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Homologous recognition promoted by RecA protein via non-Watson–Crick bonds between identical DNA strands

(recombination/recognition of homology/triplex DNA/hydrogen bond)

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ABSTRACT **The RecA protein of *Escherichia coli* forms a nucleoprotein filament that promotes homologous recognition and subsequent strand exchange between a single strand and duplex DNA via a three-stranded intermediate. Recognition of homology within three-stranded nucleoprotein complexes, which is probably central to genetic recombination, is not well understood as compared with the mutual recognition of complementary single strands by Watson–Crick base pairing. Using oligonucleotides, we examined the determinants of homologous recognition within RecA nucleoprotein filaments. Filaments that contained a single strand of DNA recognized homology not only in a complementary oligonucleotide but also in an identical oligonucleotide, whether their respective sugar-phosphate backbones were antiparallel or parallel, and a filament that contained duplex DNA showed the same polymorphic versatility in the recognition of homology. Recognition of self by a filament that contains a single strand reveals that RecA filaments can recognize homology via non-Watson–Crick hydrogen bonds. Recognition of multiple forms of the same sequence by duplex DNA in the filament shows that it primarily senses base-sequence homology, and suggests that recognition can be accomplished prior to the establishment of new Watson–Crick base pairs in heteroduplex products. However, unlike the initial recognition of homology, strand exchange is stereospecific, requiring the proper antiparallel orientation of complementary strands.**

Homologous genetic recombination is a reaction of broken DNA molecules: breaks are repaired by aligning complementary bases to restore precisely the integrity of the original DNA sequence. There appear to be two paradigms by which such repair occurs (1). The simpler paradigm involves the splicing of overlapping sequences, in which case rejoining results directly from the renaturation of two complementary single-stranded ends. A second paradigm, revealed by the action of RecA protein, is more complicated, since homology is recognized not by the pairing of two single strands but rather by the pairing of a single strand with a fully duplex homolog. Recent research has supported the hypothesis that both the recognition of homology and the strand exchange that follows are mediated by triple-helical intermediates (1–7). RecA protein creates such triplex intermediates from nonspecialized sequences, viz., sequences other than homopurine–homopyrimidine tracts, which create a different kind of triplex DNA (8–11). The structure of the triplex intermediate that is produced by RecA protein, which is under active investigation, is not known (1, 12, 13).

The starting point for the basic reactions of RecA protein is the right-handed helical nucleoprotein filament that it makes on single-stranded DNA (14, 15). Such a filament will pair with naked complementary single strands or with naked duplex DNA. In the former case, pairing produces heteroduplex DNA that remains in the filament; in the latter case, pairing leads to strand exchange, which leaves heteroduplex DNA in the filament and produces a displaced single strand that is identical in sequence to the (+)-strand contained in the filament (16, 17). Whereas the ultimate formation of stable heteroduplex joints in homologous recombination is indisputably based on Watson–Crick base pairing, the process of recognition and the structure of the intermediates are not well understood. In the experiments described below, we used oligonucleotides to examine the determinants of homologous recognition within RecA nucleoprotein filaments.

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METHODS

Materials. RecA protein was purified as described (18). Polynucleotide kinase was from New England Biolabs and proteinase K was from Boehringer Mannheim.

DNA Substrates. Circular single-stranded DNA (ssDNA) of phage M13 was prepared as described (19, 20). Single-stranded oligonucleotides were synthesized, end-labeled, and purified as described (ref. 1; see Table 1 and Fig. 1). Standard reaction conditions and assays are described in legends.

RESULTS

Polymorphic Recognition. Circular ssDNA [designated as (+)-strands by convention], which had been coated with stoichiometric levels of RecA protein in the presence of ATP, an ATP-regenerating system, 1 mM Mg^{2+}, and buffer, was incubated in the presence of 15 mM Mg^{2+} with the standard set of 5′-^{32}P-labeled 33-mer oligodeoxynucleotide substrates (Table 1 and Fig. 1).

To block nonspecific interactions of labeled homologous oligonucleotides with the nucleoprotein filament, we added excess unlabeled heterologous oligonucleotide at 10 times the nucleotide concentration of DNA in the filament. The size of the homologous target in the filament was only 1/200th of the M13 ssDNA genome and the labeled homologous oligonucleotides that were paired with that site were in only 2-fold molar excess to that site. To detect complexes of the labeled oligonucleotide with the nucleoprotein filament, we used Ultrafree-MC cellulose filters (Millipore). When samples were centrifuged through these filters at room temperature, oligonucleotide bound to the nucleoprotein filament was captured on the membrane, whereas free 33-mer oligonucleotides were not (1).

