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Putative Three-stranded DNA Pairing Intermediate in recA Protein-mediated DNA Strand Exchange: No Role for Guanine *N*-7*

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As an early step in DNA strand exchange reactions, the recA protein aligns homologous sequences within two DNA molecules to form a putative triple-stranded intermediate. In virtually all models for three-stranded DNA proposed to date, hydrogen bonds involving the *N*-7 position of guanine have played a prominent structural role. To determine whether the *N*-7 position of guanine is required for triple helix and heteroduplex formation in the recA protein-mediated DNA pairing reaction, guanine was completely replaced by the base analog 7-deazaguanine in both strands of the duplex DNA substrate using polymerase chain reaction. This modified double-strand DNA was reacted with unmodified single-strand DNA *in vitro*. The 7-deazaguanine-substituted DNA functioned as well as the unsubstituted DNA in recA protein-mediated DNA three-strand exchange reactions. Strand exchange reactions involving four strands also proceeded normally when three of the four strands contained 7-deazaguanine rather than guanine. In fact, the rate of strand exchange improved somewhat when the modified DNA substrates were used. This indicates either that the *N*-7 position of guanine is not essential for the formation of the putative triple-stranded DNA pairing intermediate, or that a three-stranded (or four-stranded) structure is not an obligate intermediate in recA protein-mediated DNA strand exchange.

The recA protein promotes a set of DNA strand exchange reactions *in vitro* that mimic the formation and extension of heteroduplex DNA during recombinational DNA repair *in vivo*. One of the best characterized reactions is the three-strand exchange reaction, involving circular single-stranded DNA (ssDNA)[1] and homologous linear duplex DNA (see Cox and Lehman, 1987; Radding, 1989; Roca and Cox, 1990, for reviews). recA protein also promotes a reciprocal strand exchange reaction involving two duplexes, one of which contains a single-strand gap (a four-strand exchange) (Fig. 1). These reactions can be divided into three experimentally distinguishable phases. The first step is the cooperative binding of recA protein to ssDNA or to the gapped duplex (DNA1), to produce a right-handed helical nucleoprotein filament. In the presence of ATP, the DNA in this filament is underwound to 18 nucleotides or base pairs per turn and extended with an axial base separation of 5.1 Å (Stasiak and Egelman, 1988; Radding, 1989; Roca and Cox, 1990). The recA protein binds to the deoxyribose phosphate backbone, leaving the bases free in the major filament groove to interact with a second DNA molecule (Dombroski *et al.*, 1983; Leahy and Radding, 1986; Di Capua and Müller, 1987). In the second step (synapsis), the nucleoprotein filament binds a homologous linear duplex DNA molecule (DNA2) and aligns it with the first. In the final phase of the reaction, recA protein promotes a unidirectional branch migration reaction coupled to ATP hydrolysis to produce stable, heteroduplex products (Cox and Lehman, 1981a, 1981b; Kahn *et al.*, 1981; West *et al.*, 1981).

The structure of the DNA pairing intermediate formed in the second step is unknown, and it remains one of the key issues impeding a full understanding of the mechanism of the strand exchange reaction. Presumably, homologous alignment of the two DNAs involves a complementary interaction mediated by interstrand hydrogen bonding. A pairing intermediate was first detected by Radding and colleagues (DasGupta *et al.*, 1980; Bianchi *et al.*, 1983) and was originally called a paranemic joint. Howard-Flanders *et al.*, (1984) proposed that the pairing involved interwinding of the two DNAs to form a right-handed, three- (Fig. 1*A*) or four- (Fig. 1*B*) stranded, helical DNA structure. The proposed interstrand hydrogen-bonding patterns in the Howard-Flanders model were based on the ideas of McGavin (1971) and Wilson (1979).

A variety of observations have provided evidence for the existence of a triple-helical pairing intermediate in three-strand exchanges. The most direct are several elecron microscopy studies (Stasiak *et al.*, 1984; Register *et al.*, 1987; Griffith *et al.*, 1990; Umlauf *et al.*, 1990; Bortner and Griffith, 1990) that have provided evidence that all three strands are closely juxtaposed and interwound at an early stage of the strand exchange reaction. Obtaining detailed information about this three-stranded structure has been hampered by its apparent instability under most conditions when recA protein is removed. There are also a number of published and unpublished experimental clues that the structure is quite dynamic under strand exchange conditions (Umlauf *et al.*, 1990; Bortner and Griffith, 1990). Perhaps for these reasons, the growing literature addressing this problem has yielded a variety of experimental results that are not always readily reconciled.

Many of the studies listed above employ DNA substrates for which steps in strand exchange subsequent to pairing are

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[1] The abbreviations used are: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; FI DNA, supercoiled closed circular form of DNA as isolated from *E. coli* cells; FIII DNA, linear double-stranded DNA; SDS, sodium dodecyl sulfate; AMT, 4′-amino-4,5′,8-trimethylpsoralen; bp, base pairs; kbp, kilobase pairs; SST, single-stranded tail; PCR, polymerase chain reaction; SSB, single-stranded DNA binding protein.

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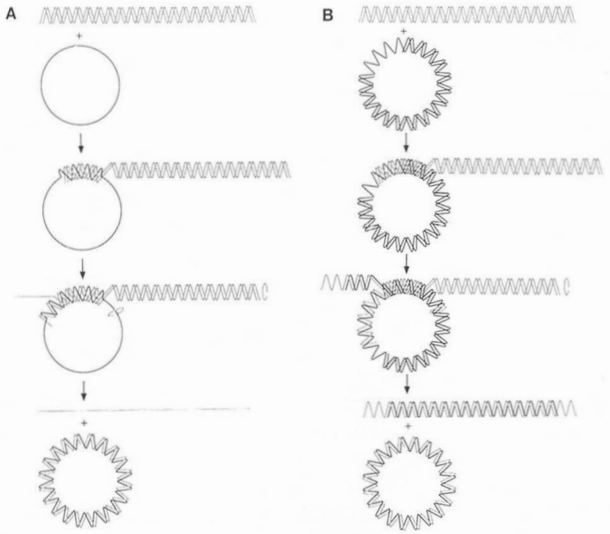


