An extended DNA structure through deoxyribose-base stacking induced by RecA protein

(homologous genetic recombination/NMR/NMR spectroscopy/transferred nuclear Overhauser effect)

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ABSTRACT The family of proteins that are homologous to RecA protein of *Escherichia coli* is essential to homologous genetic recombination in various organisms including viruses, bacteria, lower eukaryotes, and mammals. In the presence of ATP (or ATPγS), these proteins form helical filaments containing single-stranded DNA at the center. The single-stranded DNA bound to RecA protein is extended 1.5 times relative to B-form DNA with the same sequence, and the extension is critical to pairing with homologous double-stranded DNA. This pairing reaction, called homologous pairing, is a key reaction in homologous recombination. In this NMR study, we determined a three-dimensional structure of the single-stranded DNA bound to RecA protein. The DNA structure contains novel deoxyribose-base stacking in which the 2′-methylene moiety of each deoxyribose is placed above the base of the following residue, instead of normal stacking of adjacent bases. As a result of this deoxyribose-base stacking, bases of the single-stranded DNA are spaced out nearly 5 Å. Thus, this novel structure well explains the axial extension of DNA in the RecA-filaments relative to B-form DNA and leads to a possible interpretation of the role of this extension in homologous pairing.

Homologous genetic recombination plays critical roles in both evolution and maintenance of a functional genome. RecA protein is essential to homologous recombination in *Escherichia coli* (1, 2), and promotes ATP-dependent joint-molecule formation from homologous double-stranded DNA and single-stranded DNA through "homologous pairing" *in vitro* (3, 4). Homologous pairing by RecA protein has been extensively studied for more than a decade. How single-stranded DNA recognizes sequence homology in double-stranded DNA has been a central question in these studies. Based on studies using chemical probing, electron microscopy, modification of base sequences, mutant RecA proteins, and others, various models such as triplex formation have been proposed to explain the mechanism of recognition of homology (see refs. 5–12 for reviews). However, little information is available on the three-dimensional structures of DNA during homologous pairing, information that is essential for a clear view of the mechanism of homologous recognition.

At the first stage of homologous pairing, RecA protein binds to single-stranded DNA in the presence of ATP, and then double-stranded DNA binds to the nucleoprotein complex for searching for homology (13, 14). Electron microscopic studies revealed that RecA protein forms helical filaments on the single-stranded DNA. Biochemical studies showed that such filaments formed in the presence of ATP ("presynaptic filament") are molecular machines for homologous pairing of the single-stranded DNA in the filaments with naked double-stranded DNA that is then taken up into the filament (14, 15). Under certain conditions, RecA protein forms a filament on double-stranded DNA, whose shape is very similar to that of the filament formed on single-stranded DNA (14, 16, 17). In these RecA filaments, both single-stranded and double-stranded DNA are extended 1.5 times as compared with B-form DNA. In spite of low degrees of amino acid sequence homology, eukaryotic homologs of RecA protein, the Rad51 proteins from *Saccharomyces cerevisiae* and *Homo sapiens*, and the functional homolog UvsX protein from *coli-phage* T4 form helical nucleoprotein filaments that have a shape that is nearly identical to bacterial RecA protein, as revealed by electron microscopy (18–20). In the experiments described here, we determined a three-dimensional structure of single-stranded DNA bound to RecA protein, which revealed a novel stacking of deoxyribose and bases.

MATERIALS AND METHODS

Oligodeoxyribonucleotides and RecA Protein. Oligonucleotides were synthesized on a DNA synthesizer (EXPEDITE; Millipore) followed by the purification with reversed-phase column cartridges (Oligo-pak SP; Millipore), or purchased from Cruachem (Kyoto) or Genset (Tokyo). Undesirable organic impurities and metal ions were removed by using cation ion exchange resins (AG 50W-X8, Chelex 100; Bio-Rad). The purified oligonucleotides were lyophilized rapidly and stored at −20°C. DNA concentrations were determined by absorbance measurements at 260 nm and are expressed in moles of entire molecules rather than moles of nucleotide residues.