The RecA nucleoprotein filament containing a (+)-strand of DNA recognized not only a complementary (−)-strand oligonucleotide but also a homologous (+)-strand oligonucleotide, whether their respective sugar-phosphate backbones were antiparallel or parallel (Fig. 2 *A* and *B*). The low retention of a heterologous oligonucleotide (oligonucleotide V, Table 1) indicated that recognition was dependent on sequence homology (Fig. 2*A*).

Table 1. Sequences of oligodeoxynucleotides

No.	Sequence (5′ to 3′)	No. of complementary sites in M13 ssDNA with x% homology				
		100%	90%	70%	60%	50%
I	ACA-GCA-CCA-GAT-TCA-GCA-ATT-AAG-CTC-TAA-GCC	0	0	0	0	15
II	ACG-GTT-TTT-CGC-CCT-TTG-ACG-TTG-GAG-TCC	0	0	0	0	16
III	TTT-ACG-ATT-ACC-GTT-CAT-CGA-TTC-TCT-TGT	0	0	0	1	19
IV	CGA-TTG-ATG-CGT-ACC-AGC-TTA-CCG-AAG-TTA	0	0	0	0	15
V	ACC-CAC-TCG-TGC-ACC-CAA-CTG-ATC-TTC-AGC	0	0	0	1	14

I–III are homologous (+)-strand sequences located at positions 207, 5742, and 6024, respectively, in the M13 genome (21). IV and V are heterologous sequences. Complementary sites in M13 were sought I–V by using the DNANALYZE program (30). x% homology represents the minimum level of Watson–Crick base-pair matches that were demanded to score any site in the M13 (+)-strand as a complementary site.

In other experiments, we observed a higher background of nonspecific retention of oligonucleotides, either when the circular ssDNA was heterologous or when 100-fold excesses of each homologous oligonucleotide were used to reduce the specific activity (Fig. 3*A*). However, these two ways of assessing the nonspecific background gave similar values (Fig. 3). After subtracting each kind of blank and averaging the net values, we calculated that the number of moles of oligonucleotide retained per mole of nucleoprotein filament was 1.3 for the complementary (−)-strand oligonucleotide, 0.66 for the identical (+)-strand oligonucleotide, and 0.92 and 0.78, respectively, for (−)-strand and (+)-strand oligonucleotides with reversed backbones. A stoichiometric relationship approaching 1 oligonucleotide bound per molecule of M13 ssDNA in the RecA filament suggests that there were not multiple sites of association.

Other controls showed that RecA protein and ATP were required for homology-specific retention of all oligonucleotides (Fig. 3*A*). Although homology-specific recognition of all four oligonucleotides occurred, only those complexes formed between two antiparallel complementary strands survived deproteinization with SDS and proteinase K (Fig. 3*A*).

Self-Recognition. The homology-specific retention of the identical (+)-strand oligonucleotide was confirmed with sequences derived from two other loci in the M13 genome: 0.62 mol of oligonucleotide II and 0.6 mol of oligonucleotide III (see Table 1) were bound per mol of RecA nucleoprotein filament.

In addition, by a computer search, we looked for fortuitous complementary sequences in the M13 DNA for the three homologous (+)-strand oligonucleotides (I–III, Table 1), as well as for two heterologous oligonucleotides (IV and V). At degrees of homology between 100% and 70%, we found no sites complementary to any of these five oligonucleotides. Only when a search was made for sites that were 60%

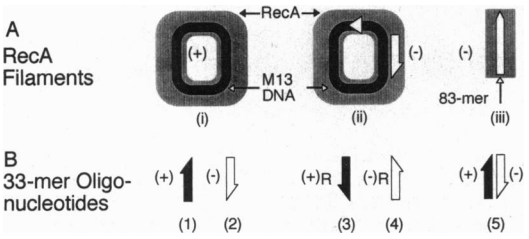


FIG. 1. Substrates. (*A*) RecA nucleoprotein filaments were formed on M13 circular ssDNA (*i*) or on the latter to which a complementary 33-mer oligonucleotide had been annealed (*ii*), or on an 83-mer oligonucleotide (*iii*). (*B*) The standard set of oligonucleotide variants of the sequence at a single site. Except as otherwise noted, oligonucleotide I [(+) (see Table 1)], its complement (−), and their variants with reversed sugar-phosphate backbones [(+R) and (−R)] made up the standard set of oligonucleotides used in all of the experiments described. All oligonucleotides were synthesized in a DNA synthesizer (Applied Biosystems model 391).

