Second-Site RecA−DNA Interactions: Lack of Identical Recognition

Pernilla Wittung,*,‡ L. Rochelle Bazemore,§ Masayuki Takahashi,|| Bengt Nordén,‡ and Charles Radding§

Department of Physical Chemistry, Chalmers University of Technology, S-41296 Gothenburg, Sweden, Groupe d'Etude Mutagénèse et Cancérogenèse, UMR216 CNRS and Institut Curie, F-91405 Orsay, France, and Department of Genetics, Yale University School of Medicine, New Haven, Connecticut 06510

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ABSTRACT: The RecA protein plays crucial roles in recombination and repair. *In vitro*, it polymerizes on single-stranded DNA and promotes homologous recognition of duplex DNA and subsequent strand exchange. How the RecA filament recognizes homologous duplex DNA is not yet clear. Recent research has indicated the possibility of recognition between identical DNA strands in the RecA filament which may be involved in a triple-stranded structure prior to strand exchange. Here we address this type of recognition by the RecA filament with a variety of physical techniques. By a gel retardation assay, we find interaction of identical DNAs in RecA filaments to be strongly dependent on the DNA length. Fluorescence measurements (emission quenching and resonance energy transfer) show that two identical DNA strands do not make tight contacts in the RecA complex and are similar in magnitude to heterologous interactions. This conclusion is supported by caloriometric measurements, which show a large exothermic enthalpy change upon the recognition of complementary strands by the RecA filament, but not for binding of identical strands. Spectroscopic techniques, linear and circular dichroism, indicate that the complexes between RecA and pairs of either identical or complementary DNA strands still have rather similar overall structures. The present study thus reveals no significant interactions between identical single strands of DNA in the RecA filament *in vitro*.

RecA protein is the prototype of a universal class of proteins that play essential roles in homologous recombination (Ogawa et al., 1993; Story et al., 1993; Shinohara et al., 1993; Sung, 1994; Kowalczykowski & Eggelston, 1994). By polymerizing on single-stranded DNA, *Escherichia coli* RecA protein forms a right-handed helical nucleoprotein filament. This complex can recognize homology in duplex DNA, with which it assimilates to form a three-stranded nucleoprotein filament that can be several kilobases in length (Stasiak et al., 1984). The filament effects a switch of base pairs, creating heteroduplex DNA and a third strand, which it displaces in the 5′-to 3′-direction, a process which is fueled at a later stage by ATP hydrolysis (Howard-Flanders et al., 1984; Cox, 1994).

How the RecA single-stranded DNA filament recognizes duplex DNA molecules (Shibata et al., 1979; McEntee et al., 1979; West et al., 1980) is one of the central riddles of homologous recombination. Current models of the recognition process fall into two categories: base-pair models in which an interacting duplex melts locally to form a new set of Watson−Crick bonds with a third strand, and base-triplet models in which a single strand in the RecA filament recognizes homology within duplex DNA without breaking the Watson−Crick hydrogen bonds. Nonenzymatic precedents exist for both these recognition models, but formation of these structures requires special conditions (thermal D-loop formation; Beattie et al., 1977) or particular sequences (pyrimidine-purine-pyrimidine antiparallel triplexes; Moser & Dervan, 1987; Felsenfeld et al., 1957; Rajagopal & Feigon, 1989). In contrast to the pyrimidine triplexes, the RecA-driven reaction requires homologous rather than specific sequences, and like strands must be parallel in order to undergo exchange.

Recent experiments suggested that the RecA filament may use non-Watson−Crick interactions for recognition (Rao & Radding, 1993, 1994; Rao et al., 1995). Experiments with RecA single-stranded DNA filaments showed that not only were complementary oligonucleotides recognized when added to the RecA−DNA filament, but also oligonucleotides identical to the strand in the RecA filament were recognized. This type of recognition, termed self-recognition, was deduced from gel-shift and filter-binding assays.

In the work presented here, we used several physical methods to test further the idea that RecA protein can promote the recognition of identical DNA strands. We used fluorescence quenching of 2-aminopurine to detect base interactions, fluorescence resonance energy transfer to test for targeting to a specific site, isothermal titration calorimetry to assess interaction enthalpies, and linear and circular dichroism to probe the overall structures of RecA−DNA complexes.

EXPERIMENTAL PROCEDURES

Materials. RecA protein was purified as described (Shibata et al., 1981). ATPγS was from Boehringer Mannheim, and polynucleotide kinase was from New England Biolabs.

DNA Substrates. Single-stranded oligonucleotides were synthesized, end-labeled, and purified as described (Rao et al., 1993). Oligonucleotide sequences used are listed in Table 1.