FIG. 1. **Putative three- and four-stranded pairing intermediates formed during recA protein-mediated DNA strand exchange.** A schematic representation of strand exchange involving either (*A*) three or (*B*) four strands. The pairing intermediate forms at the initiating end of the duplex substrate. In the four-strand reaction the initial pairing occurs within the gap of DNA-1; *i.e.* a four-strand exchange is initiated as a three-strand exchange. Exchange requires rotation of the two DNAs as shown, and a short three- or four-stranded structure may migrate with the branch point. Continued rotation leads to product formation. The continued existence of a four-stranded structure during branch migration (as depicted here) has not been demonstrated, although some indirect evidence has been obtained that the three-stranded structure persists throughout the reaction.

topologically blocked, and the pairing intermediates formed are highly unstable when recA protein is removed. In the presence of ATP, pairing intermediates can be detected within 1–2 min after the addition of homologous duplex DNA to preformed recA-ssDNA nucleoprotein complexes (Wu *et al.*, 1982; Kahn and Radding, 1984; Schutte and Cox, 1987, 1988). The duplex DNA in these complexes is extended and underwound to facilitate alignment with the ssDNA (Wu *et al.*, 1983; Schutte and Cox, 1988). The extent of this underwinding (Schuttee and Cox, 1988), the properties of observed changes in rates of ATP hydrolysis (Schutte and Cox, 1987), and some electron microscopy measurements (Register *et al.*, 1987; Umlauf *et al.*, 1990) all indicate that formation of these intermediates is very efficient and that alignment can extend over thousands of base pairs.

To stabilize the structures so that they can be better analyzed by electron microscopy, the pairing intermediates have been fixed by psoralen photocross-linking prior to removal of recA protein in two studies. Bortner and Griffith (1990) observed that the two DNAs could be cross-linked only over short regions (200–300 bp) although the duplex DNA was often incorporated into the recA-ssDNA nucleoprotein filaments over much longer distances. In contrast, Umlauf *et al.* (1990) observed homology-dependent cross-linking over much longer distances (a few hundred to more than 5,000 bp) and provided evidence that the two DNAs (three strands) within these joints are helically interwound. The differences in the results obtained in these two studies may reflect multiple differences in the experimental protocols. The long paired structures observed by Umlauf *et al.* (1990) may simply be extended versions of a shorter triplex that may form rapidly during normal strand exchange and move along the DNAs coincident with the branch point as the branch migration phase of the reaction proceeds (Radding, 1991).

In contrast to these unstable three-stranded structures, Hsieh *et al.* (1990) and Rao *et al.* (1991) have reported a three-stranded structure that remains stable after recA protein removal as measured by thermal stability and/or resistance of all three strands to nuclease digestion. This structure forms only at the distal end of the linear duplex DNA (the end where strand exchange terminates), and their relationship to the unstable joints and the strand exchange reaction has not been determined.

Whereas three-strand pairing intermediates are readily detected, four-strand intermediates have been detected and reported only recently (Conley and West, 1989, 1990; Lindsley and Cox, 1990; Chiu *et al.*, 1990). The four-stranded pairing interaction is much weaker, and is not detected unless the second duplex substrate is supercoiled. Since four-strand exchanges are initiated within a single-strand gap in DNA1 (*i.e.* four-strand exchanges begin as three-strand exchanges) and recent physical studies indicate that there are binding sites for only three strands within the recA filament (Takahashi *et al.*, 1989; Müller *et al.*, 1990; Takahashi *et al.*, 1991), the role of the four-stranded pairing interaction is unclear.

Based on the established structural parameters of recA-DNA complexes, any three-stranded DNA formed within recA protein filaments must have a novel structure. Stable three-stranded DNA structures (such as pyrimidine-purine-pyrimidine triplexes and H-DNA) reported to date (Moser and Dervan, 1987; Hanvey *et al.*, 1988; Htun and Dahlberg, 1989) are restricted to polypyrimidine or polypurine sequences and the two like strands are antiparallel. In the structure formed by the action of recA protein, like strands must be parallel, and the structure must form in a sequence-independent fashion. In addition, the base pairs (or triplets) must be separated by 5.1 Å, with 18 base pairs (triplets) per helical turn.

The work described above falls somewhat short of a clear demonstration that a novel triple-helical DNA structure is an obligate intermediate in recA protein-mediated DNA strand exchange. Nevertheless, a strong case for the existence of such a structure is being built.

Another approach to understanding the structure of the three-stranded pairing intermediated is to examine hydrogen-bonding patterns within the base triplet. The H-bonding patterns in a number of proposed models for multistranded DNA are presented in Fig. 2 (for G-C pairings only). A prominent feature of all of these models is the presence of one or more non-Watson-Crick hydrogen bonds to guanine

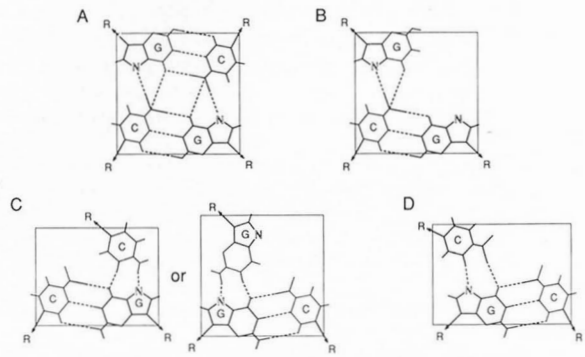


FIG. 2. **Models for interstrand hydrogen-bonding in three- and four-stranded DNA structures.** Only G-C pairings are depicted. In each case, guanine *N*-7 is denoted by an *N*; no other atoms are spelled out. *A*, the four-stranded DNA structure proposed by McGavin (1971) and Wilson (1979); *B*, the three-stranded variant of the McGavin/Wilson structure proposed by Howard-Flanders *et al.* (1984); *C*, the three-stranded pairing intermediate proposed by Hsieh *et al.* (1990); *D*, H-DNA (Hanvey *et al.*, 1988).