RecA protein was purified as described by Shibata *et al.* (21, 22), with a minor modification, and dialyzed against 20 mM Tris·Cl (pH 7.5) buffer containing 6.7 mM $MgCl_2$ and 150 mM NaCl. By use of ultrafiltration, we concentrated RecA protein and replaced the solvent by a deuterium buffer {20 mM [uniform ^2H] Tris·Cl, pH 7.1 (pH values were uncorrected for isotope effects)/6.7 mM $MgCl_2$/150 mM NaCl}; i.e., the protein solution was centrifuged at 3,000 rpm for 0.5–2 hr at 4°C in a Centriprep cartridge (30-kDa cut-off; Amicon), followed by dilution with the deuterium buffer. We repeated this process several times. The sample was then lyophilized, stored at −20°C, and redissolved in D_2O (99.96%; Euriso-top) before use. The activity of the preparation of RecA protein was

Abbreviations: NOE, nuclear Overhauser effect; NOESY, NOE spectroscopy; TRNOE, transferred NOE.
Data deposition: The atomic coordinates have been deposited in the Protein Data Bank, Chemistry Department, Brookhaven National Laboratory, Upton, NY 11973 (3rec).
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assessed by assaying the single-stranded DNA-dependent AT-Pase activity, which was not changed by lyophilization.

Just after the purification, the concentrations of RecA protein were first determined by the Folin phenol–reagent method described by Lowry *et al.* (23), with bovine serum albumin as a standard. After preparation for NMR spectroscopic observations, as just described, the concentrations were determined again by the Bradford method (Bio-Rad), with untreated RecA protein as the standard. The amounts of RecA protein are expressed as moles of 38-kDa polypeptide.

NMR Spectroscopy. One- and two-dimensional spectra were measured on a Bruker AMX600 spectrometer at 20°C–37°C in 20 mM [uniform ^{2}H] Tris·Cl buffer (pH 7.0) containing 6.7 mM $MgCl_2$ and 150 mM NaCl in D_2O.

For one-dimensional nuclear Overhauser effect (NOE) difference spectra an objective proton was irradiated for 0.5 sec prior to a 90° read pulse, and the free induction decays were subtracted from those of off-irradiated scans. The water signal was presaturated for 1.5 sec. The spectra were recorded with 6,024 Hz of spectra width and 16 k data points. Total measuring time was 29 min.

To attenuate undesirable protein resonances in the transferred NOE spectroscopy (NOESY) spectrum, we applied a short $T_{1\rho}$ filter (20 ms, $\gamma B_1 = 3$ kHz) before a standard NOESY pulse scheme (pre-sat. $-90°_x - SL_y - 90°_{-x} - g - 90° - t_1 - 90° - \tau - g - 90° - $ Acq.; g, a gradient pulse; ref. 24). The mixing time was varied randomly over 8% of the designated mixing time to suppress zero-quantum artifacts in transferred NOESY spectra (25). The spectra were acquired with a total of 1,024 (t_2) × 400 (t_1) complex points and spectral width of 6,024 Hz. The free induction decays were apodized with a 90°-shifted skewed sinebell function (skew parameter 2) before Fourier transformation in both dimensions. All two-dimensional data sets were processed and analyzed with the program FELIX, version 2.3 (Biosym Technologies, San Diego) using Silicon Graphics workstations.

