Formation of Joint DNA Molecules by Two Eukaryotic Strand Exchange Proteins Does Not Require Melting of a DNA Duplex*

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We have examined whether DNA strand exchange activities from nuclear extracts of HeLa cells or Drosophila melanogaster embryos have detectable helicase or melting activities. The partially purified recombinases have been shown to recognize homologous single strand and double strand DNA molecules and form joint molecules in a DNA strand exchange reaction. The joint molecule product consists of a linear duplex joined at one end by a region of DNA heteroduplex to a homologous single strand circular DNA. Using two different partially duplex helicase substrates, we are unable to detect any melting of duplex regions under conditions that promote joint molecule formation. One substrate consists of a 32P-labeled oligonucleotide 20 or 30 bases long annealed to M13mp18 circular single strand DNA. The second substrate consists of a linear single strand region flanked at each end by short duplex regions. We observe that even in the presence of excess recombinase protein or after prolonged incubation no helicase activity is apparent. Control experiments rule out the possibility that a helicase is masked by reannealing of displaced single strand fragments. Based on these findings and other data, we conclude that the human and D. melanogaster recombinases recognize and pair homologous sequences without significant melting of duplex DNA prior to strand exchange.

DNA strand exchange is an essential feature of models for homologous recombination based on genetic data from prokaryotes and fungi (1–3). Nevertheless, the biochemical mechanisms of homologous recombination remain obscure. The ability to carry out strand exchange in vitro appears to be unique to proteins that have been shown to be essential for homologous recombination. Examples include the recA protein from Escherichia coli (reviewed in Ref. 4), the uvsX protein from bacteriophage T4 (5–7), and the rec1 protein from the fungus Ustilago maydis (8). A general assay for strand exchange involves the recognition and pairing of homologous single strand and double strand DNA molecules and the formation of joint molecules held together by a region of DNA heteroduplex. Study of DNA strand exchange by E. coli recA protein has provided insight into one complex pathway of recombination in living cells. The recA protein is required for genetic recombination, DNA repair, and SOS induction in E. coli (reviewed in Ref. 4). RecA protein can pair a number of homologous DNA substrates in a strand exchange reaction and form heteroduplex DNA several thousand bp long. Efficient strand exchange by recA protein requires stoichiometric amounts of recA protein and E. coli single strand-binding protein, SSB, as well as Mg2+ and ATP.

We have previously described partially purified strand exchange activities termed recombinases from the human cell line RPMI-1788 (9) and from Drosophila melanogaster embryos (10). We have also partially purified a strand exchange activity from HeLa cells (this study). Characterization of strand exchange by these three activities shows marked similarities. The formation of joint molecules requires the co-incubation of recombinase, linear duplex DNA, and homologous single strand DNA. The recombinases require Mg2+ but no exogenous ATP, and they exhibit polarity of strand displacement in a 3' to 5' direction (see Fig. 1A). Under electron microscopy the joint molecules appear as a long linear duplex connected at one end to a single strand circular DNA by a short duplex region that represents the heteroduplex (9, 10). In some molecules, a short single strand tail representing the displaced strand is observed at the junction of the linear and circular portion of the joint molecules. We have demonstrated the presence of a third strand in the joint molecules in several ways, thereby ruling out an exonuclease and reannealing mechanism (9, 10). Human recombinase can form joint molecules containing heteroduplex tracts at least 150 bp in length. Joint molecules formed by recombinase from Drosophila embryos can have heteroduplex tracts considerably longer, at least 650 bp in length. Recently, several other DNA strand exchange activities have been isolated, from mitotic and meiotic cells of Saccharomyces cerevisiae (11, 12) and from cultured human T lymphoblasts (13). These recombinases pair linear duplexes and homologous single strand circular DNA and form joint molecules whose structures have been confirmed by electron microscopy. Several other less well-characterized DNA pairing activities have been described from human cells (14–17) and from rodent cells and Drosophila (18).

A favored mechanism for the recognition and pairing of homologous sequences during recombination involves melting of duplex DNA in order to facilitate the recognition of the bases. Recombinase proteins may utilize an associated helicase activity and transiently melt duplex DNA while carrying out strand exchange in vitro. In this regard, it has been reported that recA and uvsX proteins can function as weak helicases over short regions less than 30 bp in length (19, 20). In this paper we examine whether the human and Drosophila recombinases exhibit in addition to strand exchange activity, a DNA helicase or melting activity. We report that under...