DNA Substrates for Fluorescence Experiments. Oligonucleotides were synthesized on an Applied Biosystems

* Corresponding author. Fax: 46-31-7723858. Phone: 46-31-7723857. E-mail: wittung@phc.chalmers.se.
‡ Chalmers University of Technology.
§ Yale University School of Medicine.
|| UMR216 CNRS and Institut Curie.
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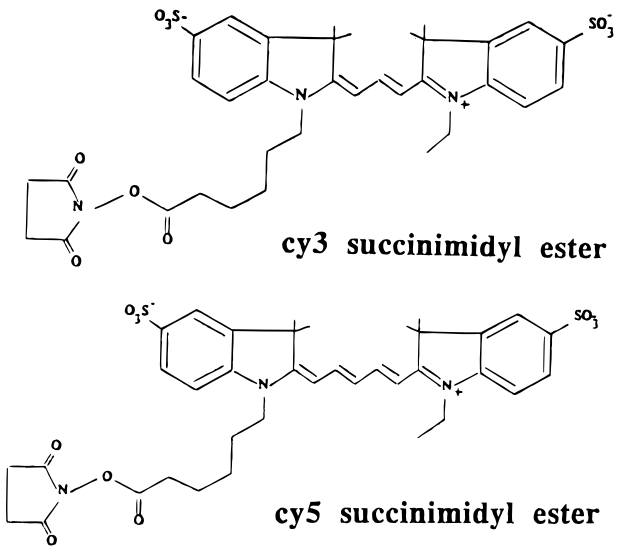
Table 1: DNA Sequences Used in This Study

label	sequence
(1) 83mer(−)	5′-TTG ATA AGA GGT CAT TTT TGC GGA TGG CTT AGA GCT TAA TTG CTG AAT CTG GTG CTG TAG CTC AAC ATG TTT TAA ATA TGC AA-3′
(2) 33mer(−)	5′-GGC TTA GAG CTT AAT TGC TGA ATC TGG TGC TGT-3′
(3) 26mer(−)	5′- GGC TTA GAG CTT AAT TGC TGA ATC TG-3′
(4) 20mer(−)	5′- GGC TTA GAG CTT AAT TGC TG-3′
(5) 83mer(+)	5′-TTG CAT ATT TAA AAC ATG TTG AGC TAC AGC ACC AGA TTC AGC AAT TAA GCT CTA AGC CAT CCG CAA AAA TGA CC T CTT ATC AA-3′
(6) 33mer(+)	5′-ACA GCA CCA GAT TCA GCA ATT AAG CTC TAA GCC-3′
(7) 26mer(+)	5′-ACA GCA CCA GAT TCA GCA ATT AAG CT-3′
(8) 20mer(+)	5′-ACA GCA CCA GAT TCA GCA AT-3′
(9) het 1	5′-ACC CAC TCG TGC ACC CAA CTG ATC TTC AGC-3′
(10) het 2	5′-CGA TTG GCG CGT ACC AGC TTA CCG CCG TTA CAG-3′
(11) het 3	5′-ACC CAC TCG TGC ACC CAA CTG ATC TTC AGC TAG-3′
(12) 43mer(−)[a]	5′-CGG ATG GCT T**X**G **X**GC TT**X** ATT GCT GAA TCT GGT GCT GAG CTC-3′
(13) 83mer(het)	5′-CGG TAC GTC ACG AGG TGG TGA GAT CAT CGA CAC GAG TAA ACG GAG TAC TGC CGT GTG CAG TTA CTG GAC TAC CGA CTA GCG CA-3′

[a] X = 2-aminopurine (fluorescent analog of adenine).

Scheme 1



DNA synthesizer (Model 380B) at Keck Biotechnology Resource Laboratory of Yale University. The 83mer(−) and 83mer (het) oligonucleotides were synthesized with a 3′ primary amine on a C_6 linker, supplied on phosphoramidate support by Glen Research. The 83mer(+) oligonucleotide was synthesized with the same primary amine on the 5′ end. The oligonucleotides were labeled with cy3 and cy5 succinimidyl esters (cyanine dyes purchased from Amersham) (Scheme 1) by reacting 5 mM DNA in 100 μL of 250 mM carbonate buffer, pH 9, with one packet of dye overnight at room temperature in the dark. Labeled oligonucleotides were purified as described in Rao et al. (1993). The absorbances at 260, 552, and 650 nm were used to calculate the concentrations of DNA, cy3, and cy5, respectively. All oligonucleotides had 100% incorporation of dye. 2-Aminopurine monomers were purchased from Glen Research and incorporated into a 43mer oligonucleotide at the Keck Biotechnology Resource Laboratory of Yale University.

Methods and Reaction Conditions. (A) Gel Retardation Assay. Nucleoprotein filaments were formed by incubating RecA protein (10 μM) with an 83mer, 43mer, or 33mer oligonucleotide (12 μM bases; sequences 1, 12, and 2, Table 1) at 37 °C for 12 min in 33 mM Pipes, pH 7.0, 1.2 mM magnesium acetate, 2 mM dithiothreitol, 100 μM bovine serum albumin, and 1 mM ATPγS. Pairing reactions were initiated by bringing the concentration of magnesium acetate up to 15 mM and adding 200 μM heterologous oligonucleotide as carrier (oligonucleotide 9, Table 1) and the appropriate amount of 5′-^{32}P-labeled oligonucleotide (identical or complementary sequences of various lengths, or heterologous 33mers; sequences 1−11, Table 1). The reaction continued at 37 °C for 10 min, except as noted; then aliquots were withdrawn, diluted 10-fold, and put on ice to stop the reaction. Immediately thereafter, 10% (v/v) glycerol, 0.1% (w/v) bromophenol blue, and 0.1% (w/v) xylene cyanol (final concentrations) were added to the aliquots. Aliquots were subjected to electrophoresis in a 0.55% agarose gel at 200 mA at room temperature with 40 mM Tris−acetate, pH 7.5, and 10 mM magnesium acetate as buffer. The gel was dried on DE-81 ion-exchange chromatography paper and autoradiographed. Counts associated with complexes as well as free oligonucleotides were quantitated with a PhosphorImager (Molecular Dynamics). Experiments performed with glutaraldehyde fixation include an additional 10 min incubation at 37 °C with 0.1% glutaraldehyde prior to electrophoresis.

