Mechanisms by Which Bloom Protein Can Disrupt Recombination Intermediates of Okazaki Fragment Maturation*

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Bloom syndrome is a familial genetic disorder associated with sunlight sensitivity and a high predisposition to cancers. The mutated gene, Bloom protein (BLM), encodes a DNA helicase that functions in genome maintenance via roles in recombination repair and resolution of recombination structures. We designed substrates representing illegitimate recombination intermediates formed when a displaced DNA flap generated during maturation of Okazaki fragments escapes cleavage by flap endonuclease-1 and anneals to a complementary ectopic DNA site. Results show that displaced, replication protein A (RPA)-coated flaps could readily bind and ligate at the complementary site to initiate recombination. RPA also displayed a strand-annealing activity that hastens the rate of recombination intermediate formation. BLM helicase activity could directly disrupt annealing at the ectopic site and promote flap endonuclease-1 cleavage. Additionally, BLM has its own strand-annealing and strand-exchange activities. RPA inhibited the BLM strand-annealing activity, thereby promoting helicase activity and complex dissolution. BLM strand exchange could readily dissociate invading flaps, e.g. in a D-loop, if the exchange step did not involve annealing of RPA-coated strands. Use of ATP to activate the helicase function did not aid flap displacement by exchange, suggesting that this is a helicase-independent mechanism of complex dissociation. When RPA could bind, it displayed its own strand-exchange activity. We interpret these results to explain how BLM is well equipped to deal with alternative recombination intermediate structures.

Bloom protein (BLM) is a member of the RecQ family of 3′-5′ helicases that assist in maintaining genome stability. Mutation or loss of function of the BLM protein causes Bloom syndrome (BS), an autosomal recessive disease characterized by sunlight sensitivity, proportional dwarfism, and a high predisposition toward many different types of cancer (1). Cells with BLM deficiency show increased chromosomal abnormalities, including hyper-recombination, elevated rates of sister chromatid exchange, and the abnormal accumulation of replication intermediates, resulting in an increase in the overall level of genomic instability (2–4). Knock-out of BLM in mice causes embryonic lethality, whereas some mutations produce live mice prone to tumorigenesis (5, 6).

BLM plays a role in several critical genome maintenance pathways. Immunodepletion of Xenopus BLM inhibits the replication of DNA in reconstituted nuclei, suggesting that BLM is directly involved in DNA replication (7). Telomere proteins TRF2 and TRF1 colocalize with BLM in immortalized cells lines and regulate its helicase activity in vivo, signifying a role for BLM in telomere maintenance (8). BLM assists in the recovery of stalled replication forks and in the prevention of repeat expansion by stabilizing repeated sequences (9–13). Additionally, BLM has been proposed to promote proper intermediate resolution and suppress crossovers in the homologous recombination pathway (14).

BLM potentially assists in resolution of illegitimate recombination intermediates through the utilization of three main enzymatic activities. BLM possesses a helicase function, driven by a DNA-dependent ATPase activity, observed in the presence of single-stranded DNA (15). Its helicase activity is structure-specific, preferentially unwinding substrates that simulate replication and recombination intermediates such as forked duplexes and four-way junctions with Holliday structure (10, 16–18). Always displaying a 3′ to 5′ polarity, it prefers to unwind partial duplex substrates that are <71 nucleotides long and has difficulty with both substrates longer than 259 nucleotides and with blunt-ended DNA duplexes (10, 19). BLM helicase activity is also stimulated by ssDNA-binding protein RPA in a length-dependent manner (19). BLM stimulates topoisomerase IIα to relax negatively coiled DNA, a DNA configuration that may affect the maximum length of DNA substrates that BLM can unwind (20). The two proteins can also form a complex that helps to suppress inadvertent recombination and breaks up DNA base mismatches while facilitating the resolution of recombination intermediates (14).

BLM and other RecQ family members can also utilize a recently discovered strand-pairing activity to promote the annealing of complementary DNA (21, 22). Like RecQ family member Werner protein, BLM contains a conserved domain called RQC (RecQ C-terminal) that forms a winged-helix structure exploited to bind DNA (23). Additionally, BLM contains a...
motif called HRDC (helicase and RNase D C-terminal) that when folded creates another DNA binding site (24, 25). The multiple DNA binding domains allow BLM to bind two or more single-stranded DNA oligomers simultaneously, bringing them together and facilitating strand annealing. This property of BLM would seem counteractive of its role in breaking up recombination intermediates, because with the annealing property BLM can actually promote the binding of short (<50 nt) uncleaved DNA flaps to complementary regions, thus creating illegitimate recombination intermediates. The strand-annexing property has been reported to work in conjunction with helicase activity in the presence of ATP to promote strand exchange (21). It has been shown that BLM possesses the ability to catalyze branch migration unidirectionally through long stretches of DNA and act on recombination intermediates containing a Holliday junction in an ATP-dependent manner (10).

BLM binds and stimulates cleavage activity of the flap endonuclease (FEN1) (26). The primary role of FEN1 is thought to be removal of the RNA primer of Okazaki fragments by flap cleavage. This suggests that BLM is a component of the DNA replication machinery. Current models of Okazaki fragment processing suggest that a subset of flaps becomes 20–40 nucleotides long prior to cleavage (27). At that length, single-stranded binding protein RPA can bind to the flap and inhibit FEN1 cleavage. The helicase/nuclease Dna2, having a cleavage activity that is stimulated by RPA, will bind and cleave the long flap (27). This will leave a flap of ~5–7 nucleotides, which FEN1 will subsequently cleave to form a ligatable nick (28). A likely role of BLM is to prevent these longer single-stranded flaps from forming productive recombination intermediates at ectopic sites before they are correctly processed by Dna2.