Analysis of NMR Data and Structural Calculation. NOE intensities in two-dimensional spectra at mixing times of 100, 120, 180, or 200 msec were integrated and calibrated using those of intra-residue C[H5]–C[H6] crosspeaks as references, except in the case of d(TAG). For d(TAG), the intensity of H2′–H1′ crosspeak was used as a reference. Interproton distance of H2′–H1′ holds a nearly constant value between C2′-*endo* (2.99 Å) and C3′-*endo* (2.73 Å). All intensities were converted to distance restraints using the $I/I_0 = (r_{ij}/r_0)^{-6}$ relation, where I = peak intensity, r_{ij} = distance between i and j protons, I_0 = peak intensity of the reference and r_0 = distance of the reference. All restraints were classified as short ($r \leq 3.5$ Å), medium (3.5 Å $< r \leq 4.5$ Å), and long (4.5 Å $< r$) and set the upper and lower bound ± 0.3 Å, ± 0.4 Å, and ± 0.5 Å, respectively. The additional distance restraints appeared in longer mixing time (≤ 200 msec) and repulsive distance restraints were incorporated during the structural refinement.

All structural calculations were carried out by the use of X-PLOR, version 3.1 (26). The refinement protocol followed essentially the procedures outlined in the X-PLOR manual. After a short cycle of energy minimization, simulated annealing calculations were initiated at 1,000 K and run for 18 ps. The temperature was then lowered to 100 K with 25 K step size and 1.5 ps dynamics during each cooling step. The structure was then minimized for 200 cycles. Because the sugar puckering was ill determined, the obtained structure was further minimized by use of the revised parameters published by Parkinson *et al.* (27).

Assignments for all nonexchangeable protons of DNA (except stereospecific assignments of H5′ and H5″) were obtained by analysis of double quantum filtered correlated spectroscopy (DQF-COSY), total correlated spectroscopy (TOCSY), rotating-frame Overhauser effect spectroscopy (ROESY), and ^{1}H-^{31}P correlated spectroscopy (^{1}H-^{31}P COSY) spectra using

standard sequential assignment techniques (28). Stereospecific assignments of H5′ and H5″ were carried out during the process of structural refinement.

RESULTS

Transferred NOE Analysis of Single-Stranded Oligodeoxyribonucleotides Bound to RecA Protein. The structure of single-stranded DNA induced by the binding to RecA protein in the presence of ATPγS was analyzed by means of the transferred NOE (TRNOE) using short (3–6-mer) oligodeoxyribonucleotides: d(TAG), d(CGA), d(TACG), and d(TGACAT). The TRNOE allows us to analyze structures of small ligands bound to large molecules when the exchange between bound and free states is fast enough (29–33).

We added RecA protein stepwise to the solution containing oligodeoxyribonucleotides and ATPγS (an unhydrolyzable ATP analog). The chemical shifts of the resonances were slightly moved and the signals were slightly broadened in one-dimensional ^{1}H-NMR spectra after the addition of RecA protein. We observed no resonances derived from the bound state, which should have appeared if the exchange rate were slow. Signals from RecA protein were hardly detected due to
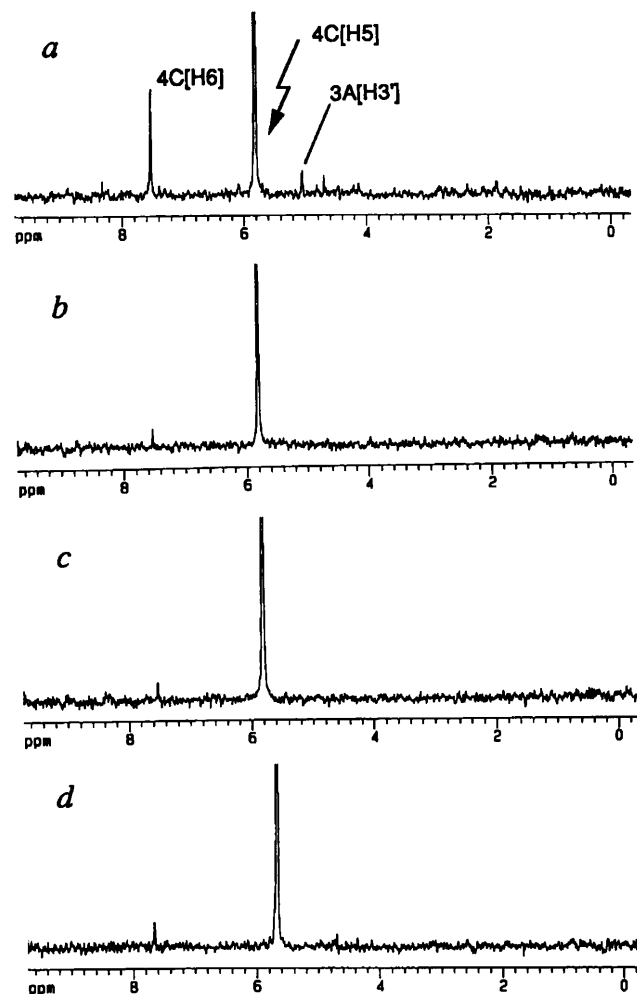

