The eukaryotic homologs of RecA protein are central enzymes of recombination and repair, and notwithstanding a high degree of conservation they differ sufficiently from RecA to offer insights into mechanisms and biological roles. The yield of DNA strand exchange reactions driven by both Escherichia coli RecA protein and its human homolog HsRad51 protein was inversely related to the GC content of oligonucleotide substrates, but at any given GC composition, HsRad51 promoted less exchange than RecA. When 40% of bases were GC pairs, the rate constant for strand exchange by HsRad51 was unmeasurable, whereas the rate constants for homologous pairing were unaltered relative to more AT-rich DNA. The ability of HsRad51 to form joints in the absence of net strand exchange was confirmed by experiments in which heterologous blocks at both ends of linear duplex oligonucleotides produced joints that instantly dissociated upon deproteinization. These findings suggest that HsRad51 acting alone on human DNA in vivo is a pairing protein that cannot form extensive heteroduplex DNA.

Human Rad51 protein (HsRad51) is a member of the universally distributed class of RecA proteins that play important roles in homologous recombination and recombinational repair (1). In prokaryotes, the RecA proteins play roles in recombination, post-replication repair, and the repair of double-strand breaks (2). In eukaryotes, members of this class play roles in meiotic recombination, double-strand break repair (3, 4), and possibly immunoglobulin switch recombination (5). In the mouse, the requirement of Rad51 for embryonic survival (6, 7) reveals a vital role that has not been found in prokaryotes and lower eukaryotes.

RecA protein from Escherichia coli promotes a search for homology by a single strand of DNA, and initiates an exchange between that strand and homologous duplex DNA. To carry out those complicated interactions, RecA protein forms a helical nucleoprotein filament on single-stranded DNA; and Rad51 from Saccharomyces cerevisiae and Homo sapiens form nucleoprotein filaments that resemble the one formed by RecA (8, 9). Once the nucleoprotein filament has been formed, RecA requires no cofactors other than ATP to promote a rapid search for homology and an extensive strand exchange. Yeast, frog, and human Rad51, as well as human Dmc1, a homolog that is specifically expressed in meiosis, are DNA-dependent ATPases that carry out homologous pairing and strand exchange reactions that resemble those catalyzed by RecA protein (10–14). However, the eukaryotic homologs differ from RecA in several notable respects. None of the eukaryotic enzymes appears to manifest the kinetic barrier to binding to duplex DNA (15), which in the case of RecA favors the loading of protein on single-stranded DNA; all hydrolyze ATP at a rate that is at least an order of magnitude lower than hydrolysis by RecA, and all promote recombination reactions much more slowly than RecA (10, 11, 12, 14). Observations on human Rad51 showed that both phases of the recombination reaction, homologous pairing and strand exchange, are markedly slower than the corresponding phases of the RecA reactions (13).

Auxiliary proteins play important roles in the reactions catalyzed by the eukaryotic homologs of RecA. Replication protein A, the eukaryotic heterotrimeric single-stranded DNA-binding protein appears to stimulate the formation of nucleoprotein filaments by yeast and human Rad51 (10, 16, 17), and recent experiments have demonstrated a physical interaction between human Rad51 and human replication protein A. Rad52 protein also appears to play an early role in stimulating reactions of yeast and human Rad51 (10, 18–20). In yeast and man, Rad52 has been shown to interact physically with Rad51 (3, 21–23), as well as with replication protein A (24, 25). Rad54 protein, another product of the episomal group of yeast genes that encodes Rad52 and Rad51 proteins, is a DNA-dependent ATPase, whose catalytic rate constant is 3 to 4 orders of magnitude greater than that of Rad51. The yeast and human homologs of Rad54 interact physically with the respective Rad51 proteins, and yeast Rad54 strongly stimulates homologous pairing by Rad51 (26–28).

The low ATPase activity of the human Rad51, its slow rates of pairing and strand exchange (13), and its limited ability to carry out extensive strand exchange (12) led us to postulate that it has a relative inability to “open” duplex DNA and to investigate the operationally and kinetically separable phases of homologous pairing and strand exchange (29). The experiments reported here show that human Rad51 can catalyze homologous recognition and the formation of homologous joints in the absence of net strand exchange and suggest that the function of Rad51 itself is limited to recognition of homology and initiation of strand invasion. These studies may provide insights into the roles of other components of the recombination machinery in eukaryotes, the underlying mechanism of homologous recognition, and the regulation of recombination.

EXPERIMENTAL PROCEDURES

Enzymes and Reagents—DNase I and dithiothreitol (DTT) were purchased from Boehringer-Mannheim; ATP and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma. RecA and replication protein A were purchased from Boehringer-Mannheim. ATP and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma. RecA and replication protein A were purchased from Boehringer-Mannheim. ATP and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma. RecA and replication protein A were purchased from Boehringer-Mannheim. ATP and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma. RecA and replication protein A were purchased from Boehringer-Mannheim. ATP and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma. RecA and replication protein A were purchased from Boehringer-Mannheim. ATP and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma. RecA and replication protein A were purchased from Boehringer-Mannheim. ATP and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma. RecA and replication protein A were purchased from Boehringer-Mannheim. ATP and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma. RecA and replication protein A were purchased from Boehringer-Mannheim. ATP and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma. RecA and replication protein A were purchased from Boehringer-Mannheim. ATP and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma. RecA and replication protein A were purchased from Boehringer-Mannheim. ATP and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma. RecA and replication protein A were purchased from Boehringer-Mannheim. ATP and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma. RecA and replication protein A were purchased from Boehringer-Mannheim. ATP and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma.
fluoride from Sigma; isopropyl-β-D-thiogalactopyranoside (IPTG) from American Bioanalytical; Q-Sepharose, native DNA cellulose, and Mono-Q HR 10/10 from Pharmacia Biotech; hydroxyapatite Bio-Gel HT from Bio-Rad; and polynucleotide kinase from New England Biolabs. RecA or Rad51 purified as described (30).