We show that BLM can disrupt recombination intermediates by helicase function. Further, we demonstrate that these intermediates can be disrupted in an ATP and helicase-independent manner by BLM strand-annexing activity and promotion of branch migration. The presence of RPA adds another dimension because it has its own strand-annexing activity that can promote flap annealing to form recombination intermediates. This activity suggests a need for BLM to inhibit illegitimate recombination during Okazaki fragment processing.

**EXPERIMENTAL PROCEDURES**

**Materials**—All oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA). Radionucleotides \([\gamma^{-32}P]ATP\) (3000 Ci/mm mol) and \([\alpha^{-32}P]dCTP\) (6000 Ci/mm mol) were bought from PerkinElmer Life Sciences. The T4 polynucleotide kinase (labeling grade), the Klenow fragment of DNA polymerase I, and ATP were from Roche Applied Science. All other reagents were of the best available commercial grade.

**Enzyme Expression and Purification**—Recombinant human FEN1 was expressed with the T7 expression plasmid pET-FCH and purified as described previously (29). Recombinant human BLM was expressed in yeast strain JEL1 transformed with pJK1. BLM was purified as described previously (15). Recombinant human DNA ligase I was cloned into T7 expression plasmid pET-15B (Novagen, WI) and was purified as described previously (30). Recombinant human RPA was provided by Dr. Mark Wold and had been purified as described previously (31).

**Oligonucleotide Substrates**—Oligomer sequences are listed in Table 1. Sequences were designed to represent a long flap Okazaki fragment maturation intermediate that annealed to complementary ssDNA to form an illegitimate recombination intermediate. Labeling and purification of substrates were performed as described below. Using standard procedures, substrates U2 and T2 were radiolabeled at the 5’-end with \([\gamma^{-32}P]ATP\) and T4 polynucleotide kinase. To 3’-end-label flap substrates F0, F3, and F20, 20 pmol of each substrate was annealed to 50 pmol of template T2 modified with a 5’-G overhang. Each flap substrate was extended by adding Klenow fragment of Escherichia coli DNA polymerase I and \([\alpha^{-32}P]dCTP\). Both the 5’- and 3’-end radiolabeled substrates were purified on 15% denaturing polyacrylamide gels containing 7 M urea.

The recombination intermediate complex containing T1, U1, F, U2, and T2 was constructed in two consecutive steps. When any F-strand was the radiolabeled substrate, T1, U1, and F were annealed together at a molar ratio of 2:4:1, whereas U2 and T2 were annealed together separately at a ratio of 4:2. For annealing, the oligomers were placed in 50 μl of annealing buffer (50 mM Tris–EDTA (pH 8.0), 50 mM KCl, and 1 mM dithiothreitol) and heated to 95 °C for 5 min. The reaction mixture was then placed at 70 °C and allowed to cool down to room temperature over the course of 2 h. The separate reactions (T1: U1:F and T2:U2) were then mixed together, placed at 37 °C for 1 h, and then allowed to incubate at room temperature overnight to achieve full annealing. Using this method, 80% of the labeled substrate was consistently incorporated into the desired complex. When U2 was the radiolabeled substrate, the molar ratios were 3:4:2:3:1 (T1:U1:F:U2:T2), respectively. When T2 was the radiolabeled substrate, the molar ratios were 3:4:2:2:1 (T1:U1:F:U2:T2), respectively.

**Enzyme Assays**—Reactions were performed in 30 mM HEPES (pH 7.5), 5% glycerol, 40 mM KCl, 0.1 mg/ml bovine serum albumin, and 4 mM MgCl2 with or without 2 mM ATP. Enzyme stocks were diluted in 30 mM HEPES (pH 7.5), 5% glycerol, 40 mM KCl, and 0.1 mg/ml bovine serum albumin. Each reaction contained 5 fmol of radiolabeled substrate in a 20-μl reaction mixture with varying amounts of the enzymes as indicated in the figure legends. All assays were incubated at 37 °C for the specified times. Any assays that required dissociation of the complex for analysis (ligase assays, etc.) were stopped by the addition of 2× termination dye (95% formamide v/v with bromphenol blue and xylene cyanole) followed by 5 min at 95 °C, and then resolved on 15% polyacrylamide, 7 M urea denaturing gels, run at 80 watts for 90 min. Any assays that required the complex to remain intact were stopped by the addition of 2× helicase dye (30% glycerol, 50 mM EDTA, 0.9% SDS, 0.25% bromphenol blue, and 0.25% xylene cyanole). These were immediately run on a native 12% polyacrylamide gel at 25 watts for 3 h. Each gel was quantitated using a PhosphorImager (Amersham Biosciences) and analyzed using ImageQuant version 1.2 software from Amersham Biosciences. In all studies, the quantitated amounts of substrates and products were utilized to calculate the percentage of product formation from the product/(product plus substrate) ratio. This method allows for the correction of any loading errors among lanes. The graphed data points were calculated by quantifying the pixel density of each band.
FIGURE 1. Creating an illegitimate recombination intermediate. A, shown is a schematic diagram of how the recombination intermediate would form in vivo. i and ii, typical Okazaki fragment replication. iii, when the distal Okazaki fragment is encountered, the RNA primer (gray) and part of the newly replicated DNA is displaced by DNA polymerase. iv, the result is a DNA/RNA flap. v/vi, if the flap remains unprocessed, it can bind to the complementary DNA strand via illegitimate recombination. Removal of the RNA followed by primer extension creates a nick. If ligated, this produces an intermediate that can disrupt DNA replication and create chromosomal aberrations. B, the final structure of our experimental recombination intermediate. T1 is the original ssDNA template utilized in Okazaki fragment replication. U1, the upstream primer, represents the proximal Okazaki fragment extended by DNA polymerase that has displaced the distal Okazaki fragment to create a flap substrate (F). The 1-bp overhang on the 3’-end of U2 creates a preferred FEN1 substrate when the recombination intermediate complex is broken up. Our three flap substrates, F20, F3, and F0 are so named because when they bind to the complementary template (T2), they form single-stranded regions between T1 and T2 of 20, 3, and 0 nucleotides, respectively. U2 is in place to provide a ligation substrate to detect annealing of the flap to T2. C1 and C2 are substrates complementary to part (C2) or all (C1) of template T2. They are utilized in experiments examining BLM-strand annealing and -exchange properties. Table 1 contains the sequences of all the complex substrates.
**Bloom Protein and Okazaki Maturation**