(B) Fluorescence Emission Measurements. For the investigation of DNA−DNA interactions, 2-aminopurine was incorporated, replacing adenines at three positions in a 43mer oligonucleotide (sequence 12, Table 1). Identical reaction conditions to those described for the gel-retardation assay were applied. Emission from 2-aminopurine was measured in the interval 330−450 nm by excitation at 310 nm in a 2 mm × 2 mm quartz cell, thermostated to 37 °C. Data were collected for the 2-aminopurine oligonucleotide bound to the RecA filament prior to addition of a second (unlabeled) 33mer DNA (sequences 2, 6, 9, or 10) and after 10 min of incubation after this addition. The experiments were carried out on an SLM 8000C spectrofluorometer. Background fluorescence from the buffer was subtracted.

(C) Fluorescence Resonance Energy Transfer Measurements. All reactions were carried out at 37 °C in the same buffer as described for the gel-retardation assay. However, the magnesium acetate concentration was kept at 1 mM throughout the reaction, and instead of ATPγS, 1.2 mM ATP was included. RecA−DNA filaments were formed by incubating RecA (3.3 μM) with 10 μM 83mer(−)-cy3 (sequence 1, Table 1) or the unlabeled equivalent for 4 min. Then 83mer(+)-cy5 (sequence 5, Table 1), 83mer(−)-cy5, the heterologous 83mer-cy5 (sequence 13), or their unlabeled

primary amine counterparts were added and allowed to react for 2 min before fluorescence spectra were taken. The spectra were collected on an SLM 8000C spectrofluorometer at an angle of 54.7° between polarizers to eliminate polarization artifacts, and with rhodamine B as a standard. For each RecA−DNA binding experiment, three mixtures were prepared: one with cy3- and cy5-labeled DNA, another with cy3-DNA plus an unlabeled strand, and finally an unlabeled strand plus cy5-labeled DNA. Emission spectra of all mixtures were measured from 550 to 720 nm upon excitation at 545 nm. In addition, samples containing cy5-labeled DNA were excited at 645 nm and emission spectra collected from 655 to 720 nm. Background signal from the buffer was subtracted. Energy transfer manifested as the sensitized emission of cy5 was calculated according to the formula:

$$F_{545}^{\text{cy3,cy5}} - (I_{545,565}^{\text{cy3,cy5}}/I_{545,565}^{\text{cy3}})F_{545}^{\text{cy3}} - (I_{645,680}^{\text{cy3,y5}}/I_{645,680}^{\text{cy5}})F_{545}^{\text{cy5}}$$

where F represents the spectrum at an excitation of 545 nm and I represents the fluorescence intensity at a particular wavelength of excitation and emission, as specified by the subscripts (Rippe et al., 1992).

(D) Isothermal Titration Calorimetry. Isothermal titration experiments were performed in a microcalorimeter from MicroCal, Inc., with the temperature set to 27 °C. The enthalpy of interaction between two molecules is measured by injecting aliquots of a solution containing one of the molecules, in this case the DNA, into the other, containing the RecA−DNA complex, in the reaction cell. The change in heat requirement of the reaction cell to maintain isothermal conditions after each injection is measured. The required heat per injection is then determined, and the molar enthalpy for the binding interaction can be calculated. The RecA−DNA interaction experiments were performed in a buffer containing 5 mM cacodylic buffer, pH 6.8, 20 mM NaCl, 10 mM magnesium acetate, and 80 μM ATPγS. The RecA concentration was 2 μM in the reaction cell. Complexes between RecA and the first DNA were preformed (in a stoichiometric ratio of 1 RecA to 3 DNA bases) by incubation for 30 min at room temperature; then a solution containing 300 μM bases of the second DNA was added by a syringe in small aliquots (5 μL).

(E) Circular Dichroism Spectroscopy. Circular dichroism (CD), defined as the differential absorption of left and right circularly polarized light, was measured on a Jasco 720 spectropolarimeter using a 1 cm path-length strain-free quartz cell at room temperature. CD spectra of 83mer(−) and 83mer(+) DNA strands (sequences 1 and 5, Table 1) were measured separately, and in complex with RecA−83mer(−) filaments. For the RecA−DNA complexes with two DNA strands, the CD obtained from a separate measurement of only the RecA−83mer(−) filament was subtracted to yield the CD contribution purely from the second added DNA. Reactions were performed at room temperature in cacodylic buffer, pH 6.8, 20 mM NaCl, 5 mM magnesium acetate, and 50 μM ATPγS using 3 μM RecA and 9 μM of bases of oligonucleotides. Spectra were measured from 200 to 320 nm, and averaged over 8 scans.

