

Possible association of BLM in decreasing DNA double strand breaks during DNA replication

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Bloom's syndrome (BS) is a rare genetic disorder and the cells from BS patients show genomic instability and an increased level of sister chromatid exchange (SCE). We generated *BLM*^{-/-} and *BLM*^{-/-}/*RAD54*^{-/-} DT40 cells from the chicken B-lymphocyte line DT40. The *BLM*^{-/-} DT40 cells showed higher sensitivity to methyl methanesulfonate and elevated levels of SCE as expected. The targeted integration frequency was also increased remarkably in *BLM*^{-/-} cells. The SCE frequency increase in *BLM*^{-/-} cells was considerably reduced and the enhanced targeted integration observed in *BLM*^{-/-} cells was almost completely abolished in *BLM*^{-/-}/*RAD54*^{-/-} cells, indicating that a large portion of the SCE in *BLM*^{-/-} cells occurs via homologous recombination, and homologous recombination events increase with the defect of BLM function. The *BLM*^{-/-}/*RAD54*^{-/-} cells showed a slow growth phenotype and an increased incidence of chromosome-type breaks/gaps while each single mutant showed relatively small numbers of chromosome-type breaks/gaps.

Keywords: *BLM*/chromosome-type breaks/HR/*RAD54*/SCE

Introduction

Bloom's syndrome (BS) is a rare genetic disorder characterized by small size, sunlight sensitivity, immunodeficiency and male infertility. The cells from BS patients show an abnormally high incidence of sister chromatid exchange (SCE) compared with cells from normal individuals (Chaganti *et al.*, 1974; reviewed by German, 1993). The gene responsible for BS, *BLM*, encodes a protein belonging to the RecQ helicase family, which consists of 1417 amino acids with seven conserved helicase motifs (Ellis *et al.*, 1995).

Recently, Werner's syndrome (WS) responsible gene (*WRN*) has been cloned and revealed to encode a RecQ

homolog (Yu *et al.*, 1996). WS is a genetic disorder characterized by premature aging, and the cells from WS patients also show genomic instability (Gebhart *et al.*, 1988; reviewed by Martin, 1982) but not increased SCEs.

In *Saccharomyces cerevisiae*, a sole gene encodes the RecQ homolog, *SGS1* (*slow growth suppressor 1*), whose mutant allele was identified as a suppressor of the slow growth phenotype of *top3* mutants (Gangloff *et al.*, 1994). The *sgs1* mutants show hyper-recombination and chromosome missegregation (Watt *et al.*, 1995, 1996). It has been shown that Sgs1 interacts physically with DNA topoisomerase (topo) II and topo III (Gangloff *et al.*, 1994; Watt *et al.*, 1995) and genetically with topo I and III (Gangloff *et al.*, 1994; Lu *et al.*, 1996). Topoisomerases are essential for DNA replication, transcription, chromosome condensation and segregation (reviewed by Wang, 1996).

Biochemical analysis of BLM, WRN and Sgs1 showed that they were all ATP-dependent helicases with 3'→5' directionality (Lu *et al.*, 1996; Gray *et al.*, 1997; Karow *et al.*, 1997; Bennett *et al.*, 1998). BLM, WRN and Sgs1 are similar in length and share limited sequence homology outside the helicase domain (Gangloff *et al.*, 1994; Ellis *et al.*, 1995; Yu *et al.*, 1996). *BLM* and *WRN* partially suppressed the increased homologous and illegitimate recombination in the *sgs1* mutants (Yamagata *et al.*, 1998), suggesting that structures and functions are partially conserved among these proteins.

The most characteristic feature of BS cells is a high incidence of SCE. Although the phenomenon of SCE has long been recognized, the molecular mechanism behind it, especially the SCE in BS cells, remains unclear. There are two major models to explain SCE. First, SCE is mediated by homologous recombination (HR) (Painter, 1980; Cleaver, 1981; Sonoda *et al.*, 1999). Secondly, topo II causes transient double-stranded DNA breaks (DSBs) during replication, and the proximity of DNA breaks on sister chromatids may result in incorrect rejoining, causing an SCE (Ishii and Bender, 1980; Pommier *et al.*, 1985; Heartlein *et al.*, 1987; Dillehay *et al.*, 1989).