DNA Substrates—The single-stranded oligonucleotides used in this study were designated as either (−) or (+)-strand. For example, A16(−) is a single-stranded oligonucleotide while A16(+) is its complementary strand. In the experiments reported here the strand used to form Rad51 or RecA filaments was designated as the (−)-strand. Sequences of 83-mer oligonucleotides A16(−) (16% GC) and W16(−) (40% GC) have been described earlier (13). The sequences of C16(−) (25% GC), R16(−) (35% GC), and G16(−) (71% GC) are as follows: C16(−) (25% GC): 5′-ATATCTCTTCTTAGGTTATCAGTTTATCGATTCTTCTTTA-3′ (5′-ATATCTCTTCTTAGGTTATCAGTTTATCGATTCTTCTTTA-3′; R16(−) (35% GC): 5′-GTTGCCAGTACGGTATTCTCTTCTTGCGAAGTTT-3′ (5′-GTTGCCAGTACGGTATTCTCTTCTTGCGAAGTTT-3′); G16(−) (71% GC): 5′-GGTTGGCGATGGGAGGTGGTGGGCTAGGGGGCTTAGGGGGGAG-3′ (5′-GGTTGGCGATGGGAGGTGGTGGGCTAGGGGGCTTAGGGGGGAG-3′).

HsRad51 was loaded on a Mono-Q (HR10/10) column. A steep gradient of 0.1 M potassium phosphate buffer, pH 7.4, 0.5 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 10% glycerol), was passed through Q-Sepharose (100 ml) pre-equilibrated with buffer R51. The column was washed, and bound protein was eluted by a linear gradient of 0.1–1 M potassium phosphate buffer, pH 7.4, 0.5 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 10% sucrose, 1 mM of l-lysine, 0.5 M KC1) followed by mild sonication. The lysate obtained on centrifugation was fractionated and stored at −80 °C.

For DNA exchange reaction, the (−)-strand used to form filament was unlabeled, whereas the duplex had fluorescein at the 3′ end and rhodamine at the 5′ end of the complementary (−)-strand. Quenching in fluorescein 2′-OH group was detected by use of a PhosphorImager (Molecular Dynamics) using ImageQuant software.

Annealing Assay—Annealing was assayed as described (13). 32P-Labeled (−)-strand oligonucleotides at 5 μM were incubated with 1.2 μM RecA or HsRad51 in reaction buffer R containing 1 mM MgCl2 for 5 min. Complementary (+)-strands were then added at 3 μM to the respective tubes and incubated further for 1 min. The reaction was quenched by addition of stop solution containing 30 μl unlabeled (−)-strand and electrophoresed through 12% polyacrylamide. Products were quantitated by use of a PhosphorImager.

DNase I Protection Assay—HsRad51 or RecA (1.2 μM) were preincubated with 3 μM 5′-32P-labeled oligonucleotides in buffer R (total volume 15 μl), as mentioned above for 5 min at 37 °C. DNase I (final concentration of 0.15 units/μl) was then added and incubation was continued for 2 min. The reaction was ended by addition of stop solution containing 25 μg of yeast tRNA. DNA was then precipitated by addition of cold 10% trichloroacetic acid and acid soluble radioactivity in the supernatants was measured.

ATPase Activity—Reaction mixtures contained 25 μM Heps, pH 7.4, 1 mM DTT, 100 μM of bovine serum albumin/ml, 6 mM MgCl2, 1.0 μCi of [32P]ATP, and 0.5 mM ATP. HsRad51 or RecA (1.3 μM) was incubated with 30 μM single-stranded oligonucleotides at 37 °C and the reaction was arrested at different time intervals by the addition of ATPase stop solution (final concentration of 1 mM ATP, 1 mM ADP, 25 mM EDTA). Three μl of the reaction mixture was processed for detection of ATP hydrolysed by thin layer chromatography as described (30). ATPase activity was expressed as moles of ATP hydrolyzed per mole of protein/min.

Fluorometric Assays for Pairing or Strand Exchange—Pairing or strand exchange were detected by two separate fluorometric assays as described before (13, 29). For pairing, 1.2 μM HsRad51 or RecA was preincubated with 3 μM 5′ fluorescein-labeled (−)-strand in reaction buffer R (total volume 150 μl) at 37 °C for 5 min. MgCl2 was then increased to 30 mM followed by the addition of duplex that contained rhodamine at the 5′ end of the complementary (−)-strand. In the strand exchange reaction, the (−)-strand used to form filament was unlabeled, whereas the duplex had fluorescein at the 3′ end of the (−)-strand and rhodamine at the 5′ end of the (−)-strand. Quenching in fluorescein intensity in the pairing assay and enhancement in the strand exchange assay were monitored every 2 s at 520 nm upon excitation at 493 nm.