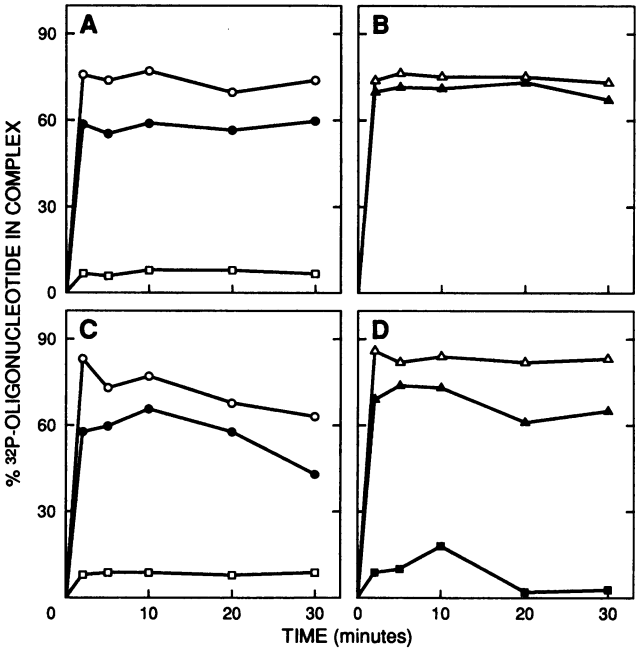


FIG. 2. Polymorphic recognition of homology. (*A* and *B*) Homologous recognition by a RecA nucleoprotein filament containing ssDNA. Filaments were formed by incubating 20 μM M13 circular ssDNA with 6.7 μM RecA at 37°C for 12 min in 33 mM Pipes, pH 7.0/1.2 mM $Mg(OAc)_2$/2 mM dithiothreitol/1.2 mM ATP/8 mM phosphocreatine containing creatine kinase (10 units/ml) and bovine serum albumin (100 μg/ml). Five separate pairing reactions were initiated by bringing $Mg(OAc)_2$ to 15 mM, adding 200 μM heterologous single-stranded oligonucleotide as carrier (30-mer IV in Table 1) and 0.2 μM 5′-^{32}P-labeled oligonucleotide [one of the standard set of four homologous oligonucleotides (Fig. 1*B*) or a 5′-^{32}P-labeled heterologous control (V in Table 1)]. As the reaction continued at 37°C, 5-μl aliquots were diluted into 95 μl of 30 mM Pipes, pH 7.0/15 mM $Mg(OAc)_2$/2 mM dithiothreitol/1.2 mM ATP and immediately centrifuged at room temperature through Ultrafree-MC cellulose filters (Millipore) at 2000 × g for 6 min. An additional 50 μl of dilution buffer was added as a wash, with subsequent centrifugation for 3 min. Counts retained on the filters were expressed as percent total input. (*C* and *D*) Homologous recognition by a RecA nucleoprotein filament containing duplex DNA. Filaments were formed with 5 μM RecA and 15 μM M13 circular ssDNA to which a complementary 33-mer had been annealed [Fig. 1 (*ii*)]. Reactions were initiated, stopped, and assayed as above, except that unlabeled heterologous carrier oligonucleotides were at 150 μM, labeled single-stranded oligonucleotides were at 0.15 μM, and labeled duplex oligonucleotide was at 0.3 μM. Six separate pairing reactions were done with ^{32}P-labeled oligonucleotides: one with each of the four homologous oligonucleotides (Fig. 1*B*), one with heterologous V (Table 1), and one with a duplex made by annealing (+)-strand I with its complement. ●, (+) sequence (Fig. 1); ○, (−) sequence; ▲, (+)R sequence; △, (−)R sequence; ■, duplex oligonucleotide described above; □, heterologous oligonucleotide IV.