(F) Linear Dichroism Spectroscopy. Linear dichroism (LD) is defined as the differential absorption of linearly polarized light, parallel and perpendicular, to the flow direction in a Wada-type Couette flow cell (Nordén et al., 1992b). LD detects deviation from isotropic distribution of light-absorbing molecules. Upon binding of RecA to single-stranded oligonucleotides, a negative LD around 260 nm arises consistent with the perpendicular orientation of the bases relative to the long axis of the RecA filament. LD was measured on a Jasco 500A spectropolarimeter with the samples oriented by a shear gradient of 1800 s^{-1} (600 rpm). Filaments with 1 μM RecA and 3 μM (in bases) 83mer oligonucleotides (sequence 1 or 5, Table 1) were formed using reaction conditions described for the gel retardation assay. Incubations were performed at room temperature. After initial LD measurement, a second DNA strand, either 83mer (sequence 1or 5) or 33mer (sequence 2, 6, 10, or 11), was added directly to the sample chamber and further incubated, and the LD was measured again.

RESULTS

Length Dependence for the Binding of Identical Oligonucleotides to RecA Filaments. By using an agarose gel-shift assay with radioactively labeled oligonucleotides, we investigated the binding efficiency of a second DNA molecule added to RecA−DNA filaments. The lengths of both the primary DNA oligonucleotide (on which RecA polymerizes) and the secondary DNA oligonucleotide added to RecA were varied. The sequences of the oligonucleotides were chosen to compare the binding of complementary and identical DNA strands (Table 1). We found that with the present sequences complementary oligonucleotides as short as 20 bases added as the second DNA were fully recognized by a RecA−DNA filament containing either an 83mer, 43mer, or a 33mer DNA strand. In contrast, binding of identical sequences, added as the second DNA strand to RecA−DNA filaments, fell drastically for oligonucleotides shorter than 33 bases, independent of the length of the primary DNA strand within the RecA filament (Figure 1a,b). No binding at all to RecA−DNA filaments could be detected for 26mer and 20mer identical oligonucleotides in our assays. Very similar results were obtained if, prior to electrophoresis, the RecA−DNA complexes were fixed with glutaraldehyde (results not shown). No significant binding of three heterologous DNA sequences (oligonucleotides 9−11, Table 1) added as the second DNA to RecA−DNA filaments was observed.

The RecA complexes with complementary DNA strands migrated as one distinct band in the agarose gel for all oligonucleotide lengths studied. In contrast, the RecA complexes with two identical DNA oligonucleotides were found to migrate as a somewhat smeared band, with minor bands moving with similar mobility to RecA−DNA filaments containing only one DNA strand, indicating that some dissociation may have occurred during electrophoresis (data not shown). This smearing decreased as the length of the second added oligonucleotide increased, in agreement with the observation above that the stability of complexes of identical strands increased with oligonucleotide length.

The time dependence of uptake of identical DNA strands of various lengths by RecA-83mer filaments was investigated. We found that maximal binding occurred within the first 10 min of incubation, and that further incubation did not significantly destabilize the RecA filaments formed (data not shown). For the shorter DNA sequences (26mer and

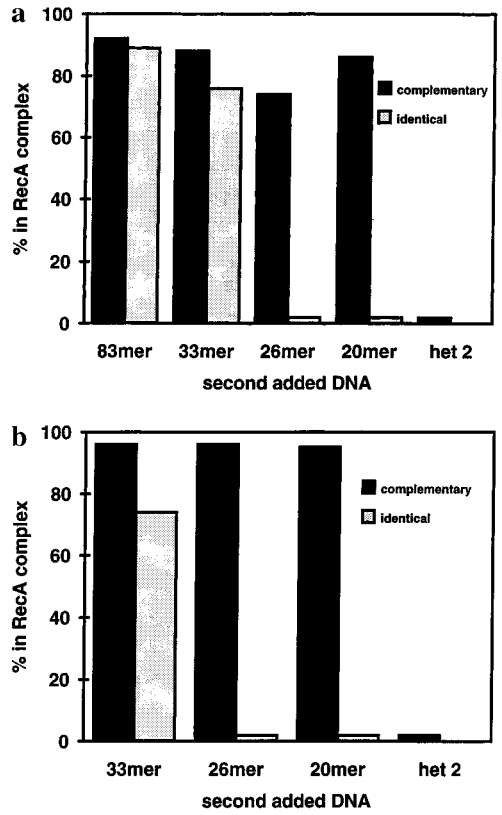


FIGURE 1: Gel retardation data showing the binding of oligonucleotides of various lengths to RecA−DNA filaments. (a) RecA−83mer(−) filaments (sequence 1, Table 1, in the filament; sequences 1−4, 5−8, and 10 added as second strands); (b) RecA−33mer(−) filaments (sequence 2, Table 1, in the filament; sequences 2−4, 6−8, and 10 added as second strands).

