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Occurrence of Three-stranded DNA within a RecA Protein Filament*

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A RecA protein-generated triple-stranded DNA species can be observed by electron microscopy, within narrowly defined conditions. Three-stranded DNA is detected only when initiation of normal DNA strand exchange is precluded by heterologous sequences within the duplex DNA substrate, when ATP is hydrolyzed, and when the DNA is cross-linked with a psoralen derivative prior to removal of RecA filaments. When adenosine 5′-*O*-(thiotriphosphate) is used, only the product hybrid duplex DNA can be cross-linked within the RecA filament. The third strand is either displaced or interwound in a conformation that does not permit cross-linking. When ATP is hydrolyzed by RecA, all three strands are cross-linked within the filament in a complex pattern that suggests a dynamic structure. This structure is altered when RecA protein is removed before cross-linking. Hsieh *et al.* (1990) and Rao *et al.* (1991, 1993) have proposed, on the basis of nuclease protection and chemical modification studies, that a stable triple-stranded DNA species can persist after removal of RecA protein. We have been unable to visualize these triple-stranded structures by the methods used in the present investigation. When RecA removal was followed immediately by interstrand cross-linking, only the two strands of the hybrid duplex DNA were cross-linked.

RecA protein is essential for recombinational DNA repair and homologous recombination in *Escherichia coli*. It promotes a DNA strand exchange reaction *in vitro* involving circular single-stranded DNA (ssDNA)[1] and homologous linear duplex DNA. Studies (Roca and Cox, 1990; Kowalczykowski, 1991; Radding, 1991; Cox, 1994)[2] have provided evidence for up to four reaction phases. The first phase is the cooperative assembly of RecA on ssDNA. In the presence of ATP or ATPγS (a nonhydrolyzable ATP analog), this results in a right-handed helical filament with a pitch of 95 Å and an axial rise per nucleotide of 5.1 Å. There are 6 RecA monomers and 18 nucleotides per helical turn. The second phase is the homologous alignment of a duplex DNA with the ssDNA in the nucleoprotein filament. When the topology of the DNA substrates permits, the second phase is followed closely by the transfer of the complementary strand of the duplex DNA to the ssDNA to form a short (≈1000 bp) region of hybrid or heteroduplex DNA.[3] This third phase is facilitated by binding energy within the filament and requires bound ATP but not ATP hydrolysis (Menetski, *et al.*, 1990; Rehrauer and Kowalczykowski, 1993). The limited strand exchange in phase 3 is bidirectional, blocked by structural barriers in the DNA, and limited to three DNA strands. In the final phase, this short segment of hybrid DNA is extended in a branch migration coupled to ATP hydrolysis (Cox, 1994). The extension is unidirectional (5′ to 3′ with respect to the single-stranded DNA) (Konforti and Davis, 1992; Jain *et al.*, 1994), bypasses structural barriers in the DNA (Kim, *et al.*, 1992a; Rosselli and Stasiak, 1991), and accommodates four DNA strands (Kim, *et al.*, 1992b).

The mechanism by which RecA facilitates the alignment of homologous sequences in phase 2 is being addressed in several laboratories. RecA binds to the phosphate backbone of the ssDNA, leaving the bases exposed and accessible for base pairing in the helical groove of the RecA filament (Di Capua and Müller, 1987; Dombroski *et al.*, 1983; Leahy and Radding, 1986). Structural studies and nucleolytic protection experiments have demonstrated that there are distinct DNA binding sites within the RecA filament that can simultaneously bind up to three DNA strands (Chow *et al.*, 1988; Kubista *et al.*, 1990; Pugh and Cox, 1987a, 1987b; Takahashi *et al.*, 1989, 1991).

Two types of models have been postulated for the mechanism by which RecA mediates homologous recognition between two DNA molecules. These can be loosely summarized as "pairing before strand separation" *versus* "strand separation before pairing" (Stasiak, 1992). A triplex DNA species could be an intermediate in either type of model (Fig. 1).

Type I models arise from ideas originally set forth by Howard-Flanders and colleagues (1984) and propose that a novel DNA triplex structure exists as the initial pairing intermediate in RecA protein-promoted DNA strand exchange reactions (Howard-Flanders *et al.*, 1984; Hsieh, *et al.*, 1990). In the scheme above, this triplex would be the product of phase 2. The triplex DNA would be a "pre-strand switch" triplex, with the two strands of the substrate duplex still joined by Watson-Crick base pairs and the ssDNA in the RecA filament interacting via non-Watson-Crick hydrogen bonds. The strand switch could result in formation of a distinct "post-strand switch" triplex, in which the strands of the hybrid DNA would be Watson-Crick base paired and the nascent-displaced strand would interact via non Watson-Crick pairing.

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[1] The abbreviations used are: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; FI DNA, supercoiled closed-circular form of plasmid or bacteriophage DNA as isolated from *E. coli* cells; FIII DNA, linear double-stranded DNA; AMT, 4′-aminomethyl-4,5′,8-trimethylpsoralen; HMT, 4′-hydroxymethyl-4,5′,8-trimethylpsoralen; TMP, 4,5′,8-trimethylpsoralen; bp, base pairs; kbp, kilobase pairs; ATPγS, adenosine 5′-*O*-(thiotriphosphate); SSB, the single-stranded DNA binding protein of *E. coli*; M13 mp8(+), circular single-stranded genome of bacteriophage M13 mp8.

[2] W. A. Bedale and M. M. Cox, unpublished data.