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conditions of efficient joint molecule formation by the recombination from the negative strand of M13mp18 viral DNA. We conclude that these eukaryotic recombinases recognize and pair homologous DNAs and form joint molecules without melting duplex DNA. The implications of this result for the mechanism of strand exchange by recombinases are discussed.

MATERIALS AND METHODS

Purification of a Strand Exchange Activity from HeLa Cells—A partially purified strand exchange activity from HeLa cells was prepared as follows. HeLa cells obtained from Dr. Bernard Moss, National Institute of Allergy and Infectious Diseases, Bethesda, MD, were grown to a density of 1 x 10^9 cells/ml in spinner culture in Eagle's spinner-modified minimal essential medium supplemented with 5% horse serum, 2 mM glutamine and containing 100 units/ml penicillin and 100 μg/ml streptomycin (Biologifs, Inc.). HeLa cultures were tested and found to be negative for mycoplasma. Cells were centrifuged at 100,000 g for 1 h and resuspended and dialyzed against 100 mM NaCl. The resulting clear extracts were prepared from approximately 10^9 cells and form joint molecules in a strand exchange assay described below. The crude extract was diluted with an equal volume of Buffer A (1% gelatin, 1 mM DTT, 500 mM NaCl, 1 mM EDTA, 10% glycerol, and 0.2 mM spermidine) and added to 60 ml of S-Sepharose Fast Flow chromatography resin (Pharmacia-LKB Biotechnology Inc.). An oligonucleotide, 0.7 nmol (220 ng) of the 5'-32P-labeled 57-mer, was 5'-end-labeled with 300 pCi of [γ-32P]-ATP (Du Pont-New England Nuclear, 6000 Ci/mmol) and 10 units of T4 polynucleotide kinase (Pharmacia PLC) and annealed to 1.2 μmol of single strand DNA (2). The resulting substrate had a specific activity of 1.5-2.0 x 10^9 cpm/1.2 x 10^9 pmol (40 ng) of M13mp18 DNA. All DNA concentrations are expressed as moles of nucleotides as well as weight.

For the linear helicase substrates, the gel-purified DNA containing an annealed 32P-labeled 57-mer (7.5 x 10^9 pmol, 250 ng) was digested with 25 units of BamHI, 20 units of Smal or 20 units of KpnI restriction enzyme (New England Biolabs) according to manufacturer's instructions in a volume of 30 μl for 10 min at 37 °C and used directly in helicase assays.

Strand Exchange Assays—Strand exchange assays were carried out at 37 °C as described previously (9) using 150 pmol (50 ng) of M13mp18 viral DNA (New England Biolabs) and 150 pmol (50 ng) of BamHI-linearized M13mp18 replicative form DNA and 100 ng of HeLa protein or 40 ng of Drosophila protein in the presence of 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM MgCl2, 1 mM ATP, and an ATP-regenerating system unless otherwise specified. E. coli recA and SSB proteins, obtained from Dr. Stephen Kowalczykowski, Northwestern University, were carried out under identical conditions except that the recA protein was incubated in assay buffer in the presence of single strand DNA alone for 5 min at 37 °C followed by the addition of SSB protein (23). After incubation for an additional 2 min, the double strand DNA was added, and incubation was continued at 37 °C for the times indicated in the text. In assays where SSB was omitted, the double strand DNA was added after a 2-min preincubuation of recA and single strand DNA. Protein concentrations are stated in the text. Reactions were stopped in 1% SDS, 10 mM EDTA, 0.04% bromphenol blue in 10 mM Tris acetate, pH 8.0, and 1 mM ethidium bromide at 0.6 V/cm for 14-16 h at room temperature. Quantitation of strand exchange assays was accomplished by densitometer scanning of Polakoid 66 negatives using an LKB 2202 Ultrascan densitometer and is expressed as the amount of linear duplex converted to joint molecule product.

Helicase Assays—32P-Labeled partially duplex substrate, 120 pmol (40 ng), was incubated at 37 °C with HeLa or Drosophila recombinase (300 or 40 ng of protein, respectively) under strand exchange assay conditions. Helicase assays were carried out as described above except that DNA substrates were preincubated for 2 min at 37 °C in assay buffer with recA alone prior to the addition of SSB and further incubation as indicated in the text. In assays where SSB was omitted, no preincubation with SSB protein was carried out. Protein concentrations are stated in the text. Reactions were stopped in 1% SDS, 10 mM EDTA, and tracking dye as described for strand exchange assays and electrophoresed on a

Joint molecules without melting duplex regions 20 bp long. We conclude that these eukaryotic recombinases recognize and pair homologous DNAs and form joint molecules without melting duplex DNA. The implications of this result for the mechanism of strand exchange by recombinases are discussed.