FIG. 1. One-dimensional TRNOE difference spectra of an oligodeoxyribonucleotide. (*a* and *b*) Spectra of 1.1 mM d(TGACAT) and 54 μM RecA protein in the presence of 1.1 mM ATPγS (*a*) or ADP (*b*) in D_2O at 37°C. (*c*) Spectrum of the DNA solution before the addition of RecA protein and ATPγS or ADP. (*d*) DNA was replaced by RNA; 1.1 mM r(UGACAU) in the presence of RecA protein and ATPγS. The cytosine H5 proton of DNA or RNA was irradiated for 0.5 sec before a 90° read pulse.

severe signal broadening. From $T_{1\rho}$ measurements as a function of the spin-lock field strength, we have determined the dissociation rate constants for the oligodeoxyribonucleotide–RecA complex. The value for d(TGACAT) at 30°C was 40,000 ($\pm$4,000) s^{-1}, which would be fast enough compared with the chemical shift scale and the cross-relaxation rate (T.N. and Y.I., unpublished observation).

ATP is an essential cofactor for RecA protein-mediated homologous pairing. ATP is hydrolyzed by RecA protein during the reaction and hydrolysis of ATP decreases the affinity of RecA protein to DNA. When ATP is replaced by ATPγS, RecA protein promotes homologous pairing of single-stranded and double-stranded DNA molecules equally well (34), and presynaptic filaments formed in the presence of ATPγS under optimum conditions for homologous pairing resemble those formed in the presence of ATP (35). We found that RecA protein induced TRNOEs of the above oligodeoxyribonucleotides in the presence of ATPγS (Fig. 1*a*), and that the crosspeaks of the transferred NOESY spectra were intense and well resolved (see Fig. 2). Intermolecular crosspeaks between RecA protein and oligodeoxyribonucleotides were not observed, probably because of severely broad signals of RecA protein. On the other hand, in the absence of RecA protein, little NOEs of oligodeoxyribonucleotides were detected (Fig. 1*c*), indicating that the TRNOEs depend on interactions of the oligodeoxyribonucleotides with RecA protein.

The NOEs Are Caused by Specific Binding of DNA to Activated RecA Protein. First, we examined whether the observed interactions between DNA and RecA protein had essential characteristics in common with homologous pairing, specifically a requirement for ATPγS and a preference for DNA over RNA.

Consistent with both the requirement of ATP (or ATPγS) for the formation of active presynaptic filaments and the reduction of affinity for DNA upon hydrolysis of ATP to ADP, TRNOEs of the oligodeoxyribonucleotides induced by the addition of RecA protein were significantly reduced when ATPγS was replaced by ADP (Fig. 1 *b* vs. *a*).

RecA protein binds to RNA with much less affinity than to DNA (36, 37). We have observed that the intensity of TRNOE signals was significantly decreased when DNA was replaced by RNA with the same sequence except for the replacement of U for T (Fig. 1*d*).

These observations indicate that the NOEs observed here are caused by specific interactions of RecA protein with DNA that are the same as those responsible for homologous pairing.