[3] Duplex DNA that arises from completely complementary sequences will be referred to as hybrid DNA, whereas duplex DNA products containing mismatches will be referred to as heteroduplex DNA.

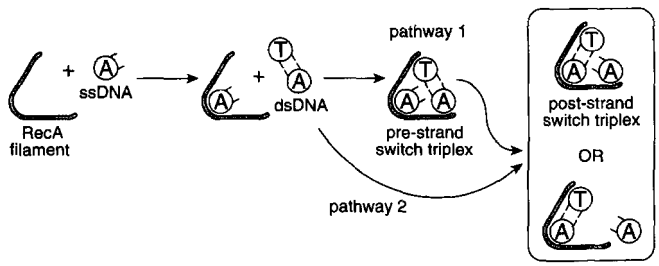


FIG. 1. **Alternative models for DNA pairing during RecA-mediated DNA strand exchange.** *Pathway 1*, a pathway involving formation of a "pre-strand switch" triplex DNA; *pathway 2*, pathways involving a concurrent alignment and strand switch. A "post-strand switch" triplex DNA is a plausible intermediate in both cases.

In type II models, homologous recognition and DNA strand exchange (phases 2 and 3 above) are concurrent (Adzuma, 1992). A local strand separation in the duplex DNA allows base pairing between the complementary strand of the duplex DNA and the incoming single-stranded DNA within the RecA filament. The binding energy within the filament effectively stabilizes the product hybrid DNA. Alternative plausible fates for the third strand lead to variations in type II models. The third strand could be immediately displaced from the RecA filament, it could remain transiently wound around the product duplex but not interact with it, or it could be transiently hydrogen-bonded to the hybrid duplex in the major groove. The latter case gives rise to the possibility that a triplex DNA could be a discrete intermediate in a type II pathway. Any type II triplex, however, would be a "post-strand switch" triplex.

Regardless of when it is formed, a RecA-mediated triplex DNA structure must have parallel like strands and be sequence independent as long as the DNAs are homologous. These factors distinguish it from the well characterized stable DNA triplexes that are formed non-enzymatically (Hanvey *et al.*, 1988; Moser and Dervan, 1987). The RecA-generated species has been called recombinant (or R-form) DNA. A triple-stranded DNA that may be analogous to R-form DNA but is formed in the absence of proteins has recently been reported (Shchyolkina *et al.*, 1994).

Indirect measurements have revealed that thousands of base pairs of homology can be aligned at early stages of the DNA strand exchange reaction (Bianchi *et al.*, 1983; Schutte and Cox, 1987, 1988). Electron microscopy with psoralen-photocross-linked RecA synaptic complexes has provided unambiguous evidence for the alignment and close juxtaposition of three DNA strands over thousands of base pairs (Bortner and Griffith, 1990; Umlauf *et al.*, 1990). However, these approaches do not distinguish between type I or type II models to the extent that three strands could be transiently juxtaposed within the filament in either case.

Chemical modification of synaptic complexes formed in the presence of ATPγS confirmed that the base pairing within the parental dsDNA is disrupted. One strand of the duplex DNA is Watson-Crick base paired to the ssDNA within the RecA filament, and the homologous strand is displaced (Adzuma, 1992). This work favors a type II model, at least when ATPγS is present. Characterization of deproteinized DNA pairing intermediates by nucleolytic digestion, chemical modification, and thermal stability studies has provided evidence for the presence of a stable triplex DNA that has many of the characteristics of a post-strand switch triplex (Rao *et al.*, 1991, 1993; Chiu *et al.* 1993; Burnett *et al.*, 1994). No evidence that uniquely favors the existence of a pre-strand switch triplex has been reported.

There is no shortage of models depicting what an R-form triplex might look like. Recent evidence involving base analog-substituted substrate duplex DNAs in RecA-mediated DNA strand exchange reactions and chemical modification of putative three-stranded DNA structures in the presence or absence of RecA filaments reveals that hydrogen bonding to the N-7 of guanine and adenine is not crucial in stabilization of any intermediate in DNA strand exchange (Adzuma, 1992; Jain *et al.*, 1992; Rao *et al.*, 1993; Jain, 1994). In contrast, Hoogsteen base pairing to the N-7 of purines is essential for non-enzymatic triplex DNA formation (Hanvey *et al.*, 1988; Moser and Dervan, 1987).[4] Umlauf (1990) proposed that triple-stranded recombination intermediates utilize two additional hydrogen bonds between the Watson-Crick base pairs in the major groove of a duplex DNA and a paired third strand (Umlauf, 1990). The effects of enzymatic methylation of the N-6 of adenine and N-4 of cytosine on the stability of deproteinized triple-stranded intermediates support the involvement of Watson-Crick positions in base triplets (Rao *et al.*, 1993). Models similar to the Umlauf proposal have recently been refined on theoretical grounds (Zhurkin *et al.*, 1994).

The present studies extend the work of Umlauf *et al.* (1990). They examine the effects of ATP hydrolysis on formation of three-stranded DNA that can be cross-linked with psoralen derivatives and explore the nature of the structure present after deproteinization of the complexes. They were carried out to provide direct visual support for the triple-stranded DNAs characterized in many previous studies using electron microscopy. While a three-stranded DNA species can be visualized after the DNA is cross-linked within the RecA filament, we were unable to confirm the existence of a stable triplex DNA after RecA removal. Cross-linking of three strands can only occur within the filament under narrowly defined conditions.