Quantification of fluorescence resonance energy transfer (FRET) due to homologous pairing was done as described (13, 29). To detect the FRET signal, the spectrum was taken from 502 to 620 nm upon excitation at 493 nm, before the addition of duplex and after the reaction is completed.

RESULTS

The Effects of Base Composition on Recombination Reactions in Vitro—Five different substrates with varying GC content were made to explore the effect of base composition on pairing and strand exchange by HsRad51 and RecA proteins: A16 (16% GC), C16 (25% GC), R16 (50% GC), W16 (40% GC), and G16 (71% GC). In the design of these oligonucleotides, we mini-
HsRad51 Forms Paranemic Joints

Nucleoprotein filaments formed on single-stranded oligonucleotides by HsRad51 or RecA were paired with homologous duplex oligonucleotides for 1 h prior to assaying the yields of cleotides by HsRad51 or RecA were paired with homologous procedures.

mized secondary structure as described under “Experimental Procedures.”

Nucleoprotein filaments formed on single-stranded oligonucleotides by HsRad51 or RecA were paired with homologous duplex oligonucleotides for 1 h prior to assaying the yields of the reactions by gel electrophoresis (Fig. 2A). For both RecA and HsRad51 the yields were inversely related to GC content of the substrates (Fig. 2A), but at any given GC content the yield of reactions mediated by Rad51 was lower than that by RecA. The parallel functions that fit the respective data on HsRad51 and RecA suggest that a common mechanism is responsible for the inverse effect of GC content on strand exchange; but the existence of parallel functions instead of a single function suggests that the effect of base composition is not exclusively attributable to secondary structure in the single-stranded DNA. In other words, there is a common effect of GC content, but each of the two proteins contributes to a quantitatively different outcome.

Either of two general explanations might account for the common effect of GC content on the yields of reactions by HsRad51 and RecA. (a) Nucleoprotein filaments might form differently as a function of the GC content of single-stranded DNA. (b) By its stabilizing effect on duplex DNA, high GC content might affect the ability of HsRad51 and RecA to carry out any of the steps that are potentially involved in the conversion of parental homoduplex substrate into the product heteroduplex DNA, including, among others, the exchange of base pairs and the final displacement of one parental strand.

Effect of GC Content on the Interactions of HsRad51 and RecA Protein with Single-stranded DNA—We examined the effect of GC content on three different indices of interactions of HsRad51 and RecA with single-stranded DNA: ATP hydrolysis, protection of single-stranded DNA from digestion by DNase I, and annealing of complementary strands (Table I).

In the case of HsRad51, we could detect no difference in catalytic rate constants (turnover number) among a set of substrates with varying GC content. In the case of RecA protein, there was a reproducible decrease in $k_{cat}$ at the highest content of GC residues (Table I).

We determined the amount of DNase I that was sufficient to convert the $32^P$ label present at the end of these 83-mer oligonucleotides into a form that is soluble in acid. HsRad51 or RecA were allowed to form filaments on these oligonucleotides (at a ratio of protein to nucleotide residues of 1:2.5) in the presence of 1 mM MgCl$_2$ at 37 °C for 5 min, followed by the addition of DNase I, and incubation for 2 more minutes. As shown in Table I, both HsRad51 and RecA protected all three oligonucleotide substrates. There appeared to be some increased digestion of the substrate with the highest GC content, but there was no discernible trend that correlated with base composition. The assay for acid solubility of label at the end of an oligonucleotide should be sensitive because only a few cuts need to be made by DNase I to make the label acid-soluble.

For annealing reactions, filaments formed on the three single-stranded oligonucleotides were paired with their respective $32^P$-labeled complementary strands for 1 min, and annealed products were monitored by gel electrophoresis after deproteinization. For both Rad51 and RecA, the yields of the annealing reactions were similar at the varying GC compositions of the oligonucleotide substrates (Table I). In aggregate, these data do not reveal a systematic effect of GC content that is sufficient to explain the inhibitory effect of GC content shown in Fig. 2.

Stabilization of Duplex DNA and Its Effect on Pairing and Strand Exchange Activity—The pairing and exchange activity of HsRad51 was checked at different concentrations of MgCl$_2$ in the presence of 100 mM NaCl, with oligonucleotides A16 (16% GC) and G16 (71% GC) as substrates. We observed, as expected, a stimulatory effect of MgCl$_2$ at low concentrations and an inhibitory effect at high concentrations (31) (Fig. 3). There was, however, a clear difference between the effect of Mg$^{2+}$ concentration on GC-rich DNA versus AT-rich DNA. High concentrations of Mg$^{2+}$ were more inhibitory to reactions with GC-rich DNA than AT-rich DNA. This observation is consistent with the interpretation that high GC content inhibits pairing and strand exchange either because it stabilizes secondary structure in single-stranded DNA or because it stabilizes the secondary structure of parental duplex DNA, either of

---

**TABLE I**

| Substrate DNA | ATP hydrolysis ($k_{cat}$) | DNase I protection (% GC mol/mol/min) | Annealing (% GC mol/mol/min) |
|---------------|---------------------------|-------------------------------------|-----------------------------|
| % GC          | HsRad51 | RecA | HsRad51 | RecA | HsRad51 | RecA | HsRad51 | RecA |
| 16%           | 0.9     | 30   | 91      | 95   | 75      | 70   |
| 40%           | 0.8     | 31   | 92      | 94   | 95      | 80   |
| 71%           | 0.9     | 14   | 80      | 82   | 87      | 72   |