20mer), however, prolonged incubation did not result in an increased yield of complexes. Adding stoichiometrical excess of a second identical DNA, either 83mer or 33mer, increased the amount of binding to the RecA−83mer filament, but no increase was observed upon adding excess of identical 26mer or 20mer oligonucleotides (data not shown).

Interactions within the RecA Filament Studied by Fluorescence Spectroscopy. Fluorescence spectroscopy has been found useful for studying interactions between DNA strands in RecA filaments *in situ*, this is to say, without the need for deproteinization or electrophoretic separation (Wittung et al., 1994, 1995). The spectroscopic properties of a fluorescent probe attached to DNA report on the immediate environment of the probe and can thus provide information about interactions occurring in the RecA filament in real time. This technique was used to investigate the binding of identical DNA sequences by RecA filaments, and to compare directly the results to those obtained by electrophoresis.

A fluorescent analog of adenine, 2-aminopurine, was incorporated in place of adenine at three different positions in a 43mer oligonucleotide (sequence 12, Table 1). Since 2-aminopurine is an analog of adenine and can base-pair with thymine just as an adenine base does (Xu et al., 1994), it is expected to give minimal interference with the structure of the oligonucleotide. The 2-aminopurine-substituted oligonucleotide was used as the primary DNA strand in the RecA filament to report on interactions with incoming strands. Fluorescence was measured on the RecA−43mer complex alone, and after incubation with a second oligonucleotide

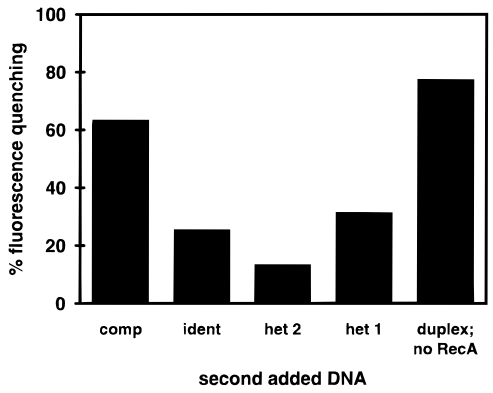


FIGURE 2: Quenching of 2-aminopurine fluorescence in substituted 43mer(−) (sequence 12, Table 1), reporting on the degree of interaction between this DNA molecule and an incoming 33mer oligonucleotide in the RecA filament (sequence 2, 6, 10, or 11). The amount of 33mers binding to the RecA−43mer filaments determined by the gel retardation assay is identical to that shown in Figure 1b for the RecA−33mer filaments.

(33mers with identical, complementary, or different sequences as the 43mer) (Figure 2). The amount of quenching of the 2-aminopurine fluorescence upon binding of the second DNA to the RecA−DNA filament may be related to the proximity of the added DNA strand to the 2-aminopurine moiety.

The degree of quenching of the 2-aminopurine fluorescence by identical DNA strands was of similar magnitude to the quenching detected when heterologous oligonucleotides were added as the second DNA (Figure 2). In contrast, we found a much larger quenching of the 2-aminopurine fluorescence with RecA−DNA complexes containing complementary DNA strands, almost as large as that obtained upon formation of a duplex with the two DNA strands in the absence of RecA. Quenching of fluorescence revealed nonspecific interactions with heterologous DNA strands that were not revealed by the gel-shift assay (compare fluorescence and gel-shift data in Figures 2 and 1b). Gel-retardation experiments were also perfomed with RecA−43mer filaments to which 33mers were added (data not shown). The results obtained for the RecA−43mer filaments were identical to that shown in Figure 1b for the RecA−33mer filaments.

Occupation of the Second Site Studied by Energy Transfer. Fluorescence resonance energy transfer between probes attached to one end of 83mer oligonucleotides was used to demonstrate the simultaneous binding and orientation of two DNA strands in the RecA filament (Rippe et al., 1992). RecA filaments were formed on the 83mer(−) oligonucleotide (sequence 1, Table 1) labeled at one end with the fluorescent probe cy3 (a cyanine dye), which acted as the donor in energy transfer. The 83mer(+), 83mer(−), or heterologous 83mer oligonucleotides (sequences 5, 1, and 13, respectively, Table 1) labeled with cy5, another cyanine dye acting as the acceptor, were added to the cy3-labeled RecA−DNA filaments. The probes were attached such that they would be on the same end of the RecA−DNA complex when complementary oligonucleotides were antiparallel and when identical or heterologous oligonucleotides were parallel. The emission maxima of cy3 and cy5 are 567 and 667 nm, respectively. Energy transfer occurred when the two probes were close in space and resulted in a decrease in cy3 emission and an increase in cy5 emission when the cy3 was selectively