MATERIALS AND METHODS

Enzymes and Reagents—*E. coli* RecA protein was purified to homogeneity and stored as previously described (Cox *et al.*, 1981). *E. coli* single-stranded DNA binding protein (SSB) was purified as described by Lohman *et al.* (1986), except that an additional step utilizing DEAE-Sepharose chromatography was included to ensure removal of single-strand exonucleases. The RecA protein and SSB concentrations were determined by absorbance at 280 nm, using extinction coefficients of $\epsilon_{280} = 0.59\, A_{280}$ mg^{-1} ml (Craig and Roberts, 1981), and $\epsilon_{280} = 1.5\, A_{280}$ mg^{-1} ml (Lohman and Overman, 1985), respectively. Restriction endonucleases were purchased from New England Biolabs. Tris buffer, Triton X-100, and ATPγS were purchased from Boehringer Mannheim. DEAE-Sepharose was purchased from Pharmacia Biotech Inc. Creatine phosphokinase, phosphocreatine, Proteinase K, low melting agarose, Nonidet P-40, *N*-laurolylsarcosine, deoxycholic acid, ATP, and 4,5′,8-trimethylpsoralen (TMP) were purchased from Sigma. 4′-Aminomethyl-4,5′,8-trimethylpsoralen (AMT) and 4′-hydroxymethyl-4,5′,8-trimethylpsoralen (HMT) were purchased from Calbiochem. The purity of the ATP and ATPγS was assayed by thin layer chromatography and were each shown to be at least 99 and 90% pure, respectively, with the major contaminant being ADP.

DNA—Circular single-stranded DNA and supercoiled circular duplex DNA from bacteriophage M13 mp8 (7,229 bp) and M13 mp8.1037 (8,266 bp) were prepared using methods previously described (Davis *et al.*, 1980; Messing, 1983; Neuendorf and Cox, 1986). The bacteriophage M13 mp8.1037 is the bacteriophage M13 mp8 with a 1037-bp sequence (*Eco*RV fragment) from the *E. coli gal* T gene inserted into the *Sma*I site. The concentrations of ssDNA and dsDNA stock solutions were determined by absorbance at 260 nm, using 36 and 50 μg ml^{-1} A_{260}^{-1}, respectively, as conversion factors. DNA concentrations are reported in terms of total nucleotides. Full-length linear duplex (FIII) DNA was derived from M13 mp8.1037 FI DNA by complete digestion with *Eco*RI or *Pst*I endonuclease, using conditions suggested by the enzyme supplier.

Strand Exchange Reaction Conditions—Reactions involving ATP were carried out 37 °C in a standard reaction buffer containing 25 mM Tris acetate (80% cation, pH 7.5), 10 mM magnesium acetate, 3 mM

[4] J. S. Lee, personal communication.

potassium glutamate, 1 mM dithiothreitol, 5% glycerol, and an ATP regeneration system (10 units ml^{-1} creatine phosphokinase, 12 mM phosphocreatine). Reaction volumes were 60 μl, and the concentrations of DNA and proteins reported below are the final concentrations after addition of all components. Single-stranded DNA (10 μM) was preincubated with RecA protein (6.6 μM) for 10 min before addition of duplex DNA (11.4 μM). After incubation of this mixture for 10 min, ATP (3 mM) and SSB (2 μM) were added to start the reactions. The ATP regeneration system maintains the ADP concentration near zero for at least 80 min.

Reactions involving ATPγS were performed at 37 °C in a standard reaction buffer containing 25 mM Tris acetate (80% cation, pH 7.5), 4 mM magnesium acetate, and 1 mM dithiothreitol. Single-stranded DNA (10 μM) was preincubated with SSB (0.9 μM) for 10 min before addition of RecA protein (6.0 μM) and ATPγS (1 mM). After incubation of this mixture for 10 min, the reaction was initiated with duplex DNA (11.4 μM). Conditions for ATP and ATPγS reactions are different so that each proceeds optimally. The ssDNA molecules are in 2-fold molar excess with respect to the dsDNA molecules in all reactions.

Electron Microscopy—Visualization of reactions by electron microscopy was carried out by spreading the entire strand exchange reaction mixture after deproteinization and dialysis. Reactions were stopped after 60 min. Uncross-linked samples were incubated with Proteinase K (1 mg ml^{-1} final) and SDS (0.5% final) at 37 °C for 30 min to remove the RecA and SSB proteins. For samples to be cross-linked before deproteinization, aliquots (20 μl) of the reaction mixture were incubated with AMT (30 μg ml^{-1} final concentration), TMP (10 μg ml^{-1} final concentration), or HMT (10 μg ml^{-1} final concentration) at 25 °C for 3 min before irradiation with long-wave UV light for 4 min at 25 °C as previously described (Jain *et al.*, 1994). Deproteinization was then carried out as for the uncross-linked samples. All samples were dialyzed into 20 mM NaCl and 5 mM EDTA for 5 h at 25 °C before spreading as previously described (Inman and Schnös, 1970).

Some samples were deproteinized prior to cross-linking. Aliquots (20 μl) of the reaction mixtures (60-min time points) were incubated with Proteinase K (1 mg ml^{-1}) and SDS (0.05%), Triton X-100 (0.5%), Nonidet P-40 (1%), *N*-laurolylsarcosine (0.1%), or deoxycholic acid (0.1%) at 37 °C for 15 min. All concentrations given are final concentrations after addition of all components. In some cases, detergent was not added, and deproteinization with proteinase K (1 mg ml^{-1}) was carried out 5 s, 1 min, or 2 min prior to addition of AMT as indicated in the specific experiment. The samples were immediately photocross-linked with AMT, dialyzed, and spread for electron microscopy as described above.