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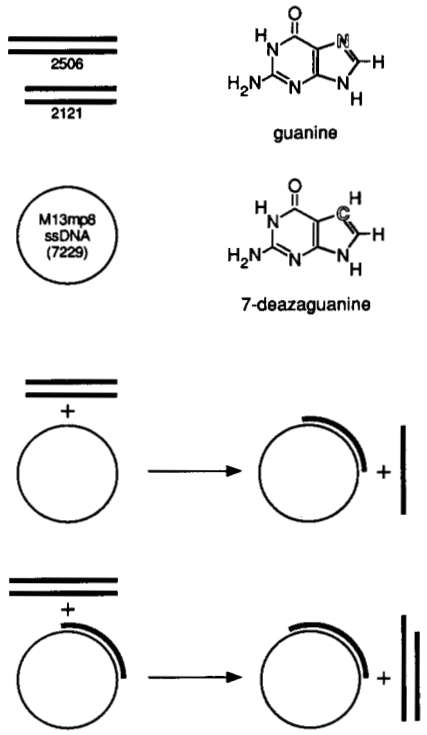


FIG. 3. **The DNA substrates used in this study, and the reactions studied in Figs. 4–8.** *Thick lines* denote strands in which guanine has been replaced by 7-deazaguanine. The *numbers* denote substrate length in nucleotides or base pairs. The structures of guanine and 7-deazaguanine are also depicted.

N-7. Interactions of this type would be essential to any homologous pairing of three or more DNA strands. If any of these models correctly depicts the structures of the three-stranded pairing intermediate formed during recA protein-mediated DNA strand exchange, and if that structure is an obligate intermediate in the reaction, then blocking the formation of hydrogen bonds at guanine *N*-7 should inhibit or block strand exchange. To examine the role, if any, of guanine *N*-7, PCR was used to synthesize duplex DNA substrates in which guanine had been completely replaced by 7-deazaguanine (Fig. 3). This substitution prevents the formation of triplex DNA structures formed by DNAs with long homopyrimidine or homopurine sequences (pyr·pur DNAs).[2] We report here that this substitution has no effect on DNA strand exchange reactions mediated by recA protein.

MATERIALS AND METHODS

Enzymes and Biochemicals—*Escherichia coli* recA protein was purified and stored as previously described (Cox *et al.*, 1981). *E. coli* single-stranded DNA binding protein (SSB) was purified as described (Lohman *et al.*, 1986). 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. *Hin*dIII restriction endonuclease was purchased from New England Biolabs. The deoxynucleotide triphosphates dATP, dTTP, dCTP, and dGTP were purchased from Pharmacia LKB Biotechnology Inc.; 7-deaza-dGTP and Tris buffer were purchased from Boehringer Mannheim Biochemicals. AmpliTaq DNA polymerase was purchased from Perkin-Elmer/Cetus. Creatine phosphokinase, phosphocreatine, proteinase K, low melting agarose, and ATP were purchased from Sigma. 4′-Amino-4,5′,8-trimethylpsoralen (AMT) was purchased from Calbiochem. Malachite green was purchased from MCB. Gelatin was purchased from United States Biochemical.

DNA—Circular single-stranded DNA and supercoiled circular duplex DNA from bacteriophage M13mp8 (7,229 bp) were prepared using methods described previously (Davis *et al.*, 1980; Messing, 1983; Neuendorf and Cox, 1986). 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. The concentrations of dsDNA in which guanine was replaced by 7-deazaguanine were determined similarly, but a conversion factor of 43 μg ml^{-1} A_{260}^{-1} was used. DNA concentrations are expressed in terms of total nucleotides unless otherwise noted. Concentrations of 7-deazaguanine-modified DNA were confirmed from phosphate assays as described below. Full-length linear duplex (FIII) DNA was derived from M13mp8 FI DNA stocks by complete digestion with *Hin*dIII endonuclease, using conditions suggested by the enzyme supplier. After digestion, residual protein was removed by extraction sequentially with phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1) followed by ethanol precipitation.

Preparation of 7-Deazaguanine-modified DNA—The base analog 7-deazaguanine was enzymatically incorporated into both strands of a 2,121-bp or a 2,506 bp fragment of M13mp8 by PCR using a Perkin-Elmer/Cetus DNA Thermal Cycler. All PCR reactions were performed in a standard reaction buffer containing 10 mM Tris-Cl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 200 μM of each nucleotide (dATP, dTTP, dCTP, and 7-deaza-dGTP), 250 nM (in total oligonucleotides) of each primer, 25 units ml^{-1} AmpliTaq DNA polymerase, and 100 ng ml^{-1} FIII M13mp8 DNA (linearized by *Hin*dIII). The total reaction volume was 100 μl. A 2,121-bp or a 2,506-bp fragment of unmodified DNA was also amplified using the PCR conditions above except that dGTP was substituted for 7-deaza-dGTP. Denaturation, annealing, and polymerization temperatures were 94, 60, and 72 °C, respectively, and amplification was carried out for 30 cycles. The PCR products were separated on a 1% agarose gel and the amplified fragment was excised and eluted using a unidirectional electroeluter (International Biotechnologies, Inc.) or the excised fragment was purified from low melting agarose by a published procedure (Sambrook *et al.*, 1989). The DNA was ethanol-precipitated and resuspended in Tris-Cl-EDTA.

Purification from low melting agarose resulted in somewhat higher yields than were obtained with electroelution, typically 2.8 and 3.8 μg of amplified fragment for the substituted and unsubstituted DNA, respectively, from a 100-μl PCR reaction. Purity of the final product was >80% (usually >90%), as estimated from densitometric scans of photographic negatives of gels stained in ethidium bromide. The densitometric scans were performed on a Zeineh Soft Laser scanning densitometer, SL-504-XL, from Biomed Instruments, Inc.

The primers used for the 2,121-bp fragment were 5′ TGGCTGCAGGTCGACGGATC 3′ and 5′ AGCGACGATTTACAGAAGCAAG 3′, yielding a fragment corresponding to positions 4,141 to 6,261 on the M13mp8 map. The G + C content of the 2,121-bp fragment was 44%. The 2,506-bp fragment used the first of the two primers above, plus 5′ CCCTACTGTTGAGCGTTGGCT 3′, yielding a fragment corresponding to positions 3,756 to 6,261 on the M13mp8 map (G + C content = 42%).

