Intrachromosomal recombination between highly diverged DNA sequences is enabled in human cells deficient in Bloom helicase

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ABSTRACT

Mutation of Bloom helicase (BLM) causes Bloom syndrome (BS), a rare human genetic disorder associated with genome instability, elevation of sister chromatid exchanges, and predisposition to cancer. Deficiency in BLM homologs in *Drosophila* and yeast brings about significantly increased rates of recombination between imperfectly matched sequences (“homeologous recombination,” or HeR). To assess whether BLM deficiency provokes an increase in HeR in human cells, we transfected an HeR substrate into a BLM-null cell line derived from a BS patient. The substrate contained a thymidine kinase (*tk*)-*neo* fusion gene disrupted by the recognition site for endonuclease I-SceI, as well as a functional *tk* gene to serve as a potential recombination partner for the *tk-neo* gene. The two *tk* sequences on the substrate displayed 19% divergence. A double-strand break was introduced by expression of I-SceI and repair events were recovered by selection for G418-resistant clones. Among 181 events recovered, 30 were accomplished via HeR with the balance accomplished by nonhomologous end-joining. The frequency of HeR events in the BS cells was elevated significantly compared to that seen in normal human fibroblasts or in BS cells complemented for BLM expression. We conclude that BLM deficiency enables HeR in human cells.

1. Introduction

Mammalian cells enjoy a variety of means for responding to and repairing DNA damage. One type of DNA lesion that must be contended with daily is the double-strand break (DSB). Failure to efficiently and accurately repair DSBs can lead to deleterious genetic rearrangements, disease, and/or cell death. Homologous recombination (HR) and nonhomologous end-joining (NHEJ) serve as two broadly defined pathways for DSB repair (reviewed in 1-10). HR, which is largely restricted to the late S or G2 stage of the cell cycle of dividing cells, typically uses a DNA template to maintain or restore genetic information and is generally considered to be accurate. In contrast, NHEJ is active throughout the cell cycle and in nondividing cells, uses no template in re-joining DNA ends, and is error-prone since it often produces sequence deletions. A distinct “nonconservative” form of HR, known as single-strand annealing (SSA), is also potentially operative throughout the cell cycle. SSA does not involve the use of a DNA template and can generate deletions via the splicing together of repeated homologous sequences with the concomitant loss of sequence between the repeats.

The consequences of HR necessarily depend on the choice of recombination partners. It seems reasonable to expect that HR pathways have been honed through evolution to allow genetic exchange only between sequences that share perfect or near-perfect homology so as to minimize the occurrence of gross chromosomal rearrangements. Consistent with such expectations, we had previously shown that spontaneous recombination in mammalian chromosomes normally occurs only between sequences sharing a considerable length and degree of homology (11-16). We found that for two closely linked sequences about 1 kb in length and displaying 20% sequence divergence, recombination was reduced over 1,000-fold compared to

recombination between nearly perfectly homologous sequences (11). We demonstrated that a significant reduction in recombination rate between two sequences can be brought about by a single mismatched nucleotide (12,14), and that a stretch of perfect homology exceeding 100 bp in length is required for efficient recombination (12). We also reported that when a spontaneous recombination event occurs within highly homologous sequences, mammalian cells fastidiously exclude adjoining mismatched bases from gene conversion tracts (15). Recently, we observed that the exclusion of mismatches from gene conversion events initiated within high homology is somewhat relaxed when the mismatches are positioned close to the DSB initiating the HR event (17). Nonetheless, the need for a stretch of high homology for the initiation of HR remains intact in proximity to a DSB (17).

Impeccable sensitivity of recombination in mammalian cells to sequence divergence would serve to protect genome integrity by preventing rearrangements between imperfectly matched (“homeologous”) sequences that pepper the genome. However, there have been reports of homeologous recombination (HeR) events between Alu family or LINE sequences that are sometimes associated with genetic diseases and recurring chromosomal rearrangements in cancers (18-27). Genetic alterations associated with such nonallelic HeR events include deletions, duplications, and inversions. It is thus clear that although HeR is rare in mammalian genomes, suppression of such events is not failsafe. To better understand conditions that might break down the barriers against HeR and potentially compromise genomic integrity, it is important to identify the proteins that are normally involved in establishing HeR barriers.

There is considerable evidence that elements of the mismatch repair (MMR) machinery, particularly the MutS homologs MSH2 and MSH6, play a role in suppression of HeR across many species (28-37). In addition to playing a pivotal role in post-replicative correction of

mispairs, MSH2 and MSH6 are components of the BRCA-1 associated genome surveillance complex (BASC) which is comprised of a variety of proteins involved in the recognition and response to a broad array of abnormal DNA structures (38-41). BASC includes BRCA-1, MSH2, MSH6, MLH1, ATM, the RAD50–MRE11–NBS1 protein complex, and BLM helicase.

How may MMR proteins, perhaps in combination with other proteins involved in DNA metabolism, protect against HeR? There is evidence that collaboration between MMR proteins and a RecQ family helicase may be key. In *E. coli*, RecQ helicase has been shown to suppress illegitimate, ectopic recombination between short sequences (42). In eukaryotes, elevated levels of HeR (in the form of SSA) have been reported in *Drosophila* strains mutated in BLM, a member of the RecQ family of helicases (43), and elevated levels of HeR have also been reported in yeast mutated in Sgs1, the sole yeast RecQ homolog (44-46). It has been proposed (31,45-47) that, in yeast, the Msh2-Msh6 heterodimer (and perhaps the Msh2-Msh3 heterodimer) works in conjunction with Sgs1 helicase to participate in a process termed “heteroduplex rejection” that dismantles recombination intermediates formed between imperfectly matched DNA sequences. Although details of heteroduplex rejection remain incompletely understood, it seems likely that the Msh2 complex has the task of detecting mismatches while Sgs1 helicase activity reverses the formation of mismatched heteroduplex through strand unwinding. In our earlier work, we had obtained evidence for an unwinding of mismatched heteroduplex intermediates as a means for avoiding HeR in mammalian cells (15), so it is not unreasonable to suspect that suppression of HeR in mammalian cells entails a helicase and is accomplished in a manner similar to that believed to operate in yeast and *Drosophila*. If this latter supposition has merit, then one might predict that a deficiency in BLM helicase should elevate the rate of HeR in mammalian cells.