The cross-linking density resulting from psoralen-long-wave UV irradiation was determined using RecA-coated gapped circles, which contained a 2,159-kbp double-stranded region. After the psoralen treatment, these molecules were deproteinized as described above and then completely denatured by heating at 70 °C for 15 min in 20 mM NaCl, 5 mM EDTA, 5% HCHO. Samples were then cooled on ice prior to spreading. When cross-linked DNA is completely denatured, each cross-over of the single strands can be assumed to represent a cross-link. Examination of such molecules by electron microscopy therefore allows an approximate determination of the cross-linking density between duplex strands. In the present case, the cross-linking densities were high enough to often produce cross-links close enough together that the denatured material appeared to contain undenatured segments (the limit of sample resolution is such that ssDNA and dsDNA cannot be differentiated if segments are less than 50 bp in length. To allow for this effect in the determination of the cross-linking density, the size of "undenatured" regions were determined by measurement and a correction applied as previously described (Schnös and Inman, 1987). Our estimate for the cross-linking density resulting from AMT treatment of dsDNA within a RecA filament is about 10 cross-links/kbp. This is reduced to 3.8 cross-links/kbp in dsDNA treated with TMP in absence of RecA.

RESULTS

Experimental Design—To characterize DNA pairing intermediates, we used circular ssDNA and duplex DNA with homology restricted to the 5′- or 3′-end (with respect to the + or homologous strand) as shown in Fig. 2. The 5′- and 3′-ends are also referred to as proximal and distal, respectively, with the proximal end being the initiation point in a normal DNA strand exchange reaction. A region of heterology at one end of the dsDNA prevents the homologous strand from being completely displaced and allows the intermediates to accumulate. In previous studies, stable triple-stranded DNA intermediates have been reported only when homology was restricted to the 3′- or distal end (Rao *et al.*, 1991, 1993; Chiu, *et al.* 1993; Burnett *et al.*, 1994).

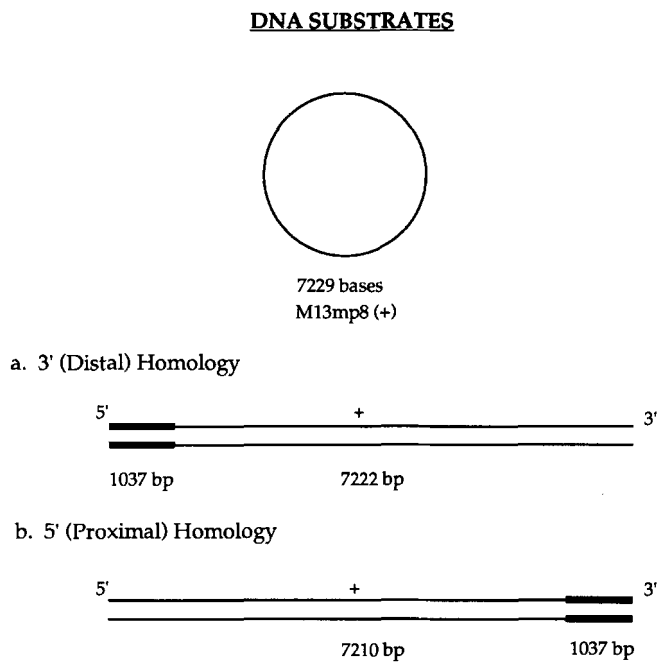


FIG. 2. **DNA substrates used in this study.** The *numbers* denote the size of the M13 mp8 single-strand circle or the length of the homologous (*thin lines*) and heterologous (*thick lines*) regions in the duplex DNA substrates. At the heterologous end of each duplex molecule, there are very short sequences with homology to M13 mp8 (not shown), which total 7 (*a*) and 19 (*b*) bp. Neither of these regions is long enough to take part in DNA pairing reactions under the conditions used for these experiments.

The Homologous Strand of the Duplex DNA Is Displaced in Paired DNA Intermediates—Synaptic complexes of circular ssDNA and partially homologous dsDNA were formed by RecA in the presence of ATP and then deproteinized. The entire reaction mixture at 60 min was spread and visualized by electron microscopy. Deproteinized samples were not cross-linked. Complexes of circular single-stranded DNA that was paired with either the 5′- or 3′-region of homology in the duplex DNA appeared similar, and representative joint molecules are shown in Fig. 3, *A* and *B*, respectively. The double-stranded region within the circle represents the region of hybrid DNA, the single-stranded tail is the displaced homologous strand of the dsDNA, and the double-stranded tail depicts the unexchanged duplex DNA. If homology extended to the the proximal end, the joint molecules represented 90% (84 of 93) of the total molecules observed in two repeated experiments. On the distal end, the joint molecules were 28% of the total (88 of 313) examined over two experiments. There was no evidence of triple-stranded structures in any of the joint molecules examined in these experiments. There have been no triple-stranded structures observed in uncross-linked joint molecules in any of the many experiments carried out in our laboratories over the past 6 years.