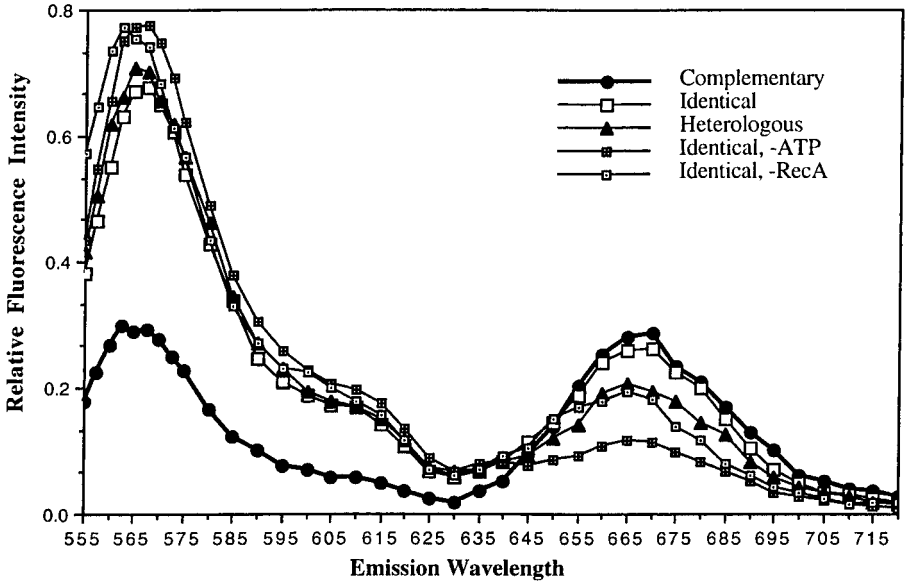


FIGURE 3: Emission spectra in the presence of RecA of complementary, identical, and heterologous 83mers labeled with cy3 and cy5, reflecting fluorescence resonance energy transfer. The specific pairings were RecA−83mer(−)-cy3 (sequence 1, Table 1) plus 83mer(+)-cy5 for complementary, 83mer(−)-cy5 for identical, or 83mer-cy5 for heterologous interactions (sequences 5, 1, and 13). Excitation was at 545 nm. Fluorescence intensities are relative to a rhodamine B standard. Raw data are shown here; Table 2 contains corrected data after subtraction of the background cy3 and cy5 emissions based on reactions containing only one or the other probe.

Table 2: Calculated Energy Transfer, Expressed as an Increase in cy5 Emission, between DNA Strands in RecA Filaments

RecA−DNA complex	added DNA	% increase in emission
RecA−83mer(−)	83mer(+)	440
RecA−83mer(−)	83mer(−)	179
RecA−83mer(−)	83mer(het)	236
RecA−83mer(−)	83mer(−), *−ATP*	41

Table 3: Enthalpies (Calories per Mole of DNA Base) for Interactions of RecA−DNA Complexes with a Second Oligonucleotide, and for Interactions of RecA with an Oligonucleotide in the First DNA Binding Site

RecA−DNA complex	added DNA	ΔH (kcal/mol)[a]
RecA−33mer(−)	33mer(+)[b]	−3.8
RecA−33mer(−)	33mer(−)[b]	not detectable
RecA	33mer(−)	−1.5
RecA	83mer(−)	−2.0

[a] Estimated error is ±0.3 kcal/mol. [b] 10 μM heterologous 30mer oligonucleotide (sequence 9) present upon titration of the second strand.

excited at 545 nm (Figure 3). In Figure 3, one can see the strong quenching of cy3 at 567 nm and the enhancement of cy5 at 667 nm due to energy transfer upon addition of a complementary oligonucleotide to the RecA−DNA filament. Variations in the background emission by cy3 and cy5 obscure the differences in energy transfer between complementary and identical reactions, but once the background is subtracted (see Experimental Procedures for details), the differences are clear (Table 2). Identical and heterologous interactions in the RecA filament yield roughly equal enhancements of cy5 emission which are only half those of complementary interactions, but are 5 times larger than in the absence of ATP. No energy transfer is seen in the absence of RecA.

Energetics of RecA−Filament Interactions. Isothermal titration calorimetry is useful for thermodynamic analysis of protein−DNA interactions (Wiseman et al., 1989; Freire et al., 1990; Ladbury, 1995). The release of heat detected upon mixing reagents is converted to molar interaction enthalpy, and when followed as a function of mixing ratio may also provide equilibrium association constants and thus reaction entropy. We studied the energetics of RecA−33mer(−) filaments (sequence 2, Table 1) binding a second 33mer oligonucleotide that was either complementary or identical to the first DNA under similar conditions to those that produced gel-shifts. There was no detectable release of heat associated with identical recognition, whereas for complementary recognition within the RecA filament a large negative reaction enthalpy was found (3.8 kcal/mol of base, Table 3). Also shown in Table 3 are interaction enthalpies (calories per mole of DNA bases) obtained for the primary binding of RecA to two oligonucleotides (*i.e.*, to the first DNA binding site) which suggest no significant variation depending on the length of the oligonucleotide.

Structure of RecA Complexes with Identical Strands. Circular dichroism was measured in an attempt to assess the conformation of the second DNA strand in a RecA−DNA complex containing identical 83mer DNA strands compared to a complex containing complementary 83mer DNA strands. CD of the primary RecA−DNA filament was subtracted from the total CD of the RecA complex with two strands, to give only the CD contribution from the second DNA. No significant differences in the overall of conformation of identical and complementary DNA added as the second DNA to RecA filaments were observed (Figure 4). In both cases, the CD spectrum of the second DNA indicates a right-handed DNA conformation, but it is somewhat different compared to the CD of the free DNA strands. This difference can be ascribed to a stretched and unwound DNA conformation within the RecA filament (Takahashi, 1989; DiCaupa et al., 1982; Kubista et al., 1990).