It has indeed been recognized for some time that BLM helicase is involved in the regulation of recombination in mammalian cells (48,49). The reported roles for BLM in recombination in mammals are varied and complex, and perhaps in some instances seemingly conflicting. Bloom Syndrome (BS) is a genetic disorder caused by BLM deficiency. One consistent feature seen in BS patients and in mouse models for BS is a greatly elevated level of sister chromatid exchanges (SCEs) (50-52). We have shown (53) that depletion of BLM helicase via RNA interference in cultured normal human fibroblasts skews the mode of DSB repair away from NHEJ and toward recombinational repair in the form of SSA or crossovers, which would predictably increase SCEs and possibly other rearrangements. Recent work by others has shown that destabilization of BLM protein brought about by deficiency in TopBP1 leads to an increase in the levels of SCEs in cultured human cells (54). An increase in SCEs and crossover events provoked by BLM deficiency should result in a global increase in rates of loss of heterozygosity (LOH) which, in turn, may contribute to the cancer predisposition seen in BS patients. Indeed, an escalation of LOH has been attributed directly to BLM deficiency (55,56).

In addition to an increase in SCEs, an increase in translocations and chromosomal radial formation is also commonly seen in BS cells, and most radials are formed between nonhomologous chromosomes (57). These latter observations indicate an escalation in promiscuous rearrangements in association with BLM deficiency, and these rearrangements may portend a role for BLM in blocking HeR in human cells.

Is there any direct evidence that BLM deficiency increases the frequency of HeR in mammalian cells? To our knowledge, this issue has been addressed in a single previous study (58) in which it was concluded that HeR is *not* elevated in either BLM-deficient mouse cells or in cells derived from a BS patient. The assays used in this latter study of mouse and human cells

involved monitoring either extrachromosomal recombination among sequences on transfected plasmids, or recombination events occurring between one chromosomal sequence and one transfected sequence.

In the current study, we present the first investigation into whether HeR is enabled between two sequences located within a chromosome in BS cells. We report that BS cells can in fact carry out intrachromosomal HeR between two sequences displaying a high level of divergence (19% mismatch). Moreover, complementation of BS cells by re-expression of BLM helicase significantly suppressed the ability of cells to carry out HeR. We did not recover any HeR events from normal human fibroblasts. Our work thus reveals that BLM helicase blocks HeR in human cells. Lifting of the barrier against HeR may be an important factor in the profound cancer predisposition seen in BS patients.

2. Materials and Methods

2.1 Cell Culture

Normal human fibroblast cell line GM00637 was obtained from the NIGMS as was human fibroblast cell line GM08505 which was derived from a BS patient and is null for BLM expression. Cell line GM08505 is homozygous for a six bp deletion/seven bp insertion at nucleotide 2,281 of the open reading frame of the BLM gene, a mutation referred to as “*blm*^{Ash},” which produces a frameshift and a stop codon. Cells were cultured in alpha-modified minimum essential medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum. Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂.

2.2 Repair substrates

Substrates pLB4 and pBR3 (Fig. 1) were described previously (59). Each substrate contains a *tk-neo* fusion gene (referred to as the “recipient”) disrupted by the insertion of a 22-bp oligonucleotide that contains the 18-bp recognition sequence for endonuclease I-SceI. In addition to the fusion gene, substrate pLB4 contains a 2.5-kb HindIII fragment that includes a complete functional herpes simplex virus type 1 (HSV-1) thymidine kinase (*tk*) gene. This “donor” fragment served as a potential HR partner for the recipient gene. The *tk* donor on pLB4

Fig. 1. DSB repair substrates pLB4 and pBR3. Each substrate contains a *tk-neo* fusion gene that is disrupted by a 22-bp oligonucleotide containing the 18-bp recognition site for endonuclease I-SceI (underlined sequence). Sites of cleavage by I-SceI are indicated. BamHI (B) and HindIII (H) sites are shown. Substrate pLB4 contains a 2.5-kb HindIII fragment (open rectangle) containing a complete HSV-1 *tk* gene, while pBR3 contains a 1.4-kb HindIII fragment (diagonally striped rectangle) containing a complete HSV-2 *tk* gene. The direction of transcription of both the *tk* gene and *tk-neo* fusion gene in each substrate is from left to right as drawn. PCR primers used to amplify DSB repair products for subsequent sequence analysis are indicated by short horizontal arrows.

shares about 1.7 kb of homology with the *tk* portion of the recipient. The *tk* donor and the *tk-neo* recipient are oriented as direct repeats, with the direction of transcription going from left to right, as drawn in Fig. 1. Due to several scattered mismatches, the donor on pLB4 displays a very low level (< 1%) of sequence divergence from the *tk* portion of the recipient (Fig. 2).

Substrate pBR3 contains a 1.4-kb HindIII fragment containing a complete functional HSV-2 *tk* gene. This fragment displays 19% sequence divergence from the *tk* portion of the recipient and served as a potential HeR partner for the recipient gene. As in pLB4, the donor and recipient reside as direct repeats.