Transferred NOE Analysis of DNA Bound to RecA Protein. The patterns of NOE crosspeaks exhibited by oligodeoxyribonucleotides tested in this study have common features. Unusually intense interresidue crosspeaks between H3′ and H8/H6 were observed in transferred NOESY spectra, whereas by contrast, few, if any, interresidue crosspeaks between H1′ and base protons were detected (Fig. 2*B*). We also observed relatively weak sequential H2′–H8/H6 and H2″–H8/H6 NOEs of comparable intensity (Fig. 2*A*). These are a remarkable contrast to those expected for B-form or A-form DNA (28).

Based on these NOE data, we did structural calculations applying a simulated annealing protocol by use of X-PLOR (26). The final structure for each oligodeoxyribonucleotide was well defined as shown in Fig. 3, the result for d(TACG) as an
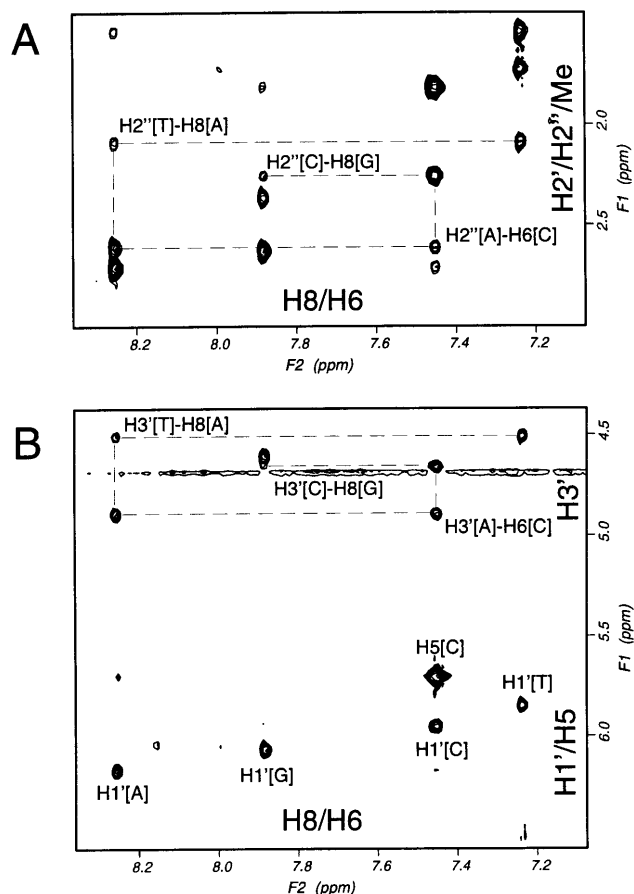

FIG. 2. Two-dimensional transferred NOESY spectra of an oligodeoxyribonucleotide. Two-dimensional transferred NOESY spectra of 0.80 mM d(TACG), 97 μM RecA protein, and 0.80 mM ATPγS at 180 msec mixing time at 25°C. The regions of H8/H6 and H2′/H2″ are shown in *A*, and those of H8/H6 and H3′/H1′ in *B*.
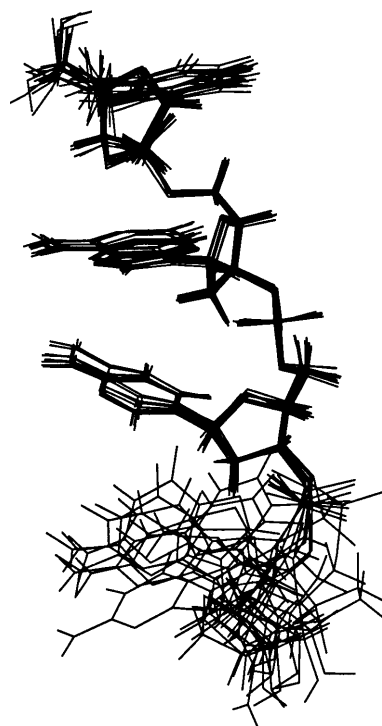