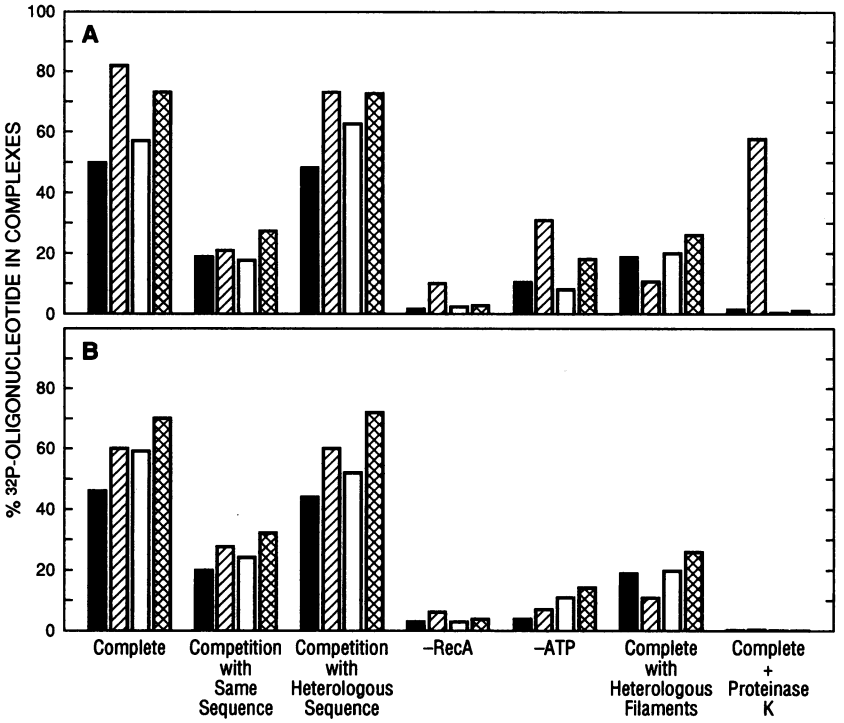
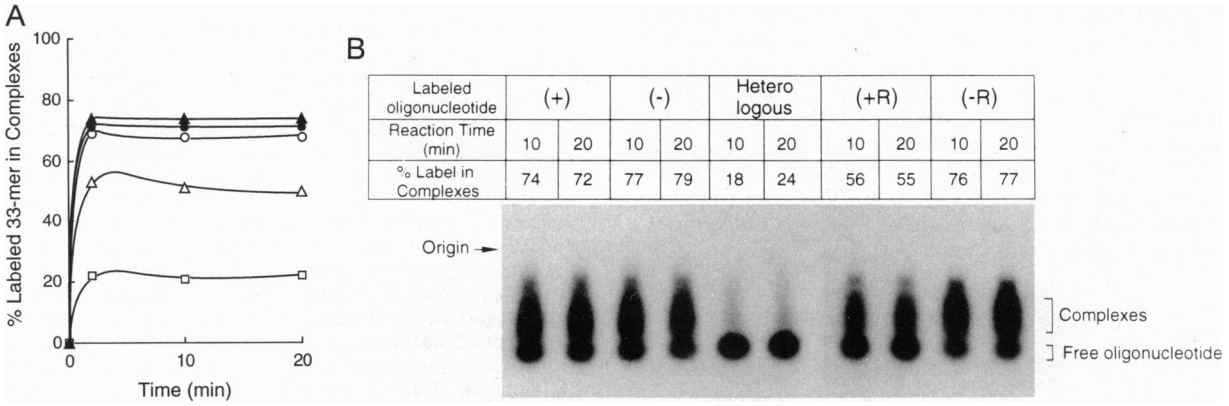


FIG. 3. Specificity of the recognition reactions. (*A*) RecA nucleoprotein filaments containing ssDNA. Complete pairing reactions were done between M13 circular ssDNA and 5′-^{32}P-labeled oligonucleotides under standard conditions as described for Fig. 2 *A* and *B*. The reactions were assayed at 15 min in duplicates, the average of which is expressed on the ordinate. In controls lacking RecA protein, an equal volume of RecA buffer was substituted: 50 mM Tris·HCl, pH 7.5/0.5 mM EDTA/5 mM dithiothreitol/10% (vol/vol) glycerol. This control otherwise contained all components of a standard reaction mixture, which included bovine serum albumin (100 μg/ml). All competition reactions were done in the presence of a 100-fold excess (20 μM) of unlabeled competitor oligonucleotide. φX174 circular ssDNA replaced M13 DNA in the reactions labeled "Complete with Heterologous Filaments." In the last set of controls, samples were deproteinized at 37°C for 30 min with SDS (0.4%) and proteinase K (400 μg/ml). (*B*) RecA nucleoprotein filament containing duplex DNA. Pairing reactions were done as described for Fig. 2 *C* and *D*. Filled bars, (+) sequence; hatched bars, (−) sequence; open bars, (+)R sequence; cross-hatched bars, (−)R sequence.