**TABLE 1**

| Oligonucleotide sequences (5’-3’) |
|----------------------------------|
| The double underlining represents annealed regions of exchange substrates and templates. The single underlining represents annealed regions of upstream primers and templates. Un-annealed regions are in boldface. |

| Upstream primers | 26-mers | 20-mers |
|------------------|---------|---------|
| U1               | GGA CCA TGG TAC GCC GTA TTA TCT TCA CAA CTT TGA | TCA GTA TTA AAG CTA TGG GTA |
| U2               | GGA CCA TGG TAC GCC GTA TTA TCT TCA CAA CTT TGA | TCA GTA TTA AAG CTA TGG GTA |

| Templates | 51-mers | 57-mers |
|-----------|---------|---------|
| T1        | GGA CCA TGG TAC GCC GTA TTA TCT TCA CAA CTT TGA | TCA GTA TTA AAG CTA TGG GTA |
| T2        | GGA CCA TGG TAC GCC GTA TTA TCT TCA CAA CTT TGA | TCA GTA TTA AAG CTA TGG GTA |

| Flaps | 63-mers | 46-mers | 43-mers |
|-------|---------|---------|---------|
| F20   | GGA CCA TGG TAC GCC GTA TTA TCT TCA CAA CTT TGA | TCA GTA TTA AAG CTA TGG GTA |
| F3    | GGA CCA TGG TAC GCC GTA TTA TCT TCA CAA CTT TGA | TCA GTA TTA AAG CTA TGG GTA |
| F0    | GGA CCA TGG TAC GCC GTA TTA TCT TCA CAA CTT TGA | TCA GTA TTA AAG CTA TGG GTA |

| Exchange substrates | 57-mers | 37-mers |
|---------------------|---------|---------|
| C1                  | GGA CCA TGG TAC GCC GTA TTA TCT TCA CAA CTT TGA | TCA GTA TTA AAG CTA TGG GTA |
| C2                  | GGA CCA TGG TAC GCC GTA TTA TCT TCA CAA CTT TGA | TCA GTA TTA AAG CTA TGG GTA |

and subtracting the measurable background pixel density. The percentages of strand annealing, strand exchange, and helicase activities were calculated as the amount of labeled DNA in the final product band divided by the total amount of DNA from all bands in the gel lane.

In most assays, 100 fmol of BLM was found to be sufficient for demonstrating BLM-related activities. At 50 fmol, RPA is approximately equimolar to the recombination intermediate complex because of all of the excess DNA needed to ensure that all (or almost all) of the labeled substrate is in complex. Because each assay contained ~50 fmol of single-stranded DNA, 50 fmol of RPA was used in all non-titration assays to maintain the 1:1 RPA:ssDNA ratio.

**RESULTS**

Creating Illegitimate Recombination Intermediates—We hypothesize that normal eukaryotic DNA replication produces intermediates that can lead to illegitimate recombination. Here we examine the functional activities of BLM and how they work on such intermediates to promote genome stability.

The creation of a long flap during Okazaki fragment processing and participation of the flap in recombination are diagramed (Fig. 1A). We designed a series of complexes that simulate this illegitimate recombination intermediate (Fig. 1B). The basic structure consists of two templates (T); T1, which is the template for Okazaki fragment synthesis, and T2, the alternative template to which the displaced flap can anneal. The primer U1, upstream with respect to synthesis, represents the newly synthesized Okazaki fragment displacing the flap. It is annealed to the 3'-end of template T1 and forms a 1-nucleotide 3' overhang flap at the annealing point of the downstream flap substrate F, creating the favored FEN1 cleavage substrate. The upstream primer U2 represents the newly synthesized DNA from utilization of template T2. This has been included in the complex such that, when the flap substrate (F) is annealed, U2 and F can be ligated together to represent an additional step in the recombination reaction. Although flaps processed during Okazaki fragment maturation contain small RNA primers at their 5'-ends, the F flap DNA oligonucleotides were designed as we would presume the recombined flap structure to be prior to ligation in vivo. Even though annealing of the flap inhibits FEN1 endonucleolytic cleavage, RNase H or FEN1 exonucleolytic activity should degrade the RNA at the end of the flap. The resulting DNA terminus would then be free for ligation to create a stable recombination intermediate. Substrates F20, F3, and F0 annealed to T1 are designed to represent un-cleaved flaps created during Okazaki maturation. When also inappropriately annealed to T2 they form crossovers with ~20, 3, and 0 nucleotides of single-stranded DNA between the two templates, respectively, depending on whether partial melting occurs in complexes with flap substrates F3 and F0. These central nucleotides were not complementary to any part of the templates, allowing them to remain single-stranded. For flap substrates, the 3'-end was complementary to the 5'-end of the template T1. The 5'-end that forms the flap can be annealed to template T2 to form the illegitimate recombination intermediate. There are two substrates used for strand exchange experiments. Exchange substrate C1 is the complementary sequence to template T2. When utilized during strand exchange, it will anneal to cause the dissociation of both the flap and U2 from template T2. C2 represents the correct pairing partner for the section of T2 encompassing the downstream ssDNA portion as well as the annealing region of the flap substrates. If annealed, C2 would displace the flap substrates only, leaving U2 bound to T2 and creating a ligatable substrate. The sequences of the strands used to produce all of these structures are given in Table 1.