**Fig. 2.** Pairing and strand exchange by HsRad51 and RecA versus GC content. Reactions were carried out and analyzed as described under “Experimental Procedures” with substrates containing the indicated content of G and C nucleotide residues. A, pairing and strand exchange measured by gel electrophoresis versus GC content of the substrates. B, homologous pairing measured by FRET versus pairing and strand exchange as measured by gel electrophoresis. Steady state FRET assays were performed independently for each of the substrates and for both enzymes, and the values were plotted versus those obtained by gel electrophoresis in A. ○, HsRad51; ●, RecA.
Discrimination of the Respective Effects of GC Content on Homologous Pairing and Strand Exchange—Assays based on FRET can distinguish the initial rapid second order pairing of homologous pairs. In each case the strand used in the Rad51 filament, with (upper row) or without (middle and last row) mismatches, was the complement of the bottom strand shown in the diagrams. The strand exchange reaction was carried out and analyzed as described under “The Gel Assay for Pairing and Strand Exchange” (see “Experimental Procedures”). The filaments were formed on 3 μM single-stranded oligonucleotides and were reacted with 2.5 μM homologous duplexes. The first two rows are controls. The upper row shows the reaction in which mismatches were present in the filament strand, resulting in the conversion of a perfectly matched duplex substrate into mismatched heteroduplex. The middle row shows the opposite, conversion of a mismatched duplex substrate to a perfectly base-paired heteroduplex product. The last row shows the experiment done to test the effect of helix-destabilizing mismatches in isoenenergetic exchanges in which the mismatched duplex substrate and mismatched heteroduplex product were identical. Numbers in parentheses indicate the result obtained in a second independent experiment. Transversions were made in W16(−→) and W16(−→) to produce the mismatched substrates. Substrates containing two, six, and seven mismatches, respectively, had transversions at positions 24 and 60; and 12, 24, 36, 48, 60, and 72; and 6, 18, 30, 42, 54, 66, and 78 (see Ref. 32).

| Number of Mismatches | 0 | 2 | 6 | 7 |
|----------------------|---|---|---|---|
| **Duplex Substrates & Products** | **Strand Exchange (%)** | | | |
| WT | | | | |
| mm | 10 | 5 | 1 | 1 |
| mm | 10 | 34 | 53 | 91 |
| WT | | | | |
| mm | 10 | 9(12) | 14(14) | 18(20) |

A Stimulatory Effect of Base Mismatches in Isoenergetic Strand Exchanges—One expects a thermodynamic effect of mismatched bases on the yield of products of pairing and strand exchange, reflecting the net gain or loss of Watson-Crick base pairs in the reaction. We designed experiments in which strand exchange promoted by HsRad51 resulted in either a net loss, a net gain, or no change in the number of base pairs. This was accomplished by varying the strand in which base substitutions were located.

When substitutions were located in the single strand used to make filaments and these were reacted with perfectly paired duplex DNA (Table II, row 1), the product of strand exchange contained mismatched base pairs, which results in a net energetic loss. As expected, the yield of the reaction decreased as the number of mismatches increased (Table II, row 1). By contrast, when base substitutions were located in the (+)-strand of the duplex substrate and the latter was reacted with filament containing a (+)-strand that lacked substitutions, the reaction produced a net increase in Watson-Crick base pairs and the yield increased as the number of mismatches increased (Table II, row 2).

Our aim, however, was to examine the situation in which an isoenergetic exchange might occur with a duplex 83-mer that had been destabilized by up to 7 mismatched pairs of bases. This was accomplished by the substitution of bases in the strand that was complementary to the strand in the filament, in which case the duplex substrate and the duplex product had precisely the same number and kinds of mismatched bases. As the number of mismatches increased up to 7, the yield of this isoenergetic reaction increased by 80%. Thus, the yield of products of pairing and strand exchange promoted by human Rad51 was inhibited by three forces that stabilize duplex DNA, namely, high GC content, Mg2⁺, and perfectly matched base pairs.

**Discrimination of the Respective Effects of GC Content on Homologous Pairing and Strand Exchange**—Assays based on FRET can distinguish the initial rapid second order pairing of a single strand with duplex DNA from the subsequent, slower, first order strand exchange (15, 29, 32). In the pairing assay, fluorescein is conjugated to one end of the strand that is used to make a filament, and rhodamine is conjugated to the complementary strand in parental duplex DNA. When pairing takes place, the two dye-conjugated strands come into apposition and photoexcitation of fluorescein leads to non-radiative transfer of energy to rhodamine, which then fluoresces at its emission wavelength (Fig. 4A). Transfer of energy is also manifested as a quenching of light emitted by fluorescein at its own emission wavelengths, and for measurements of kinetics the quenching of fluorescein emission is much more readily measured (see Ref. 29). In the pairing assay, the two dyes remain in apposition even after strand exchange takes place. Thus the yield measured at any moment represents the sum of synaptic intermediates (Wcw) plus one of the products of strand exchange (Wc), which is equal to the cumulative fraction of substrate molecules that have undergone homologous pairing (Fig. 4A).

In the version of the assay that detects strand exchange, the two dyes are conjugated instead to the complementary strands of the parental duplex oligonucleotide (Fig. 4B). At the starting point of this assay, energy transfer is maximal and the fluorescence of fluorescein is quenched. As strand exchange separates the strands, energy transfer diminishes and the fluorescence of fluorescein is enhanced (Fig. 4B).