Linear dichroism, using rotating cylinders generating shear flow to align the DNA molecules hydrodynamically, showed similar magnitudes of increase in orientation whether adding a complementary or an identical 83mer to preformed RecA−

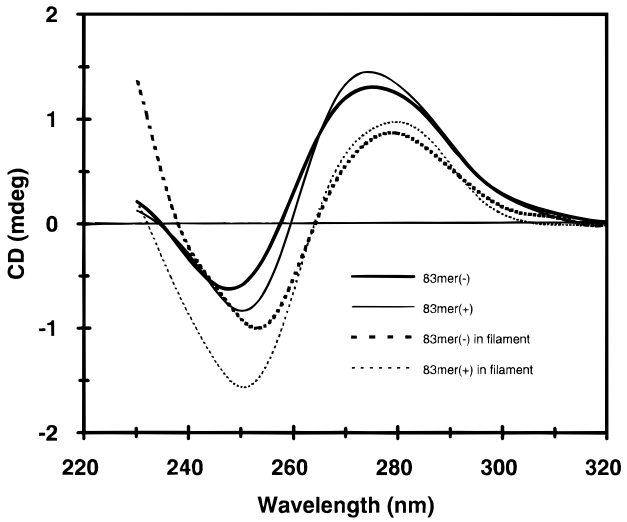


FIGURE 4: Circular dichroism as an indicator of the avarage secondary structure of the 83mer(−) and 83mer(+) oligonucleotides (sequences 1 and 5, Table 1), free in solution or bound as the second DNA to RecA−83mer(−) filaments. The CD contribution from the primary RecA−83mer(−) filament was subtracted to obtain the CD for only the second bound DNAs.

83mer(−) complexes (sequence 1, Table 1; Figure 5a). This shows the RecA−DNA filament to be able to bind another 83mer regardless of its sequence and adopt a stiffer structure with a higher degree of alignment in the flow.

We also monitored by LD the interaction of RecA−83mer filaments with 33mers of various sequences. Some negative increase in LD intensity at 260 nm was observed upon addition of identical or heterologous DNA strands, verifying the binding of all these DNAs (Figure 5b). This increase in negative LD signal indicates that the bases of the second bound DNA are also (as observed for the first DNA in RecA) oriented perpendicular to the RecA−fiber axis. The change in LD was somewhat larger upon adding identical DNA strands compared to heterologous strands.

In contrast, adding a complementary 33mer to RecA−83mer filaments diminished the LD signal (Figure 5b). Starting with either (+) or (−) strands of the 83mer (sequence 1 or 5, Table 1) in RecA showed identical results upon adding the complementary 33mer (data not shown). We may speculate that this somewhat unexpected result may be due to the very tight binding of a complementary 33mer to only the target sequence (in the center of the 83mer), in contrast to the binding of identical or heterologous DNA sequences which is looser and may occur anywhere along the 83mer strand in the RecA filament, not preferentially at the target sequence. The pitch of RecA filaments is suggested to be different between fiber structures of RecA complexes with single-stranded DNA compared to RecA complexes with double-stranded DNA (Hewat et al., 1991; Nordén et al., 1992a). Thus, an explanation for the reduced LD is that the formation of a double-stranded DNA structure inside an established RecA−ssDNA filament could subject the helical filament to forces which may give rise to disorder seen as poor alignment in the shear flow. (This observation will be further explored in other studies.)

DISCUSSION

In order to understand the mechanism of recognition of homologous DNA molecules mediated by the RecA protein, we have studied the binding of identical oligonucleotides