Studies on HR in *S.cerevisiae* defined the *RAD52* epistasis group of genes, which are constituents of a pathway for the repair of DSBs by HR (reviewed by Game, 1993; Shinohara and Ogawa, 1995). The *rad51*, *rad52* and *rad54* mutants, which are involved in the *RAD52* epistasis group, show similar phenotypes including high sensitivity to ionizing radiation and a reduced mitotic recombination frequency, and recent studies indicated that these genes are conserved in function from yeasts to vertebrate cells (Bezzubova *et al.*, 1997; Essers *et al.*, 1997; Sonoda *et al.*, 1998; Yamaguchi-Iwai *et al.*, 1998).

In this study, to elucidate the molecular mechanism to increase SCEs in BS cells and to examine whether the increased SCEs are mediated via HR, we generated

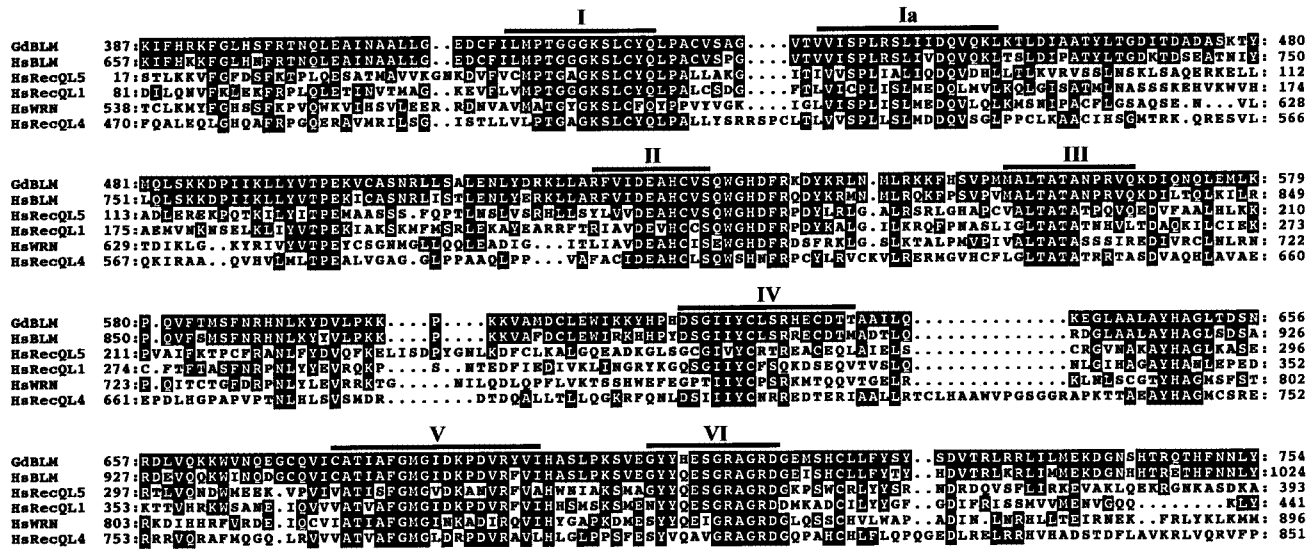


Fig. 1. Amino acid alignments in the helicase domain of chicken BLM (GDBLM) and human (Hs) BLM, WRN, RecQL1, RecQL4 and RecQL5. Thick lines indicate helicase motifs.

BLM^{-/-} and *BLM*^{-/-}/*RAD54*^{-/-} cells from the chicken B-lymphocyte line DT40 and characterized their phenotypes.