Stabilization of Triple-stranded DNA Intermediates at the Distal End of the Duplex DNA Requires Psoralen Cross-linking—Next, we used a psoralen cross-linker, AMT, to covalently link the strands prior to deproteinization and spreading of the joint molecules. Psoralens undergo photochemical addition to adjacent pyrimidines in opposite DNA strands to form covalent interstrand cross-links (Kanne *et al.*, 1982; Cimino *et al.*, 1985). Such cross-links can be visualized and counted by electron

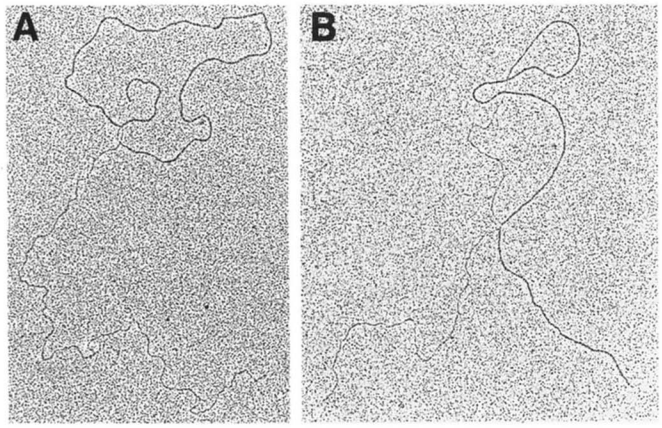


FIG. 3. **The homologous strand of the duplex DNA is displaced in paired DNA intermediates.** RecA-mediated DNA strand exchange reactions were carried out in the presence of ATP with circular ssDNA (10 μM), linear dsDNA with 5′ or 3′ homology (11.4 μM), 6.6 μM RecA, and 2 μM SSB as described under "Materials and Methods." The samples were prepared and visualized by electron microscopy. *A*, a representative paired complex involving linear dsDNA with proximal homology; *B*, a representative paired intermediate involving linear dsDNA with distal homology. The double-stranded circular DNA corresponds to the hybrid DNA formed in the strand exchange reaction, and the long single strand represents the displaced homologous strand. The duplex tail reflects the unreacted DNA within the duplex substrate.

microscopy if the duplex is completely denatured, since the single DNA strands are held together at many points (corresponding to cross-links). In the context of our experiments, several neighboring instances of a single strand cross-linked to a duplex identifies the region between the cross-links as triple-stranded DNA by inference, even though all three strands may not coincide in the spread sample. Reactions were again carried out in the presence of ATP and examined after 60 min.

With homology at the proximal end of the duplex DNA, the cross-linked joint molecules appeared similar to the uncross-linked intermediates. There was no indication of any triple-stranded DNA structures in any of the 62 paired complexes observed in two independent experiments (Fig. 4*A*). The joint molecules represented 95% of the total molecules (duplex DNA substrates and paired complexes) examined. A similar result, the absence of triple-stranded DNA in joint molecules with hybrid DNA extending to the proximal end, was obtained after only 15 min of reaction (data not shown).[5]

In contrast, when homology was at the distal end of the duplex DNA, the AMT-cross-linked joint molecules consisted of three types. The first class of intermediates (25% of the paired complexes observed) were indistinguishable from the uncross-linked intermediates shown in Fig. 3*B* (Table I). These intermediates were observed in 12 independent experiments; the detailed counts in Table I come from three of these, one chosen at random from experiments carried out with each of three different psoralen derivatives.

The second type of paired complexes contained regions of triple-stranded DNA extending to the end of the duplex DNA