Long Flaps Can Readily Form Recombination Intermediates—Okazaki fragment flaps exceeding 27 nucleotides in length are proposed to be coated by the single-stranded binding protein RPA (27, 32, 33). We assessed whether an RPA-coated flap would anneal to an ectopic site and ligate to an adjacent primer. For the measurement, we formed two different structures. The annealing of these structures is the readout of the experiment. The first was composed of oligomers T1, U1, and F20, to simulate the dsDNA Okazaki fragment/template DNA with the ssDNA unprocessed flap. The second was composed of oligomers U2 and T2 representing the complementary ssDNA to which the unprocessed flap might bind. We found that RPA possesses its own strand-anealing activity and will promote annealing of the flap complex to the complementary template in the absence of BLM (Fig. 2A). This is seemingly contradictory to earlier reports that RPA disrupts the formation of recombination intermediates by both actively promoting strand unwinding after their formation, and by preventing the anneal-
ing of single-stranded DNA in the presence of annealing advocates Werner protein and BLM (34). Our results demonstrate that RPA promotes strand unwinding as well. The combined effects should produce an equilibrium in which the rate of RPA-induced ssDNA strand annealing is equivalent to the rate of RPA-induced dsDNA dissociation (Fig. 2B). In the absence of BLM, only 5.8% of the labeled flap F20 annealed to template T2 after 120 min without RPA present, whereas 35.7% of the flap annealed with 50 fmol of RPA present (1:1 ratio with DNA), a 6-fold increase. Conversely, 23% of the normally stable complex was deconstructed in 120 min in the presence of RPA. However, over 73% of the fully annealed complex was also ligated by human DNA ligase I, showing that RPA-coated strands are readily ligatable (data not shown). This observation is consistent with earlier reports showing that RPA stimulates the activity of DNA ligase I (35). These findings demonstrate that RPA-coated flaps, expected to be produced during Okazaki fragment processing, can equilibrate with an ectopic site to readily form recombination intermediates.

**BLM Strand-annealing activity Promotes Recombination**

Three RecQ members, including BLM, possess a strand-pairing activity in which they promote single-stranded DNA annealing (21, 36). Because our recombination intermediate complex model is derived from an unprocessed ssDNA annealing to a complementary ssDNA strand, we wanted to test whether the addition of BLM would actually accelerate the formation of the intermediate. This action would counteract BLM helicase activity, which works to disrupt the complex. For the measurement, we utilized the same structures that were used to measure RPA strand annealing (flap substrate T1:U1:F20 and complementary template U2:T2).

We found that BLM greatly accelerated the formation of the recombination intermediate complex compared with the formation in the absence of enzyme. Specifically, 73% of the label, which was on F20 of the unprocessed flap complex, was found in the recombination intermediate complex after 10 min, compared with only 1.1% in the reaction without BLM (Fisher’s exact test, \( p < 0.001 \); data not shown). Over 8 h were necessary for the unassisted reaction to approach the level of strand annealing seen in the presence of BLM (data not shown). ATP was then added to the reaction to ascertain the simultaneous effects of the helicase and annealing activities. After an early spike in the formation of the recombination intermediate, equilibrium between BLM helicase activity and BLM strand annealing was achieved at \( 10 \) min, as the amount of formed recombination intermediate leveled off at \( 30\% \) of the labeled DNA (Fig. 3A). A slow increase in the intermediate over the next 2 h may have been due to some depletion of ATP by BLM helicase activity. Alternatively, the helicase activity may be less stable than the annealing activity.
RPA Directly Blocks BLM Strand-annealing Activity—Others have found that RPA inhibits the strand-annealing property of BLM family members RECQ4 (37) and RECQ5β (38). Additionally, RPA also prevents the re-annealing of ssDNA substrates after BLM helicase activity dissociates them, presumably by binding the ssDNA and blocking re-annealing (34). Because we found that RPA actually facilitates annealing of ssDNA in the absence of BLM, we decided to test whether the effect seen previously was due to RPA blocking the strand-annealing activity of BLM. This interpretation suggests that BLM strand annealing does not counteract its helicase activity in vivo.