Results

BLM targeting constructs and generation of *BLM*^{-/-} and *BLM*^{-/-}/*RAD54*^{-/-} clones

A chicken *BLM* cDNA fragment was amplified from chicken testis RNA by RT-PCR using primers based on mouse *BLM* sequences. The N-terminal region and the C-terminal region of chicken *BLM* were obtained by 5'-RACE and 3'-RACE, respectively. We obtained an ~3.5 kb chicken *BLM* cDNA fragment containing the helicase domain. The helicase domain of chicken BLM shares 84.4% identity with that of human BLM at the amino acid level and 39.1, 47.5, 35.1 and 46.5% identity with those of human WRN, RecQL/DNA helicase Q1 (RecQL1), RecQL4 and RecQL5, respectively (Figure 1).

An ~4 kb genomic DNA fragment was amplified by long-range PCR using primers synthesized according to the cDNA sequences within the helicase domain. To generate *BLM* targeting constructs, either histidinol (his), blasticidin (bsr) or hygromycin (hyg) resistance genes were inserted into the genomic sequence as shown in Figure 2A. Targeted integration of these constructs is expected to delete the helicase motif III, which is highly conserved among the RecQ helicase family. Two *BLM* targeting constructs were sequentially transfected into DT40 cells. Disruption of the *BLM* gene was verified by Southern blotting (Figure 2B). Northern blotting using N-probe indicated expression of truncated mRNA (Figure 2C).

Since HR was reduced in *RAD54*^{-/-} cells (Bezzubova *et al.*, 1997), we generated *BLM*^{-/-}/*RAD54*^{-/-} clones from a *BLM*^{-/-} clone. The two *RAD54* targeting constructs containing selection markers of hygromycin or puromycin were sequentially transfected into a *BLM*^{-/-} clone. The disruption of the *RAD54*^{-/-} gene was also verified by Southern blotting (data not shown).

Proliferative properties of BLM^{-/-}, *RAD54*^{-/-} and *BLM*^{-/-}/*RAD54*^{-/-} cells

To examine the proliferative property, we monitored growth curves of wild-type, *BLM*^{-/-}, *RAD54*^{-/-} and *BLM*^{-/-}/*RAD54*^{-/-} cells (Figure 3). *BLM*^{-/-} and *RAD54*^{-/-} cells proliferated at a slightly lower rate than wild-type cells, while *BLM*^{-/-}/*RAD54*^{-/-} cells proliferated at a considerably lower rate than either single mutant. The doubling times of these cells were 7.4 ± 0.2 , 8.2 ± 0.1 , 8.9 ± 0.2 and 16.0 ± 0.5 h for wild-type, *BLM*^{-/-}, *RAD54*^{-/-} and *BLM*^{-/-}/*RAD54*^{-/-} cells, respectively.

Sensitivity of BLM^{-/-} cells to methyl methanesulfonate (MMS), VP16 and camptothecin (CAM)

To examine the sensitivity of *BLM*^{-/-} cells to MMS, they were grown in medium containing various concentrations of MMS. The *BLM*^{-/-} cells were sensitive compared with wild-type cells (Figure 4A). Thus, we next examined the sensitivity of *RAD54*^{-/-} and *BLM*^{-/-}/*RAD54*^{-/-} cells to MMS. *RAD54*^{-/-} cells were moderately sensitive, like *BLM*^{-/-} cells, and *BLM*^{-/-}/*RAD54*^{-/-} cells were highly sensitive to MMS.

Since the yeast homolog for BLM, Sgs1, physically interacts with topo II and topo III and functional interaction between Sgs1 and topo I has been shown genetically, we examined the sensitivity of *BLM*^{-/-} cells to the topo II inhibitor, VP16, which is known to induce DNA strand breaks and SCEs (Tominaga *et al.*, 1986) and to the topo I inhibitor, CAM (Hsiang *et al.*, 1989). As shown in Figure 4B and C, *BLM*^{-/-} cells showed no significant difference in VP16 or CAM sensitivity compared with wild-type cells.

