Interactions of Three Strands in Joints Made by RecA Protein†

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ABSTRACT: RecA protein from *Escherichia coli* has been used to form a triple-stranded DNA structure from either single-stranded M13 DNA or a single-stranded oligonucleotide plus a duplex oligonucleotide with a hairpin loop. The secondary structure of purified deproteinized triplex was examined by probing with DNase I, P1 nuclease, potassium permanganate, and diethyl pyrocarbonate. The two strands destined to form heteroduplex DNA showed the same patterns of chemical modification and enzymatic digestion as control duplex DNA, indicating that they formed a normal duplex substructure. However, the nascent outgoing strand showed properties consistent with a novel triplex structure: most of its purine residues, especially adenines, were hyperreactive to all probes. The patterns of digestion by DNase I and P1 nuclease indicated that the nascent outgoing strand was not a freely mobile or single-stranded branch but rather was still interacting with the newly formed heteroduplex DNA. On the basis of the planar base triads proposed previously (Rao et al., 1993) and energy minimization of a third strand in the major groove of B-form DNA, we derived a model that helps to rationalize the properties revealed by chemical and enzymatic probing.

Escherichia coli RecA protein is the prototype of a widespread class of recombination proteins (Bishop et al., 1992; Ogawa et al., 1993; Roca & Cox, 1990; Shinohara et al., 1992, 1993; Story et al., 1993). By polymerizing on single-stranded DNA in the presence of ATP, RecA protein extends the single strand and forms a right-handed nucleoprotein filament that subsequently recognizes and incorporates long stretches of homologous duplex DNA. Within this helical triple-stranded complex, further interactions, fueled ultimately by the hydrolysis of ATP, lead to an exchange of base pairs, formation of a new heteroduplex molecule, and complete separation of one strand of the original duplex molecule (Kowalczykowski, 1991; Radding, 1988, 1991; Roca & Cox, 1990; West, 1992). Howard-Flanders et al. (1984) first proposed that triple-stranded DNA is the central intermediate in the kind of homologous recombination just described. Evidence of three-stranded regions within the nucleoprotein filament was provided by electron microscopic studies (Bortner & Griffith, 1990; Register et al., 1987; Stasiak et al., 1984, 1991; Umlauf et al., 1990). Biochemical observations indicated that a three-stranded form of DNA is also present in some deproteinized intermediates. RecA nucleoprotein intermediates that involved several kilobases of homologous sequences yielded stable deproteinized products whose relative resistance to digestion by nucleases indicated the existence of triplex DNA (Rao et al., 1990). The intermediates that yielded such products include joints found late in the course of strand exchange between fully homologous molecules, joints formed when the completion of strand exchange was blocked by a downstream heterologous region (proximal joints), and joints formed when the initiation of strand exchange was blocked by an upstream heterologous region (distal joints;[1] Rao et al., 1990, 1991). Moreover, intermediates that were deproteinized and isolated from a RecA reaction contained a preferential binding site for RecA protein from which strand exchange could be promptly restarted by fresh protein (Rao et al., 1990). Hsieh et al. (1990) observed thermostable products when they deproteinized RecA joints involving only 56 nucleotide residues of homology at the distal[1] end of linear duplex DNA.

The existence of stable triplex DNA in nonuniform sequences is surprising, however, in view of extensive studies on the spontaneous formation of triplex DNA by oligonucleotides, which requires runs of homopurine and homopyrimidine sequences (Conney et al., 1988; Moser & Dervan, 1987; Praseuth et al., 1988). Indeed, when RecA protein forms triplex joints from single-stranded oligonucleotides and duplex DNA, the joint does not survive deproteinization unless the duplex DNA is superhelical (Ferrin & Camerini-Otero, 1991; B. Jwang and C. M. Radding, unpublished observations). In order to explore the homologous interactions of three strands that are promoted by RecA protein, we have studied the stable product that results upon deproteinization of joints made when the duplex substrate is a hairpin oligonucleotide (Rao et al., 1993). In the following, we report further observations made by chemical and enzymatic probing of this three-stranded product. These observations support the view that homologous recognition can occur via a three-stranded intermediate.

MATERIALS AND METHODS

Enzymes and Chemicals. RecA protein was purified from *E. coli* as described by Shibata et al. (1981). Single-stranded DNA binding protein (SSB)[2] was purified as described by Lohman et al. (1986). T4 polynucleotide kinase was purchased from New England Biolabs. P1 nuclease from *Penicillium*

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[1] RecA protein promotes strand exchange in a directional fashion. The complementary strand from linear duplex DNA is progressively paired in the 3′ to 5′ direction with a circular plus strand within the RecA filament. This directionality defines the *proximal* and *distal* ends of the duplex DNA. Accordingly, when homology is limited to the proximal end of a linear duplex substrate, we term the joints *proximal joints*, and when homology is limited to the distal end, we term the joints *distal joints*. In proximal joints, RecA protein initiates strand exchange and propagates the exchange up to the barrier presented to the heterologous distal end of the molecule; RecA protein cannot initiate strand exchange in distal joints, but rather maintains these joints at steady state by a continuous cycle of association and dissociation (Radding, 1991; Wu et al., 1984; Burnett et al., submitted for publication).

[2] Abbreviations: DEPC, diethyl pyrocarbonate; SSB, *E. coli* single-stranded DNA binding protein.

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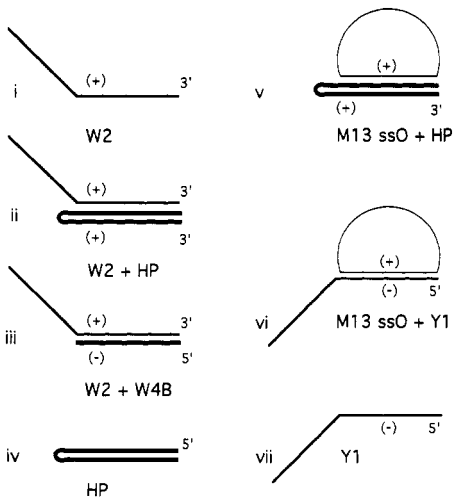

FIGURE 1: Substrates. W2 and Y1 are 70-mer oligonucleotides; HP is a self-complementary 70-mer hairpin oligonucleotide. M13 ssO stands for circular single-stranded M13 DNA. Homology is defined in relation to some segment of the sequence in phage M13 viral or plus strand. A complementary sequence is designated as complementary or (–); an identical sequence is designated as anticomplementary or (+). The complementary arm of the hairpin oligonucleotide used in these experiments had a 5′ end. In chemical or enzymatic probing experiments in which the hairpin oligonucleotide (HP) was labeled at its 5′ end, cleavage in the complementary arm produced fragments that migrated toward the bottom of gels during electrophoresis, whereas cleavage in the anticomplementary arm produced fragments that migrated nearer to the top of the gel.

citrinum was purchased from United States Biochemicals. Proteinase K and DNase (RNase-free from bovine pancreas) were from Boehringer Mannheim. Dimethyl sulfate, diethyl pyrocarbonate, potassium permanganate, and piperidine were bought from Aldrich Chemical Co.

DNA Substrates. M13 circular single-stranded DNA was prepared as described (Cunningham et al., 1980). The amount of contaminating linear single-stranded DNA was less than 5% as judged by gel electrophoresis. Oligonucleotides were synthesized by an Applied Biosystems DNA synthesizer (Model 380B) at the Keck Biotechnology Resource Laboratory at Yale.