BLM Helicase Activity Disrupts the Recombination Intermediates—BLM helicase activity has been demonstrated to prefer specific DNA structures that represent recombination intermediates, such as Holliday junctions (10), D-loops (16), and bubble substrates (17), to standard duplex DNA. To analyze the substrate specificities of BLM helicase, we tested its activities on the recombination intermediate model substrates...
BLM helicase activity was measured using two different methods of detection. The first was based on flap cleavage by FEN1. As BLM dissociates the recombination intermediate, the single-stranded flap normally resulting from Okazaki fragment strand displacement will be re-created. Because BLM also stimulates FEN1 cleavage activity (26, 39), the characteristic FEN1 cleavage product should rapidly appear, and the resulting radiolabeled ssDNA can be detected using PAGE.

Under our measurement conditions FEN1 readily cleaved the flap when its 5′ terminus was free, and not part of the recombination intermediate complex, even without BLM stimulation. However, once the flap was incorporated into the recombination intermediate, FEN1 would not cleave the flap unless both BLM and ATP were present (Fig. 4A). This demonstrated that BLM helicase activity is capable of dissociating the recombination intermediate to allow correct flap cleavage. BLM helicase activity is partially inhibited on the intermediates with F3 and F0 when compared with the F20 intermediate (Fig. 4B). This suggests that BLM interaction with the substrate prior to helicase activity is partially obstructed when three or fewer nucleotides of ssDNA are available for binding. BLM stimulation of FEN1, which does not require ATP, is insufficient to dissociate the recombination intermediate for FEN1 cleavage. Overall, the results show that BLM can readily access flap recombination intermediates and use helicase activity to free their 5′-ends for FEN1 cleavage.

The second method of helicase detection utilized the nick between the upstream primer U2 and the annealed 5′ portion of the flap. If the recombination intermediate were not broken up by BLM helicase activity, then the nicked substrate would remain as a substrate for DNA ligation. The ligated strands would migrate slower than the un-ligated flap, with partial ligation resulting in two distinct bands in a PAGE separation. We saw effective disruption of the intermediate by BLM, with lesser activity on the substrates with the zero and three nucleotide single-strand regions (data not shown). Overall, the results showed how BLM could prevent covalent joining of a flap 5′ terminus to a site of illegitimate recombination.

RPA was not included in the previous set of experiments, because it inhibits FEN1 cleavage (27). However, RPA has been shown to have a stimulatory effect on BLM helicase activity (19, 40). Using a native gel to resolve complex structures, we found that addition of RPA up to ten times in excess over the labeled substrate had no effect on BLM helicase activity for complexes using flap F20, which is 38 nucleotides in length (data not shown). It should be noted, however, that the positive effect of RPA on BLM helicase activity is proportional to the length of the duplex DNA being unwound (19). Thus, these results show that RPA does not stimulate helicase activity on short intermediates; however it may be of assistance to BLM helicase disruption of longer intermediates.

BLM-catalyzed Strand Exchange Can Potently Suppress Recombination—BLM single strand DNA-pairing activity has been demonstrated to work with BLM DNA helicase activity to perform coordinated strand exchange (21). To discern whether BLM can initiate strand exchange on our recombination inter-

FIGURE 4. BLM could dissociate the recombination intermediate to promote FEN1 cleavage of the flap. A, the kinetics of BLM helicase activity on an intermediate is shown. Complexes were constructed as described under “Results.” Radiolabeled at the 3′-end, flap F can only be cleaved by FEN1 when the recombination intermediate is dissociated. Lanes 1–6 represent cleavage of an FEN1-preferred substrate. Lanes 7–12 show the same substrate now in complex. Lanes 13–30 include 100 fmol of BLM, 2 mM ATP, and complexes containing F20 (lanes 13–18), F3 (lanes 19–24), and F0 (lanes 25–30), respectively. FEN1 cannot cleave the flap unless the complex is broken up by BLM helicase activity. As described under “Experimental Procedures,” disruption of the recombination intermediate by BLM helicase activity was performed at 37 °C in the presence or absence of ATP for the following times: 0, 1, 2, 5, 10, and 20 min. After addition of 2× termination dye and 5 min of boiling at 95°C, the samples were run on a 15% polyacrylamide denaturing gel for 1.6 h at 80 watts. Each lane contained 5 fmol of radiolabeled flap substrate. The asterisk indicates which substrate is radiolabeled. B, the percentage of radiolabeled flap displaced by BLM helicase activity that was cleaved by FEN1 at each time point in A. Data were normalized as demonstrated under “Experimental Procedures.” Points are plotted for the percentage of labeled substrate cleaved by FEN1 versus time. ○, FEN1-preferred flap complex, U1:T1:F20; ●, F20* recombination intermediate, FEN1/BLM (no BLM helicase activity); ○, F20* recombination intermediate, FEN1/BLM/ATP; □, F3* recombination intermediate, FEN1/BLM/ATP; △, F0* recombination intermediate, FEN1/BLM/ATP.
mediate complexes, we designed two single-stranded DNA oligomers complementary to template T2. The first oligomer, called C1, is the exact complement to template T2. The second (C2), complements T2 from its 5'-end through to the section that is bound by flap F20.

We hypothesized that BLM strand-annealing activity would bring the free ssDNA ends of T2 and C1 together. When the annealed flap (F20) is encountered, it would be displaced via branch migration facilitated by BLM strand-exchange activity, creating a double-stranded DNA complex comprised of T2 and C1. To test this, we combined the recombination intermediate complex containing 5 fmol of radiolabeled flap substrate with 25 fmol of C1 and 100 fmol of BLM at 37 °C and measured strand exchange at time intervals between 0 and 30 min. Strand exchange is denoted by the appearance of a fast-moving band in each lane, corresponding to the T1:U1:F20:U2:T2* complex (Fig. 5A).