homologous or less did we find partially complementary sites in ssDNA. The numbers of such sites were similar for all five sequences (Table 1). Since homologous (+)-strand oligonucleotides and heterologous oligonucleotides did not differ with respect to partially complementary sites in M13 ssDNA, we attribute the specific binding of (+)-strand oligonucleotides by the M13 (+)-strand in RecA filaments to homology-dependent self-recognition.

Recognition Between Oligonucleotides Assessed by Both a Filter Assay and a Gel Retardation Assay. To confirm that RecA filaments can recognize both identical and complementary sequences, we did experiments in which both single strands were oligonucleotides. This further eliminated concerns about fortuitous recognition at other sites and, in addition, made it possible to assay the products by agarose gel electrophoresis. Filaments were formed on an 83-mer oligonucleotide in the middle of which was located a sequence of 33 nucleotides that was complementary to (+)-strand oligonucleotide I (Table 1 and Fig. 1). The imbedded homologous 33-mer sequence was incubated with the standard set of 33-mers (Fig. 1). To stabilize the RecA filament formed on the 83-mer and to minimize the possible simulta-



Labeled oligonucleotide	(+)		(-)		Hetero logous		(+R)		(-R)	
Reaction Time (min)	10	20	10	20	10	20	10	20	10	20
% Label in Complexes	74	72	77	79	18	24	56	55	76	77

FIG. 4. Polymorphic recognition between oligonucleotides. (*A*) Filter assay. Nucleoprotein filaments were formed by incubating RecA protein (7.2 μM) with an 83-mer oligonucleotide [(−) sequence] (12 μM) under standard conditions (legend to Fig. 2) except for the substitution of adenosine 5′-[γ-thio]triphosphate for ATP. The sequence of the 83-mer was 5′-TTGATAAGAGGTCATTTTTGCGGATGGCTTAGAGCT-TAATTGCTGAATCTGGTGCTGTAGCTCAACATGTTTTAAATATGCAA-3′. The letters in boldface correspond to the complement of (+)-strand oligonucleotide I (Table 1). Five separate pairing reactions were initiated by adding 15 mM magnesium acetate, 120 μM heterologous single-stranded carrier oligonucleotide (30-mer oligonucleotide IV, Table 1) and 10 μM 5′-^{32}P-labeled homologous oligonucleotides from the standard set (Fig. 1) or 10 μM 5′-^{32}P-labeled heterologous oligonucleotide V (Table 1). At various times, 8-μl aliquots were diluted into 900 μl of 30 mM Tris·HCl, pH 7.5/15 mM magnesium acetate and immediately filtered through alkali-treated filters (4). The filters were then washed with 1 ml of the same buffer. Counts retained on the filters were expressed as the percentage of total input (+) sequence (●), (−) sequence (○), (+)R sequence (△), (−)R sequence (▲), or heterologous oligonucleotide V (Table 1) (□). (*B*) Gel retardation assay. From the above reactions 30-μl aliquots were withdrawn at various times, diluted with 270 μl of 30 mM Hepes, pH 7.2/15 mM magnesium acetate, and subsequently made to contain 10% (vol/vol) glycerol, 0.1% (wt/vol) bromophenol blue, 0.1% (wt/vol) xylene cyanol. Aliquots (30 μl) were electrophoresed in a 0.55% agarose gel at 200 mA and 350 V (17.5 V/cm) at room temperature with 40 mM Tris acetate, pH 7.5/10 mM magnesium acetate as buffer. The buffer was circulated at about 300 ml/hr through a peristaltic pump. The gel was dried on a DE-52 ion-exchange sheet and autoradiographed. Counts associated with complexes as well as the free oligonucleotide were quantitated with a PhosphorImager (Molecular Dynamics).