2.3 Insertion of repair substrates into cell lines

Cell line pLB4/11, previously described (60), was derived from normal human fibroblast cell line GM00637 and contains a single integrated copy of recombination substrate pLB4 (Fig. 1). Derivatives of GM00637 containing substrate pBR3 and derivatives of the BLM-null cell line GM08505 containing pLB4 or pBR3 were isolated by electroporating 5×10^6 cells from the appropriate cell line with 3 μ g of the particular substrate (linearized with XhoI). Cells plus DNA were suspended in 800 μ l of phosphate-buffered saline, placed in a 0.4 cm gap electroporation cuvette, and electroporated using a Bio-Rad Gene Pulser set at 1000 V, 25 μ F. Cells from each electroporation were plated into a 175 cm² flask and allowed to grow for two days under no selection. Cells were then plated at a density of 1×10^6 cells per 75 cm² flask into medium supplemented with hygromycin at 100 μ g/ml (GM00637 cells) or 200 μ g/ μ l (GM08505 cells). After 14 days of growth, hygromycin-resistant colonies were picked. These clones were propagated and screened by Southern blot analysis to identify cell lines containing a single stably integrated copy of the transfected plasmid substrate.

2.4 Complementation of BLM-null cells

As previously described by others (61), a cDNA designated R12 had been identified that encodes human BLM. We recovered the R12 cDNA on a NotI DNA fragment cut from plasmid

pOPRSVI-BLM (62) and inserted it into the NotI site within the multi-cloning site of pcDNA3.1/Zeo(+) (Invitrogen) to form pBLM/Zeo. On this latter plasmid, BLM expression is under the control of a CMV promoter. Plasmid pBLM/Zeo was used to complement the BLM-null cell line A5-1 which was derived from GM08505 cells and contains a stably integrated copy of HeR substrate pBR3. pBLM/Zeo was linearized with FspI and 3 µg of the linearized construct were electroporated into 5×10^6 A5-1 cells using a Bio-Rad Gene Pulser set at 1000 V, 25 µF. Following electroporation, cells were plated into a 175 cm² flask and allowed to grow for two days under no selection. Cells were then plated at a density of 1×10^6 cells per 75 cm² flask into medium supplemented with zeocin (75 µg/ml) to select for stable transfectants. After 10-14 days, colonies were recovered, propagated, and assayed for BLM expression by Western blot. A similar procedure was used to establish a derivative of A5-1 cells containing an integrated copy of the empty vector pcDNA3.1/Zeo(+).

2.5 Western blots

Western blots were used to assess the level of BLM expression in clones stably transfected with pBLM/Zeo or the empty vector pcDNA3.1/Zeo(+). Cells were harvested by trypsinization and cell lysates were prepared for Western blotting analysis by standard procedures. BLM antibody ab476 (Abcam) was used as a primary antibody, and HRP-conjugated anti-rabbit IgG antibody NB730-H (Novus Biologicals) was used as a secondary antibody. To assess protein loading, α -tubulin monoclonal antibody T1568 (Sigma–Aldrich) was used as a primary antibody in conjunction with HRP-conjugated goat anti-mouse IgG antibody sc-2005 (Santa Cruz Biotechnology) as a secondary antibody. HRP signal was detected using an ECL Select Western Blotting Detection Kit (GE Healthcare).

2.6 Recovery of DSB-induced HR and NHEJ events

Plasmid pCMV3xnlS-I-SceI (“pSce”) expresses endonuclease I-SceI in mammalian cells and was generously provided by M. Jasin (Sloan Kettering). To induce a DSB at the I-SceI site within an integrated DNA construct in a cell line, pSce was transiently transfected into cells via electroporation. Five million cells from a particular cell line and 20 µg of pSce were resuspended in 800 µl of phosphate-buffered saline and placed in a 0.4 cm gap electroporation cuvette. A Bio-Rad Gene Pulser was used to deliver a pulse of 700 V and 25 µF. Following electroporation with pSce, cells were plated into 75 cm² flasks under no selection. The precise density of cells plated into 75 cm² flasks was determined empirically to produce a reasonable density of colonies for subsequent harvesting after genetic selection. Several additional aliquots of cells were plated into 25 cm² flasks at 100 cells per flask to assess viability in terms of colony forming units. Forty-eight hours after plating, cells in 75 cm² flasks were refed medium supplemented with 1000 µg/ml active G418 to select for DSB repair events. Cells plated for viability assessment were allowed to continue to grow under no selection. Colonies in all flasks were counted 10 to 14 days after plating. Colonies under selection were expanded for further analysis.

2.7 PCR amplification and DNA sequence analysis

A segment of the *tk-neo* fusion gene spanning the I-SceI site was amplified from 500 ng of genomic DNA isolated from G418-resistant clones using primers AW85 (5'-TAATACGACTCACTATAGGGCCAGCGTCTTGTCATTGGCG-3') and AW91 (5'-GATTAGGTGACACTATAGCCAAGCGGCCGAGAACCTG-3'). AW85 is composed of nucleotides 308–327 of the coding sequence of the HSV-1 *tk* gene, numbering according to (63),

with a T7 forward universal primer appended to the 5' end of the primer. AW91 is composed of 20 nucleotides from the noncoding strand of the neomycin gene mapping 25 through 44 bp downstream from the neomycin start codon, with an Sp6 primer appended to the 5' end of the primer. PCR was carried out using Ready-To-Go PCR beads (GE Healthcare) and a “touchdown” PCR protocol as previously described (17). PCR products were expected to be ~1.4 kb in length unless detectable deletions or insertions had occurred. Prior to sequencing, PCR products were treated with shrimp alkaline phosphatase and exonuclease I (USB). PCR products were then sequenced from a T7 or Sp6 primer by Eton Bioscience, Inc. (Research Triangle Park, NC). Sequence analysis was performed using Sci-Ed Central software.

2.8 Southern blotting analysis

Genomic DNA samples (8 µg each) were digested with appropriate restriction enzymes and resolved on 0.8% agarose gels. DNA was transferred to nitrocellulose membranes and hybridized with ³²P-labeled *tk* probes as previously described (64).