FIG. 3. Superposition of calculated structures of an oligodeoxyribonucleotide, d(TACG). One hundred structures were calculated independently by the use of simulated annealing protocol (X-PLOR; ref. 26). The 10 lowest energy structures are best fitted at the T-A-C region. Total number of NOE constraints is 59; 39 for intraresidue NOEs and 20 for interresidue NOEs. The root-mean-square deviation of the T-A-C region is 0.30 Å. All residues shows similar deoxyribose-base stacking, whereas the fourth residue (G) is disordered because of few NOE constraints due to signal overlapping. There is no violation to the final structure.
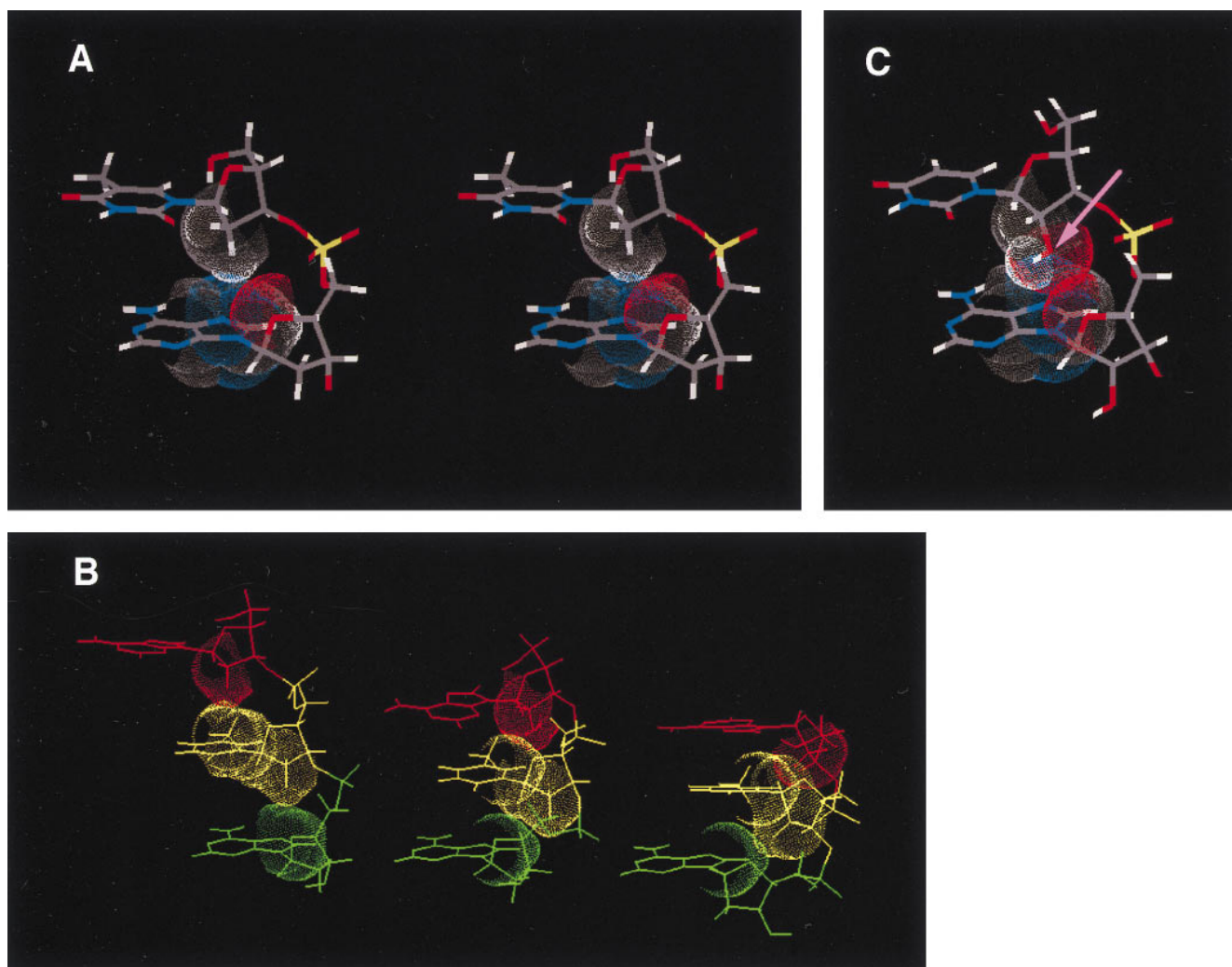
FIG. 4.   An extended single-stranded DNA structure induced by RecA protein. (*A*) Stereoview of the representative structure of RecA protein-bound DNA [sequence: d(TA)]. Van der Waals contact surface of a 2′-methylene moiety of a 5′ side residue (T) and those of O4′, C4, C5, N7, C8, H8, and N9 of the 3′ side residue (A) are shown. A structure deduced from the calculation by use of a simulated annealing method was refined using revised parameters as published (27). (*B*) Side views of the structure of DNA in RecA protein-bound form (*Left*), B-form (*Middle*), and A-form (*Right*). Van der Waals contact surfaces between adjacent residues are shown. In B- or A-form DNA the whole structure must be disrupted on the process of strand exchange because of its close packing between adjacent residues. (*C*) A hypothetical RNA structure in the RecA protein-bound form. H2″ is replaced upon a hydroxyl group (indicated by arrow) in RNA.