All oligonucleotides were 5′-labeled by T4 polynucleotide kinase which was then heat-inactivated at 70 °C for 10 min, according to the procedure recommended by New England Biolabs. Labeled oligonucleotides were then separated from unincorporated label by centrifugation through Chroma Spin-30 columns (from Clontech Laboratory, Inc.).

The nomenclature and sequences of oligonucleotides and joint molecules were as follows (see Figure 1 for schematic diagrams):

W2 (Figure 1, substrate i) consisted of 33 nucleotides at the 3′ end (underlined) which were identical to residues 207–239 of M13 phage DNA (van Wezenbeek et al., 1980). The 37 residues at the 5′ end constituted a random sequence:

5′-GTA CAG ACG ATC AGA TCC GAC GTT GCT TAA TTG AGC

TAC AGC ACC AGA TTC AGC AAT TAA GCT CTA AGC C-3′

HP (Figure 1, substrate iv) was a 70-residue, self-complementary, hairpin oligonucleotide whose sequence corresponded to the 33 residues at the 3′ end of W2 (above). The loop region was made up of four cytosine residues. The 5′ end of this oligonucleotide was complementary to the sequence in circular single-stranded M13 DNA and therefore is termed the *minus strand*; the 3′ end is correspondingly termed the *plus* or *anticomplementary strand*:

5′-GGC TTA GAG CTT AAT TGC TGA ATC TGG TGC TGT CC

3′-CCG AAT CTC GAA TTA ACG ACT TAG ACC ACG ACA CC

W4B (see Figure 1, substrate iii) was a 28-nucleotide sequence that was complementary to the 3′ end of W2:

5′-GGC TTA GAG CTT AAT TGC TGA ATC TGG T-3′

Y1 (Figure 1, substrate vii) was a 70-mer single-stranded oligonucleotide. The 33 nucleotides at the 5′ end were complementary to nucleotides 6390–0015 in the sequence of single-stranded M13 DNA (van Wezenbeek et al., 1980). The 5′ sequence was linked *via* 4 cytosine residues to a sequence of 33 nucleotides at the 3′ end that had the same sequence as the anticomplementary arm of HP (see above):

5′-AAT AGT AGT AGC GTT AAC ATC CAA TAA ATC ATA CCC

CAC AGC ACC AGA TTC AGC AAT TAA GCT CTA AGC C-3′

Substrate vi was made by heating 383 μM circular single-stranded M13 DNA with 8 μM Y1 oligonucleotide in the presence of 50 mM sodium chloride at 90 °C for 2 min and then at 65 °C for 30 min, followed by cooling to room temperature. Substrate vi was also formed by RecA protein: 20 μM circular single-stranded M13 DNA was incubated with 0.84 μM Y1 oligonucleotide in the presence of 6.7 μM RecA protein for 15 min. It was then deproteinized and purified in the same way as the triplex substrate described below.

Standard Reaction Conditions for Formation of Triplex Intermediates. (a) Formation of Triplex with Circular Single-Stranded DNA. Thirty micromolar circular single-stranded DNA was incubated with 10 μM RecA protein and 2.4 μM single-stranded DNA binding protein at 37 °C for 10 min, in a reaction mixture containing 33 mM Tris·HCl (pH 7.5), 12 mM magnesium chloride, 2 mM dithiothreitol, 1.2 mM ATP, 8 mM phosphocreatine, 10 units/mL creatine phosphokinase, and 100 μg/mL bovine serum albumin. Triplex formation was started by adding 1.2 μM HP, followed by incubation for 20 min at 37 °C. The reaction was stopped by addition of SDS to 0.5% and proteinase K to 100 μg/mL, followed by incubation at 37 °C for 10 min. Triplex formation was scored by assay D (Rao et al., 1990).

The deproteinized mixture was passed through a Sepharose 2B column to separate the triplex from unreacted hairpin duplex. The bed volume of the column was around 7 mL for a sample volume of 300 μL. Fractions of 4 drops were collected; the void volume fractions were scanned for radioactivity by Cerenkov counting, and they were then pooled for later experiments. The isolated triplexes were used on the day of preparation, no longer than 4 h after gel filtration.

(b) Formation of Triplex with a Single-Stranded Oligonucleotide. In a 200-μL reaction, 1 μM single-stranded oligonucleotide (W2) was incubated in the standard reaction mixture described above, with the following changes: SSB was absent, the concentration of magnesium was 1 mM instead of 12 mM, and RecA protein was incubated with oligonucleotide for 12 min. Hairpin oligonucleotide (HP) was then added, and the concentration of magnesium was raised to 20 mM, or other concentrations as indicated in particular experiments. Incubation was continued for 15 min after which the reaction was stopped and the products were deproteinized as described above.

The deproteinized triplex was then isolated by electrophoresis at 4 °C in an 8% native polyacrylamide gel that had been pre-run at 20 V/cm for 30 min. The buffer (TBE) was 90 mM Tris, pH 8.3, 90 mM borate, and 2 mM EDTA. Following electrophoresis at 15 V/cm until the bromophenol blue was three-fourths from the bottom of the gel, X-ray film was exposed to the gel for 10 min to detect the triplex product. The part of the gel corresponding to the position of triplex was excised and electroeluted at 4 °C in TBE buffer. The sample was concentrated to 100 μL in a Centricon 10 column (Amicon).

CHEMICAL MODIFICATION AND ENZYMATIC REACTIONS

Substrates for chemical probing included (1) triplex DNA with ^{32}P label at the 5′ end of the single-stranded oligonucleotide or at the 5′ end of the hairpin oligonucleotide, (2) 5′-labeled single-stranded oligonucleotide, and (3) 5′-labeled hairpin oligonucleotide. Approximately 2 pmol (in nucleotide) of substrate was mixed with a chemical DNA modifier in a buffer of 40 mM Tris·HCl (pH 7.5), 1 mM $MgCl_2$, and 50 μg of tRNA/mL. In the case of reactions involving M13 single-stranded DNA and its triplex, 1 nmol (in nucleotide) of single-stranded DNA was present. The final volume was 100–250 μL.

$KMnO_4$. This reagent was added at 8 μg/mL to a reaction mixture of 100 μL and incubated at 25 °C for 10 min. The reactions were then stopped by adding 1 μL of β-mercaptoethanol, 5 μg of tRNA, 0.3 M sodium acetate (pH 7.5), and 2 volumes of absolute ethanol followed by incubation at −20 °C for 30 min. The DNA was recovered by precipitation with 70% ethanol, washed twice with ethanol, and redissolved for analysis by gel electrophoresis as described below. Since the sequences of the oligonucleotides are known, the patterns of cleavage at specific nucleotide residues could be inferred in several cases (see Figures 4 and 6, for example).

Osmium tetroxide (OsO_4) at 22 mM, together with 2 μL of pyridine, was added to the reaction mixture held at 25 °C for 2 min. The reaction was stopped by adding 20 μg of tRNA, 0.3 M sodium acetate (pH 7.5), and 2 volumes of absolute ethanol. The DNA was recovered as described above.

Diethyl Pyrocarbonate (DEPC). DNA was incubated with 2% DEPC at 25 °C for 5 min. The reaction was stopped, and the DNA was recovered as described above.

Dimethyl sulfate (DMS) at 0.5% was incubated with DNA in 50 mM cacodylate buffer (pH 7.5) and 4 mM $MgCl_2$ at 25 °C for 2 min. The reactions were stopped, and the DNA was recovered as described above.

Those samples that had been modified by $KMnO_4$, DEPC, or OsO_4 were then heated with 10% piperidine at 90 °C for 25 min. Piperidine was removed completely by lyophilization, which was repeated twice, each time after the addition of 40 μL of water. All sequencing ladders were produced according to Maxam and Gilbert (1980).