3. Results

3.1 Recovery of intrachromosomal DSB repair events from normal and BS cells

To gain a better understanding of the role that BLM helicase may play in averting HeR during DSB repair in human cells, we stably transfected substrates pLB4 and pBR3 (Fig. 1) into the BLM-null cell line GM08505 as well as into the normal human cell line GM00637. We identified cell lines F1-6 and C3-2 as derivatives of GM08505 containing a single integrated

copy of pLB4, and lines A5-1 and A1-2 as derivatives of GM08505 containing a single copy of pBR3. Cell line pLB4/11, previously described (60), is a derivative of GM00637 containing a single copy of pLB4 while cell lines C8, D4, and D7 are derivatives of GM00637 each containing an integrated copy of pBR3.

DSB repair events can be recovered by electroporating cells containing pLB4 or pBR3 with pSce to induce a DSB and, subsequently, selecting for cells in which restoration of *tk-neo* gene function confers G418-resistance. In substrate pLB4, the *tk* donor sequence shares a high degree of homology (>99%) with the *tk* portion of the closely linked *tk-neo* recipient gene which is disrupted by the insertion of the recognition site for endonuclease I-SceI. Substrate pLB4 allows the recovery of HR or NHEJ following DSB induction by I-SceI. In substrate pBR3, the *tk* donor and *tk* portion of the fusion gene display 19% sequence divergence and so pBR3 allows the recovery of HeR as well as NHEJ following DSB induction by I-SceI. For either pLB4 or pBR3, recovery of an NHEJ event requires only that the proper reading frame be restored to the *tk-neo* fusion gene (60). To a first approximation, therefore, one in three NHEJ events is recoverable.

Cell lines derived from GM08505 or GM00637 and containing substrate pLB4 or pBR3 were electroporated with pSce, and DSB repair events were recovered by selecting for G418-resistant colonies (Table 1). The frequency of colony recovery from cell line pLB4/11 was 4.8×10^{-3} , consistent with our previously published results for both pLB4/11 and a second GM00637-derived cell line containing pLB4 (53). In comparison, the colony frequencies for BLM-null cell lines C3-2 and F1-6 (containing pLB4) were 1.4×10^{-5} and 1.8×10^{-5} (Table 1).

Table 1

Recovery of DSB repair events from normal and BS fibroblasts

Cell line	Substrate	BLM status ^a	No. of trials ^b	Cells plated into selection (10 ⁶) ^c	Colonies	Colony frequency (10 ⁻⁵) ^d
pLB4/11	pLB4	positive	4	0.7	3377	482.0
C3-2	pLB4	null	2	7.6	105	1.4
F1-6	pLB4	null	2	3.2	58	1.8
C8	pBR3	positive	1	0.3	248	82.7
D4	pBR3	positive	1	5.8	242	4.2
D7	pBR3	positive	1	0.3	101	33.7
A5-1	pBR3	null	7	29.3	357	1.2
A1-2	pBR3	null	1	9.7	70	0.7

^aBLM- positive cell lines were derived from normal human fibroblast cell line GM00637; BLM-null cell lines were derived from BS fibroblast cell line GM08505.

^bNumber of independent electroporations with the I-SceI expression vector pSce.

^cTotal number of viable cells plated for all trials for each cell line.

^dCalculated as total number of colonies divided by total number of viable cells plated into selection

The frequencies of colony recovery from GM00637-derived cell lines C8, D4, and D7 (containing pBR3) were 82.7×10^{-5} , 4.2×10^{-5} , and 33.7×10^{-5} , respectively. In comparison, the colony frequencies for BLM-null cell lines A5-1 and A1-2 (containing pBR3) were 1.2×10^{-5} and 0.7×10^{-5} respectively. The significance of the precise values for colony frequencies for the various cell lines was not clear since a variety of factors likely contribute to this measurement; nonetheless, two matters were apparent from the data in Table 1. First, the colony frequencies for derivatives of the BLM-null GM08505 cell line were lower than those for derivatives of the normal fibroblast cell line GM00637. Second, whereas colony recovery from normal cells containing pBR3 was substantially reduced compared to colony recovery from normal cells containing pLB4, the colony recovery for BLM-null cells was similar regardless of whether the cells contained pBR3 or pLB4. One possible interpretation of this latter observation

is that the degree of homology between the donor and the recipient gene in the integrated constructs had a greater impact on DSB repair in normal cells than in BS cells

3.2 Characterization of DSB events generated using HR substrate pLB4 in normal and BS cells

Following DSB-induction within the integrated copy of substrate pLB4 in cell lines F1-6 (derived from GM08505, BLM-null) and pLB4/11 (derived from GM00637, BLM-positive), representative G418-resistant clones were recovered and analyzed to determine whether each particular clone arose via HR or via NHEJ (Table 2).

For each clone analyzed, a segment of DNA surrounding the repaired DSB was PCR-amplified and sequenced. Evaluation of sequence data was aided by the 13 scattered mismatches

Table 2

Categorization of DSB events recovered from normal and BS fibroblasts containing HR substrate pLB4

Cell line	BLM status ^a	No. of trials ^b	Events analyzed	HR events			NHEJ
				Total	GC ^c	HDD ^d	
pLB4/11 ^e	positive	4	127	97 (76%) ^f	52	45 (46%) ^g	30
pLB4/11 ^h	positive	2	172	126 (73%)	82	44 (35%)	46
F1-6	null	2	58	50 (86%)	23	27 (54%)	8

^aCell line pLB4/11 was derived from BLM-positive normal human fibroblast line GM00637 while cell line F1-6 was derived from BLM-null cell line GM08505.

^bNumber of independent electroporations with the I-SceI expression vector pSce.

^cGene conversions

^dHomology-dependent deletions

^eData in this row was generated in current study.

^fNumbers in parentheses in this column represent the percentage of analyzed events that were categorized as HR.