In a previous detailed analysis of RecA reactions by these assays, we were able by computer simulation to fit the data to the two-step kinetic scheme shown in Fig. 4 (29). For that analysis, we assumed that the quenching of fluorescence from fluorescein was the same in the intermediate (Wcw) and product (Wc). The analysis was based on three assays, one that detected Wcw plus Wc, one that detected only Wcw, and one...
that detected only Wc. The coherence of the analytical data did not reveal any major flaws in the analytical procedures and assumptions. We used the same methods and assumptions to analyze the reactions of HsRad51 described below. Justification for use of the same methods and assumptions is provided by the parallel behavior of RecA and HsRad51 (Figs. 2 and 5 below), and by the fit that we obtained of computer-generated simulations to kinetic data on HsRad51 (data not shown).

As shown in Fig. 2, oligonucleotides with increasing GC content progressively inhibited the yield of Rad51 reactions as measured by gel electrophoresis. At 71% GC content, the yield was negligible. From the oligonucleotides studied in Fig. 2, we chose those with 40 and 16% GC to examine the effects of GC content on the phases of the reaction promoted by both Rad51 and RecA (Fig. 5). These two oligonucleotides showed no indication of effects of base composition on the formation of filaments (Table II).

In the case of Rad51, 40% GC content roughly halved the yield of homologous pairing relative to 16% GC content (Fig. 5A), consistent with the data in Fig. 2. By contrast, the fluorometric assay that detects the physical separation of fluorescent dyes on the ends of strands, showed that the same increase in GC content from 16 to 40% reduced strand exchange to unmeasurable levels (Fig. 5B).

With the same substrates, we compared the effects of increasing GC content on homologous pairing and strand exchange by RecA protein (Fig. 5, C and D). The results confirm the general conclusion drawn from Fig. 2 that increasing GC content inhibits RecA reactions as well as HsRad51 reactions, but to a lesser degree at any particular GC content. The comparison also shows more specifically that at 40% GC content, where strand exchange promoted by Rad51 was virtually eliminated, as measured by the fluorescence assay, RecA protein still promoted substantial exchange.

We analyzed the data in Fig. 5, A and B, by computer simulation, using Kinsim software as described previously (29). The derived rate constants are shown in Table III. Consistent with visual inspection of the data, the increase from 16% GC to 40% GC reduced $k_2$, the rate constant for strand exchange by 2 orders of magnitude. By contrast, $k_1$ and $k_1^{-1}$, the forward and reverse rate constants for homologous pairing were not significantly changed. This calculation suggests that the apparent decrease in homologous pairing at 40% GC (Fig. 5A) is attrib-
Table III: Kinetic analysis of homologous pairing and strand exchange catalyzed by HsRad51 from substrates with 16 or 40% GC content

| DNA   | $k_1$ (10$^{-3}$ s$^{-1}$) | $k_{-1}$ (10$^{-3}$ s$^{-1}$) | $K_{eq1}$ (10$^{3}$ M$^{-1}$) | $k_2$ (10$^{-2}$ s$^{-1}$) | $h_{-2}$ (10$^3$ M$^{-1}$ s$^{-1}$) |
|-------|--------------------------|-----------------------------|----------------------------|--------------------------|-----------------------------|
| A16 (16% GC) | 1.5 ± 0.1 | 2.2 ± 0.3 | 6.6 ± 0.7 | 2.7 ± 0.6 | 1.0 |
| W16 (40% GC) | 1.2 ± 0.2 | 1.6 ± 0.3 | 8.0 ± 1.0 | ≤10$^{-2}$ |    |

utable to the elimination of strand exchange, which otherwise drives the reaction to the right. In other words, at 40% GC content, which is approximately the average base composition of human DNA, HsRad51 promotes homologous pairing, but no net strand exchange.

Evaluation of the Fluorometric Assays Versus the Gel Electrophoresis Assay—As just described, with substrates that contained 40% GC, strand exchange promoted by Rad51 was completely inhibited according to the fluorometric assay (Fig. 5B). However, at the same GC content, the assay by gel electrophoresis registered 30% of product (Fig. 2A). This discrepancy required further comparisons of the two assay systems.

The physical basis of the fluorometric assays has been described above. These assays can be done in two ways. One can measure FRET by exciting fluorescein at its absorption maximum and observing the resulting “sensitized” emission of light from rhodamine, or one can monitor the quenching of light emitted from fluorescein as a result of the energy that it has lost by non-radiative transfer to rhodamine. In the experiments described in this section we used both methods.

The fluorometric assays are carried out in solution without deliberate disruption of protein-DNA complexes. The assay by gel electrophoresis requires deproteinization of intermediates and products, and consequently has different properties as shown in the following experiments. The electrophoretic assay detects the homology-dependent incorporation of a labeled single strand into a heteroduplex structure or the displacement of one labeled strand from parental duplex DNA. By its nature, this assay is incapable of distinguishing homologous pairing from strand exchange. The required deproteinization of the reaction mixture prior to electrophoretic analysis creates uncertainty about how much of the measured outcome is attributable to strand exchange that occurred in solution and how much to spontaneous branch migration that occurred after deproteinization.