P1 Nuclease. The reaction conditions were the same as in the chemical reactions described above. After the addition of 10^{-2} unit of enzyme in 100 μL, each reaction mixture was incubated at 37 °C for 2 min. (One unit of enzyme, as defined by the supplier, was the amount of enzyme required to catalyze the hydrolysis of 1 μmol of 3′-AMP per minute at 37 °C, pH 7.2.) The reaction was stopped by the addition of 20 μg of calf thymus DNA; the DNA was recovered by ethanol precipitation and washed twice with 70% ethanol.

DNase I. Reactions (100 μL) were carried out at 37 °C for 2 min, at either pH 7.5 or pH 5.0; 0.08 unit of DNase was used at pH 7.5, and 0.2 unit was used at pH 5 (1 unit is defined as the amount of enzyme that effects an absorbance increase of 0.001/min under the assay conditions specified by the supplier). The amounts of enzyme required had been determined by a series of titration experiments.

All DNA samples were resuspended in formamide loading buffer containing 90% formamide, 0.05% bromophenol blue, and 0.05% xylene cyanol (Sambrook et al., 1989). Equal numbers of radioactive counts were loaded on a 300 × 400 × 0.4 mm sequencing gel containing 8 M urea and 40% formamide (ultrapure grade from American Bioanalytical), which had been prerun until the temperature of the gel was 50 °C. After electrophoresis, the gel was fixed in a solution of 10% methanol/10% acetic acid and dried on a Whatman no. 1 filter. X-ray film was exposed to the gel at −80 °C with a Cronex Lightning-Plus intensifying screen (Dupont). The relative intensity of bands was measured by a PhosphoImager scanner (Molecular Dynamics).

Generation of a Computer Model of the Protein-Free Triplex. A B-DNA model of our hairpin oligonucleotide was built using the program DNA-FIT (written by J. Warwicker, Yale University). Starting from the base sequence of the hairpin oligonucleotide, we generated the B-form DNA model and a second copy of the plus strand. Individual nucleotides of the plus strand were then rotated in the plane of the original base pair until the proposed hydrogen-bonding scheme (Rao et al., 1993) was satisfied (see Discussion). As far as possible, the proposed hydrogen bond distances were kept close to 2.7 Å; however, in the cases of G-GC and T-AT triads, the distances were approximately 3.2–3.3 Å. Once these constraints were applied, the distance between two consecutive sugar–phosphate units in the third strand was too long to link the nucleotide residues of the third strand: the distance between O3′ and C3′ was about 2–5 Å, depending on the nucleotide sequence. To link the nucleotides of the third strand, the triplex model was energy-minimized using X-PLOR (Brünger, 1990) while keeping the original duplex and the bases in the third strand fixed.

RESULTS

Characterization of the Formation of Triplex Oligonucleotides. We have previously described the pairing of a hairpin oligonucleotide with circular single-stranded M13 DNA (Rao et al., 1993). In order to study all three strands in the triple-stranded intermediate, we replaced the circular single-stranded DNA in some experiments by a single-stranded oligonucleotide, which can be analyzed more readily than the larger single strand. We started with a 70-nucleotide-long oligonucleotide whose 3′ half was homologous to a hairpin oligonucleotide (see Figure 1, substrates i, ii, and iii).

The single-stranded oligonucleotide was first incubated with RecA protein in the presence of 1 mM $MgCl_2$ to form a nucleoprotein filament, following which the magnesium ion concentration was raised to 20 mM, and duplex oligonucleotide was added. After incubation, the reaction mixture was deproteinized by SDS and proteinase K before being loaded onto a native polyacrylamide gel to separate triple-stranded intermediate from unreacted substrates. A more slowly migrating band (indicated by the open arrowhead in Figure 2) was labeled when either the hairpin oligonucleotide or the single-stranded oligonucleotide was radioactive. The material isolated from this band by electroelution had a melting transition near 60 °C, as determined by heating samples of the isolated material and then analyzing them again by gel electrophoresis (data not shown); this melting transition was

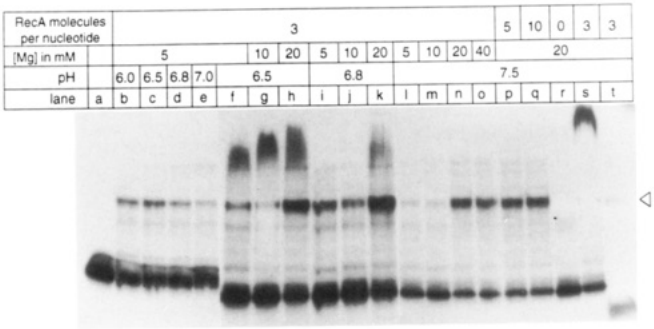

FIGURE 2: Optimal conditions for formation of triplex by RecA protein. The substrates used in this set of reactions were the single-stranded oligonucleotide W2 (Figure 1, i) and the hairpin oligonucleotide HP (Figure 1, iv). The reaction conditions were those described under Materials and Methods except for those variations indicated at the top of the figure. Labeled single-stranded oligonucleotide was incubated at the pH indicated with different amounts of RecA protein, following which the concentration of Mg^{2+} in the final reaction was raised to that indicated. Lane a, marker, single-stranded oligonucleotide W2 alone. Lane s, the reaction condition was the same as lane n except that proteinase K was not added. Lane t was a reaction with the hairpin DNA labeled at its 5′ end rather than the single-stranded DNA. The specific activity of labeling of HP was much less than W2; therefore, the bands were less intense than the others. The autoradiogram is a composite of three sets of experiments. In lanes f–h and k, the top band (above the putative triplex band marked by an arrowhead) corresponds to incompletely deproteinized product as indicated by a similar band that was present when proteinase K was omitted (lane s).

similar to that observed previously for the triplex formed by circular single-stranded DNA and the hairpin oligonucleotide (Rao et al., 1993).

Titration with RecA protein showed that the minimal amount of RecA protein required was three molecules per residue of single-stranded oligonucleotide (data not shown), which is greater than the stoichiometric requirement when long single strands are used, presumably because RecA protein does not bind tightly to short oligonucleotides (Leahy & Radding, 1986) (Figure 2, lanes n, p, q, and r). The formation of the triple-stranded intermediate required RecA protein (Figure 2, lane r). When proteinase K was omitted, a more slowly migrating band was seen that appeared to represent incompletely deproteinized triplex (Figure 2, lane s, and see figure legend). In the presence of 5 mM $MgCl_2$, the optimal pH for formation of the triple-stranded intermediate was around 6.5 (compare lanes b–e), whereas at 20 mM $MgCl_2$, the optimal pH was closer to neutrality and the yield was higher (lanes h, k, and n). An increase in the concentration of ATP beyond 1.2 mM did not affect the yield of joints. Spermidine at 2 mM could replace 20 mM $MgCl_2$ and produced the same amount of joints (data not shown).

Thus, in all subsequent experiments, we formed the triple-stranded intermediate by using three molecules of RecA protein per nucleotide residue of single-stranded oligonucleotide, and the reactions were carried out at pH 7.5 with 20 mM $MgCl_2$. The strong band (open arrowhead in Figure 2), which appeared only in the presence of RecA protein, was excised and electroeluted from the native polyacrylamide gel for use in chemical and enzymatic probing experiments.

Digestion of the Triple-Stranded Intermediate by DNase I. The triple-stranded intermediate was labeled either at the 5′ end of the single-stranded oligonucleotide or at the 5′ end of the duplex oligonucleotide. After digestion by DNase I, the product was precipitated by ethanol and loaded onto a denaturing polyacrylamide gel containing urea and formamide (see Materials and Methods).