Increased SCE frequency in BLM^{-/-} DT40 cells was reduced by disruption of the *RAD54* gene

Lymphoblastoid cells from BS patients show a 10- to 15-fold higher number of SCEs than cells from normal individuals (Chaganti *et al.*, 1974; reviewed by German, 1993). Thus, we examined the frequency of SCE in *BLM*^{-/-}

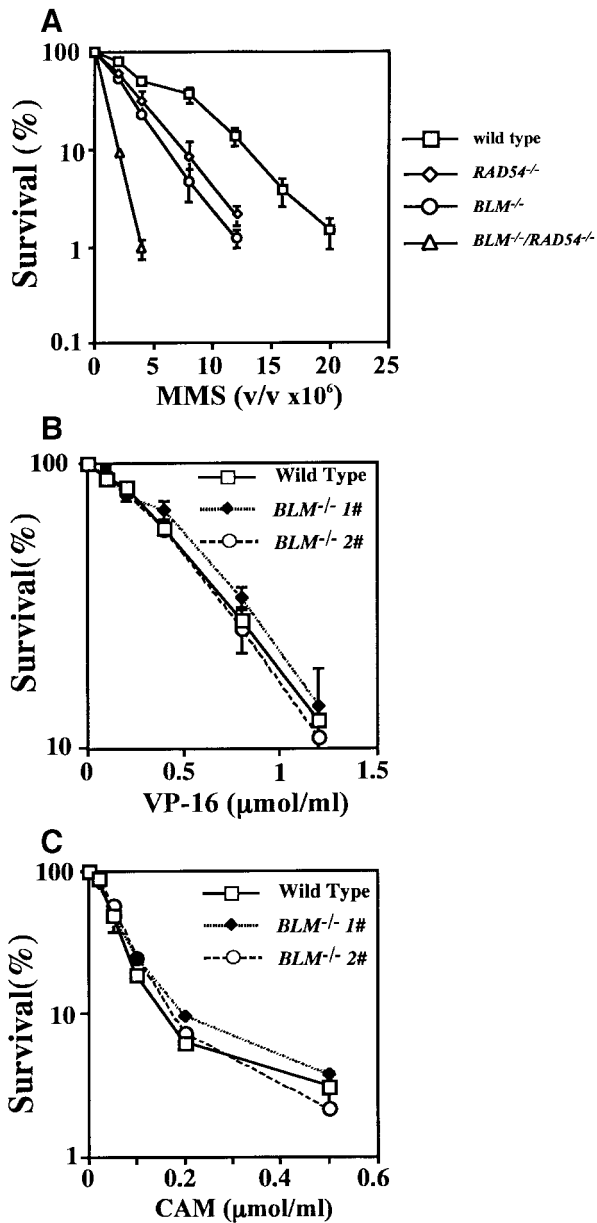


Fig. 4. Sensitivity of cells with various genotypes to MMS, VP16 and CAM. Cells were treated with the indicated concentration of MMS (A), VP16 (B) or CAM (C) as described in Materials and methods. (A) The concentration of undiluted MMS (99%) is 1. The data show the average of the results from three independent experiments.

or 82% (59/72) for BLM^{-/-} cells (Table I). Disruption of the RAD54 gene of BLM^{-/-} cells decreased the targeted integration frequency to 1.7% (1/60), indicating that the targeted integration in BLM^{-/-} cells is Rad54-dependent HR.

Cell cycle analysis of BLM^{-/-}/RAD54^{-/-} cells

The growth curves shown in Figure 3 indicated that disruption of BLM and RAD54 had a synergistic effect on cell growth. Thus, we analyzed the cell-cycle phase distribution of asynchronous cells by flow cytometry. The cell-cycle phase distribution patterns for wild-type, RAD54^{-/-} and BLM^{-/-} cells were essentially the same, although the population in G₂-M phase was increased slightly in RAD54^{-/-} and BLM^{-/-} cells compared with wild-