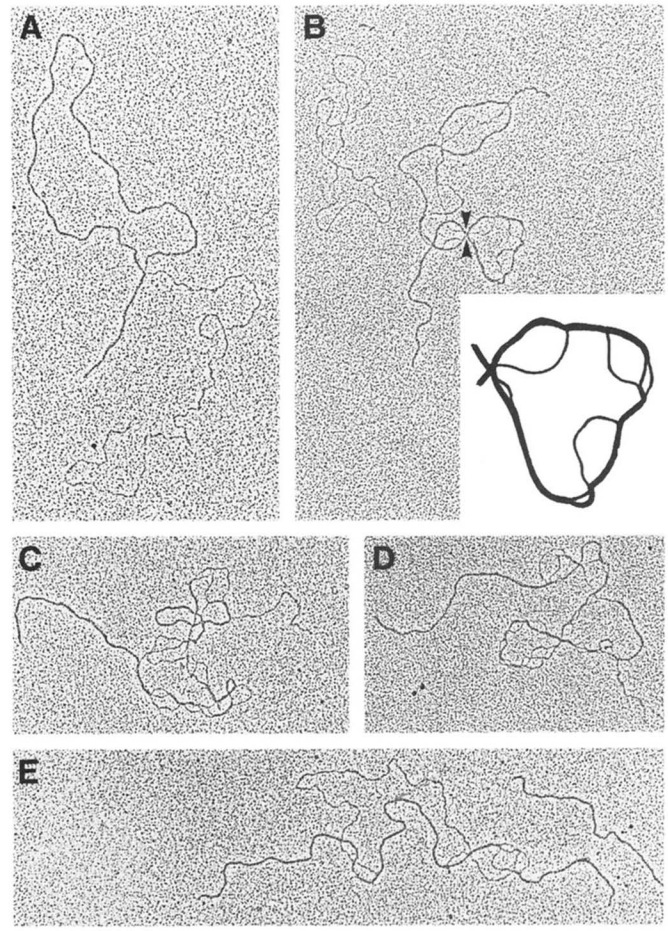


FIG. 4. **Electron micrographs of AMT cross-linked DNA intermediates.** DNA strand exchange reactions were carried out in the presence of ATP as described under "Materials and Methods." *A*, cross-linked DNA intermediates formed with circular single-stranded DNA and duplex DNA with proximal homology; *B–E*, cross-linked paired DNA intermediates involving circular ssDNA and duplex DNA with distal homology. Three representative paired complexes formed in DNA strand exchange reactions that proceed from the 3′ homologous free end of the duplex DNA are shown in *B–D*. Although the DNA strand paths in these molecules are complex, there are many consecutive regions that are consistent with a triple-stranded structure. The *inset* in *B* represents our interpretation of one of these triple-stranded regions between the *two arrows*, with multiple cross-links between the single-stranded DNA and the duplex DNA. Pairing of the ssDNA with an internal homologous region in the duplex DNA is depicted in *E*.

substrate (Fig. 4, *panels B–D*). The structures are complicated, and it is difficult to unambiguously trace the path of the three strands in most of the triple-stranded DNAs. The three-stranded DNA contained alternating substructures in which all three-DNA strands appear to be aligned, in which one strand is displaced from a duplex DNA region and in which all strands are single stranded. The *inset* within Fig. 4*B* shows our interpretation of a region in the micrograph showing these features. The spreading conditions used to prepare the molecules shown in Fig. 4 do not cause significant denaturation of normal dsDNA. However, in the triple-stranded regions, there were repeated examples of short regions where all three strands appeared to be in a single-stranded form. This suggests that the triple-stranded structure was in a strained conformation within the filament and unraveled to the extent permitted by the cross-linking upon RecA removal. These triple-stranded intermediates represented 56% of the paired DNA molecules (Table I).

The third class of synaptic complexes were three-stranded structures identical to the second, except that they involved

[5] When these same reactions (duplex DNA substrates with homology at the proximal end) were cross-linked at earlier reaction times, a substantial portion of the substrate DNA was found in complex aggregates containing many DNA molecules, precluding a careful analysis. The aggregates probably reflect the coaggregation process described by Gonda and Radding (1986). The interactions producing the aggregates were transient, as most did not survive the spreading process if not cross-linked. The aggregates were generally resolved into simple joint molecules between 10 and 30 min into the reaction. The simple joint molecules found at early time points, with hybrid DNA extending to the proximal end, did not contain triple-stranded DNA that could be cross-linked.

TABLE I

Psoralen photocross-linked paired DNA intermediates

RecA-mediated DNA strand exchange reactions involving circular ssDNA and linear duplex DNA with distal homology were carried out for 60 min in the presence of ATP as described under "Materials and Methods." The reaction mixtures were cross-linked with AMT, TMP, or HMT. The percent of each DNA species observed by electron microscopy is given as the percent of the total number of molecules counted. The total number of molecules counted in the reactions cross-linked with AMT, TMP, and HMT were 395, 128, and 239, respectively. Triple-stranded DNA is defined as DNA that has several neighboring instances in which the single-stranded DNA is cross-linked to a dsDNA region. Odd molecules include gapped duplex DNA, point contacts between circular ssDNA and linear dsDNA, and other complicated molecules that we interpret to arise from broken or nicked substrates.

DNA Species Observed	Total molecules		
	AMT	TMP	HMT
	%		
Intermediates			
Displaced homologous strand	9	12	17
Triple-stranded DNA (involving end of dsDNA; Fig. 4, *B–D*)	20	19	13
Internal triple-stranded DNA (Fig. 4, *E*)	7	6	10
Odd	15	16	16
Linear duplex DNA	49	48	44

TABLE II

Cross-linking of DNA intermediates after deproteinization

RecA-promoted DNA strand exchange reactions involving circular ssDNA and linear duplex DNA with distal homology were carried out in the presence of ATP. Reaction mixtures at 60 min were deproteinized with Proteinase K and a detergent or Proteinase K alone and then immediately cross-linked with AMT. The percentages reported reflect the percent of a given DNA species out of the total paired intermediates counted. This measurement does not include unreacted DNA substrates. *N* is the number of paired intermediates observed. The total number of DNA molecules counted (including DNA substrates), for the reactions treated with Proteinase K and detergents, Proteinase K (5 s), Proteinase K (1 min), and Proteinase K (2 min) are 509, 195, 176, and 93, respectively.

	Intermediates		*N*
	Triple-stranded	Homologous strand displaced	
	%		
Proteinase K and detergent[a]	0	100	107
Proteinase K, 5 s	49	51	84
Proteinase K, 1 min	9	91	65
Proteinase K, 2 min	3	97	31

[a] The data for four independent reactions treated with Proteinase K and Triton X-100, Nonidet P-40, *N*-laurolylsarcosine, or deoxycholic acid have been combined since the results were similar for all four reactions.

pairing of the circular ssDNA with an internal region of homology within the duplex DNA (Fig. 4*E*). They were similar to the three-stranded structures previously characterized by Umlauf *et al.* (1990). These intermediates represented 19% of the paired complexes observed (Table 1).

The stabilization of the triple-stranded structures formed in RecA-mediated DNA strand exchange reactions is dependent on AMT cross-linking. As we decreased the psoralen concentration 20-fold (1.5 μg ml^{-1} final concentration), the fraction of intermediates in classes 2 and 3 decreased significantly. Photocross-linking with a 100-fold lower concentration of AMT (0.3 μg ml^{-1} final concentration) almost eliminated detection of the three-stranded DNA structures (data not shown).