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RESULTS

A schematic diagram of a strand exchange assay is shown in Fig. 1A. Strand exchange by partially purified recombinases from HeLa cells and Drosophila embryos involves the pairing of homologous DNA substrates, in this case M13mp18 double strand linear DNA and homologous M13mp18 single strand circular DNA. The product of strand exchange is a joint molecule in which the two DNA substrates are joined by a region of DNA heteroduplex. The product of strand exchange by recombinase can be visualized by ethidium bromide staining of agarose gels. These eukaryotic recombinases can utilize with equal efficiency linear duplexes generated by restriction enzyme cleavage that yields flush ends or ends having short 3' or 5' overhangs (9, 10).

The substrate for DNA melting or helicase activity is shown in Fig. 1B. A short 32P-labeled oligonucleotide 20 or 30 bases long and labeled at the 5'-end was annealed to complementary M13mp18 single strand circular DNA at a site adjacent to the polylinker cloning site (see "Materials and Methods"). The gel-purified substrate was then incubated with human or Drosophila recombinase activity under conditions identical to those used for efficient strand exchange. The appearance of displaced 32P-labeled oligonucleotide was monitored by autoradiography after electrophoresis on 12% non-denaturing polyacrylamide gels. As mentioned previously, although strand exchange by recombinase does not require ATP, all helicases described so far have an absolute requirement for nucleoside triphosphate hydrolysis. Therefore, in order to compare relative strand exchange and helicase activities, both assays were performed in the presence of ATP and an ATP regenerating system as well as in the absence of ATP. Strand exchange assays utilized 50 ng each of the linear duplex and single strand substrates while helicase assays used slightly less DNA, 40 ng of the annealed duplex substrate.

The extensively studied prokaryotic helicases require a region of single strand DNA adjacent to the duplex to initiate unwinding. These helicases have a distinct polarity of translocation along the single strand DNA in a 5' to 3' or 3' to 5' direction (reviewed in Ref. 26). The helicase substrate shown in Fig. 1B has extensive regions of single strand DNA flanking both the 5' and 3' sides of the annealed oligonucleotide allowing one to detect a broad spectrum of helicases independent of their directionality.

The results of a strand exchange assay and helicase assay for the recombinase from HeLa cells is shown in Fig. 2A and B, respectively. Strand exchange by HeLa recombinase results in the appearance of a prominent product band migrating on agarose gels slower than either the single strand or double strand DNA substrates. We have shown previously that this represents the joint molecule product of strand exchange by recombinase. Quantitation by densitometer scanning of the strand exchange data shown indicates that 27% of the linear duplex corresponding to 40 pmol was converted to joint molecules by human recombinase in a 5-min incubation at 37 °C. Strand exchange experiments were also carried out in the absence of ATP with similar results (data not shown).

In contrast to the efficiency of strand exchange by human recombinase, analysis of the helicase assay revealed no significant displacement of either a 20- or 30-base oligonucleotide from annealed substrates in the presence or absence of ATP after a 5-min incubation at 37 °C (Fig. 2B). In fact, no displaced oligonucleotides above the low level present initially in control samples, lanes 1 and 2, were observed by densitometer scanning. Heat denaturation of both 32P-labeled 20- and 30-bp substrates indicates that most of the annealed oligonucleotide can be melted and recovered as full length fragments in our assay.

Small amounts of rapidly migrating 32P label were observed (8.8% of total label in lane 3 and 15% of total label in lane 5). While it is possible that this label results from contaminating phosphatase or nuclease activities acting on displaced single strand fragments, we do not believe this to be the case. First, the small amount of free unannealed oligonucleotide present in the initial substrate in control reactions without recombinase (lanes 1 and 2) is present to the same extent in the extract-treated samples (lanes 3 and 5). The presence in the extract of a phosphatase or nuclease activity specific for single strand DNA would have removed the label present in these oligonucleotides. Second, we assayed directly for the presence of a contaminating single strand-specific phosphatase or nuclease activity in the HeLa extracts. A short oligonucleotide 30 bases long was labeled at the 5' end with [γ-32P]ATP and purified by passage over a desalting column. An amount of the oligonucleotide, 0.8 pmol, corresponding to the amount of annealed oligonucleotide used in helicase assays was incubated with HeLa recombinase under conditions used for
agarose gels containing ethidium bromide. Assay conditions are described under "Materials and Methods." Reactions were stopped with SDS and EDTA and electrophoresed on conditions described under "Materials and Methods." Reactions were stopped with SDS and EDTA and electrophoresed on a 12% nondeaturing acrylamide gel and not from single-strand fragments.