Using the same oligonucleotide substrates with varying GC content as studied in Fig. 2A, we measured the steady state level of homologous pairing as measured by FRET for both Rad51 and RecA. A plot of the FRET values versus the electrophoretic values for each substrate of a given GC concentration revealed a linear correlation encompassing the data obtained with both Rad51 and RecA (Fig. 2B). Thus, these two assays revealed a remarkable concordance in relation to GC content.

We then compared strand exchange, as measured by enhanced emission from fluorescein, with the measurement provided by the electrophoretic assay. From concurrent observations designed to test the polarity of strand exchange (17), we had identified substrates that showed different kinetics according to the fluorometric assay. One substrate was the 83-mer A16 containing 16% GC bases. Two other substrates were chimeric molecules in which one end was GC-rich and the other was AT-rich, with an average GC content of 50%. In oligonucleotide RG1, the 3’ end of the strand in the Rad51 filament was GC-rich, whereas in oligonucleotide RG2 the 5’ end was GC-rich. These were reacted with their respective duplex counterparts.

The time course of strand exchange with each of these substrates was compared directly by the fluorometric and the gel assays (Fig. 6). Relative to the AT-rich reference oligonucleotide A16, the yield with oligonucleotide RG1 was reduced 3-fold and that of RG2 was reduced more than 5-fold as measured by the fluorometric assay. The outcome was quite different when measured by the gel assay. There was no difference observed between substrates RG1 and RG2, and their yields were reduced only about 20% relative to the reference oligonucleotide A16.

The observations described above show that the data obtained by the electrophoretic assay correlate with those obtained by the measurement of homologous pairing by FRET, but do not correlate with those obtained by the fluorometric assay for strand exchange. The simplest interpretation is that with oligonucleotides as substrates of reactions promoted by Rad51, the electrophoretic assay provides a better measure of homologous pairing than it does of strand exchange. We suggest that after deproteinization, intermediates that have not completed exchange in the presence of Rad51 undergo spontaneous branch migration resulting in either the regeneration of substrates or the production of additional strand exchange products. Thus, as in the fluorometric assay for homologous pairing (see previous section above), the electrophoretic assay...
at any point in time measures the sum of some fraction of existing synaptic intermediates (Wcw) plus products of strand exchange (We + w), which is proportional to the cumulative fraction of substrate molecules that have undergone homologous pairing (see Fig. 4). If strand exchange is blocked, as it is in substrates containing 40% GC base pairs (Fig. 5B), the electrophoretic assay still measures some fraction of the synaptic intermediate because of the spontaneous branch migration that can occur upon deproteinization.

Homologous Pairing of Oligonucleotides with Heterologous Ends—As a further test of the ability of human Rad51 to promote homologous pairing without strand exchange, we examined reactions of 83-mer oligonucleotides with blocks of 10 heterologous nucleotides at both ends. The single-stranded oligonucleotide in these experiments was A16(−) which has 16% GC base pairs. The duplex oligonucleotide, GC10, was derived from A16 by substituting heterologous blocks of 10 GC base pairs at each end. As a standard for comparison, reactions were also carried out between A16(−) and the completely homologous duplex oligonucleotide (Fig. 7B).

The terminal blocks of heterologous sequences were as effective in blocking strand exchange as a completely heterologous sequence (Fig. 7C), whereas homologous pairing was still substantial (Fig. 7A). In the assay for pairing, the amplitude of the change in fluorescence was about half of that observed for completely homologous substrates (Fig. 7B). This relative decrease in apparent yield of the pairing reaction was the same as seen when strand exchange was eliminated in substrates containing 40% GC base pairs (Fig. 5). Some major part of this decreased amplitude of the pairing reaction is probably an indirect consequence of blocking strand exchange, as described above.

After pairing reactions reached their limits we added SDS to deproteinize the reactions and as quickly as possible we resumed measuring the fluorescence output. When the substrates had heterologous ends, the addition of SDS resulted in the immediate restoration of fluorescence to the level seen prior to pairing (Fig. 7A). Thus the synaptic intermediates in this case were unstable in the absence of protein and whatever bonds mediated homologous recognition were immediately broken. The same treatment of a reaction of completely homologous substrates (Fig. 7B) resulted in the restoration of only about a fifth of the fluorescence emission, indicating that a smaller fraction of molecules were in the synaptic state. In the reaction of completely homologous molecules any that had gone on to completion of strand exchange would not contribute upon deproteinization to restoration of the initial fluorescence amplitude.

DISCUSSION

Base Composition Versus Secondary Structure in Single Strands—In a previous study of the effect of base composition on strand exchange promoted by RecA protein, Gruss et al. (33) reported several effects of high GC content on the overall process of strand exchange promoted by RecA protein. One effect of high GC content appeared attributable to more stable secondary structure in single-stranded DNA. Since single-stranded DNA-binding protein, which removes secondary structure from single-stranded DNA, was only partially able to overcome the inhibitory effect of high GC content, factors other than secondary structure presumably contributed to the effect of GC bases. Gruss et al. (33) identified another effect, manifested by inhibition when a GC-rich region in single-stranded DNA was adjacent to the site of initiation of strand exchange. Those observations identified effects of GC-rich sequences on the formation or structure of nucleoprotein filaments but did not exclude effects on strand exchange itself.
Rad51 protein. When the substrates contained 40% GC, the forward rate constant for strand exchange was unmeasurable, whereas the forward and reverse rate constants for homologous pairing were unchanged relative to DNA with a much lower GC content. However, the inhibitory effect of GC content did not end there. When the substrate was 71% GC, Rad51 was unable to promote any significant homologous pairing, as reflected either by the electrophoretic assay (Fig. 2) or by the specific fluorescence assay for homologous pairing (17).