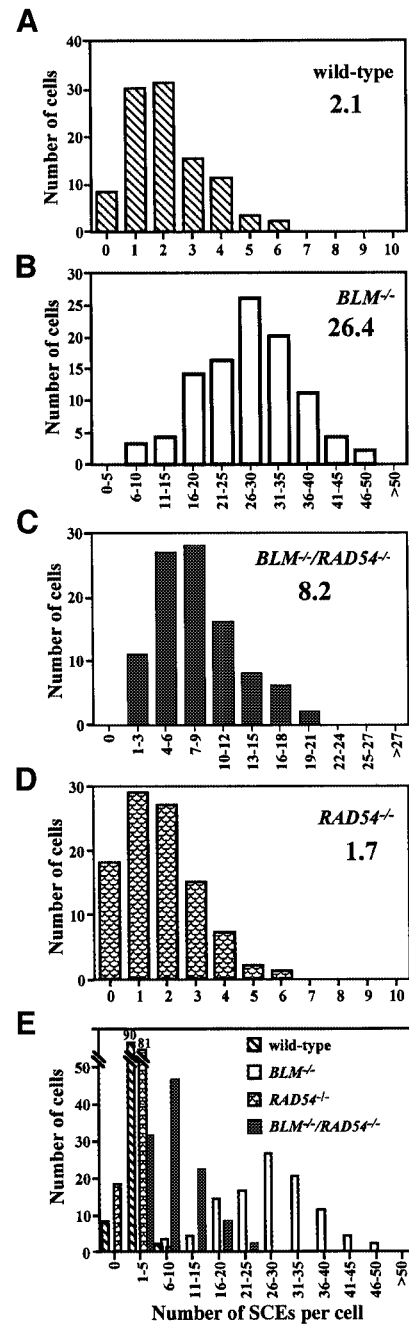


Fig. 5. Histograms of SCE in wild-type, RAD54^{-/-}, BLM^{-/-} and BLM^{-/-}/RAD54^{-/-} cells. SCEs in the macrochromosomes were counted. Histograms show the frequency of cells with the indicated number of SCEs per cell. The mean number of SCEs per cell is shown in the upper right corner. (A) Wild-type cells; (B) BLM^{-/-} cells; (C) BLM^{-/-}/RAD54^{-/-} cells; (D) RAD54^{-/-} cells; (E) a superimposed figure of (A), (B), (C) and (D) expressed on the same scale.

type cells (Figure 7). In contrast, BLM^{-/-}/RAD54^{-/-} cells showed a remarkable increase in the G₂-M phase population.

Chromosome-type breaks/gaps are increased in BLM^{-/-}/RAD54^{-/-} cells

We next analyzed chromosomal breakage. DT40 cells display a stable karyotype with a modal chromosome number of 80 in total, which comprises 11 autosomal

macrochromosomes, the ZW sex chromosomes and 67 microchromosomes (Sonoda *et al.*, 1998). Since alterations in the minichromosomes are difficult to assess, analysis of chromosomal breakage was limited to the 11 autosomal macrochromosomes and the Z chromosome. *BLM*^{-/-} and *RAD54*^{-/-} cells showed slightly larger numbers of chromatid- and chromosome-type breaks/gaps than wild-type cells (Table II). *BLM*^{-/-}/*RAD54*^{-/-} cells exhibited many more chromosomal aberrations than either single gene mutant, that is, chromosome-type breaks/gaps were specifically increased in the double mutants.

Discussion

BLM^{-/-} DT40 cells show phenotypes characteristic of human BS cells

The *BLM*^{-/-} DT40 cells showed the slow growth phenotype, a higher sensitivity to MMS and an elevated frequency of SCE, which are characteristic phenotypes of human BS cells (reviewed by German, 1993). The helicase domain of chicken BLM showed 84.4 and 83.8% identity

to those of human and mouse BLM, respectively. These results suggest that the structure and function of BLM are conserved in vertebrates.

The results shown in Figure 5 indicate for the first time that a considerable portion of the SCEs in *BLM*^{-/-} cells are formed via a process requiring Rad54 function, HR. The mechanism of SCE in *BLM*^{-/-}/*RAD54*^{-/-} cells is not clear at present. One possibility is that Rad54B, a homolog of Rad54, functions in place of Rad54 (Hiramoto *et al.*, 1999) and another possibility is that the SCEs are formed by topo II (Ishii and Bender, 1980; Heartlein *et al.*, 1987; Dillehay *et al.*, 1989). These possibilities should be addressed in a future study.