In control experiments, DNase I digested both arms of the free duplex hairpin oligonucleotide, except the loop region which is composed of four cytosine residues (Figure 3A, lane F), but only poorly digested the single-stranded oligonucleotide (lane A). These control observations are consistent with the specificity of DNase I, which efficiently cleaves only B-form DNA (Drew, 1984). When triplex oligonucleotide was made by incorporating a hairpin oligonucleotide whose 5′ end was labeled, the digestion pattern shown in lane E was observed. The products in the lower part of the gel result from cleavage in the arm of the hairpin that is complementary to the single-stranded oligonucleotide W2 (see Figure 1, ii). The digestion pattern in that part of the gel was the same as that of the duplex hairpin control (lane F). However, the anticomplementary strand, represented by the bands at the top of the gel, showed a digestion pattern that appeared to differ at several sites from the duplex control: The ratio of intensities of several bands within lane E differed from the ratios of corresponding bands in lane F. Similar differences were seen in several repetitions of this experiment, as well as in the sensitivity of triplexes formed by a hairpin oligonucleotide and single-stranded M13 DNA (Rao et al., 1993).

In the triplex, the digestion pattern of the 3′ half of W2 (upper portion of lane B) appeared to be the same as that of the 3′ end of the corresponding annealed duplex control (Figure 3, lane D;[3] cf. Figure 1, iii) and that of the control hairpin DNA (upper half of lane F). The 5′-overhanging half of the single-stranded oligonucleotide in the triplex (Figure 3, lower half of lane B; cf. Figure 1, ii) and in the partially duplex control (Figure 3, lower half of lane D; cf. Figure 1, iii) showed, as expected, the same lack of digestion as the fully single-stranded control (Figure 3, lane A).

Thus, probing of the triplex with DNase I revealed that the 3′ end of the plus strand oligonucleotide W2 and the complement that was contributed by the hairpin duplex were in a duplex configuration. However, the anticomplementary strand contributed by the hairpin was also in a configuration that was sensitive to DNase I, and hence was not single-stranded, but its sensitivity appeared on multiple observations to differ in detail from the sensitivity of the duplex control.

Relative Resistance to P1 Nuclease. P1 nuclease has been shown to cleave single-stranded DNA better than double-stranded DNA (Fujimoto et al., 1974) and has been used to detect regions of unwinding (Bramhill & Kornberg, 1988; Jwang & Radding, 1992). This nuclease apparently can detect not only single-stranded DNA but also partially unwound DNA or DNA with a conformational change (Pulleyblank et al., 1986). When we probed the hairpin oligonucleotide with P1 nuclease, we found, as expected, that it could digest only the loop of the hairpin DNA (Figure 3B, lane E). In the triplex as well, the hairpin was digested mainly in the loop region (Figure 3B, lane D). Although in this case there was also some cleavage of the anticomplementary strand, it was much less than cleavage of the single-stranded loop, suggesting that the anticomplementary strand was not fully single-stranded or was not single-stranded in all molecules. Similar observations were made when mung bean nuclease was used (data not shown). These data are consistent with the observations made on the action of DNase I.

Chemical Probing. In all of the experiments described below, the single-stranded DNA was M13 circular DNA instead of an oligonucleotide, which facilitated the isolation

[3] Some bands are missing from the upper part of lane D because the oligonucleotide W4B is only 28 residues long (see Materials and Methods).

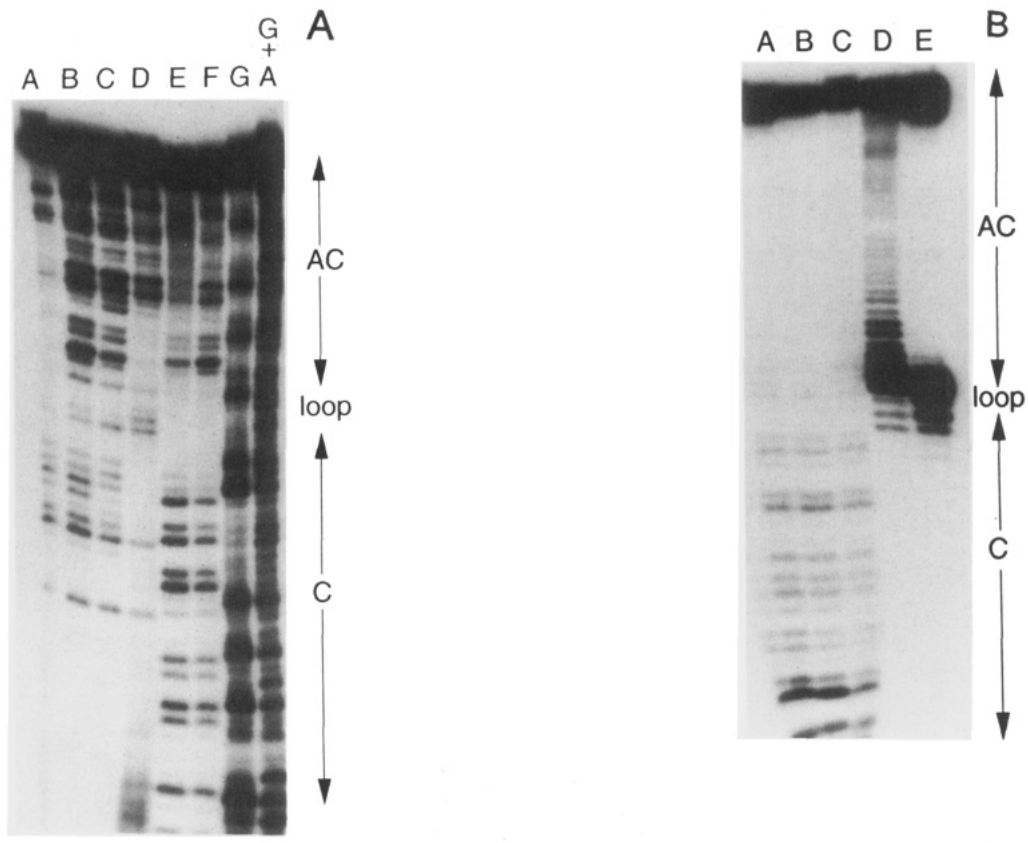

FIGURE 3: (A) Probing of triplex by DNase I: a B-form substructure and an anticomplementary strand in an unusual configuration. The pairing condition was as described under Materials and Methods. The isolated triple-stranded intermediate and other control substrates were treated with 0.08 unit of DNase I. In lanes A–D and the last two lanes, the label was at the 5′ end of W2. In lanes E and F, the label was at the 5′ end of HP. Lane A is the single-stranded oligonucleotide, W2 (Figure 1, i). Lanes B and C, the triple-stranded substrate made from labeled W2 and HP (Figure 1, ii). Lane D, a partially duplex substrate (Figure 1, iii). Lane E, the triple-stranded intermediate (Figure 1, ii) with label at the 5′ end of the hairpin DNA. Lane F, hairpin DNA alone (Figure 1, iv). Lane G, the guanine-specific Maxam–Gilbert sequencing ladder of W2 (see Materials and Methods). Lane G+A, the guanine- and adenine-specific chemical sequencing ladder. To the right of the gel, the designation AC indicates the location of fragments from the anticomplementary strand, and C indicates the location of fragments from cleavage of the complementary strand. "Loop" identifies the single-stranded loop of the hairpin oligonucleotide. (B) Probing of triplex by P1 nuclease: the anticomplementary strand is not fully single-stranded. Conditions of the reaction were as described under Materials and Methods. Lanes A and B, triple-stranded intermediate (Figure 1, ii) with ^{32}P label at the 5′ end of W2. Lane C, partially duplex substrate (Figure 1, iii) with label as in lanes A and B. Lane D, triple-stranded intermediate (Figure 1, ii) with label at the 5′ end of the hairpin DNA. Lane E, hairpin DNA alone, labeled as in lane D.

of the product of the RecA reaction. The patterns of chemical and enzymatic probing were similar for triplex substrates made with a single-stranded oligonucleotide vs circular single-stranded DNA. Additional controls were also made possible by the use of M13 single-stranded DNA (substrates vi and vii in Figure 1).