Very similar results were obtained with two other psoralen derivatives, TMP and HMT (Table I). These have lower solubilities and weaker affinities for duplex DNA than AMT (Isaacs *et al.*, 1977).

Formation of Triple-stranded DNA (Dependence on ATP Hydrolysis)—It seemed possible that RecA-generated triplex DNA is stabilized by ATPγS. To investigate the effects of ATP hydrolysis on the formation of three-stranded DNA intermediates, we paired circular ssDNA and linear duplex DNA with RecA in the presence of ATPγS. Samples were deproteinized without cross-linking. When pairing was limited to one end of the duplex DNA or the other, the paired complexes were all DNA structures with displaced, single-stranded tails, hybrid duplex DNA within the circle, and unexchanged duplex DNA tails. There was no evidence of triple-stranded DNA in any of the 989 paired complexes observed among 2371 molecules counted (data not shown). These came from 18 independent experiments, with pairing restricted to the proximal end of the duplex DNA in 10 experiments and to the distal end in 8 experiments.

For the ATPγS-mediated reactions, cross-linking with AMT prior to deproteinization had no effect on the results. All of the DNA intermediates contained displaced homologous single strands, whereas the hybrid duplex DNA regions were cross-linked. There was no indication of triple-stranded intermediates in any of the 95 and 75 paired complexes involving duplex DNA with proximal or distal homology, respectively, in two independent experiments for each reaction (data not shown). The total number of molecules counted in the experiments described above were 125 and 108, respectively. No triple-stranded DNA was observed in any reaction carried out with ATPγS, with or without DNA cross-linking.

Triple-stranded DNA Cannot Be Cross-linked after Removal of the RecA Filament—The triplex DNA species detected after deproteinization are reported to have a thermal stability similar to that of duplex DNA (Hsieh, 1990; Rao *et al.*, 1991, 1993). Duplex DNA is not denatured by the DNA spreading procedures used for our study. The absence of identifiable triplex DNA structures when cross-linking was omitted suggested that cross-linkable triplexes might survive deproteinization but that some aspect of the procedures used to prepare samples for electron microscopy might selectively disrupt them. We therefore deproteinized reactions where the three-stranded DNAs were observed above (with pairing restricted to the distal end of the duplex DNA and with ATP) and then cross-linked with AMT before preparing the samples for electron microscopy.

We disrupted the RecA filament on the paired intermediates by treatment with Proteinase K and either of two nonionic detergents, Triton X-100 or Nonidet P-40, or one of two anionic detergents, *N*-laurolylsarcosine or deoxycholic acid, for 15 min at 37 °C. Samples were then immediately cross-linked with AMT. All of the paired intermediates contained displaced single DNA strands, and no triple-stranded DNA was observed. The results with the four detergents were similar and were combined in Table II. To verify the presence of cross-linking within the duplex DNA regions of paired complexes and to confirm that the cross-linking reactions worked under the conditions used, individual reaction mixtures were heat denatured after deproteinization and AMT cross-linking. DNAs that were incubated with Proteinase K and any of the four detergents revealed degrees of cross-linking in the duplex DNA similar to those observed without the detergents. Control experiments had shown that SDS interfered with the cross-linking reaction (data not shown).

We wanted to examine the transition of triple-stranded DNA recombination intermediates detected within the filaments to complexes with displaced single-stranded DNA tails. Reaction mixtures involving circular ssDNA and linear dsDNA with distal homology were treated with Proteinase K for 5 s (psoralen added immediately after addition of Proteinase K), 1 min, or 2 min and then cross-linked with AMT. Triple-stranded structures were observed in half of the paired complexes

counted when the DNAs were cross-linked at 5 s (Table II). Incubation with Proteinase K for 1 min significantly reduced the number of three-stranded complexes, and 2 min of digestion almost eliminated the triple-stranded structures (Table II). Evidently, removal of RecA leads to an altered triple-stranded structure that is not cross-linkable, or the triple-stranded structure is lost immediately because the third strand is no longer found to be associated with the hybrid DNA. The DNA intermediates appeared to be dotted with remnants of the RecA filament as observed by electron microscopy (data not shown). We verified that the duplex DNA regions within the paired complexes were cross-linked in the presence of Proteinase K, so the results do not reflect an inhibition of the cross-linking process.

DISCUSSION

As previously reported (Umlauf *et al.*, 1990), a triple-stranded DNA species, reflecting homologous pairing between a single strand and a duplex DNA, can be cross-linked within a RecA filament and visualized by electron microscopy. The present study demonstrates that this species can be detected only in a limited set of circumstances. The three-stranded DNA is found only when complete DNA strand exchange is precluded (*e.g.* by heterologous sequences at the proximal end of the linear duplex DNA). The triple-stranded DNA is also observed only when ATP is hydrolyzed and cannot be detected once the RecA filament is removed. No three-stranded DNA that can be cross-linked with AMT is observed by electron microscopy during normal DNA strand exchange reactions between completely homologous DNA substrates or when pairing is restricted to the proximal end of the duplex DNA substrate.

There is one major point of agreement among all of the studies in which the status of individual DNA strands within RecA-paired complexes could be assessed. Under most conditions, the RecA filament tends to stabilize the *products* of DNA strand exchange. When ATPγS was employed in the present study, only the hybrid duplex DNA was cross-linked within paired regions at either end of the duplex DNA. This result is in close agreement with that of Adzuma (1992). When pairing occurred on the proximal end of the duplex DNA, the hybrid DNA was cross-linked exclusively even when ATP was hydrolyzed. The homologous strand of the duplex is evidently displaced rapidly during strand exchange with ATP if the structure of the DNA substrates permits.