BLM suppresses homologous recombination

Disruption of the *BLM* gene resulted in a remarkable increase in the targeted integration frequency (Table I), indicating that specific inhibitors for BLM, if any exist, would increase the targeted integration frequency of higher eukaryotic cells. As expected, the elevated targeted integration in *BLM*^{-/-} cells was abolished by disruption of *RAD54*. In budding yeast *sgs1* disruptants, the frequency of illegitimate recombination (Yamagata *et al.*, 1998) as well as HR was elevated (Gangloff *et al.*, 1994; Watt *et al.*, 1996). In addition, an increase in illegitimate recombination was observed in *Escherichia coli recQ* mutants (Hanada *et al.*, 1997). However, in the *BLM* disruptants,

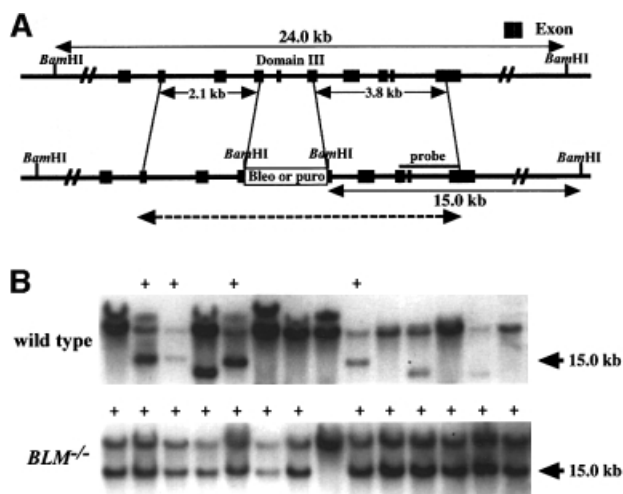


Fig. 6. Southern blot analysis of drug-resistant clones. (A) Schematic representation of the wild-type allele and the targeted allele. The dotted line corresponds to the targeting construct. (B) The DNA samples were digested with *Bam*HI and hybridized to the probe shown in (A). A typical Southern blot is shown. Arrowheads indicate the position of the fragment representing the targeted allele. +, targeted integration.

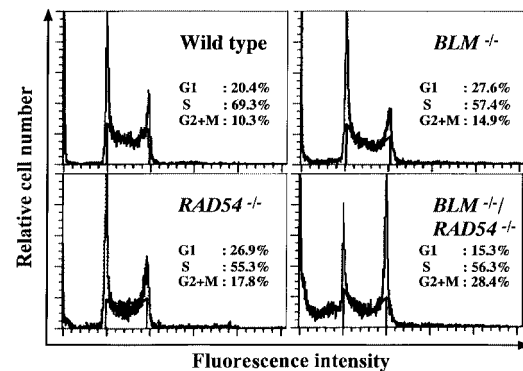


Fig. 7. Cell cycle analysis by flow cytometry. Distribution patterns of asynchronous culture of wild-type, *BLM*^{-/-}, *RAD54*^{-/-} and *BLM*^{-/-}/*RAD54*^{-/-} cells in the cell cycle.

Table I. Targeted integration frequency

Targeting construct	Targeted integrated colonies/tested colonies				
	Wild type	<i>BLM</i> ^{-/-} 1#	<i>BLM</i> ^{-/-} 2#	<i>RAD54</i> ^{-/-}	<i>BLM</i> ^{-/-} / <i>RAD54</i> ^{-/-}
RECQL1-puro					
expt 1	7/24	21/24	22/24	0/48	–
expt 2	12/36	33/36	32/36	0/48	–
total	19/60 (32%)	54/60 (90%)	54/60 (90%)	0/96 (0)	–
transfection efficiency	2.8×10^{-5}	2.3×10^{-5}	1.9×10^{-5}	2.2×10^{-5}	
RECQL1-bleo					
expt 1	6/24	20/24	–	0/48	0/24
expt 2	10/36	39/48	–	0/48	1/36
total	16/60 (27%)	59/72 (82%)	–	0/96 (0)	1/60 (1.7%)
transfection efficiency	2.2×10^{-5}	1.6×10^{-5}		2.1×10^{-5}	1.3×10^{-5}

RECQL1 targeting constructs were transfected into cells of the genotypes indicated, and targeted integration events following selection were determined by Southern blot analysis and PCR.