Determining the Concentration of 7-Deazaguanine-substituted DNA—We could not locate an extinction coefficient for 7-deazaguanine-substituted dsDNA in the literature. The extinction coefficients of 7-deaza-dGTP and dGTP are quite similar (13,400 and 13,700, respectively, at 260 nm), leading to the expectation that the presence of the modified nucleotide would not have a large effect on the absorption of the DNA. However, the substituted DNA was stained only weakly by ethidium bromide [an observation also made by Innis (1990)], so that on an agarose gel it gives the appearance of much less DNA being present than the absorption at 260 nm would suggest. Latimer and Lee (1991) have recently shown that 7-deazaguanine-substituted DNA binds ethidium bromide almost as well as normal DNA, but the fluorescence of the bound ethidium bromide is quenched. The only fluorescence observed is derived from ethidium intercalated between two adjacent A-T base pairs, so that the observed fluorescence for a given substituted DNA will depend upon sequence and A-T content. The substitution of 7-deazaguanine for guanine in DNA does not appear to have a substantial effect on the overall structure of the DNA (Latimer and Lee, 1991).

We therefore determined the extinction coefficient of the modified duplex DNA fragment used in the present experiments by comparing the A_{260} and phosphate concentration of several samples. The malachite green method for phosphate analysis (Hess and Derr, 1975) was used to measure inorganic phosphate concentrations with slight modifications. All of the glassware used either for reagent storage or the reactions was phosphate-free. Two to four nmol of DNA (as

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

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estimated from A_{260} measurements assuming a conversion factor of 50 μg ml^{-1} A_{260}^{-1}) was mixed with 100 μl of 10% Mg $(NO_3)_2$ in 95% ethanol and ashed over an open flame. HCl (0.5 ml of a 1 N solution) was added to each tube and heated at 100 °C for 15 min. All samples were then neutralized by the addition of 0.5 ml of 1 N NaOH. A 1:1 solution of 3% ammonium molybdate-0.06% malachite green (0.5 ml) was added and absorbance at 650 nm was measured after 10 min. Values were compared to a standard curve prepared using dilutions of a stock solution of KH_2PO_4.

Phosphate determinations were carried out on 7-deazaguanine-substituted DNA on 4 different days over 6 months, using four different PCR preparations. The determinations indicated that the concentration of the substituted DNA was 86 ± 3% (average of 15 total determinations) of that expected assuming the extinction coefficient was the same as unsubstituted DNA. We therefore assumed the extinction coefficient was 7.6×10^3 M^{-1} cm^{-1}, yielding a conversion factor of 43 μg ml^{-1} A_{260}^{-1}, as reported above. As a control, 15 phosphate determinations were also carried out on PCR-amplified unsubstituted M13mp8 dsDNA. On average, these yielded values for DNA concentration that were 104 ± 4% of that expected based on the extinction coefficient of B-DNA (6.7×10^3 M^{-1} cm^{-1}) at 260 nm.

Strand Exchange Reaction Conditions—All reactions were performed at 37 °C in a standard reaction buffer containing 25 mM Tris acetate (80% cation, pH 7.5), 10 mM magnesium acetate, 3 mM potassium glutamate, 1 mM dithiothreitol, 5% glycerol, and an ATP regeneration system (10 units ml^{-1} creatine phosphokinase, 12 mM phosphocreatine). Protein and DNA concentrations are reported in the text and figure legends. Single-stranded DNA was preincubated with recA protein for 10 min before addition of duplex DNA. After incubation of this mixture for 10 min, ATP (3 mM) and SSB were added to start the reactions. Final reaction volume is provided in the text.

Agarose Gel Assays—Aliquots (10 μl) of strand exchange reactions described above were removed at each time point, and the reactions were stopped by the addition of 5 μl of gel loading buffer (25% glycerol, 15 mM EDTA, 0.025% bromphenol blue, 5% SDS). These aliquots were stored on ice until after the last time point was taken. Samples were electrophoresed overnight in an 0.8% agarose gel at 2–2.5 V cm^{-1}.

Electron Microscopy—Samples for electron microscopy were obtained by either elution of DNA product bands from agarose gels or by spreading the entire strand exchange reaction mixture as noted in the text. Elution of DNA from agarose gels was carried out using a unidirectional electroeluter (International Biotechnologies, Inc.) according to the manufacturer's instructions. The samples were incubated with proteinase K (1 mg ml^{-1} final) and SDS (1% final) at 37 °C for 30 min. Some samples were cross-linked with 4′-amino-4,5′,8-trimethylpsoralen (AMT) prior to spreading. Aliquots (20 μl) of the strand exchange reactions at the 40-min timepoint were immediately mixed with AMT (30 μg ml^{-1}, final concentration), incubated at 25 °C for 3 min, and irradiated with long-wave UV light for 5 min at 25 °C (Umlauf *et al.*, 1990). The UV light was generated with two fluorescent black light tubes. Samples were placed 8 cm below the light source. Intensity of light ranged between 2 and 4 milliwatts/cm^2 at 365 nm (as measured with a Blak-ray J-221 long-wave UV monitor). A glass microscope slide was placed over the sample to filter out UV irradiation below 320 nm. All cross-linked samples were dialyzed into 20 mM NaCl and 5 mM EDTA overnight at 25 °C before spreading as previously described (Inman and Schnös, 1970). These samples were checked for AMT cross-linking density and for triple strandedness by denaturing the samples at 70 °C in the

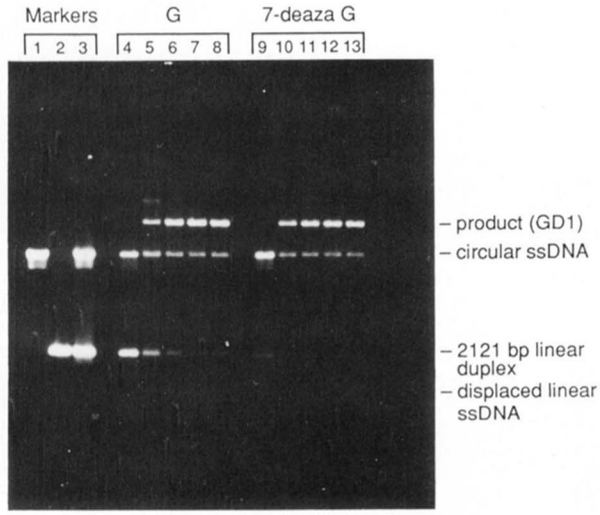


FIG. 4. **Three-strand exchange reactions.** Reactions were carried out under standard reaction conditions as described under "Materials and Methods" and included (in 60 μl of total volume) 6.7 μM recA protein, 20 μM M13mp8 circular ssDNA, 2.0 μM SSB, and either 20 μM (unmodified) or 17.2 μM (7-deazaguanine-substituted) dsDNA fragments (2,121 bp in length). Markers (from *left* to *right*) are circular M13mp8 (+) ssDNA, 2,121-bp linear duplex DNA, and gapped duplex DNA with a 2,121-bp paired region (GD1). *Lanes 4–8,* reaction with unmodified DNA; *lanes 9–13,* reaction with 7-deazaguanine-substituted dsDNA. Timepoints for each reaction shown correspond to 0, 10, 20, 40, and 60 min (*left* to *right*).