We saw no evidence for triple-stranded structures after deproteinization using the methods described in this investigation. Only the hybrid duplex can be cross-linked with AMT in the deproteinized intermediates. The characterization of these deproteinized species with chemical and enzymatic probes also indicates that the two strands representing the hybrid duplex are joined by Watson-Crick base pairs (Rao *et al.*, 1991; Chiu *et al.*, 1993; Rao *et al.*, 1993; Burnett *et al.*, 1994). The third strand in the stable triplexes may be hydrogen-bonded in the major groove of this product duplex DNA (Rao *et al.*, 1993) but not in a conformation suitable for cross-linking with a psoralen derivative.

It is worth pointing out that there is no direct evidence in any study for a DNA triplex existing prior to the actual strand switch. In all of the putative triplex DNA structures described to date for RecA reactions, including the stable triplexes detected after deproteinization, the Watson-Crick pairing occurs between the strands destined to form the hybrid DNA product. In effect, there is no evidence for a type I model. Triplex DNA described to date appears to be a transient *product* of DNA strand exchange, existing only until the displaced strand can be unraveled from the product duplex and persisting only when that unraveling is precluded by DNA structure. The relationship of this species to the process by which two DNA molecules are initially aligned is unclear.

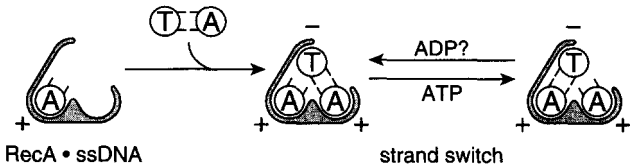


FIG. 5. **A model for DNA pairing within a RecA filament that is hydrolyzing ATP.** The single minus (−) strand alternates between complementary (+) strand partners without formation of a base-paired triplex DNA.

The cross-linking patterns observed in the circumstances where we do observe three-stranded DNA suggest a dynamic structure (Fig. 5). The single minus strand in the complex may be positioned so that it can pair alternatively with either of the complementary plus strands. Psoralen and its derivatives most readily cross-link antiparallel strands that are joined by Watson-Crick base pairs (Cimino *et al.*, 1985). The linking of three strands (when ATP is hydrolyzed and the third strand cannot be displaced) suggests that the paired region is divided into small subdomains in which the minus strand is alternately paired with either complement. Such a pattern was documented in Umlauf *et al.* (1990). The alternation could be facilitated by ATP hydrolysis. When ATP is bound (or ATPγS), the hybrid duplex is favored. ATP hydrolysis may lead to rapid displacement of the third strand (Burnett *et al.*, 1994). If the extra strand cannot be displaced, bound ADP, or the absence of nucleotide, may permit the minus strand to switch back and pair with its original complement in the substrate duplex. This would yield a mixture of the "pre-switched" and "post-switched" states, in the terminology of Burnett *et al.* (1994).

This kind of switching by the minus strand could be viewed as a sampling mechanism in the homology search required to align the two DNA molecules. However, DNA pairing occurs efficiently without ATP hydrolysis (at least *in vitro*). The switching may instead reflect conformation changes in a relatively tight complex that would normally lead to displacement of the third strand. The third strand is at least transiently displaced in distal joints because it is susceptible to exonuclease I digestion (Bedale *et al.*, 1993). The same process may contribute to dissociation of DNA joints that are not productive (Burnett *et al.*, 1994).

A triplex species in which the bound single-stranded DNA interacts with the major groove of the incoming duplex DNA still seems like a plausible, if not probable scenario for DNA pairing. The lack of direct evidence for a "pre-switch" triplex could simply reflect a short lifetime, with the strand switch to hybrid DNA occurring rapidly once alignment is achieved. The like-strand pairing recently detected by Rao and Radding (1993) suggests that the two sites in the filament where the plus strands bind are close enough for these strands to interact.

We cannot rule out the possibility that a triple-stranded DNA structure remains after RecA removal. However, our inability to visualize triplex DNA after deproteinization does establish some additional structural parameters. First, the triple-stranded structure must be less stable under some or all conditions than duplex DNA. No uncross-linked triplex DNA species survives our spreading procedures, even though the same procedures do not disrupt duplex DNA. Although the triplex DNA has been reported to be thermally stable (Hsieh *et al.*, 1990; Rao *et al.*, 1991, 1993), the surface tension to which DNA is subjected during spreading might preferentially destabilize uncross-linked triplex DNA. We note that the R-form DNA described by Shchyolkina *et al.* (1994) is much less stable than normal duplex DNA. Second, the cross-linking of the

hybrid duplex DNA strands after deproteinization indicate that these strands are Watson-Crick base paired, consistent with chemical modification patterns recently reported (Chiu *et al.*, 1993). The third strand is clearly not in a conformation that permits it to be AMT-cross-linked to either of the other two strands. Finally, the change in cross-linking patterns brought about by deproteinization also allows us to conclude that the structure of the DNA species present after deproteinization is not the same as the three-stranded DNA pairing intermediate that can be cross-linked within the RecA filament. Whatever its detailed structure, the relationship of the deproteinized species to events that occur within the RecA filament remains to be determined.

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