^gNumbers in parentheses in this column represent the percentage of HR events that were categorized as HDD

^hData in this row is from reference 53.

between the donor *tk* sequence and the *tk* portion of the recipient fusion gene (Fig. 2). If, in a particular clone, one or more of the mismatched nucleotides had been transferred from donor to recipient, then the clone was categorized as having arisen via HR. If a deletion or insertion of nucleotides was found at the site of the DSB in a particular clone and no mismatches had been transferred from the donor to recipient sequence, then the clone was categorized as having arisen via NHEJ. Events categorized as HR were further assessed to be gene conversions or crossovers (or SSA events), based on distinct patterns produced on a Southern blot (Fig. 3). Because we cannot always distinguish between crossovers and SSA, we use the term “homology-dependent deletions” (HDD) to refer collectively to these latter two types of events (Fig. 3). Either event displays a loss of sequence between the *tk* donor and the *tk*-neo fusion gene along with an accurate, in-frame joining of the *tk* donor and fusion gene. A summary of the analysis of G418-resistant clones is presented in Table 2.

As revealed by the data in Table 2, when compared to DSB repair events recovered from cell line pLB4/11 in our current studies, events recovered from cell line F1-6 appeared to be comprised of a somewhat higher fraction of HR as opposed to NHEJ ($p = 0.17$ by a two-sided Fisher exact test). The data presented on the second row of Table 2 is derived from a previous study of ours (53). If we pool the previous data with our current data set for pLB4/11, then the difference in the portion of HR events recovered from lines F1-6 versus pLB4/11 is a bit more apparent ($p = 0.063$ by a two-sided Fisher exact test). This outcome is consistent with the view that BLM deficiency may produce a hyperrecombination phenotype.

The data in Table 2 is also suggestive of an increased percentage of HDD among the HR events recovered from F1-6 compared with pooled data set for pLB4/11 ($p = 0.082$ by a two-sided Fisher exact test). This outcome is consistent with the view that BLM serves to promote

pLB4

Gene conversion:

HDD:

BamHI Digests

P HDD GC GC

4.5 kb –
3.9 kb –

pBR3

Gene conversion:

HDD:

SfuI Digests

P HDD HDD GC

4.0 kb –

2.1 kb –

Fig. 3. Southern blot analysis of recombination events. On the left side of the upper and lower panels are schematic illustrations of gene conversion and homology-dependent deletion (HDD) events for HR substrate pLB4 and for HeR substrate pBR3. HDD potentially include both crossovers and SSA events. Open rectangles represent HSV-1 *tk* sequences, diagonally striped rectangles represent HSV-2 *tk* sequences. BamHI (B), HindIII (H), and SfuI (S) sites are shown. For each substrate, gene conversions can be distinguished from HDD based on distinct restriction digest patterns. To the right of each schematic is a representative Southern blot displaying the restriction pattern for a parent cell line (P) containing the particular substrate and the patterns diagnostic for gene conversions (GC) and HDD. The blots were hybridized with *tk*-specific probes.

resolution of HR events as noncrossovers (gene conversions).

Another difference between events recovered from F1-6 and pLB4/11 was revealed upon sequence analysis of gene conversion tracts. Based on the 13 mismatches between donor and recipient (Fig 2), we could make an assessment of the length of each conversion tract by asking how many marker mismatches had been transferred from donor to recipient. In accord with our

previous work (53,60), most gene conversion tracts recovered from normal cells were “short.” We define a “short” tract as one involving the transfer of no more than one or both of the DSB-proximal markers, the mismatches labeled “4” and “5” in Fig. 2, which are positioned 21 bp apart on the donor. Indeed, 47 out of 52 gene conversion tracts recovered from pLB4/11 in our current work were short, displaying the transfer of only mismatch “4” and/or “5.” In contrast, only 13 out of 23 conversion tracts from F1-6 were short, the balance of the tracts displaying transfer of additional more distal markers, and this difference in the portion of short tracts was highly significant ($p = 0.0015$ by a two-sided Fisher exact test). Two of the 23 gene conversion tracts recovered from F1-6 displayed transfer of all 13 markers, encompassing at least 1211 bp of donor sequence, while none of the 52 gene conversion tracts from pLB4/11 were this length. (Characteristics of gene conversion tracts recovered from cell lines F1-6 and pLB4/11 are summarized in Supplementary Table 1.)

Sequence analysis of NHEJ events recovered from cell lines F1-6 versus pLB4/11 revealed a difference in the use of microhomology. For the eight NHEJ clones recovered from F1-6, only two DNA junctions involved microhomology, and in each case it was a single base (A). For pLB4/11, 25 out of 30 NHEJ events used microhomology, with the length of microhomology ranging from one to six bases. Comparing NHEJ events recovered from F1-6 versus pLB4/11, the difference in the portion of events that used microhomology was highly significant ($p = 0.0035$, by a two-sided Fisher exact test). The deletion sizes for NHEJ events recovered from F1-6 ranged from 4 bp to 34 bp, with a median deletion size of 16 bp. For pLB4/11, the deletion sizes ranged from 1bp to 400 bp, with a median deletion size of 22 bp. (Characteristics of NHEJ events recovered from lines F1-6 and pLB4/11 are summarized in Supplementary Table 2.)

3.3 Characterization of DSB events generated using HeR substrate pBR3 in normal and BS cells

Following DSB-induction within integrated substrate pBR3 in cell line A5-1 (derived from GM08505, BLM-null) and cell lines C8, D4, and D7 (derived from GM00637, BLM-positive), representative G418-resistant clones were recovered and analyzed to determine whether each particular clone arose via HeR or via NHEJ. For each clone analyzed, a segment of DNA surrounding the repaired DSB was PCR- amplified and sequenced as above. In pBR3, the donor and recipient display 19% sequence divergence and are thus homeologous with numerous mismatches. The mismatches between donor and recipient sequences in pBR3 in the region proximal to the I-SceI recognition site in the recipient are illustrated in Fig. 4. If a G418-resistant clone displayed the transfer of one or more of these mismatches from donor to recipient, then the clone was categorized as having arisen via HeR. As above, a deletion or insertion at the DSB site in the absence of transfer of mismatches defined a clone as having arisen via NHEJ. HeR events could be further categorized as gene conversions or HDD by Southern blots (Fig. 3). A summary of the analysis of G418-resistant clones is presented in Table 3.