example. Thus, we conclude that the obtained structure of each oligodeoxyribonucleotide is the major species that mainly contributes to the TRNOE crosspeaks. The calculated structures for all the tested oligodeoxyribonucleotides with variations in sequence and length have a common substructural feature, suggesting that the DNA structure defined in this study is not specific to a sequence or to the size of oligodeoxyribonucleotides.

Fig. 4*A* illustrates a refined molecular model for the structure of single-stranded DNA bound to RecA protein in the presence of ATPγS. If we assume that a helical axis is perpendicular to the base planes as indicated by linear dichroism (38), the axial rise per base is nearly 5 Å (1.5 times that of B-form DNA).

DISCUSSION

By TRNOE analysis, we have determined a three-dimensional structure of single-stranded DNA that has been extended by binding to RecA protein in the presence of ATPγS. The most prominent feature of the DNA structure is in the manner of base stacking. In the normal forms of DNA, adjacent bases are stacked by a van der Waals contact. In contrast, in the RecA

protein-bound form, the 2′-methylene moiety of each deoxyribose is located above the base of the next residue in place of the normal base–base stacking, and the bases of the single-stranded DNA are separated by nearly 5 Å (Fig. 4*A*). This spacing agrees well with the 50% extension of single-stranded DNA in presynaptic filaments observed by electron microscopy (Fig. 4*B*; refs. 14 and 39), and the present observations reveal the structural basis for that extension. Interactions between a methylene moiety and an aromatic ring were observed in various biomacromolecules (see ref. 40 for review). There is, to our knowledge, no prior report of extended DNA structures maintained by deoxyribose-base stacking through a methylene-base interaction. On the other hand, another type of deoxyribose-base interaction is found in Z-form DNA (41): the cytidine O4′ oxygen is situated above the six-membered ring of guanine at d(CpG) steps.