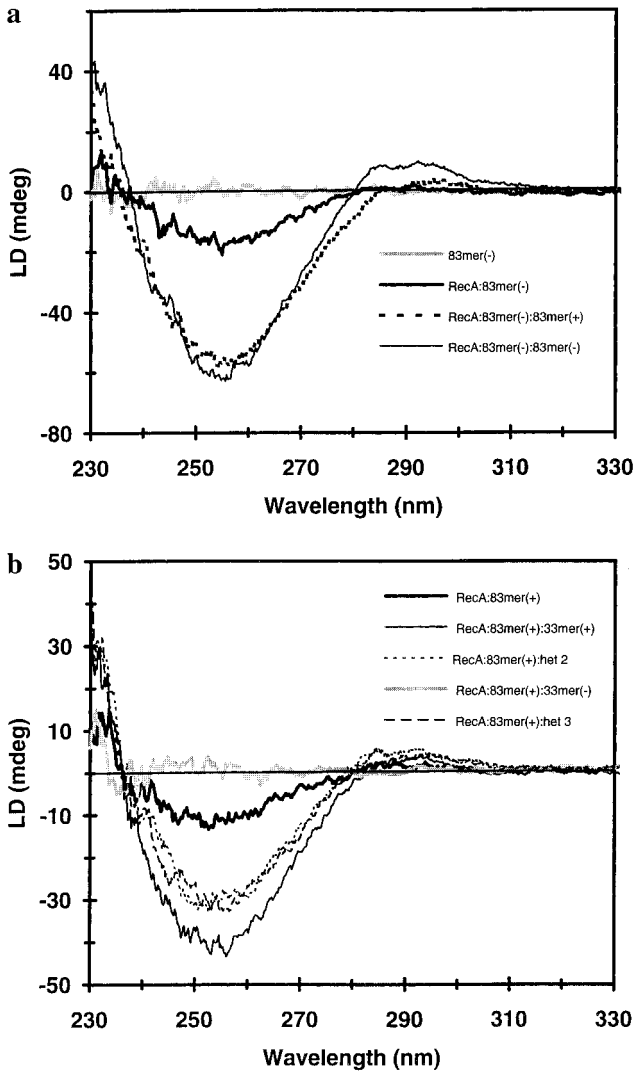


FIGURE 5: Linear dichroism obtained by a shear flow of 600 rpm of RecA−83mer filaments before and after adding a second DNA strand. The observed negative signal around 260 nm correlates with the perpendicular orientation of the DNA bases in the RecA filament. (a) 83mer(−) as first DNA in RecA filament; 83mer(−) or 83mer(+) (sequence 1 or 5, Table 1) as second added DNA. (b) 83mer(+) as first DNA in RecA filament; various 33mers (sequence 2, 6, 10, or 11) added as second DNA.

by RecA−DNA filaments, as well as the structure of and base-proximity in such protein−DNA complexes. The results obtained were compared with corresponding data obtained with oligonucleotides of complementary or nonrelated DNA sequences.

The binding of identical DNA sequences to RecA−DNA filaments (verified by a gel-retardation assay) is found to depend on the length of the added DNA, with a sharp decrease in the stability of the complex formed with sequences less than 33 bases long. In contrast, the binding of complementary DNAs to RecA−DNA filaments is efficient even for very short sequences. This indicates that the interaction between identical DNA sequences in RecA is weak compared to the protein-promoted recognition between complementary DNA sequences.

Weak interactions between identical DNA sequences in RecA were also observed by fluorescence measurements. Fluorescent probes incorporated in the DNA (a 43mer oligonucleotide) show RecA-mediated interactions between DNA strands with complementary sequences to be significantly stronger than between identical DNA strands. Non-

specific, weak interactions of the RecA−DNA filaments with DNA of any sequence are detected, and are similar in magnitude to the interactions between identical DNAs in RecA.

Energy transfer between probes attached to the ends of the 83mer DNA strands was also used to study interactions between the two strands in the RecA−DNA filament. The observed difference in energy transfer between complementary oligonucleotides compared to between identical or heterologous oligonucleotides may have several explanations. The proportion of RecA−DNA complexes with the second site filled could be higher when adding complementary strands, due to stabilization by the formation of base-pairs. Furthermore, the complementary strands may be in closer proximity, resulting in more efficient energy transfer. In RecA complexes with identical or heterologous DNA strands, the strands may not be in register, or could be oriented both parallel and antiparallel to each other, such that only part of the RecA−DNA complexes have the probes situated at the same end.

A somewhat larger energy transfer was observed upon adding heterologous 83mers compared to identical 83mers to RecA−DNA filaments, which could reflect a sequence preference by RecA in the second DNA binding site. Alternatively, it may suggest the presence of secondary structure in the identical oligonucleotide that may hinder binding to some extent. The heterologous 83mer, however, had more predicted secondary structure than the identical 83mer according to the GCG stemloop software (Wisconsin Package, version 8.1, Genetics Computer Group).

Calorimetric data show no interaction energy associated with recognition between identical DNA strands in RecA, in contrast to a large enthalpy found for complementary DNA interactions in the RecA filament. These results correlate well with the fluorescence data, where both the quenching behavior and the energy transfer results suggest that much stronger interactions occur in RecA between complementary DNA strands than between identical or heterologous DNA strands.

The LD and CD results obtained using RecA−DNA filaments with 83mer DNA strands show that, at least for RecA−DNA complexes with 2 oligonucleotides 83 bases or longer, the structure of the protein filaments and the arrangement of the DNA strands within the filament are independent of DNA sequence (complementary or identical). CD shows the second added DNA to RecA−DNA complexes to adopt a right-handed conformation in the case of both identical and complementarty strands. The slight modification of the shape of the CD spectrum of bound DNA relative to free may be related to a stretching of the DNA strand when bound in RecA. LD shows that also the DNA bases of the second added strands are almost perpendicular to the RecA fiber axis, verified for pairs of both identical and complementary DNA strands in the RecA filaments. Thus, the bases of the first and the second DNA molecules are in proximity to be base-paired, if possible.

The physical data presented here do not support the previous conclusion (Rao & Radding, 1993, 1994) that the RecA protein can promote the recognition of identical DNA sequences. While this work was in progress, data were presented which revealed that the binding of oligonucleotides to the second DNA binding site in a RecA filament is strongly influenced by DNA sequence (Martin & Kowalczykowski, 1996).

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