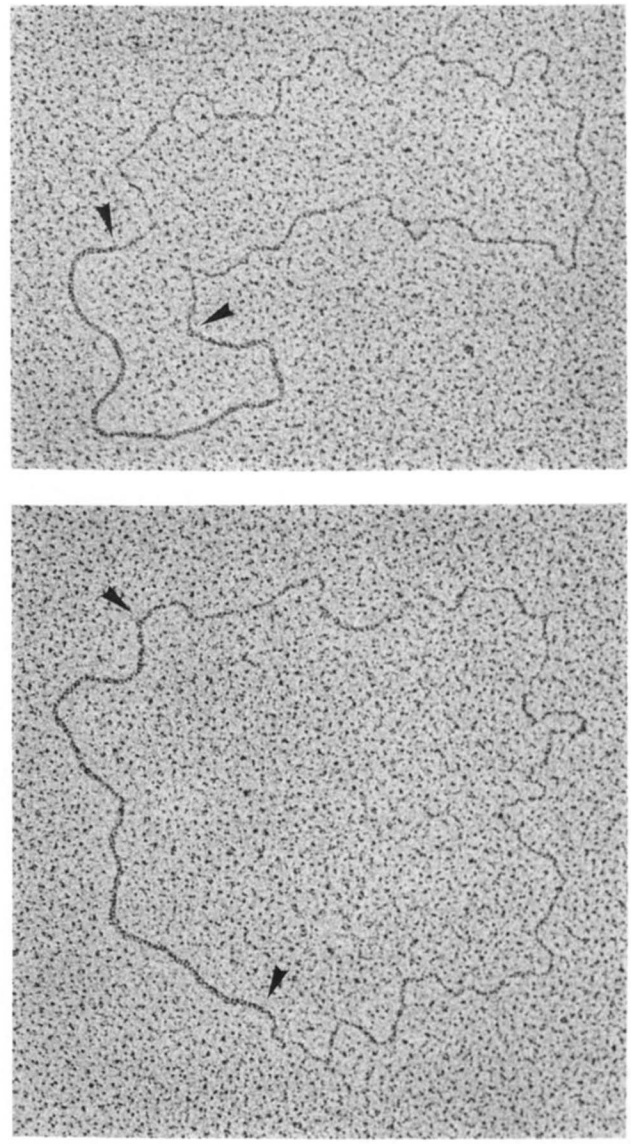


FIG. 5. **Gapped duplex products of the DNA strand exchange reaction depicted in Fig. 4, *lanes 9–13*.** A strand exchange reaction was carried out as described under "Materials and Methods" and in the legend to Fig. 4. The reaction product comigrating with gapped duplex circles at the 40 min timepoint was cut out and eluted from a gel as described under "Materials and Methods." The DNA was spread for electron microscopy and two typical molecules are shown. The ends of the 2,121-bp duplex regions are indicated by *arrows*. In these molecules, the short strand contains 7-deazaguanine in place of guanine.

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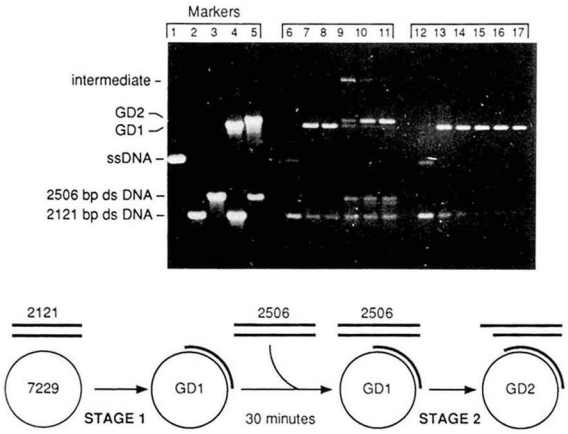


FIG. 6. **Four-strand exchange reactions.** Reactions were carried out in two stages as described in the text and illustrated in the schematic. Standard reaction conditions were used (see "Materials and Methods"). Reactions (*stage 1*) contained in 70 μl total volume: 3.3 μM recA protein, 10 μM M13mp8 ssDNA, 1.0 μM SSB, and 17.2 μM 7-deazaguanine-substituted dsDNA fragments (2,121 bp). At 30 min, *stage 2* was initiated by adding the 7-deazaguanine-substituted 2,506-bp dsDNA fragment to a final concentration of 17.2 μM. Markers are: *lane 1,* circular M13mp8 ssDNA; *lane 2,* 2,121-bp dsDNA fragment; *lane 3,* 2,506-bp dsDNA fragment; *lane 4,* gapped duplex DNA with 2,121-bp paired region (GD1); *lane 5,* gapped duplex DNA with a 2,506-bp paired region (GD2). Lower bands in *lanes 4* and *5* are the linear dsDNA fragments in *lanes 2* and *3,* respectively. *Lanes 6–11* depict the two stage reaction at timepoints 0, 10, 25, 40, 50, and 70 min, respectively. The 40-min timepoint corresponds to 10 min after the initiation of *stage 2. Lanes 12–17* show a control reaction that is identical to that in *lanes 6–11* (with the same timepoints) except that the 2,506-bp linear dsDNA fragment (7-deazaguanine-substituted) was not added.

presence of 5% formaldehyde. Holliday intermediates formed in the four-strand exchange reactions were verified by partially denaturing the samples in a mixture containing 7.3% HCHO, 14.3% formamide, 0.05 M NaOH, and 0.016 M Na_2CO_3 at 50 °C for 10 min, to open up the crossover junction.