Sequence analysis (see Fig. 4) revealed unequivocally that 16 out of 71 G418-resistant clones recovered from BLM-null cell line A5-1 arose as products of accurate HeR, with the balance having arisen from NHEJ events (Table 3). In sharp contrast, zero out of a total of 86 G418-resistant clones recovered from the BLM-positive cell lines C8, D4, and D7 arose from HeR. The difference in recovery of HeR from the BLM-null A5-1 line versus each of the BLM positive cell lines C8, D4, and D7 was statistically significant ($p = 3.5 \times 10^{-2}$, 2.3×10^{-3} , and 5.3×10^{-4} , respectively, by two-sided Fisher exact tests). If we pool the data from the three BLM-positive cells, the difference in HeR recovery from BLM-null versus BLM-positive cells was highly significant ($p = 1.1 \times 10^{-6}$ by a two-sided Fisher exact test). All 16 HeR events from

Table 3

Categorization of DSB events recovered from normal and BS fibroblasts containing HeR substrate pBR3

Cell line	BLM status ^a	No. of trials ^b	Events analyzed	HeR events			NHEJ
				Total	GC ^c	HDD ^d	
C8	positive	1	16	0	0	0	16
D4	positive	1	31	0	0	0	31
D7	positive	1	39	0	0	0	39
A5-1	null	7	71	16	0	16	55

^aBLM-positive cell lines C8, D4, and D7 were derived from normal human fibroblast cell line GM00637; BLM-null cell line A5-1 was derived from BS fibroblast cell line GM08505.

^bNumber of independent electroporations with the I-SceI expression vector pSce.

^cGene conversions.

^dHomology-dependent deletions.

A5-1 were HDD, and sequencing analysis revealed that the site of joining between donor and recipient in each event was located downstream from the DSB site, as expected. Based on nucleotide differences between donor and recipient, we could determine that the crossover site varied among the clones and ranged from about 18 bp to more than 350 bp downstream from the position of the DSB, with no obvious preference for the precise site of sequence joining. The locations of crossover sites are indicated in Fig. 4.

Sequence analysis (data not shown) of NHEJ events recovered from A5-1 revealed that 16 of 55 events used microhomology and, similar to the situation with NHEJ events recovered from BLM-null cell line F1-6 above, this differed significantly ($p = 1.43 \times 10^{-8}$ by a two-sided Fisher exact test) from the NHEJ events recovered from the GM00637-derived cell lines containing pBR3 in which 57 out of 72 events involved microhomology. These results provided corroborating evidence suggesting that dependency of NHEJ on microhomology is reduced by BLM deficiency. Deletion sizes for NHEJ events from A5-1 ranged from 1 bp to 958 bp, with an average deletion size of 111 bp and a median size of 10 bp. Deletion sizes for NHEJ events

from GM00637-derived lines ranged from 1 bp to 1037 bp, with an average deletion size of 110 bp and a median size of 22 bp.

3.4 *HeR recovery depends on BLM deficiency*

A striking result revealed in Table 3 was the significant difference in recovery of HeR among the DSB events recovered from BLM-null versus BLM-positive cells. The data presented in Table 3 thus suggested that BLM deficiency in human cells lifts a barrier to HeR, similar to the impact elicited by deficiencies of BLM homologs in yeast and *Drosophila*.

To explore this finding more rigorously, and to ascertain that the ability of A5-1 to carry out HeR was indeed due to BLM deficiency, we stably transfected cell line A5-1 with the BLM expression vector pBLM/Zeo to complement the BLM defect. Western blot analysis showed that three stable transfectants (named BLM A1, BLM A9, and BLM B12) had levels of BLM expression comparable to that seen in normal human fibroblast cell line GM00637 (Fig. 5). As an additional control, cell line A5-1 was transfected with the empty expression vector pcDNA3.1/Zeo(+) to establish cell line C17.

Fig. 5. Western blot demonstrating complementation of BLM expression in a BS cell line. Expression of BLM was monitored for cell lines A5-1 (lane 1), C17 (lane 2), GM00637 (lane 3), BLM A1 (lane 4), BLM A9 (lane 5), and BLM B12 (lane 6). As described in the text, cell line C17 was produced by transfecting BLM-null cell line A5-1 with empty vector pcDNA3.1/Zeo(+) and was expected to be null for BLM. Cell lines BLM A1, BLM A9, and BLM B12 were produced by transfecting A5-1 with pBLM/Zeo and each cell line displayed BLM expression comparable to that seen in normal cell line GM00637. Each lane of the blot was loaded with 30 µg of protein.

Following the establishment of the BLM-complemented cell lines and control cell line C17, each of these lines was transfected with pSce to induce a DSB, and G418-resistant clones were recovered and analyzed. As presented in Table 4, the overall frequency of recovery of

Table 4

Recovery and characterization of DSB events from BS fibroblasts and BLM-complemented BS fibroblasts containing HeR substrate pBR3

Cell line	BLM status ^a	No. of trials ^b	Colony frequency (10 ⁻⁵) ^c	Colonies analyzed	HeR events		
					Total	GC ^d	HDD ^e
A5-1	null	7	1.21	71	16	0	16
C17	null	5	2.60	110	14	7	7
BLM A1	positive	3	1.22	16	0	0	0
BLM A9	positive	4	1.36	81	4	1	3
BLM B12	positive	5	0.22	27	2	1	1

^aCell line C17 was produced by transfecting BLM-null cell line A5-1 with an empty expression vector while the three BLM-positive cell lines were produced by stably transfecting A5-1 with a BLM expression vector.

^bNumber of independent electroporations with the I-SceI expression vector pSce.

^cCalculated for each cell line as the total number of colonies recovered from all trials divided by total number of viable cells plated into selection for all trials.