Determination of the amount of 5'-32P-label retained in the annealed substrate after incubation with HeLa extract clearly demonstrates that the partially purified recombinase activity from HeLa cells contains very low levels of contaminating nucleases or phosphatases. Quantitation of the data in Fig. 2B establishes that 80-90% of the 32P label in the annealed substrate was released from the oligonucleotide indicating that contaminating single strand-specific phosphatases or nucleases are present at very low levels. We conclude that the 32P label released by contaminating activities in the extract originate from the annealed duplex and not from single-strand fragments.

The inability to demonstrate a helicase activity in the human recombinase fraction was not due to insufficient protein or incubation times. In experiments using human recombinase, we detected no helicase activity when a 5-fold excess of recombinase protein over the amount required for strand exchange was used or when incubation times were increased to 20 min (data not shown). Under the conditions of strand exchange used here, initiation of strand exchange and formation of joint molecules is essentially complete in 10 min.

In Fig. 3, we show the results of similar strand exchange and helicase assays with purified E. coli recA protein. Standard strand exchange substrates, 150 pmol of M13mp18 linear duplex DNA and 120 pmol of M13mp18 single strand DNA were incubated in ATP-containing strand exchange buffer in the presence of 0.9 μM recA protein for 10 or 60 min at 37 °C.

The results of similar assays for strand exchange and helicase activities using Drosophila recombinase are shown in Figs. 3, A and B, respectively. Quantitation by densitometer scanning of the strand exchange assay reveals that 66% (100 pmol) of the linear duplex DNA is converted to joint molecules in a 5-min incubation in the presence of ATP (Fig. 3A, lane 2). After a 5-min incubation under identical assay conditions, no helicase activity is detected (Fig. 3B). Less than 10% of the 32P label in the case of the 20-mer (lane 2) and less than 15% of the 32P label in the case of the 30-mer (lane 5) are present at the solvent front. The omission of ATP had no effect on strand exchange (data not shown) or helicase assays (lanes 3 and 6).

Fig. 2. Under conditions that promote strand exchange, the HeLa recombinase does not melt short duplex regions. A, strand exchange assay with HeLa recombinase containing 150 pmol of M13mp18 viral DNA and 150 pmol of M13mp18 linear duplex. Assay conditions are described under "Materials and Methods." Reactions were stopped with SDS and EDTA and electrophoresed on agarose gels containing ethidium bromide. Lane 1, control without HeLa protein; lane 2, addition of 100 ng of HeLa recombinase protein. B, helicase assays were carried out with HeLa recombinase under conditions described under "Materials and Methods." Reactions were stopped with SDS and EDTA and electrophoresed on a 12% nondeaturing polyacrylamide gel. Helicase substrates were a 32P-labeled 20-base oligonucleotide annealed to M13mp18, lanes 1, 3, and 4, or a 32P-labeled 30-base oligonucleotide annealed to M13mp18 DNA, lanes 2, 5, and 6. The 20- and 30-bp substrates were combined and heated at 100 °C for 3 min prior to electrophoresis (extreme left lane).

Fig. 3. Under conditions that promote strand exchange, Drosophila embryo recombinase does not melt short duplex regions. A, strand exchange reactions were carried out as described in Fig. 2A. Lane 1, control without Drosophila protein; lane 2, 40 ng of Drosophila protein. B, helicase assays were carried out as described in Fig. 2B. DNA substrate was a 20-bp duplex, lanes 1–3, and a 30-bp duplex, lanes 4–6. The 20- and 30-bp substrates were combined and heated at 100 °C prior to electrophoresis (extreme left lane).
As a positive control, the helicase substrates used in the experiments described above were incubated with bacteriophage T4 gene 41 and 61 proteins (Fig. 5). As established by others, the gene 41 and 61 proteins act as a primase for DNA replication (reviewed in Ref. 27). The gene 41 protein also has a 5' to 3' helicase activity that is stimulated by gene 61 protein and utilizes helicase substrates similar to those used in this study (28). When the annealed M13 substrates containing a 32P-labeled 20- or 30-base fragment was incubated with 41 and 61 proteins under appropriate conditions, the oligonucleotides were efficiently displaced by the T4 proteins (Fig. 5, lanes 3 and 5).