RESULTS

Experimental Design—To begin to investigate potential non-Watson-Crick hydrogen bonding patterns within the putative three-stranded DNA pairing intermediate in recA protein-mediated DNA strand exchange, we decided to generate DNA substrates containing base analogues that lack one or more hydrogen bond acceptor or donor groups. The analogue 7-deazaguanine was chosen for this study because (*a*) hydrogen bonds to guanine *N*-7 play a prominent role in virtually every published model for three- and four-stranded DNA structures, (*b*) 7-deaza-dGTP is readily substituted for dGTP in DNA polymerase reactions (Innis *et al.,* 1988), (*c*) the substitution eliminates H-bonding potential without introducing a bulky group into the DNA, and (*d*) there are good commercial sources for 7-deaza-dGTP with a purity that is compatible with PCR. PCR was chosen as the method of choice for generating relatively long duplex DNAs in which the guanine nucleotides had been completely replaced with the analogue. The lengths of the duplex fragments used as substrates were chosen as those that could be generated in high yield by PCR. Attempts to generate linear duplexes 2–3 times longer led to greatly reduced yields and problems in purifying the PCR product. For control experiments, unmodified DNA fragments of the same size and sequence were also generated by PCR, using the same protocols as those used to produce the substituted DNAs. This was done to control for the possibility of artifacts introduced by the PCR method used to generate the DNA substrates.

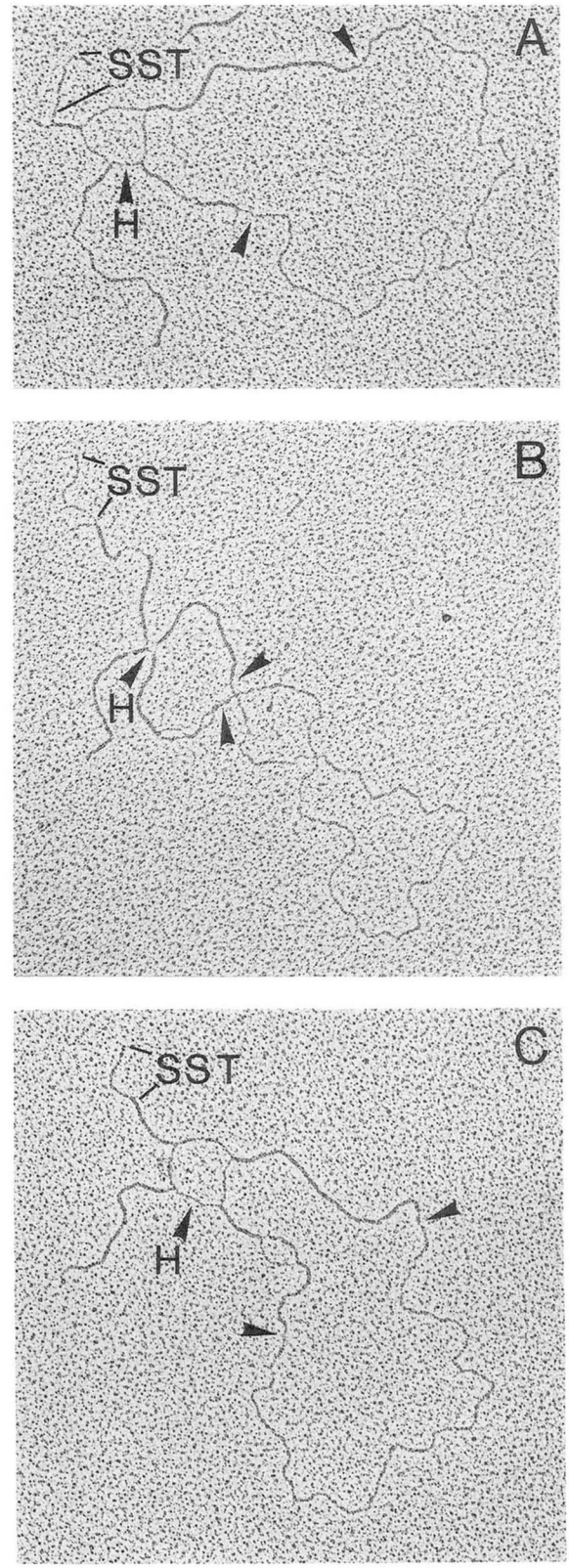


FIG. 7. **Holliday structures generated during the four-strand exchange reaction of Fig. 6.** A four-strand exchange reaction was carried out as described in the legend to Fig. 6. Aliquots were taken from the reaction at 40 min (Fig. 6, *lane 9*), cross-linked, deproteinized, denatured, and spread as described under "Materials and Methods." Three representative Holliday structures are shown. In each case the Holliday junction (*H*), single-stranded tail (*SST*) on one arm, and the ends of the duplex DNA in the circle (*arrows*) are indicated. In molecules *A* and *C,* all four strands are clearly visible at the denatured junction.

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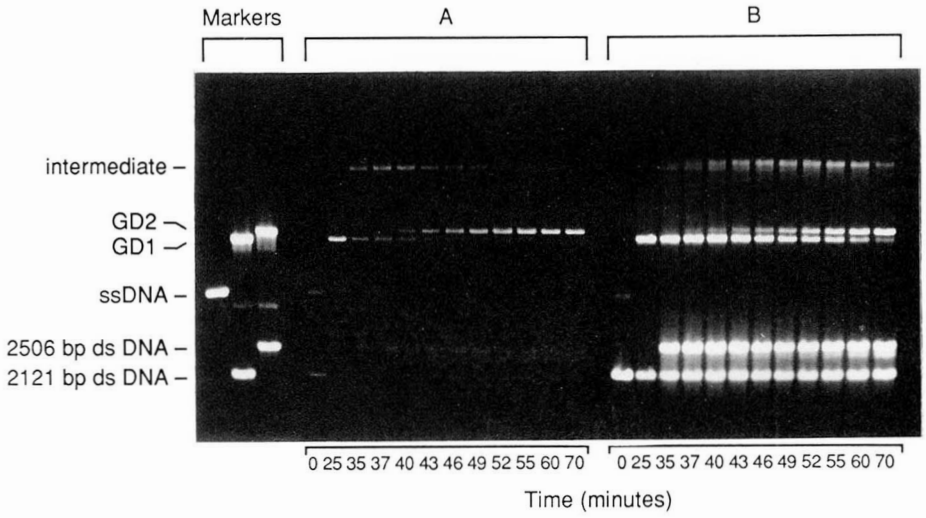