This order of effects in relation to increasing GC composition, the complete elimination of strand exchange first, and then the elimination of homologous pairing, is the opposite of what one might expect if GC content were interfering only with the formation of presynaptic filaments, in which case the inhibition of homologous pairing and subsequent strand exchange should go hand in hand: without homologous pairing there can be no strand exchange. On the other hand, in the case of RecA protein, we know that pairing can occur without any net strand exchange (34); and the present experiments show that the same is true of Rad51. The observed order of effects of increasing GC content can be rationalized readily if, as has been suggested (35), the switching of base pairs is a step that is common to both recognition of homology and strand exchange. Hsieh et al. (36) estimated that homologous recognition promoted by RecA protein requires only about a half dozen base pairs. Thus, to form a homologously aligned synaptic complex with 83 nucleotide residues in each strand, perhaps only a small subset of bases needs at any instant to have switched from parental to heteroduplex pairs, whereas the completion of strand exchange by the separation of one parental strand from its erstwhile complement obviously requires that all parental base pairs be ruptured. If increasing GC content progressively inhibits the switching of base pairs, it follows that net strand exchange would be abolished before homologous recognition were eliminated.

Formation of Paranemic Joints by HsRad51—Acting on circular single-stranded DNA and circular duplex DNA, RecA protein promotes the recognition of homology and the formation of joints whose stability depends on the continued binding of the protein (34, 37). Such joints are true paranemic structures in which the linking number of parental duplex and parental single strand is zero, and no net strand exchange can occur. When a region of homology is flanked on both sides by heterologous sequences, joints with similar properties are formed and are also called paranemic joints (34). The experiments described here show that human Rad51 protein also promotes the formation of this kind of paranemic joint from substrates whose ends are heterologous. By chemical probing, Adzuma (35) observed that when RecA protein forms paranemic joints in the presence of ATPγS, a non-hydrolyzable analog of ATP, bases are found in a switched heteroduplex configuration. In the present experiments on HsRad51, the instantaneous dissociation of paranemic joints upon deproteinization means either that in the presence of ATP, no bases had switched, or more likely, that switched bases were in an unstable or dynamic state from which they could readily revert to the parental state. This is reminiscent of the properties of other joints formed by RecA protein that cannot progress to net strand exchange but instead undergo continuous turnover (38, 39).

Function of Rad51 in Vitro Versus in Vivo—The reaction carried out by Rad51 protein with substrates that contain 40% GC bases leads to an intermediate that also resembles a paranemic joint, one in which no net strand exchange takes place. A direct comparison showed that whereas 40% GC content completely inhibited strand exchange by Rad51 (Fig. 5B), RecA was still able to perform strand exchange, which supports the view that HsRad51 has a relative deficiency in the ability to promote strand exchange. On the basis of reconstitution experiments in which the E. coli RuvAB complex dissociated RecA from joint molecules and then accelerated branch migration, West and colleagues (40) suggested that RuvAB is primarily responsible for the extension of heteroduplex DNA in vivo. On the other hand, RecA acting alone in vitro catalyzes extensive heteroduplex formation, and using the energy of ATP hydrolysis RecA has an impressive ability to push strand exchange through sizable heterologous insertions (41, 42, 43). On the basis of the present results it would seem that the ability of human Rad51, as well as human Dmc1 (14), to promote the formation of heteroduplex DNA is much more limited. Without the action of other proteins, the function of human Rad51 and human Dmc1 may be limited to homologous pairing. In relation to the observed blockage in vitro of strand exchange promoted by HsRad51 when the substrate had 40% GC base pairs, it is interesting to note that the average GC content of human DNA is 39% (44). The ability of RecA and its homologs to promote strand exchange to greater or lesser degrees presumably reflects in part an essential mechanistic coupling between the switching of base pairs and either homologous recognition itself or the stabilization of a homologous joint. RecA and its homologs are key players in finding homology and initiating strand invasion; they probably extend and stabilize D-loops as well, but the subsequent creation of extensive heteroduplex DNA, particularly following the formation of Holliday structures is probably the task of other enzymes.

Origins of replication tend to be AT-rich, as are promoter regions which are hotspots for the initiation of meiotic recombination in yeast (45). Thus AT-rich sequences in the genome are sites of initiation of replication and recombination, presumably because of the lower stability of AT base pairs. The striking dependence of human Rad51 and Dmc1 on high AT content of DNA is consistent with their postulated biological roles in initiating recombination.

Acknowledgments—We are grateful to Efim Golub and Oleg Kovalenko for helpful comments on the manuscript, Jan Zulkeski for data processing, and Zhufang Li for technical assistance.