(*1*) *Potassium Permanganate* (*$KMnO_4$*). $KMnO_4$ has been used to detect whether a particular region is unwound or single-stranded (Glover & Pulleyblank, 1990). Usually permanganate modifies thymine residues in genuine single-stranded DNA (Rubin & Schmid, 1980). We used it to probe triple-stranded joints. In this set of experiments, the single-stranded DNA was M13 circular viral strand (+), and the radioactive label was on the 5′ end of the hairpin DNA. Under standard reaction conditions, $KMnO_4$ modified residues throughout the anticomplementary strand of the hairpin in the triple-stranded joint (Figure 4A, lane C), but surprisingly, the bases modified were mostly purines. Moreover, quantitation of the reactivities of individual purines showed that adenine was usually more strongly modified than guanine (Figure 4B). Although adenine residues were extensively modified in our experiment, there were two thymine residues in the middle of the strand that were also modified by $KMnO_4$, whereas the modification of two other thymine residues was suppressed relative to the single-stranded control (Figure 4B). The single-stranded DNA control was modified at thymine residues, indicating that $KMnO_4$ was behaving as expected under the conditions of our experiment (Figure 4A, lanes G or H, and Figure 4B, lower trace). The duplex hairpin was almost untouched by $KMnO_4$ with the exception of a T residue right next to the loop of 4 C's (Figure 4A, lanes A and B).

These observations rule out the possibility that the target of enzymatic and chemical probing was simply a mixture of single-stranded and double-stranded DNA, in which case the amount of material cleaved by treatment with $KMnO_4$ might have been reduced, whereas the pattern of cleavage should have been normal.

In this set of experiments, a Y-shaped or branched control was included (Figure 1, vi). It was made by annealing circular single-stranded M13 DNA with a single-stranded oligonucleotide whose 5′ moiety was homologous to a different site on the M13 DNA and whose 3′ moiety had the same sequence as the anticomplementary strand of the hairpin oligonucleotide. The modification of the 3′ moiety of the branched structure by $KMnO_4$ was quite different from that of both the single-stranded control and the identical anticomplementary arm of the hairpin oligonucleotide in the triple-standed joint. There was a general depression of modification at neutral pH (Figure 4A, lane E). In general, there were several guanine residues slightly modified in addition to those thymine residues that

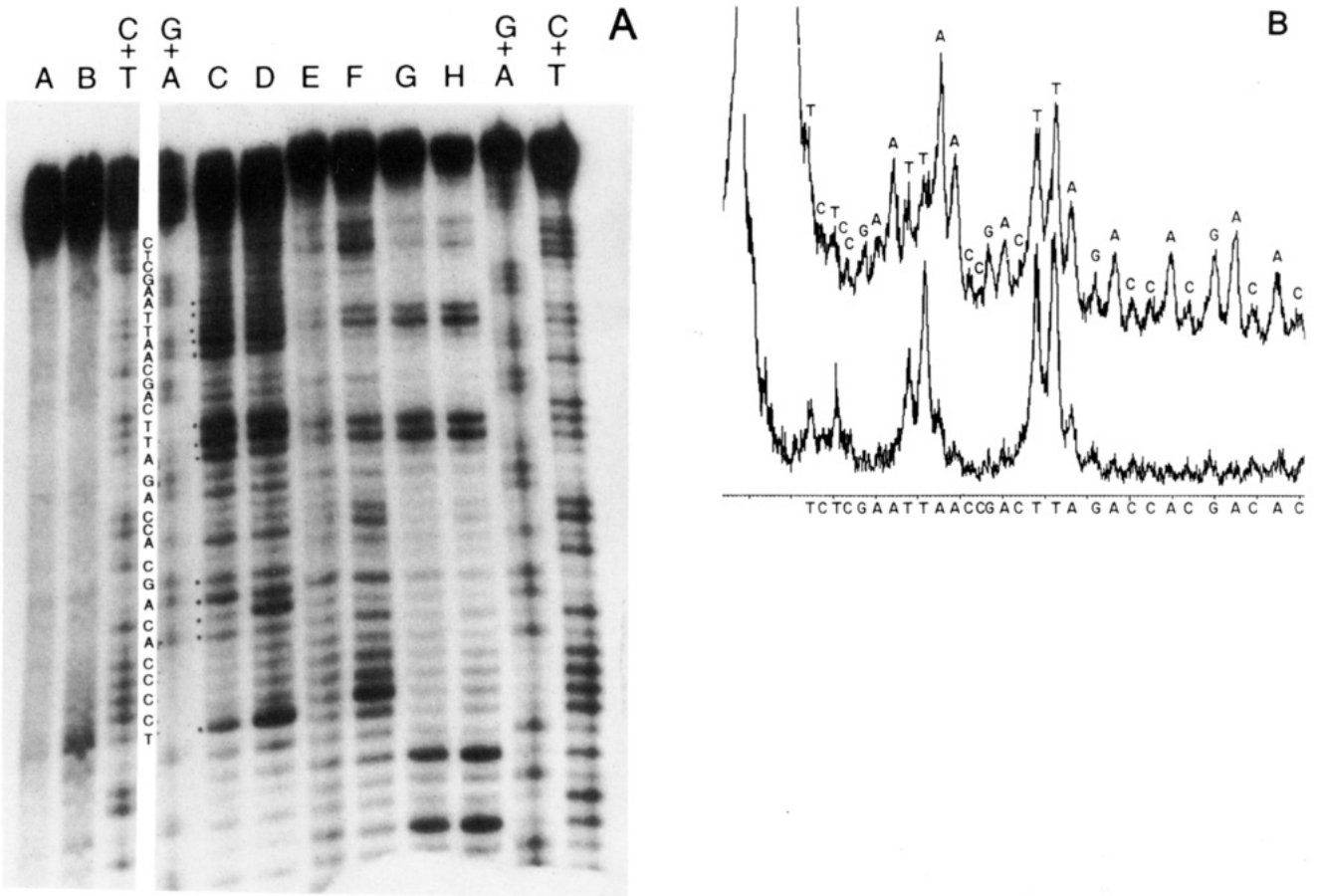

FIGURE 4: Altered specificity of cleavage by $KMnO_4$ of the anticomplementary strand in triplex DNA. (A) Polyacrylamide gel analysis of the substrates modified by $KMnO_4$ at pH 7.5 and 5. In lanes A–D, the label was at the 5′ end of HP (Figure 1, iv): lanes A and B, HP (Figure 1, iv) at pH 7.5 and 5, respectively; lanes C and D, triple-stranded intermediate (substrate v) at pH 7.5 and 5. In lanes E–H, the label was at the 5′ end of Y1 (Figure 1, vii): lanes E and F, partially duplex substrate (Figure 1, vi) at pH 7.5 and 5, respectively; lanes G and H, single-stranded oligonucleotide Y1 (Figure 1, vii) at pH 7.5 and 5. Lane C+T and lane G+A, pyrimidine-specific and purine-specific chemical sequencing ladders, respectively. The C+A and G+A lanes between lanes B and C are chemical sequencing ladders of HP, and those following lane H are sequencing ladders of Y1. The mobilities of bands from these two oligonucelotides were not identical because half of each had a different sequence. (B) Upper portion: A quantitative trace of lane C, the anticomplementary arm of the hairpin oligonucleotide in triple-stranded substrate v (Figure 1). Lower portion: A quantitative trace of lane G, the 3′ moiety of single-stranded oligonucleotide Y1 (Figure 1, vii), which has the same sequence as the anticomplementary strand of the hairpin. The trace was accomplished by use of PhosphoImager ImageQuant Software.