FIG. 8. **Comparing the rates of four-strand exchange reactions with 7-deazaguanine-substituted DNA vs. unmodified DNA.** Markers: *lane 1*, circular M13mp8 ssDNA; *lane 2*, gapped duplex DNA (GD1) and the 2,121-bp linear fragment; *lane 3*, gapped duplex DNA (GD2) and the 2,506-bp linear duplex DNA fragment. The additional band in each lane represents a small amount of linear M13mp8 ssDNA present in most circular ssDNA preparations. Reaction A is the reaction in Fig. 6 (*lanes 6–11*) with additional timepoints added as indicated. Reaction B is the same reaction, with unmodified duplex DNA substrates. Reaction conditions and reagent concentrations are described under "Materials and Methods" and in the legend to Fig. 6. Total reaction volume in each case was 140 μl.

These duplex DNA fragments were reacted with unmodified M13mp8 circular ssDNA or gapped duplex DNA in which the circular strand is unmodified in the three- and four-stranded reactions depicted in Fig. 3. The use of the unmodified circular strand was necessitated by the lack of methods to generate substituted circular single strands. Therefore, two strands in a three-strand exchange and three of the strands in a four-strand exchange are modified. The three-strand exchange reaction generates a gapped duplex product, in which the short strand is modified.

The reactions were monitored with the agarose gel assay of Cox and Lehman (1981a). Since 7-deazaguanine-substituted DNA had not been used previously in strand exchange reactions, the substrates, intermediates, and products were also identified and extensively analyzed by electron microscopy.

The concentrations of the analogue-containing dsDNA fragments were slightly different than the concentrations of the unmodified fragments used in the controls. The concentrations used reflect a characterization of the substituted DNA that initially indicated that its UV absorption properties were the same as for the unmodified DNA. The reported concentrations reflect a more elaborate characterization (described under "Materials and Methods") carried out after most of the experiments were complete.

In these experiments it is important to keep in mind that the 7-deazaguanine-containing duplex DNA does not stain well with ethidium bromide. This can give the erroneous impression that the substituted substrate DNA is present at much lower concentrations than the unmodified DNA. The problem appears to be less pronounced with hybrid DNAs containing only one modified strand; the duplex product of this strand exchange reaction binds ethidium bromide much better than the completely substituted duplex fragment used as substrate.

Three-strand Exchange Reactions—Results with both substituted and unsubstituted duplex DNA substrates are presented in Fig. 4. This experiment included 20 μM ssDNA, 20 μM unmodified dsDNA or 17.2 μM 7-deazaguanine-substituted dsDNA, 6.7 μM recA protein, and 2.0 μM SSB in a final reaction volume of 60 μl. Since the M13mp8 ssDNA is 3.4 times longer than the 2,121-bp duplex fragment, the fragments are present in 1.7-fold excess (1.5-fold in the case of the substituted DNA) relative to the ssDNA in terms of molecules. The ATP regenerating system (see "Materials and Methods") was sufficient to prevent accumulation of ADP for at least 60 min.

As shown in Fig. 4 (*lanes 4–8*), the unmodified duplex fragments react efficiently with the ssDNA to produce a species that migrates at the position expected for the gapped duplex product. Somewhat unexpectedly, the reaction with 7-deazaguanine-substituted duplex DNA proceeded just as efficiently (Fig. 4, *lanes 9–13*).

To confirm that the strand exchange reaction with the substituted DNA yielded the expected product, the product band was excised from the gel and the DNA was examined by electron microscopy. Of 81 molecules examined at random, 52 were gapped duplexes with the structure shown in Fig. 5.

We wanted to eliminate the possibility that these products reflected the generation of a novel three-stranded structure (since the substituted DNA does not stain well with ethidium bromide, we could not observe the displaced linear ssDNA fragment in these gels). Since three-stranded DNA has been reported to have an appearance indistinguishable from duplex DNA in the electron microscope, some of these samples were cross-linked with AMT (as in Umlauf *et al.*, 1990), denatured, and examined. These experiments confirmed that the product had only two strands (data not shown). In seven trials of the experiment in Fig. 4, reactions with the 7-deazaguanine-containing DNA consistently proceeded with rates and extents similar to or exceeding those of the reactions with unmodified DNA.

Four-strand Exchange Reactions—This reaction requires a gapped duplex substrate and a second linear duplex with a homologous end overlapping the ssDNA gap at the end where the reaction is initiated. Because only limited amounts of substituted DNA could be generated by PCR, we did not construct gapped duplex DNA in which one strand was modified directly (the methods used to generate gapped duplexes usually proceed with low yields as described in Lindsley and Cox, 1989). To examine a four-strand reaction in which three of four strands contained 7-deazaguanine, we therefore adapted a two stage reaction strategy. In the first stage, a three-strand exchange is carried out as in Fig. 4. The gapped duplex product of this reaction, with a 2,121-bp hybrid duplex region, is here called GD1. Under the reaction conditions employed here, recA protein remains bound to this heteroduplex product, and the displaced linear ssDNA is bound by SSB (Cox *et al.*, 1990; Roca and Cox, 1990).[3] This product is therefore bound in a nucleoprotein filament that represents the required substrate for a four-strand exchange. A 7-deazaguanine-containing 2,506-bp fragment was generated by

[3] C. Ullsperger and M. Cox, unpublished results.

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PCR (see "Materials and Methods") to provide the required second substrate for the reaction illustrated in Fig. 6. After the gapped duplex GD1 was produced in the first stage, the 2,506-bp fragment was added to initiate a four-strand reaction as the second stage. One of the products of the second stage is a gapped duplex with a 2,506-bp paired region, designated GD2.