^dGene conversions.

^eHomology-dependent deletions.

colonies was not affected by BLM expression. Among the BLM-complemented cell lines, only six HeR events were recovered collectively out of a total of 124 DSB repair events. This outcome was significantly different ($p = 0.00029$ by a two-sided Fisher exact test) from the recovery of 16 HeR events among 71 DSB repair events recovered from parent line A5-1. The recovery of 14 HeR events among 110 events from the control line C17 was not statistically different ($p = 0.102$ by a two-sided Fisher exact test) from results obtained with the parent line A5-1 but represented a significantly greater recovery of HeR events than was seen with the complemented lines ($p = 0.036$ by a two-sided Fisher exact test). In summary, the results

presented in Tables 3 and 4 established that BLM-deficiency enables the occurrence of HeR in human cells.

HeR events from control line C17 and complemented lines BLM A9 and BLM B12 were analyzed by Southern blots and at the nucleotide level. The results of sequence analysis are represented in Fig. 4. Sequencing revealed that among seven HeR gene conversion tracts recovered from C17, five displayed conversion of a single mismatch upstream and four mismatches downstream from the DSB site. Including the first and last converted mismatches, these five tracts were minimally 11 bp in length. Another gene conversion tract from C17 displayed conversion of seven upstream and four downstream mismatches, while the final gene conversion tract displayed conversion of eight mismatches upstream and four downstream from the DSB. For these latter two tracts, the distances spanned on the donor were 68 bp and 87 bp, respectively. The single gene conversion tract recovered from line BLM A9 displayed conversion of two mismatches upstream and four downstream from the DSB, spanning 38 bp on the donor. The conversion tract from BLM B12 displayed conversion of 1 upstream and 4 downstream mismatches, spanning 10 bp on the donor. The HeR HDD events recovered from C17, BLM A9, and BLM B12 displayed crossover sites located from 15 bp to 309 bp downstream from the DSB site, with no obvious preference for the precise site of crossing-over (Fig. 4).

4. Discussion

BS, a rare autosomal disorder caused by mutations in the BLM helicase, is associated with short stature, genomic instability and a marked increase in risk for cancer of all types

(50,51,66,67). A hallmark of BS, and actually the only objective criterion for diagnosis of BS (67) other than sequencing the gene for BLM, is a large (about ten-fold) increase in the frequency of SCEs observed in cultured cells from patients. Additional chromosomal abnormalities associated with BS include an increase in DSBs, translocations, and the formation of chromosomal radials. Such observations of chromosomal abnormalities in BS have contributed to the assessment that BLM normally plays a role in “quality control” of recombination (reviewed in 68,69).

Consistent with the notion that BLM is involved in recombination quality control, *Drosophila* BLM and the yeast BLM homolog Sgs1 have been shown to suppress HeR (43-46). In the case of *Drosophila*, BLM was reported to suppress HeR in the form of SSA (43). In contrast, a previous report (58) concluded neither murine nor human BLM plays a similar role in blocking HeR. This latter finding was perhaps unexpected, but it was also possible that the outcome of the study was shaped by the use of recombination assays in which one or both of the recombining sequences were not actually located within a mammalian chromosome. We had previously shown (11) that homology requirements for recombination in mammalian cells can differ significantly depending on whether the recombining DNA sequences reside within a chromosome or exist as extrachromosomal entities. In our current work, we reevaluated the role of human BLM in blocking HeR using an assay that surveyed recombination between two chromosomal sequences. We report here that BLM deficiency in human cells indeed enables the occurrence of intrachromosomal HeR between sequences displaying 19% divergence, indicating that human BLM does normally suppress HeR. It is possible that the higher percent sequence divergence (19%) used to study HeR in our current study compared to the level of divergence (~1.5%) used in the previous study (58) also contributed to different outcomes.

Sequence analysis of DSB repair events recovered from BLM-null cell line A5-1 containing HeR substrate pBR3 provided us with incontrovertible evidence that HeR had occurred within the genome of these cells. From the data presented collectively in Tables 1-3, one can estimate that, in BS cells, the frequency of DSB-induced HeR between sequences displaying 19% divergence was reduced only about seven-fold relative to the frequency of DSB-induced HR between highly homologous sequences. In comparison, the data in Tables 1-3 reveal that HeR was reduced several hundred-fold relative to HR in normal human fibroblasts.

The occurrence of HeR in BLM-null cell line A5-1 did not by itself rigorously establish that BLM deficiency was responsible for allowing HeR. To gain stronger evidence for a causative role of BLM deficiency in enabling HeR, we isolated three cell lines derived from A5-1 in which the BLM deficiency had been complemented. In each of these complemented cell lines (BLM A1, BLM A9, BLM B12) the frequency of recovery of HeR was reduced relative to parent line A5-1 (Table 4), and collectively the reduction in HeR in complemented lines was highly significant. Since transfection of A5-1 with an empty expression vector did not impact HeR recovery (cell line C17, Table 4), the results for the complemented lines provide robust evidence that BLM deficiency enables the occurrence of HeR. We also have never recovered a single HeR event from derivatives of normal human fibroblast cell line GM00637 containing pBR3 (Table 3).

We had previously shown that experimental depletion of BLM in normal human cells using RNA interference shifted DSB repair pathway choice away from NHEJ and toward HR events associated with HDD (53). The data in Table 2 in the current study is also consistent with BLM deficiency eliciting an increase in DSB repair via HR associated with HDD. An increase in HR events resolving as HDD in the face of BLM deficiency can explain the elevated

level of SCEs seen in BS patients. An argument can be made that an increase in LOH, as would be predicted to occur concomitantly with an elevated frequency of crossovers, may contribute significantly to the cancer predisposition seen in BS. It has in fact been demonstrated that an increase in LOH is engendered by BLM deficiency (55,56). The *combined* impacts of increased HDD frequency along with an increased HeR frequency that we now report may lead to translocations and radials between nonhomologous chromosomes and profoundly destabilize the genomes of BS patients, further contributing to cancer predisposition.