also appeared in the single-stranded control (Figure 4, compare lanes E and G). We suspect that the putatively single-stranded branch was not literally an unpaired branch, but rather was imperfectly paired with partially homologous sequences elsewhere in the M13 circular single-stranded DNA. This hypothesis is supported by the observation that the 3′ "tail" was digested by DNase I, which preferentially digests double-stranded DNA [Figure 5, compare the top portions of lanes C and D (the branched structure) with lanes E and F (the single-stranded oligonucleotide Y1); see Discussion for further comments]. When we used RecA protein in place of thermal annealing to make the Y-shape structure, the digestion pattern by DNase I was identical (data not shown).

We have recently shown that acid pH stabilizes triplex joints made by RecA protein (Burnett et al., unpublished observations). Therefore, we tested the effect of acidic pH and looked for a change in secondary structure by probing with $KMnO_4$. There were only two more bases modified at pH 5 when compared with pH 7.5 along the whole continuous hairpin DNA molecule. One of them was a thymine residue near the hairpin loop that showed enhanced modification after the pH change (Figure 4A, compare lane D, pH 5.0, with lane C, pH 7.5). The other one was a cytosine residue that is two bases away from the cytosine loop. The cytosine in the C loop itself was only slightly more modified at acidic pH. In the Y-shape structure, there were several cytosine residues showing enhanced modification, especially those near the junction between the double-stranded region and the "tail" region. By contrast, there was no observable change in the single-stranded control. These results strongly suggest that only those DNA regions that have formed some kind of secondary structure are affected by acidic pH. Possibly cytosine when protonated at pH 5 was excluded from the existing structure and was thus detected by $KMnO_4$.

Magnesium ions have been shown to increase the stability of a variety of DNA structures including H-DNA and nodule DNA (Häner & Dervan, 1990; Lyamichev et al., 1991; Panyutin & Wells, 1992). Therefore, we looked for possible modification of the RecA triplex structure by magnesium ions, but from 1 to 10 mM magnesium chloride, no change occurred that was detected by $KMnO_4$ (data not shown).

(*2*) *Diethyl Pyrocarbonate.* Diethyl pyrocarbonate (DEPC) has been used as a DNA structural probe because it can detect bases that are present in the B–Z junction (Johnston & Rich, 1985) and at a cruciform loop (Furlong & Lilley, 1986). It is more specific to purines because it carboxylates mainly N-7 of adenine (Leonard et al., 1971) and to a lesser extent N-7 of guanine. It has been used in detecting the syn conformation of guanine in Z-form DNA.

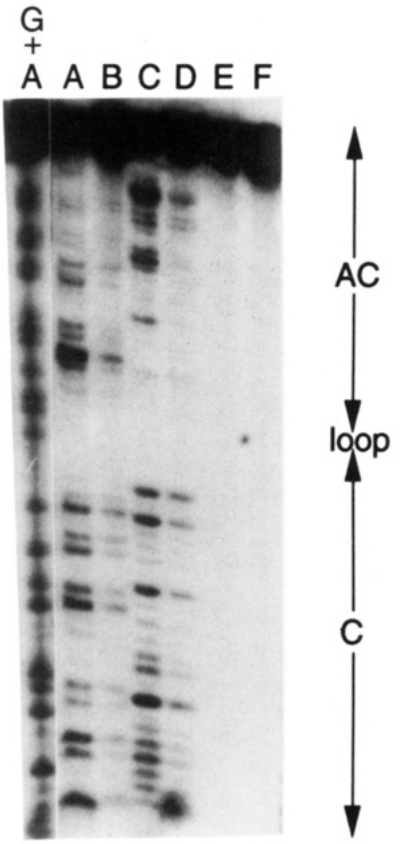

FIGURE 5: DNase probing of the single-stranded arm of a branched structure (Substrate vi, Figure 1). The first lane, designated G+A, is a sequencing ladder. Substrates in the three subsequent pairs of lanes were treated with DNase I at pH 7.5 and 5, respectively. Lanes A and B, the hairpin substrate (Figure 1, iv) labeled at its 5′ end; lanes C and D, the branched structure (Figure 1, vi) labeled at the 5′ end of oligonucleotide Y1; lanes E and F, the single-stranded oligonucleotide Y1 (Figure 1, vii) labeled at its 5′ end. The designations to the right of the photograph are the same as described in the legend to Figure 3.

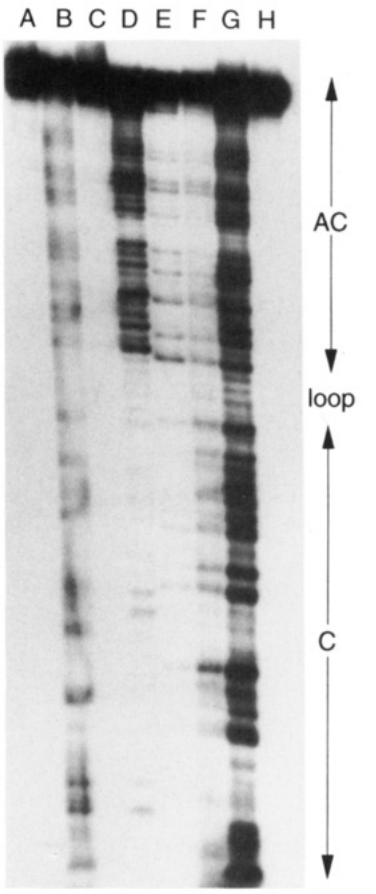

FIGURE 6: Diethyl pyrocarbonate (DEPC)-modified adenine and, to a lesser extent, guanine. In lanes A–D, the hairpin oligonucleotide HP (Figure 1, iv) was labeled at its 5′ end. Lane A, control untreated HP; lane B, purine-specific chemical sequencing marker of HP (see Materials and Methods); lane C, HP treated with DEPC; lane D, triple-stranded intermediate (Figure 1, v) modified by DEPC. In lanes E–H, the label was at the 5′ end of the single-stranded oligonucleotide Y1 (Figure 1, vii). Lane E, partially duplex substrate (Figure 1, vi) modified by DEPC; lane F, Y1 (Figure 1, vii) modified by DEPC; lane G, purine-specific sequencing ladder of Y1; lane H, control untreated Y1. Designations to the right of the autoradiogram are the same as in Figure 3.

Consistent with the result of $KMnO_4$ modification, the carboxylation reaction of DEPC occurred mainly on the adenine residues of the anticomplementary strand in the triplex DNA (Figure 6, lane D) as judged by comparison with several other lanes of this gel: lane E, which shows the modification of Y1 by DEPC, identified the adenine residues in the sequence that was common to the 3′ end of Y1 and the anticomplementary strand in the triplex DNA in neighboring lane D. When allowance is made for a slight displacement of the bands in lane D, which we attribute to the hairpin structure, one can see that the most heavily modified sites in lane D corresponded to adenine residues.