In the following experiment, we demonstrate that a helicase activity is not being masked by reannealing of a displaced fragment. We have previously shown that human recombinase, like recA protein from E. coli, can anneal complementary single strands (9). A possible explanation for the absence of detectable helicase activity is that any free oligonucleotide generated by helicase action is immediately annealed to M13 single strand DNA with an unoccupied site. Based on the specific activity of the 32P-labeled helicase substrates, we calculate that in excess of 75% of available sites are occupied by an annealed oligonucleotide at the start of helicase assays. Furthermore, we are unable to observe additional annealing of 32P-labeled oligonucleotides to helicase substrates prepared with cold oligonucleotides (data not shown). Thus, the number of vacant annealing sites is very small.

Despite the paucity of potential annealing sites, the following assay was carried out. The helicase assay was repeated in the presence of a 4-fold molar excess of M13 single strand circular DNA from an M13 clone containing a heterologous 3.5-kilobase insert from the Herpes simplex virus thymidine kinase gene. This single strand DNA, designated tkL, has an annealing site for the 32P-labeled oligonucleotides that might be displaced by a helicase activity. The tkL substrate also has a larger size and migrates on agarose gels at a position distinct from M13mp18 single strand DNA. Examination of helicase assays containing a 4-fold excess of tkL annealing sites revealed that all of the 32P-labeled oligonucleotides remained annealed to M13mp18 with no displacement of 32P-labeled DNA substrate, respectively. When the concentration of recA protein in strand exchange assays was lowered to 0.36 or 0.09 μM, no strand exchange products were observed even after 60 min incubation at 37 °C (Fig. 4A, lanes 7 and 8).

The results of helicase assays carried out in strand exchange buffer using purified E. coli recA protein are shown in Fig. 4B. When 120 pmol of the 32P-labeled 20-base duplex was incubated with 0.9 μM recA, 8% of the annealed 32P-labeled 20-mers was melted after incubation at 37 °C for 10 or 60 min, respectively (Fig. 4B, lanes 2 and 6). In the 60-min incubation, 8% of total 32P label migrated at the solvent front. In the presence of 0.36 μM recA, 4% of 32P-labeled 20-base fragments was melted after 10 or 60 min, respectively, at 37 °C (lanes 3 and 7). No helicase activity was observed at a concentration of 0.09 μM recA (lanes 4 and 8). Consistent with previous observations (19), we failed to observe melting by recA of the 30-base duplex, and we also failed to observe recA helicase activity in the presence of SSB protein which stimulates strand exchange by recA (data not shown). The absence of significant melting by recA under strand exchange conditions is not due to reannealing of melted single strand fragments by recA. This was determined by carrying out a competitive reannealing assay described in Fig. 6 in the presence of 0.9 or 0.36 μM recA protein (data not shown).

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of protein, remained unchanged. Despite the presence of excess recombinase activity we did not observe any displacement of the annealed oligonucleotide in a helicase assay nor did we observe any annealing of displaced oligonucleotide to tkL (data not shown). In contrast, the formation of joint molecules in the strand exchange assay under these conditions was efficient.

Shown in Fig. 6B is a control experiment demonstrating that the tkL single strand substrate has an annealing site for the 32P-labeled 20-base fragment and competes effectively with M13mp18 single strand DNA for displaced oligonucleotides. The 32P-labeled 20-bp helicase substrate was melted by heating at 100 °C in 10 mM Tris-Cl, pH 8.0, and 1 mM EDTA. A 4-fold molar excess of tkL single strand DNA was added to the heat-denatured sample which was brought to 100 mM NaCl and 10% (w/v) polyethylene glycol and incubated for 20 min (lane 3) or 60 min (lane 4) at 37 °C to allow reannealing. After electrophoresis on a neutral 0.7% agarose gel, autoradiography, and densitometry, the 32P-labeled 20-mer was observed to anneal to M13mp18 and tkL single strand DNAs at a ratio that reflected the relative molar concentrations of the two viral strand DNA species.