In the absence of BLM, only 6% of the radiolabeled F20 underwent strand exchange after 30 min, compared with 68% in the presence of BLM. As expected, the recombination intermediate model substrates with 20 nucleotides, 3 nucleotides, or no single-stranded DNA between the two DNA templates (F20, F3, and F0, respectively) all underwent strand exchange at approximately equal rates (4.68%/min, 5.22%/min, and 5.12%/min) during the first 15 min of the experiment, after which the levels of strand exchange plateaued (Fig. 5B). This is especially interesting following the findings that a range in the length of un-annealed flap regions can affect helicase activity. It suggests that, when BLM is obstructed from binding to a single strand to initiate helicase activity, the strand-exchange activity is preferred over the helicase activity.

The strand-exchange activity was further demonstrated utilizing complementary substrate C2 in the place of C1, such that only the flap is displaced, not upstream primer U2, creating a ligatable substrate. Using the ligatable product as an indicator of strand exchange, addition of BLM increased strand exchange 3.3-fold over the basal level after 20 min (data not shown; 72% exchanged versus 22% exchanged; two-tailed Fisher’s exact test, p < 0.001).

**The Rate-limiting Step of BLM-assisted Strand Exchange Is Strand Annealing**—We next compared the rate of strand exchange in the recombination intermediate complex to the rate of the annealing of radiolabeled T2 and C1 independent of the complex. In our system there are two individual steps to BLM-assisted strand exchange. The first step is the annealing of the exchange substrate C1 to the 5'-end of template T2 contained within the recombination intermediate (T1:U1:F20:U2: T2). This is followed by the second step, branch migration, which will displace both the flap complex and upstream primer U2 as C1 and T2 fully anneal.

In both the strand annealing and strand exchange assays, 5 fmol of radiolabeled T2 was added to 25 fmol of C1 with either 0 or 50 fmol of BLM at 37 °C, and the amounts of strand annealing (T2* and C1) and strand exchange (T1:U1:F20:U2:T2* and C1) were measured over time. In the absence of BLM, only 15% of the labeled T2 annealed to C1 after 15 min (1.0% annealed/min), while the annealing of the two oligomers in the presence of 50 fmol of BLM plateaued at 90% (9.0% annealed/min), a 9-fold increase that demonstrates BLM strand-annealing activity (Fig. 6A, lanes 1–14). In the absence of BLM, only 3% of the recombination intermediate complex underwent strand exchange after 15 min (0.20% exchanged/min), whereas after 15 min the complex strand exchange in the presence of 50 fmol of BLM again plateaued at 79% (5.27% exchanged/min, Fig. 6A, lanes 15–28).

If strand annealing were the rate-limiting step in BLM-assisted strand exchange, then the rates of strand annealing (Fig. 6A, lanes 7–12) and strand exchange (Fig. 6A, lanes 22–28) should be approximately the same. If branch migration were the rate-limiting step, then the rate of strand annealing should be greater than that of strand exchange. After 15 min, the rates of strand annealing and strand exchange in the presence of
BLM were identical (Fig. 6B), showing that strand annealing is the rate-limiting step in BLM-assisted strand exchange.

Because RPA inhibits BLM strand annealing, and because strand annealing is the rate-limiting step in BLM-assisted strand exchange, we examined the effect of RPA on BLM-assisted strand exchange. Five femtomoles of radiolabeled T2 in complex (T1:U1:F20:U2:T2*) was added to 25 fmol of exchange substrate C1 at 37 °C with either 0 or 50 fmol of BLM and in the presence or absence of 50 fmol of RPA, and the rates of strand exchange were measured over time. As expected, addition of BLM in the absence of RPA enhanced the rate of strand exchange after 15 min (Fig. 7A, lanes 1–14), from 0.27% exchanged/min to 5.3% exchanged/min, an almost 20-fold increase. However, the addition of RPA to assays containing BLM reduced the rate of strand exchange after 15 min to 2.03% exchanged/min, less than 40% of the rate seen in the absence of RPA (Fig. 7B). Furthermore, addition of RPA in the absence of BLM increased the rate of strand exchange 5-fold over basal level (1.37% exchanged/min from 0.27% exchanged/min), indicating that RPA has a strand-exchange activity of its own. This was further confirmed in assays containing both RPA and BLM, in which the rate of strand exchange after 15 min occurred at a level similar to that with RPA alone (Fig. 7B). Thus, if RPA lacked strand-exchange activity, exchange would have dropped to the basal level, and if RPA did not affect BLM strand exchange, it would have risen to the level seen with BLM alone. This is consistent with the findings that strand annealing is the rate-limiting step of BLM-assisted strand exchange and RPA inhibits BLM strand annealing. The rate-limiting step of RPA-assisted strand exchange is also strand annealing, with identical rates of annealing and exchange after 15 min (data not shown). These results suggest that the role of BLM-assisted strand exchange in the dissolution of illegitimate recombination intermediates is limited to branch migrations that do not involve initial pairing of RPA-coated single strands.