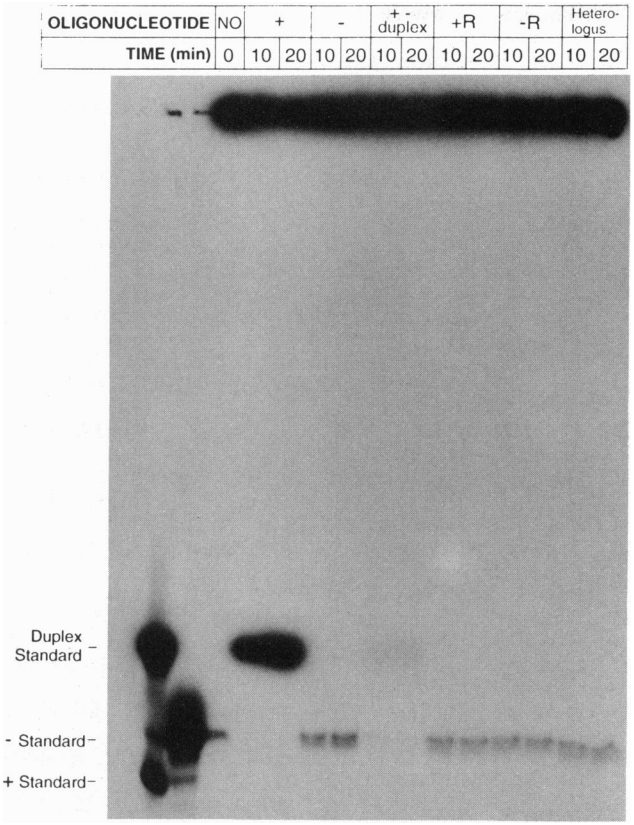


FIG. 5. Stereospecificity in strand-exchange reactions. RecA nucleoprotein filaments were formed under the standard reaction conditions containing an ATP regeneration system (see legend to Fig. 2 *A* and *B*) with 5.2 μM RecA protein and 13 μM M13 circular ssDNA to which had been annealed the 5′-^{32}P-labeled complementary 33-mer from the standard set [Fig. 1 (*ii*)]. In six separate reactions, pairing and strand exchange were initiated by adding 1.5 μM single-stranded oligonucleotide (3.0 μM in the case of duplex oligonucleotide) and 15 mM magnesium acetate. At 10 and 20 min of incubation, 30-μl aliquots were treated with 20 mM EDTA (pH 8.0), 0.5% SDS, and proteinase K (200 μg/ml) and incubated at 37°C for an additional 20 min. The samples were analyzed by electrophoresis in a polyacrylamide gel under nondenaturing conditions in a cold room (5% polyacrylamide in spacer gel; 15% polyacrylamide in the bottom gel; 89 mM Tris borate, pH 7.8/2 mM EDTA as buffer; 48 cm × 20 cm × 0.1 cm; 600 V and 150 mA; 18 hr), followed by autoradiography. The − and + standards were 33-mer single-stranded oligonucleotides of (−) and (+) sequence, respectively; the duplex standard was a 33-bp annealed product between + and − standards.

neous coating of the 33-mer by RecA protein, we coated the 83-mer first with an amount of RecA protein that was not in excess, and we used adenosine 5′-[γ-thio]triphosphate as the nucleotide cofactor.

Both a filter assay and gel electrophoresis, neither of which involved prior deproteinization, showed that the RecA nucleoprotein filament recognized all four homologous oligonucleotides (Fig. 4). By both assays, after correction for background, the molar ratios of oligonucleotide incorporated per nucleoprotein filament ranged from 0.6 to 1.0 for the four homologous oligonucleotides. Once again, the apparently stoichiometric association of homologous oligonucleotides with the filament supports the conclusion that specific homologous complexes were formed (see *Polymorphic Recognition* above).

Recognition of Homology in Triplexes Is Polymorphic. Although a kinetic barrier favors the binding of RecA protein to single-stranded vs. double-stranded DNA (22), the protein readily forms a filament on duplex DNA that contains a single-stranded tail or gap, and such filaments are active in homologous pairing and strand exchange (19, 23, 24). In the same way as described above for the filament containing ssDNA, we used the filament containing duplex DNA at a single homologous site to investigate the steric requirements for homologous recognition when three strands of DNA are involved.

The tract of duplex DNA was made either by RecA-mediated pairing or by thermal annealing of M13 circular (+)-strand DNA with the 33-mer (−)-strand oligonucleotide from the standard set [Fig. 1 (*ii*)]. M13 circular DNA with preannealed oligonucleotide was separated from free 33-mer by filtration through Sepharose 2B (1). To monitor the annealing as well as purification, we mixed the 33-mer (−)-strand oligonucleotide with about 50,000 cpm of 5′-labeled oligonucleotide of the same sequence, which did not interfere with the subsequent assays involving oligonucleotides of higher specific activity.