As described under Results above, we noted that the gene conversion tracts in BLM-null cell line F1-6 containing pLB4 were conspicuously longer than conversion tracts recovered from normal cell line pLB4/11. Ongoing work indicates significant lengthening of gene conversion tracts in a second GM08505-derived cell line. Collectively, our results suggest that BLM may limit the extent of gene conversion tracts in healthy cells. We also found that the use of microhomology in NHEJ was reduced in the absence of BLM. This result implies that facilitation of end-joining through base-pairing within microhomologies is diminished in the absence of BLM. This result is consistent with our previous observation of a reduced occurrence of GC basepairs within microhomologies used in NHEJ events recovered from BLM-depleted cells (53). Other investigators have also reported a lesser use of microhomology in NHEJ in the absence of BLM (65).

How may a deficiency in BLM bring about an increase in HeR, an increase in recombination events associated with HDD, lengthened gene conversion tracts, and lesser dependency of NHEJ on microhomology? We may unify these observations with the simple proposition that a key role for BLM helicase in healthy cells is to unwind and dismantle recombination and repair intermediates to generally constrain DNA transactions. In the absence

of BLM, DNA transactions become less restricted as DNA ends become, in a sense, “stickier,” allowing for an increased incidence of exchanges, and an increased promiscuity of exchanges. We elaborate on this idea below.

A nearly ubiquitous feature of models for DSB repair is the pairing of a single-stranded DNA tail from one end of a DSB with a DNA strand at either the other end of the DSB or with a sequence at a second, homologous locus. How stable that pairing is will influence the ultimate outcome of the repair event, perhaps profoundly. We posit that the stability or “stickiness” of such pairing is necessarily affected by the degree of match between the paired strands, and also by the presence or absence of BLM activity.

For DSB repair via recombination, it is envisioned that a 3' end of DNA invades an homologous sequence, and that the invading DNA end may be elongated via synthesis using the invaded duplex as a template. By the synthesis-dependent strand annealing (SDSA) model, the newly synthesized strand of DNA is subsequently displaced from the template and then annealed to sequence on the other side of the DSB. A role for the helicase activity of BLM in peeling the newly synthesized DNA strand from its template has been proposed (70,71). In such a role, BLM serves to limit strand elongation during SDSA, a recombination pathway that leads exclusively to gene conversions (noncrossovers). This scenario can explain how BLM deficiency can lead to a lengthening of gene conversion tracts since, in the absence of BLM, elongation of the invading 3' DNA would proceed for an extended period of time. This scenario also predicts that BLM deficiency will lead to an increase in crossover events (recovered as HDD) because if the newly synthesized DNA strand is not efficiently displaced from the template, the likelihood of formation of a Holliday junction (HJ) or double HJ intermediate will be increased. An increase in HJ formation will subsequently increase the frequency of

crossovers since crossover events require the formation of HJ intermediates. Additionally, BLM has been shown to act in conjunction with BLAP75 and Topo III alpha to catalyze “dissolution” of double HJs specifically into noncrossovers (71). The absence of BLM would therefore not only increase the formation of HJ intermediates, but also increase the incidence of processing of HJs into crossovers rather than noncrossovers.

An increased frequency of formation of HJs under conditions of BLM deficiency may increase the overall fraction of recombination events accomplished via HJs as opposed to SDSA, which would mean an increase in the fraction of gene conversion events arising via resolution of HJ intermediates. This increase in conversion via HJ resolution may also contribute to lengthened conversion tracts due to the potential for HJs to undergo extensive branch migration prior to resolution. Moreover, if one normal function of BLM is to dismantle or unwind recombination intermediates via reverse branch migration of HJs, BLM deficiency may increase the extent of branch migration.

We consider the most salient finding in our current work to be the enabling of HeR under conditions of BLM deficiency. Facilitation of HeR can be explained as an outcome of the very same mechanisms described above that lead to an increase in crossover frequency and lengthening of gene conversion tracts, with one additional consideration; BLM’s normal role in dismantling recombination intermediates in healthy human cells may be particularly robust when BLM acts on intermediates containing mismatched bases. The mechanism for prevention of HeR in human cells may be essentially analogous to models presented for heteroduplex rejection in yeast (31,45-47) in which the BLM homolog Sgs1 collaborates with mismatch repair proteins to produce a complex that recognizes mispairs in recombination intermediates and then responds by efficiently unwinding heteroduplex DNA. It has indeed been demonstrated that HeR is

elevated in mammalian cells deficient in mismatch repair protein MSH2 (33). Thus, HeR in human cells may be prevented by a BLM-dependent rejection of mispaired strands at the very earliest stage of strand invasion, at the annealing step of SDSA, and/or by reversing branch migration of Holliday junctions to undo mismatched heteroduplex. The proposed proclivity of BLM (in concert with mismatch repair proteins) for unwinding DNA strands containing mismatches can also be invoked to explain why BLM deficiency allows an increased occurrence of NHEJ events between DNA ends displaying no microhomology. Of relevance to this latter point are our observations that, similar to BLM deficiency, a deficiency in MSH2 or MLH1 in mammalian cells lessened the dependency of NHEJ on microhomology (72,73).

We have outlined a broad scheme by which BLM helicase activity may operate in mammalian cells to protect genome integrity by generally restraining DNA transactions and suppressing HeR. The scheme proposed here does not ascribe any unanticipated enzymatic activities to BLM. Our work is the first demonstration that BLM plays a role in protecting against recombination between diverged sequences in humans, leaving little doubt that BLM serves to protect against potentially detrimental rearrangements in a multifaceted fashion. The importance of BLM in preserving genome integrity, as amply demonstrated by the deleterious consequences of BLM deficiency in humans, provides plentiful motivation for continued investigations into BLM's diverse facets.

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