**BLM Helicase Activity and ATP Are Not Required to Promote Strand Exchange**—Recent publications (e.g. Ref. 21) have described the necessity of ATP and BLM helicase activity in the promotion of strand exchange. If BLM helicase activity were not required for strand exchange, then in the absence of ATP BLM would either break up the complex using only its strand exchange/strand-annealing activity, or it would not break up

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**FIGURE 6.** The rate-limiting step of BLM strand-exchange activity with the intermediate was strand annealing of the flap to the complementary template. A, the kinetics of BLM-assisted strand exchange with the intermediate. BLM-assisted strand annealing was measured on complementary ssDNA substrates (T2* and exchange substrate C1) and the recombination intermediate (T2* in complex, C1). Strand annealing precedes strand exchange in the recombination intermediate complex. Addition of 50 fmol of BLM enhanced both the rate of strand annealing of the two ssDNA substrates (6.1× increase) and the rate of strand exchange to break up the recombination intermediate (7.3× increase) after only 15 min. The bottom band in lanes 15–28 is T2*:U2, which never annealed to the flap substrate when constructing the recombination intermediate. It is also broken up via strand exchange, and band densities were included in the final analysis. As described under “Experimental Procedures,” the dissolution of the recombination intermediate via BLM strand exchange was performed at 37 °C for the following times; 0, 5, 15, 30, 60, and 120 min. The samples were then run on a native 12% polyacrylamide gel for 3 h at 25 watts. Each lane contains 5 fmol of radiolabeled flap substrate. The asterisk indicates which substrate is radiolabeled.

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the intermediate at all. Because we know that RPA inhibits BLM strand annealing and strand exchange and FEN1 cleavage, it was omitted from this set of experiments. Dissolution of the recombination intermediate results in the formation of FEN1-preferred flap complex T1:U1:F3*, so both BLM-assisted strand-exchange activity and helicase activity were detected by FEN1 cleavage of flap substrate F3* resolved on a denaturing polyacrylamide gel. We found that, in the presence of an exchange-promoting strand without ATP, BLM disrupts the recombination intermediate containing flap F3 nearly as easily as it does in the presence of ATP alone (71% versus 75% after 20 min, Fig. 8A, lanes 6 and 12, respectively). Simultaneous addition of both ATP and the exchange substrate C1 slightly increased the rate of recombination intermediate dissolution, suggesting that the two BLM activities partially complement each other (Fig. 8B).

**DISCUSSION**

In this study, we report reconstitution experiments suggesting that structures formed during eukaryotic Okazaki fragment maturation can initiate recombination. By designing a series of complexes that simulate illegitimate recombination intermediates, we were able to investigate the specific roles of BLM helicase and BLM strand-exchange activity in the dissolution of these proposed structures.

Current models suggest that a fraction of Okazaki fragment flaps become long enough to be coated by RPA and require the sequential action of Dna2 and FEN1 for proper processing (27). BLM binds to FEN1 and stimulates its cleavage activity (26). Moreover, expression of human BLM in yeast can compensate for defects in Dna2 (41). These properties suggest that BLM is a normal component of the DNA replication machinery. Because a major role of BLM is to suppress recombination, it is logical to assume that it helps to prevent long flaps from invading ectopic sites on the same or another chromosome to form illegitimate recombination intermediates. BLM activity directed at suppression of flap invasion is consistent with the high level of sister chromatid exchange observed in BLM mutant cells.

The crystal structure of the DNA-binding domain of human RPA70 bound to a short oligonucleotide indicates that the interaction site for human RPA is at least eight nucleotides (42). The binding affinity for substrates 20–30 nucleotides long is high, but sharply decreases as substrate length is decreased (43). Thus, when the flap is not cleaved immediately by FEN1 and reaches a length greater than eight nucleotides, it can be bound, albeit weakly, by RPA. The binding affinity of RPA would then increase with flap length, and more effectively suppress cleavage by FEN1.

Although RPA inhibits cleavage by FEN1, it stimulates cleavage by the nuclease/helicase Dna2 (27, 28), allowing a mechanism for flap cleavage. However, we found that the presence of RPA on the flap actually stimulated the ability of the flap to interact and subsequently anneal to an ectopic template. Moreover, once annealed, the flap was readily ligated to form a stable and irreversible recombination product. This implies that, prior to cleavage by Dna2, long flaps are effective precursors to recombination. Additionally, because Dna2 must enter the flap from a single-stranded 5’-end (44), correct processing of the

**FIGURE 7. RPA exhibits its own strand-exchange activity and inhibits BLM strand-exchange activity.** A, the kinetics of RPA-assisted strand exchange and inhibition of BLM strand exchange. BLM-assisted and RPA-assisted strand exchange was measured using the recombination intermediate [T1:U1:F20:U2:T2*] and recombination substrate C1. Addition of 50 fmol of BLM enhanced the rate of strand exchange to break up the recombination intermediate. Addition of 50 fmol of RPA to reactions lacking BLM also facilitated strand exchange, although to a lesser degree than BLM. Addition of RPA to reactions containing BLM reduced the level of measurable strand exchange to near that of RPA alone. As described under “Experimental Procedures,” the dissolution of the recombination intermediate via strand exchange was performed at 37 °C for the following times; 0, 2, 5, 15, 30, and 60 min. The samples were then run on a native 12% polyacrylamide gel for 3 h at 25 watts. Each lane contains 5 fmol of radiolabeled flap substrate. The asterisk indicates which substrate is radiolabeled. B, addition of 50 fmol of RPA or 50 fmol of BLM separately causes an increase in the level of strand exchange as measured by the creation of the dsDNA complex (T2*:C1). Addition of 50 fmol of RPA and 50 fmol of BLM jointly creates a strand exchange rate near that of RPA alone, suggesting that RPA inhibits BLM strand exchange. Data were normalized as described under “Experimental Procedures.” Points are plotted as the percentage of labeled substrate in the newly formed dsDNA complex (T2*:C1) versus time. ■, −BLM/−RPA; □, +BLM/−RPA; △, −BLM/+RPA; ▲, +BLM/+RPA.
flap by Dna2 is effectively stopped at the moment of annealing. Even though Dna2 has helicase activity (45), because loading onto a 5'-single-stranded region is a prerequisite to initiate helicase functions, annealing of the flap is an effective deterrent to Dna2 loading.