REFERENCES
1. Shinohara, A., Ogawa, H., Matsuda, Y., Ushio, N., Ikeo, K., and Ogawa, T. (1993) Nat. Genet. 4, 239–243
2. Kowalezykowski, S. C., and Eggleston, A. K. (1994) Annu. Rev. Biochem. 63, 991–1043
3. Shinohara, A., Ogawa, H., and Ogawa, T. (1992) Cell 70, 457–470
4. Bishop, D. K., Park, D., Xu, L., and Kleckner, N. (1992) Cell 70, 439–456
5. Li, M.-J., Peakman, M.-C., Golub, E. L., Reddy, G., Ward, D. C., Radding, C. M., and Maizel, N. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 10222–10227
6. Tsuzuki, T., Fujii, Y., Sakumi, K., Tominaaya, N., Nakao, K., Sekiguchi, M., Matsushiro, A., Yoshimura, Y., and Morita, T. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 6263–6268
7. Lim, D., and Hasty, P. (1996) Mol. Cell. Biol. 16, 7133–7143
8. Ogawa, T., Yu, X., Shinohara, A., and Egeland, E. H. (1993) Science 259, 1896–1899
9. Benson, P. E., Stasiak, A., and West, S. C. (1994) EMBO J. 13, 5764–5771
10. Sung, P. (1994) Science 265, 1241–1243
11. Tsuzuki, T., Fujii, Y., Sakumi, K., Tominaaya, N., Nakao, K., Sekiguchi, M., Matsushiro, A., Yoshimura, Y., and Morita, T. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 6263–6268
12. Shinohara, A., Ogawa, H., and Ogawa, T. (1992) Cell 70, 457–470
13. Gupta, R., Bazemore, L. R., Golub, E. I., and Radding, C. M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 463–468
14. Li, Z., Golub, E. I., Gupta, R., and Radding, C. M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 11221–11225
15. Pugh, B. F., and Cox, M. M. (1997) J. Biol. Chem. 272, 1326–1336
16. Sugiyama, T., Tazieva, E. M., and Kowalezykowski, S. C. (1997) J. Biol. Chem. 272, 7890–7914
17. Hays, S. L., Firmenich, A. A., and Berg, P. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 6925–6929
18. Shinohara, A., and Ogawa, T. (1998) Nature 391, 407–410
19. Shinohara, A., and Ogawa, T. (1998) Nature 391, 404–407
20. New, J. H., Sugiyama, T., Tazieva, E., and Kowalezykowski, S. C. (1998) Nature 391, 407–410
21. Haya, S. I., Firmenich, A. A., and Berg, P. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 6925–6929
22. Milne, J. T., and Weaver, D. T. (1993) Genes Dev. 7, 1755–1765
23. Shen, Z., Cloud, K. G., Chen, D. J., and Park, M. S. (1996) J. Biol. Chem. 271, 4554–4558
24. Sugiyama, T., Tazieva, E. M., and Kowalezykowski, S. C. (1997) J. Biol. Chem. 272, 1326–1336
24. Firmenich, A. A., Elias-Arnanz, M., and Berg, P. (1995) Mol. Cell. Biol. 15, 1620–1631
25. Park, M. S., Ludwig, D. L., Stigger, E., and Lee, S.-H. (1996) J. Biol. Chem. 271, 18996–19000
26. Jiang, H., Yie, Y., Houston, P., Stemke-Hale, K., Mortensen, U. H., Rothstein, R., and Kolodeck, T. (1996) J. Biol. Chem. 271, 33181–33186
27. Golub, E. I., Kovalenko, O. V., Gupta, R. C., Ward, D. C., and Radding, C. M. (1997) Nucleic Acids Res. 25, 4106–4110
28. Petukhova, G., Stratton, S., and Sung, P. (1998) Nature 393, 81–94
29. Bazemore, L. R., Takahashi, M., and Radding, C. M. (1997) J. Biol. Chem. 272, 14672–14682
30. Shibata, T., Cunningham, R. P., and Radding, C. M. (1981) J. Biol. Chem. 256, 7557–7564
31. Shibata, T., DasGupta, C., Cunningham, R. P., Williams, J. G. K., Osber, L., and Radding, C. M. (1981) J. Biol. Chem. 256, 7565–7572
32. Bazemore, L. R., Folta-Stogniew, E., Takahashi, M., and Radding, C. M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 11863–11868
33. Gruss, A., Moretto, V., Ehrlich, S. D., Duwat, P., and Dabert, P. (1991) J. Biol. Chem. 266, 6667–6669
34. Bianchi, M., DasGupta, C., and Radding, C. M. (1983) Cell 34, 931–939
35. Adzuma, K. (1992) Genes Dev. 6, 1679–1684
36. Hsieh, P., Camerini-Otero, C. S., and Camerini-Otero, R. D. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 6492–6496
37. Riddles, P. W., and Lehman, J. R. (1985) J. Biol. Chem. 260, 165–169
38. Burnett, B., Rao, B. J., Jwang, B., Reddy, G., and Radding, C. M. (1994) J. Mol. Biol. 238, 540–554
39. Reddy, G., Burnett, B., and Radding, C. M. (1995) Biochem. 34, 10194–10204
40. Eggleston, A. K., Mitchell, A. H., and West, S. C. (1997) Cell 89, 607–617
41. Bianchi, M. E., and Radding, C. M. (1983) Cell 35, 511–520
42. Kim, J.-I., Cox, M. M., and Inman, R. B. (1992) J. Biol. Chem. 267, 16438–16443
43. Rosselli, W., and Stasiak, A. (1991) EMBO J. 10, 4391–4396
44. Hartl, D. L., Freifelder, D., and Snyder, L. A. (1988) in Basic Genetics, pp. 505, Jones and Bartlett, Boston
45. Lichten, M., and Goldman, A. S. H. (1995) Annu. Rev. Genet. 29, 423–444