Observations on recognition of homology by the double-stranded DNA in the RecA filament closely paralleled those described above for the filament containing a single strand of DNA (Fig. 2 *C* and *D*; Fig. 3*B*). All four oligonucleotides of the standard set were recognized in a reaction that depended on homology, RecA protein, and ATP. After correction for background, the molar ratios of oligonucleotide incorporated per nucleoprotein filament ranged from 0.6 to 0.8 for the four oligonucleotides.

Stereospecific Strand Exchange. A labeled 5′ end was carried by the 33-mer (−)-strand oligonucleotide that was annealed to M13 circular ssDNA [Fig. 1 (*ii*)]. The annealed product was separated from free ^{32}P-labeled oligonucleotide by gel filtration. Reactions were done between this labeled DNA in RecA filaments and unlabeled 33-mer oligonucleotides from the standard set (Fig. 1*B*). Samples were deproteinized and analyzed by polyacrylamide gel electrophoresis under nondenaturing conditions.

Only the reaction of the (+)-strand oligonucleotide [Fig. 1*B* (*1*)] that was antiparallel to its oligonucleotide complement in the filament resulted in strand exchange (Fig. 5). At the end of the reaction, a large fraction of the labeled 33-mer complementary strand originally present at the double-stranded site in the RecA filament was recovered in deproteinized samples as duplex 33-mer oligonucleotide product. In the other three reactions of single-stranded oligonucleotides, although recognition was efficient (Fig. 2 *C* and *D*; Fig. 3*B*), the labeled 33-mer oligonucleotide in the filament was not displaced and no new products were recovered (Fig. 5). Little or no recognition was detectable when the oligonucleotide was duplex [Fig. 1*B* (*5*)] (Fig. 2*D*).

DISCUSSION

By two different methods, a filter assay and a gel retardation assay, we observed a surprising set of interactions of the RecA nucleoprotein filament with oligonucleotides, including the recognition of either (+)-strand or (−)-strand oligonucleotides with sugar-phosphate backbones in either antiparallel or parallel orientation. Most of these interactions are weak, as judged by the dissociation of aligned strands upon deproteinization; but these weak interactions may nonetheless play a role in the search for homology, and they reveal new aspects of the RecA nucleoprotein filament.

Of particular interest is the recognition of homology in an oligonucleotide whose sequence was identical to that of a single strand in the filament. That observation reveals that RecA protein can promote homologous recognition via non-Watson–Crick bonds. We are unable to specify the nature of these bonds that mediate recognition, but examples exist of self-pairing at acid pH of oligonucleotides and homopolymers (25–29), and a recent paper has described the self-pairing at acid pH of oligomers containing repeats of 5′-CGAT-3′ (29).

What do self-recognition and the recognition of inverted sequences [those designated (+)R and (−)R above] by the RecA filament containing a *single strand* of DNA have to do with the recognition of homology? Perhaps nothing. On the other hand, the bonds that mediate self-recognition in the single-stranded filament might be a subset of those that mediate recognition when three strands are involved. Moreover, an identical set of observations showed that the RecA filament containing a homologous *duplex* segment recognized the same set of oligonucleotides as the filament containing a single strand, which indicates that the surprising versatility of homologous recognition is preserved when three strands are aligned. Together, the two sets of data (Fig. 2 *A* vs. *C*, Fig. 2 *B* vs. *D*, and Fig. 3 *A* vs. *B*) suggest that the filament primarily senses base-sequence homology rather than other structural elements such as Watson–Crick complementarity or the directionality of the backbones of aligned chains. The versatility of homologous recognition when three strands are involved further supports the view that a triplex intermediate mediates recognition and strand exchange.

Only the interaction of two complementary strands in antiparallel orientation, the classical Watson–Crick type, survived the removal of RecA protein by SDS and proteinase K. One of the hallmarks of the right-handed nucleoprotein filament that is formed by RecA protein and single- or double-stranded DNA in the presence of ATP is the extended axial rise per base pair, 5.1 Å, which is 1.5 times greater than that of the B-form double helix (14, 15). It is presumably this architecture that supports the interactions that are not seen between free DNA molecules in solution. The versatility of homologous recognition as seen in these experiments may represent a rapid but imperfect screening mechanism that is edited at the next step by the greater specificity of strand exchange.

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