In these experiments, the concentration of ssDNA, recA protein, and SSB used in the first stage were reduced by half relative to the experiments in Figs. 4 and 5. The resulting 3.4-fold excess of duplex fragments (3.0-fold for the substituted DNA) ensures that virtually all of the ssDNA is converted to product. This minimizes the amounts of GD2 that can be produced in the second stage via a three-strand reaction involving the 2,506-bp linear duplex and any unreacted ssDNA. The amounts of recA protein are just sufficient to saturate the ssDNA or the GD1 product of stage 1, minimizing the possibility of aberrant reactions in stage 2 involving the linear displaced ssDNA fragment from stage 1. Stage 1 was carried out for 30 min, at which time stage 2 was initiated by the addition of the 2,506-bp gapped duplex DNA (17.2 μM for the 7-deazaguanine-substituted DNA and 20 μM for the unmodified duplex fragment). The ATP regenerating system in this experiment was sufficient to prevent ADP accumulation for 60 min.

Results of the four-strand exchange reaction with the 7-deazaguanine-substituted DNA are shown in Fig. 6 (*lanes 6–11*). Stage 1 is shown in *lanes 6–8*, yielding GD1 as expected. Stage 2 is shown in *lanes 9–11*. An efficient four-strand exchange reaction is seen, with production of a somewhat larger gapped duplex (GD2) while the band corresponding to GD1 disappears. In a control experiment (*lanes 12–17*) an identical reaction is shown without initiation of stage 2. This control confirms that GD1 is stable over the entire timecourse if the 2,506-bp fragment is not added.

The occurrence of a four-strand exchange is indicated by the disappearance of GD1 coincident with the appearance of GD2. The stage 2 reaction is complete within 20 min (the 50-min timepoint). Additional confirmation was obtained by electron microscopy. Aliquots were removed at 40 min, where the agarose gel indicates that substrates, products, and an apparent intermediate band were all present (Fig. 6, *lane 9*). The entire reaction mixture was cross-linked with AMT, deproteinized, dialyzed, and spread for electron microscopy. Of 479 molecules containing duplex DNA examined at random, 74% were linear duplex fragments (note that the linear duplex fragments are present in excess), 10% were gapped duplexes, 14% were Holliday junction intermediates, and 2% were tailed circles. The latter structures appeared to be molecules in which a four-strand exchange was being initiated. The presence of the Holliday junction confirms that a normal four-strand exchange is taking place. To be certain that the Holliday junctions did not represent the accidental juxtaposition of gapped duplexes and linear fragments on the grid, some of these samples were cross-linked with AMT and denatured. Typical Holliday junctions observed in this experiment are shown in Fig. 7. In 78% of the molecules where the junction had been denatured sufficiently to trace strands, all four strands were present and unbroken. In addition, in most of these molecules one of the linear duplex arms had a discernible ssDNA tail about 300–400 nucleotides in length, as expected (see Fig. 6).

Interestingly, the four-strand reaction with the 7-deazaguanine-substituted DNA appeared to be more efficient than the same reaction with unmodified DNA. The two reactions are compared with an expanded timecourse in Fig. 8. Although precise comparisons are difficult because of small differences in dsDNA concentrations and differences in ethidium bromide staining, the product GD2 appeared at significantly earlier timepoints and the reaction reached completion earlier in all of three trials of this experiment. Note that the problems that preclude exact comparisons (there is somewhat less of the modified dsDNA (fragment and it is harder to detect) would tend to lead to a relative underestimation of the rate and efficiency of the reaction with the 7-deazaguanine-substituted DNA. This supports the idea that the rate enhancement observed for this modified DNA is real.

DISCUSSION

Our primary conclusion is that interstrand hydrogen bonds involving guanine *N*-7 are not required for recA protein-mediated strand exchange. In reactions involving both three and four strands, we noted no inhibitory effects when normal duplex DNA was replaced by DNA in which all guanine residues were replaced by 7-deazaguanine. In fact, the substituted DNA exhibited an apparent rate advantage in many of the experiments. Rao and Radding[4] have recently found that methylation at guanine *N*-7 in duplex DNA does not interfere with the formation of short triplex DNA structures made by recA protein from single-stranded DNA and a hairpin duplex oligonucleotide, a result that reinforces our conclusion.

Inasmuch as hydrogen bonds to guanine *N*-7 are featured in every published model for three- and four-stranded DNAs, this result has important implications with respect to the nature of the putative pairing intermediate in recA protein-mediated strand exchange reactions. There are at least three possible interpretations of this result. First, the three-stranded intermediate may not feature hydrogen bonds to *N*-7. If this is the case, the intermediate is not adequately described by any of the models published to date for multiplex DNA structures. The second possibility is that the three-stranded structure does utilize hydrogen bonds to guanine *N*-7, but is sufficiently stable in their absence to permit strand exchange. We cannot, as yet, evaluate this possibility, although much of the homologous interaction between DNAs in most models clearly depends upon hydrogen bonding at this position. The last, and most obvious possibility is that the three-stranded intermediate either doesn't exist or is not an obligate intermediate on the reaction pathway. One alternative intermediate is one in which the strand switch to create heteroduplex is complete. This "intermediate" would resemble products, although the displaced strand might still be interwound with the heteroduplex transiently (as suggested in Umlauf *et al.*, 1990; Roca and Cox, 1990). A more stable three-stranded structure, such as that observed by Hsieh *et al.* (1990) and Rao *et al.* (1991) might form spontaneously from this structure under some conditions.

Hydrogen bonding to guanine *N*-7 may be critical to DNA-DNA interactions in the McGavin-Wilson, 1979). In the absence of a plausible alternative structure for a four-stranded DNA recombination intermediate, the results described in Figs. 6–8 for the four-strand reaction tend to argue against a critical role for a four-strand pairing intermediate. Further work is clearly required to determine the role, if any, of the weak four-stranded pairing interaction recently observed in several laboratories (Conley and West, 1989, 1990; Lindsley and Cox, 1990; Chiu *et al.*, 1990). Since this four-stranded interaction involves only a small fraction of the available DNA and is observed only when DNA2 is supercoiled, the possibility exists that it represents an interaction between the

[4] B. J. Rao and C. M. Radding, personal communication.

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duplex DNA in the filament and one strand of DNA2 derived from transient supercoil-induced unwinding of DNA over short regions. In other words, the putative four-strand interaction might involve only three of the four strands present.

The approach described here appears to hold promise for examining the structure of unusual DNA structures generated in the course of genetic recombination. It should also be useful in exploring the structure of H-DNA and other known multistranded DNAs.

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