These observations seem closely related to the apparent change in specificity of $KMnO_4$ when acting on the triplex intermediate, and suggest that the two reagents are detecting a specific conformation of the anticomplementary strand.

(*3*) *Dimethyl Sulfate* (*DMS*). Dimethyl sulfate reacts primarily with N-7 of guanine and N-3 of adenine of both single-stranded and double-stranded DNA (Maxam & Gilbert, 1980). To determine if N-7 of guanine and N-3 of adenine are involved in any of the three strands of this triple-stranded joint, we methylated the triplex made from oligonucleotides (Figure 1, ii). None of the strands in the triplex differed in modification from control single-stranded DNA or hairpin duplex DNA (data not shown). This indicates that none of the three strands participates in Hoogsteen base-pairing, which in the case of C-G·C triplets in H-DNA involves the N-7 substituent of guanine (Voloshin et al., 1988). This observation confirms the results of a previous experiment done on triplex DNA made from the hairpin oligonucleotide and single-stranded M13 DNA (Rao et al., 1993).

DISCUSSION

By using a duplex oligonucleotide with its proximal ends linked by a hairpin loop, we have isolated from reactions promoted by RecA protein a stable DNA product that survives deproteinization (Rao et al., 1993). To understand the interactions of the three strands in the complex, we used DNase I and $KMnO_4$ as probes of secondary structure. The present results extend our previous studies (Rao et al., 1993), by using oligonucleotide substrates, additional enzymatic and chemical probes, and additional controls.

Two of the Three Strands Are in the B Conformation. Experiments with DNase I revealed that both the incoming single-stranded DNA from the RecA filament and the complementary strand from the hairpin duplex DNA were in the B conformation. $KMnO_4$, DEPC, and P1 nuclease, which have been used to detect DNA conformation, are not reactive on B-form DNA. The insensitivity of these two strands toward all three confirmed that they were in a B-form substructure. This also indicates that the deproteinized product has already

undergone an exchange of strands and formed heteroduplex DNA, in agreement with related observations of Adzuma (1992; and see below for further comparisons).

The Anticomplementary Strand Is Not Single-Stranded. Since the anticomplementary strand is linked to the complementary strand by a hairpin loop, the DNase I digestion pattern in both strands can be analyzed in the same sequencing gel. The relative intensities of the bands from the anticomplementary strand, although in a different pattern, were comparable to those of the complementary strand, and were more intense than the bands in the single-stranded control, which indicates that the anticomplementary strand was not simply single-stranded.

When we analyzed the anticomplementary strand with $KMnO_4$ (Figure 4), DEPC (Figure 6), and OsO_4 (data not shown), we found, surprisingly, that most of the purines were modified. However, the single-stranded oligonucleotide control showed that each of these chemical reagents worked as expected, *viz.*, on the correct bases: $KMnO_4$ on thymine, DEPC on adenine, and OsO_4 on cytosine. Another surprise was presented by probing the Y-shaped DNA substrate. The putative single-stranded tail of the Y-shape structure did not appear to be a free single strand since DNase I digested the tail and $KMnO_4$ modified several adenine residues. There are some precedents for the modification of purines by $KMnO_4$. Sasse-Dwight and Gralla (1989) found that certain adenine residues in the open complex of *lac* and its related promoters were hyperreactive to $KMnO_4$. Glover and Pulleyblank (1990) found that guanine residues in the "single-stranded" polypurine tract of H-DNA were modified by $KMnO_4$. Under the condition that they used for probing, the adenine bases in DNA molecules may have been protonated as the pK_a value is about 6. The lack of reactivity of adenine in that case might reflect the existence of hydrogen-bonding between protonated adenine and the phosphate backbone of the triplex, a kind of interaction that has been reported recently in single-stranded DNA at acidic pH (Dolinnaya & Fresco, 1992).

In our experiments (see Results), genuine single-stranded DNA did not show any anomalous chemical sensitivity. Thus, we interpret the sensitivity of the anticomplementary strand to DNase I, and its anomalous reactivity to several chemical probes as evidence that it was not simply a single-stranded branch. The observations on the Y-shaped control support this interpretation. The reactivity of the 3′ end of oligonucleotide Y1 in the Y-shaped control also differed from that of the free single-stranded oligonucleotide, indicating that the tethered branch intracts with other regions of DNA. Although the sequence of the 3′ end of Y1 was identical to that of the anticomplementary strand of the hairpin oligonucleotide, the sensitivity of this sequence in the Y-shaped control was both quantitatively and qualitatively distinct from its sensitivity in the triplex. We conclude that the anticomplementary strand of the hairpin was neither a free branch nor a partially duplex structure like that found in the Y-shaped control.

Using DMS and $KMnO_4$, Adzuma (1992) probed the three strands of DNA in RecA synaptic complexes. The nucleotide cofactor used during the formation of complexes was ATPγS, and probing was carried out on complexes that were not deproteinized. (Our conditions, one should recall, involved ATP and deproteinized complexes.) When the strands of the original duplex were probed, the substrate was nonsuperhelical. Adzuma observed that C's, which are protected in duplex DNA, were modified by DMS in the anticomplementary or "outgoing" strand whereas C's in the complementary or

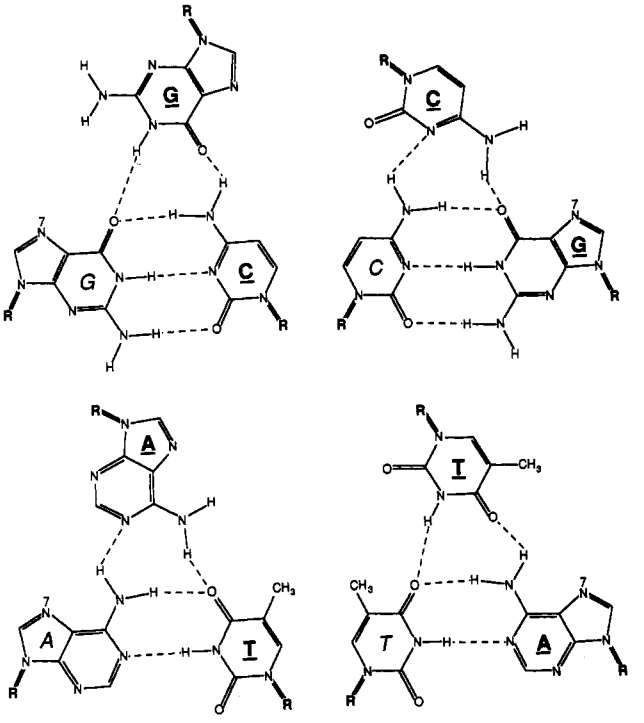

FIGURE 7: Base triad model of the triplex (Rao et al., 1993). The incoming single-stranded DNA (bases with italicized letters) is now paired with the complementary strand (bases with bold-face and underlined letters) to form a heteroduplex. The anticomplementary strand (bases in boldface and underlined letters) is situated in the major groove of the heteroduplex. All the N-7 atoms of guanines and adenines are exposed in this model. Zhurkin et al. have proposed a base triplet recognition scheme founded on electrostatic interactions that is consistent with this placement of bases (1993, and personal communication).

