexes that catalyze DNA replication and transcription. In the strand exchange reactions, very large helically arranged protein scaffolds, consisting of hundreds of protein monomers, must first form along one of the pairing substrates. What ensues is a three-step pathway. First, the RecA protein-ssDNA filament makes contact with a homologous dsDNA (joining). Second, both DNA partners are at least partially enveloped within the nucleoprotein filament, and, if the DNA topology is favorable, exchange of DNA strands then ensues (envelopment/exchange). Finally, upon completion of strand exchange, this complex is resolved and the products are released. Just as the division of strand exchange into presynaptic filament formation, synapsis, and strand exchange defines the reaction biochemically, the division of joining, envelopment/exchange, and release of products defines the reaction structurally.

Many important questions concerning the mechanism of DNA strand exchanges remain unanswered. How are the strands arranged in the RecA (or UvsX) protein-DNA filaments? What is the role of paranemic joining? As exchange progresses along the length of a protein-covered ssDNA, does the scaffolding protein (RecA, UvsX protein), which was bound to the ssDNA, remain in the filament or is there a rapid exchange of protein between the DNA-bound and the free pool at the site of exchange? In addition to the rec1 protein of *Ustilago*, will other proteins of this class be found in yeast and higher eukaryotic cells? These questions must await future studies.

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