We envision that a long flap might interact at another chromosomal site at a gap, possibly the region between Okazaki fragments. Alternatively, the errant flap could invade in a double-stranded region. Because competing strand interactions are necessary for strand invasion, the intermediate would form a D-loop. The strand-exchange property of BLM is well suited for disruption of the invading strand of a D-loop, because the exchange process would replace a strand with short homology with one having longer homology and that is already built into the structure. Moreover, even though one strand of the D-loop is coated by RPA, the strand-exchange process does not involve an initial annealing of RPA-coated strands. Instead RPA would dissociate from the one strand during the branch migration process. We suggest that the helicase function protects against flap invasion of gaps, and the strand-exchange function destabilizes D-loops.

BLM also stimulates FEN1 cleavage activity, such that in our system the flap was rapidly cleaved as soon as its 5'-end was released. This also shows that, on those substrates on which BLM has to track 3' to 5' on the flap, its binding and movement do not interfere with subsequent FEN1 5' to 3' tracking and cleavage.

At sites where helicase activity is desirable for dissolution of recombination intermediates, the powerful annealing activity would appear to limit the ability of BLM to act efficiently. However, we found that BLM-annealing activity is inhibited by RPA. RPA inhibition aside, a possible explanation for robust annealing activity is that BLM functions are a compromise in a protein that has multiple roles. It has been shown the BLM has a role in both homologous recombination (reviewed in Ref. 36) and non-homologous end-joining (reviewed in Ref. 46). Non-homologous end-joining in BS cells is highly error-prone, and repeatedly results in DNA ligation at distant sites of micro-homology, creating large DNA deletions (47). This would suggest that one role of BLM strand-annealing activity is to work with the helicase to test strand-anealing sites for binding stability. The competing activities would set up a binding equilibration to detect the best and most stable base pairing interactions. This would discourage ligation when the interacting sequences are not very stable. Extracts from BS cells also show a very low rejoinder accuracy of introduced double-stranded breaks compared with that from normal cells (46). Again the combined helicase and annealing functions could test where the annealing point for a double-strand break should be.

When the amount of single-stranded DNA between the original and the invaded templates was three nucleotides or less, the helicase activity of BLM was partially suppressed. However, this inhibition was not evident with BLM-strand annealing or strand-exchange activity. This suggests that the binding site on the BLM protein required for strand annealing differs from that used for helicase activity and that the annealing site is more easily accessible. The C-terminal domain of BLM has been shown to be essential for binding to ssDNA, without which BLM is unable to catalyze strand annealing. This region is distal to the DEAH helicase region, where the majority of BLM helicase mutations have been found (48, 49).
Bloom Protein and Okazaki Maturation

RPA present at levels sufficient to coat the substrates had an inhibitory effect on BLM strand annealing and strand exchange. As discussed above, in the absence of BLM, RPA actually improved the rate of annealing of the flap to form the recombination intermediate. These observations apparently differ from those of Machwe et al. (21), who observed that RPA stimulates the BLM helicase function and prevents the un-annealed strands from re-annealing. We conclude that the decreased re-annealing rate of strands after they are dissociated by BLM helicase is not due to slowing of re-annealing by RPA, but rather direct inhibition of BLM strand annealing by RPA. The differences between the two studies may relate to the molar excess of RPA used and the length and nucleotide composition of the strands tested in that report. Moreover, because recombination occurs in vivo, and is exacerbated in BS cells, the cellular concentration of RPA is apparently appropriate to allow strand annealing to form recombination intermediates.

It is probable that not all flaps created during Okazaki fragment replication have a 5’-free end. In the case where flap equilibrium creates a 3’-flap, BLM helicase activity cannot be utilized to dissociate any illegitimate recombination intermediates. In this case, BLM would bind to the 3’-flap and track backwards toward the original template, not the complementary template initially invaded by the flap. Still, BLM helicase could track on the single-stranded region of the gap in the complementary template, but this might be suppressed by a polymerase binding and extending the invading 3’-end. In instances like these, BLM-exchange activity offers a potential pathway to dissociate the flap and restore the original template without the need for helicase function.

BLM can dissociate a recombination intermediate using one or a combination of its activities (Fig. 9). When the correct exchange substrate DNA strand is available and the recombination intermediate is in a form such that RPA cannot bind, BLM strand-exchange activity will bind the exchange strand to the site, displacing the errant DNA flap by branch migration. The resulting flap can then be cleaved by FEN1, whose cleavage activity is coincidentally stimulated by BLM. In the absence of the exchange DNA strand, BLM helicase activity will separate the flap from the ectopic site, freeing it to be processed by the DNA-replication machinery. Our system demonstrates that BLM is well equipped to use several mechanisms to suppress recombination that can occur during DNA replication.

In conclusion, there are millions of Okazaki fragments made and joined each time a cell must duplicate its chromosomal DNA. The current view is that some of them grow long and require processing by Dna2. A sub-population of these may invade ectopic sites. BLM appears to be a part of the DNA-replication machinery that suppresses recombination that can result from such invasions.

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