Finally, we attempted to detect a helicase activity using a substrate that more closely resembles the ends of the linear duplex substrate used in strand exchange. Two observations strongly suggest that strand exchange by human and Drosophila recombinases that yields stable joint molecules initiates from the ends of the linear duplex. These observations include the ability to block strand exchange with small regions, 61 bp, of nonhomologous sequence at the ends of the linear duplex (9, 10) and the ability of recombinase to form joint molecules when homology between the linear duplex and single strand circular DNA is restricted to a short region at the end of the linear duplex.2

The helicase substrate shown in Fig. 7A was constructed by annealing a 5'-32P-labeled oligonucleotide 57 bases long to M13mp18 single strand DNA creating several unique internal restriction enzyme sites. Cleavage of the gel-purified annealed substrate with BamHI results in a long linear single strand DNA flanked at both ends by a short duplex region of 18 or 39 bp. Helicase activity that acts on duplex ends was monitored by release of the 32P-labeled 18-base fragment. An autoradiogram of the helicase assay using both HeLa and Drosophila recombinases is shown in Fig. 7B. As we observed previously, no significant helicase activity is detectable after quantitation by densitometer scanning. With one exception, no 32P-labeled oligonucleotides were displaced. While a small amount of displaced 18-base oligonucleotide in assays containing ATP and HeLa recombinase is visible in the overexposed autoradiogram shown (lane 3), this constitutes less than 1% of total annealed fragments as determined by densitometer scanning. In the absence of ATP (lane 4), no such displaced oligonucleotide is observed even though the recombinase is fully competent to carry out strand exchange in the absence of nucleotide cofactors. As we noted earlier, some 32P label was present at the solvent front. Quantitation of the autoradiogram shown in Fig. 7 indicated that in the assays containing HeLa recombinase, less than 7% of the total 32P label in the presence of ATP (lane 3) and 5% of the 32P label in the absence of ATP (lane 4) is released. The Drosophila extract released somewhat more 32P label, 14% of total label in the presence of ATP (lane 5) and 26% of total label in the absence of ATP (lane 6). For reasons that are unclear, the Drosophila recombinase removed more of the 5'-32P label from

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2 P. Hsieh, C. S. Camerini-Otero, and R. D. Camerini-Otero, unpublished observations.
residual 57-mers in the presence of ATP than in the absence of ATP (compare lanes 5 and 6). Nevertheless, the amount of the 57-mer species is exceedingly small, and ATP is not a requirement for strand exchange by the Drosophila recombinase.

The results of helicase assays using the linearized 57-bp duplex substrate are particularly relevant to the issue of melting of duplex ends by strand exchange proteins. This substrate is designed to detect an activity that would not utilize the partially duplex circular substrates, namely, a helicase that initiates from the ends of linear duplexes and has no requirement for binding to single strand DNA to initiate melting. Like the circular helicase substrates, the linear substrate detects helicases that translocate either 5' to 3' or 3' to 5' from the ends of the linear duplex.

We have performed more extensive experiments with this substrate in order to examine two parameters that might have resulted in a bias in the detection of helicase activity using the BamHI-linearized 5'-32P-labeled substrate. These parameters included the length of the 32P-labeled duplex region that could be melted by a helicase and the configuration of the ends of the linear duplex region. Helicase assays shown in Fig. 8 were carried out with human recombinase and the 5'-32P-labeled 57-bp substrate linearized at unique BamHI, Smal, or KpnI restriction sites. The BamHI-digested substrate has a 32P-labeled 18-bp duplex region with a 5' overhang; the Smal-digested substrate has a 32P-labeled 25-bp duplex region with flush ends; the KpnI-digested substrate has a labeled 41-bp duplex region with a 3' overhang. As we observed above, no melting of duplex DNA by HeLa recombinase was observed using any of these substrates.

**DISCUSSION**

We have examined whether recombinases from HeLa cells and *D. melanogaster* embryos can melt short regions of duplex DNA under conditions that promote efficient in vitro strand exchange leading to the formation of joint molecule products. Using two different helicase substrates, we have demonstrated that human and Drosophila recombinase activities have no detectable helicase activity.

The first substrate examined was a short duplex with flanking single strand regions formed by annealing a 20- or 30-base oligonucleotide to M13mp18 circular single strand DNA. Similar substrates were previously shown to be utilized by *E. coli* recA protein (19) as well as several prokaryotic helicases including T4 gene 41 protein (24), *E. coli* helicases I (29) and II (30), and *E. coli* uvrAB proteins (31) and two eukaryotic proteins, helicase B from mouse FM3A cells (32) and SV40 T antigen (33).