"recipient" strand were not. When probed with permanganate, T's in both the outgoing and recipient strands were modified, although the modification appeared to be more uniform in the outgoing strand. Probing of the strand originally in the RecA filament, termed the "incoming" strand, involved synaptic complexes made with superhelical DNA. As in the recipient strand, C's in the incoming strand were not susceptible to DMS, and $KMnO_4$ appeared to modify T's in both the incoming and recipient strands in a similar "position-dependent" way. Adzuma concluded that the DNA in synaptic complexes, made in the presence of ATPγS, had already undergone strand exchange, leaving the strands of nascent heteroduplex DNA paired by Watson–Crick hydrogen bonds, and the displaced strand in an unpaired state.

Adzuma's observations were made on synaptic complexes in the presence of RecA protein, whereas ours were made on a deproteinized product in which the anticomplementary strands was tethered by a hairpin connection. The results of the two studies agree in showing that the two respective structures represent a stage that follows the exchange of strands and the formation of nascent heteroduplex DNA. However, with respect to the deproteinized complex made from the hairpin oligonucleotide and single-stranded DNA, we believe, as reasoned above, that the anticomplementary or outgoing strand retains or reestablishes homology-dependent interactions with the nascent heteroduplex.

An Energy-Minimized Triplex Model. To explain previous observations on the triplex structure made by RecA protein (Rao et al., 1993), we proposed a planar model of base triplets (Figure 7) which is useful particularly in rationalizing data on modification of the hairpin substrate and the triplex product

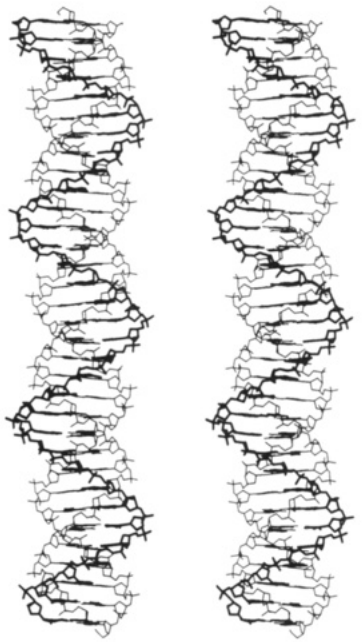

FIGURE 8: Three-dimensional model of the overall structure of the protein-free triplex. The DNA model with 33 triads is shown. The heteroduplex structure (inner) is drawn with thin bonds, and the anticomplementary strand is drawn with thick bonds. The features of this structure are described under Discussion.

by DMS. The planar model, however, did not help to explain the reactivity of the triplex to DEPC and $KMnO_4$. To improve our understanding, we sought a three-dimensional model. A triplex structure was modeled on a computer as described under Materials and Methods. Inspection of this model (Figure 8) reveals several interesting features that help to explain our data.

First, the anticomplementary strand divides the major groove of the B-form heteroduplex of DNA into two unequal halves and thus creates a new major groove and a new minor groove (Figure 8). Second, the path of the anticomplementary strand has a larger radius than that of the heteroduplex DNA, which we assumed to be B-form DNA. Nonetheless, after minimization, the deoxyribose moieties of the stretched anticomplementary strand retain the 2′-endo configuration. Thus, modeling based on the assumptions described (see Materials and Methods) showed that the sugar–phosphate backbone must be stretched in order to accommodate the interactions of bases proposed in the planar model (Figure 7). Such a structure might explain why the anticomplementary strand is more susceptible to single-stranded nucleases than duplex DNA while at the same time it is much less sensitive than single-stranded DNA. Third, most of the bases in the anticomplementary strand do not stack well either upon each other or upon the bases of the heteroduplex DNA (Figure 9). Unstacking of the bases and stretching of the sugar–phosphate backbone imply an unstable structure. Other experiments in this laboratory show that the isolated triplex is indeed a metastable structure that transforms into another structure within a couple of days, as a result of protonation (Burnett et al., unpublished observations).

Lastly, we examined the accessibility of the N-7 groups of both guanine and adenine residues on all three strands in a space-filling version of the triplex model. The model indicates that the N-7 atoms of guanine and adenine in the anticomplementary strand are in a very exposed location as the sugar–phosphate backbone and the base of this strand protrude from the triple helix (Figure 9; arrow 1). On the other hand, those in the complementary strand are less accessible because they are situated within the new major groove (arrow 2). Moreover, the N-7 atoms of the incoming strand in the heteroduplex are now almost on the bottom of the trough of the new minor groove, which is even narrower than the minor groove of the heteroduplex (arrow 3). These aspects of the model can be used to explain why N-7 atoms of guanine and adenine residues in the anticomplementary strand are hypersensitive to DEPC, $KMnO_4$, and OsO_4, whereas those of the other two strands are not. We cannot explain, however, why $KMnO_4$ preferentially oxidize purines rather than thymines in the anticomplementary strand, nor do we know how protonation stabilizes triplex joints.

Triplex DNA vs Homologous Pairing and Strand Exchange. These observations help to clarify the relationship between triplex DNA and the mechanisms of homologous pairing and strand exchange as promoted by *E. coli* RecA protein. The intermediates and the deproteinized products that can be extracted from the RecA complexes appear to bear little relationship to the triplex DNA that is formed by homopurine–homopyrimidine sequences. There are no sequence limitations in the RecA reaction, but there is no stable protein-free product except under special circumstances. In our view, the formation of protein-free complexes in which three homologous strands are interacting comes about when

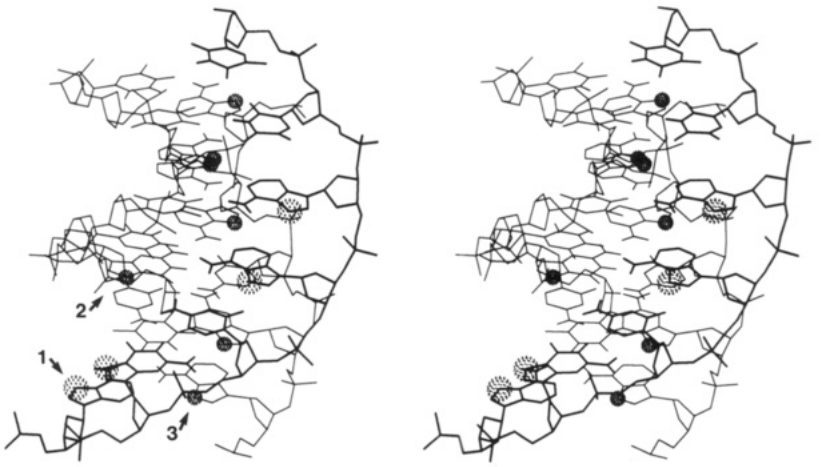

FIGURE 9: Stereoscopic view of part of the triplex model to illustrate the spatial positions of the N-7 atoms of guanine and adenine residues of the three strands. In this view, the helical axis has been tilted out of the plane of the paper to view along the new major groove (left-hand side). Thick bonds depict the anticomplementary strand and thin bonds the heteroduplex. Dotted spheres are shown around the positions of the N-7 atoms of adenine and guanine residues.

the anticomplementary strand cannot escape, either as a result of a hairpin connection or because of the equivalent lack of a free 5′ end of the anticomplementary strand in the joint originally formed by RecA protein. In both cases, RecA protein makes a three-stranded joint and promotes the switching of base pairs, creating heteroduplex DNA and an extra anticomplementary strand. When protein is removed, the covalently attached anticomplementary strand maintains or reestablishes homology-dependent interactions with the nascent heteroduplex, as shown here. We view this interaction as evidence that homologous recognition can occur by non-Watson–Crick bonds, without any sequence limitations, even in the absence of RecA protein. The protein, however, makes use of these transient interactions, as suggested by recent experiments which demonstrate that RecA protein can support homologous recognition by non-Watson–Crick bonds (Rao & Radding, 1993).

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