What is the meaning of this characteristic deoxyribose-base stacking in the RecA-induced DNA extension? RecA protein has been proposed to bind primarily to the phosphate backbone of single-stranded DNA (42). This type of intermolecular interaction probably triggers the extension of single-stranded DNA upon polymerization of RecA monomers along the DNA backbone. In addition, we propose that the hydrophobic

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deoxyribose-base stacking interaction stabilizes intramolecularly the unique DNA conformation. This mechanism presents a striking contrast to that of the widely found DNA extension upon intermolecular stacking interactions, namely, intercalation of aromatic moieties of a dye or amino acid residue between adjacent bases. In this context, it has been suggested that some intramolecular interaction contributes to stabilization of an extended DNA; protein-free DNA molecules, under stress from an external force, undergo a highly cooperative transition into a stretched structure whose length is 1.7 times that of B-form DNA (43, 44)

Judging from the DNA structure revealed by this study, RNA molecules would not form a stable complex with RecA protein, because the 2′-hydroxyl group of RNA will repel the base and the sugar of the following residue (Fig. 4*C*). This would explain previous and current observations that RNA has much less affinity to RecA protein than DNA (Fig. 1*d*).

DNA has an advantage over RNA as material to hold genetic information. A widely accepted reason has been that H2″ confers chemical stabilization on DNA compared with 2′-OH of RNA. Our study suggests another role of H2″ of DNA as genetic material: deoxyribose-base stacking, including 2′-methylene moieties of DNA is required for the binding to RecA protein and its homologs that are general and pivotal machines for homologous recombination. In addition, the deoxyribose-base stacking could be intrinsically required for a homology search between polynucleotides (see below). These newly suggested roles of 2′-methylene moieties might account for the low efficiency and fidelity of homologous recombination in an RNA virus in contrast to high efficiency and accuracy in homologous recombination in organisms with DNA genomes (45).

What is the advantage of the structure stabilized by the deoxyribose-base stacking through a methylene-base interaction? The processes of homologous recognition and strand exchange require rotation of bases so as to exchange partners in base pairs. As described above, RecA protein appears to bind primarily to the phosphate backbone of single-stranded DNA and leaves the bases free for homologous pairing (42). In DNA stabilized by deoxyribose-base stacking, the rotation of adjacent bases is less hindered sterically than in B-form or A-form (Fig. 4*B*). Such freer rotation of bases may favor both homologous pairing and strand exchange.

Another merit of the extended DNA structure is suggested by theoretical conformation analysis of triplex DNA molecules (R-form DNA), which are supposed to be formed during RecA protein-promoted homologous pairing (46–48). According to a structural prediction by the theoretical calculations, the bases of the third strand in the putative triplex DNA would incline and mispair to adjacent base pairs when DNA molecules are not extended (49).

In the presence of ATP or its unhydrolyzable analog, RecA protein binds to double-stranded DNA as well and forms a helical nucleoprotein filament. Double-stranded DNA in the RecA filament has also been found to be extended by 1.5 times as compared with B-form DNA, and to be unwound to 18.6 bp per turn (16, 17). We suppose that RecA-bound double-stranded DNA would be extended by the deoxyribose-base stacking as in the case of single-stranded DNA. We made a model-building study on double-stranded DNA including the deoxyribose-base stacking, and obtained a structure that fits the parameters of RecA filaments (T.N., unpublished work).

Finally, although the structure of a long stretch of DNA in presynaptic filaments could be different from those of the oligodeoxyribonucleotides, we believe that the three-dimensional structure revealed by this study reflects the structure of single-stranded DNA in presynaptic filaments for the following reasons: (*i*) the signals from which the structure was deduced depend on the presence of both RecA protein and ATPγS, (*ii*) the structure can be adopted by DNA but not by RNA, (*iii*) the pattern of NOE crosspeaks is independent of residues in an oligodeoxyribonucleotide and of the sequence and length of the tested oligomers, and (*iv*) the structure agrees well with the extension of single-stranded DNA in the filaments as observed by electron microscopy.

Thus, the structure of the oligodeoxyribonucleotides determined in this study provides a new model that explains how the extended form of DNA in the RecA nucleoprotein filament is stabilized and that further suggests that the functional significance of this form is to facilitate the rotation of bases.

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