Human and Drosophila recombinases did not melt the 20- or 30-bp duplex despite forming joint molecules under identical assay conditions. In control experiments, we observed that T4 gene 41 and 61 proteins efficiently melted the duplex substrates. The inability of the eukaryotic strand exchange activities to melt short duplexes was still apparent when five times the amount of recombinase required for strand exchange was used or when incubation times in helicase assays exceeded those required for maximum joint molecule formation. In addition, control experiments rule out the possibility that a helicase activity present in recombinase fractions was being masked by reannealing of displaced strands. Although the duplex regions used in these helicase assays is very short, their length is most likely not a factor in our inability to detect melting. We have observed that human and *Drosophila* recombinases can recognize homologous sequences considerably shorter than 30 bp in a strand exchange reaction where homology between single strand and double strand substrates is limited to one end of the linear duplex.

The second helicase substrate was designed to mimic the ends of the strand exchange linear duplex substrate, M13mp18 linearized with BamHI. This helicase substrate shown in Fig. 7A has a long single strand region flanked on both ends by short (18 and 39 bp) duplex regions with 5' overhangs or flush ends for strand exchange (9, 10), we failed to detect melting of linear duplexes despite altering the configuration of the ends to yield each of the three possibilities.

Two main conclusions can be drawn from our findings. First, the formation of joint molecules by human and *Drosophila* recombinases in which a linear duplex and a homologous circular single strand DNA are joined by a region of heteroduplex DNA does not occur by a two-step helicase and reannealing mechanism. Such a pathway would involve the melting of the ends of the M13 linear duplex independently of the presence of single strand DNA; subsequently, the
homologous single strand DNA could anneal to the melted strands of the linear duplex giving rise to a joint molecule. As we discussed previously for the human recombinase (9) the detection of heteroduplex tracts longer than 150 bp coupled with the inability to form joint molecules when 61 bp of nonhomologous sequence blocks the ends of the linear duplex makes a helicase and reannealing mechanism unlikely. In addition, joint molecules are formed efficiently in the absence of any nucleoside triphosphate, an absolute requirement for all known helicasas. In this paper, we demonstrate directly that under conditions of joint molecule formation, no detectable helicase activity is observed. We conclude that the formation of joint molecules by recombinase is via a strand exchange mechanism in which pairing of the homologous single strand and double strand molecules is required for initiation of strand invasion.

Our results do not exclude the possibility that a separate and distinct eukaryotic helicase might act in concert with strand exchange proteins in homologous recombination. It has been postulated that the RecBCD protein with its helicase activity, acts in concert with recA protein to mediate homologous recombination in E. coli (reviewed in Ref. 34). In addition, it has been shown that the strand exchange activity of the bacteriophage T4 recombinase, uvsX protein, is stimulated by T4 dda protein, a DNA helicase (35).

The second point regarding our findings is related to the mechanism of strand exchange by these recombinases. The ability of strand exchange proteins to act as helicasas might explain how these proteins pair homologous sequences. Melting of DNA duplex regions would allow base pairing with an invading single strand DNA.

While we cannot rule out melting by recombinase of extremely short regions of DNA less than one helical turn of DNA in length, the absence in human and Drosophila recombinases of any detectable melting of short duplex regions makes it unlikely that these recombinases melt the ends of a linear duplex in order to facilitate pairing with single strand DNA. Since strand exchange by the HeLa and Drosophila recombinases appears to initiate from the ends of the linear duplex, we conclude that a helicase activity is not involved in the early steps of pairing by these recombinases. In our hands, E. coli recA protein can act as a weak helicase over a region of 20 bp but only in the absence of E. coli SSB protein which has been shown by others to stimulate recA-mediated strand exchange (reviewed in Ref. 4). We also observe that under comparable assay conditions, the strand exchange activity of recA is not associated with a correspondingly efficient strand separating activity. These results raise the possibility that recA protein may also recognize and pair homologous DNAs without melting duplexes. Perhaps, as suggested by others, this process takes place within a three-stranded DNA intermediate (36-38). Our findings leave open the question of whether the eukaryotic recombinases unwind duplex regions without melting of the base pairs. The unwinding of duplex DNA by recA protein is well documented (39-41).

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