Table II. Spontaneous chromosomes aberrations

Genotype	Chromatid breaks/gaps	Chromosome breaks/gaps	Chromatid exchanges	Total (per cell)
Wild type	3	1	0	4 (0.013)
BLM ^{-/-}	2	6	0	8 (0.027)
RAD54 ^{-/-}	10	4	0	14 (0.047)
BLM ^{-/-} RAD54 ^{-/-}	3	46	0	49 (0.163)

Spontaneous chromosomal aberrations were analyzed in 300 cells of the genotypes indicated.

only targeted integration, i.e. HR, increased, indicating that BLM mainly suppresses HR.

Sgs1 and RecQ are the sole RecQ helicase in budding yeast and *E.coli*, respectively, and they carry out multiple functions. In contrast, *Caenorhabditis elegans*, *Drosophila melanogaster* and higher eukaryotes, including vertebrates, have multiple RecQ helicase genes (Kusano *et al.*, 1999; Sekelsky *et al.*, 1999). In human cells, five genes encoding RecQ homologs have been identified: *RECQL1* (Puranam and Blackshear, 1994; Seki *et al.*, 1994), *BLM* (Ellis *et al.*, 1995), *WRN* (Yu *et al.*, 1996), *RECQL4* and *RECQL5* (Kitao *et al.*, 1998). Recently, *RECQL4* has been shown to be a causative gene of Rothmund–Thomson's syndrome (Kitao *et al.*, 1999). Thus it seems likely that some of the five RecQ homologs share the functions carried out by Sgs1 in higher eukaryotic cells. In this context, it is interesting to determine which RecQ homolog suppresses illegitimate recombination.

Slow growth phenotype of BLM^{-/-}/Rad54^{-/-} cells is caused by DNA lesions to induce chromosome-type breaks/gaps

Flow cytometric analysis showed an accumulation of BLM^{-/-}/RAD54^{-/-} cells in G₂-M phase, and a high incidence of chromosome-type breaks/gaps was observed in these cells. It is suggested that chromosome-type breaks are caused by DSBs generated prior to and during DNA replication, while chromatid-type breaks are caused by DSBs generated following DNA replication. Previous studies showed delayed DNA-chain maturation in BS cells (Hand and German, 1975; Giannelli *et al.*, 1977). Considering both of the above, it seems likely that DSBs are generated in BLM^{-/-}/RAD54^{-/-} cells during DNA replication.

Chromosome analysis showed that ~15% of BLM^{-/-}/RAD54^{-/-} cells contain chromosome-type breaks/gaps, and this value is probably underestimated because we can only analyze the chromosomes of the cells that passed through the G₂ phase. A single unrepaired DSB is sufficient to induce cell death in yeast (reviewed by Game, 1993), and RAD51^{-/-} DT40 cells died rapidly showing chromosome-type breaks after inhibition of the expression of exogenous Rad51 (Sonoda *et al.*, 1998). Therefore, a substantial fraction of BLM^{-/-}/RAD54^{-/-} cells are dying or dead.

A possible mechanism for the increase of SCE and targeted integration in BLM^{-/-} cells

Zou and Rothstein (1997) showed that Holliday junctions accumulate spontaneously during DNA replication in mitotically growing yeast. Recently, a model for the occurrence of DSBs at arrested replication forks in *E.coli* was proposed (Seigneur *et al.*, 1998). According to this

model, Holliday junctions are formed by the annealing of two newly synthesized DNAs at arrested replication forks but are not formed via DSBs, and DSBs are formed by cleavage of the Holliday junctions. Indeed, annealed molecules consisting of two newly synthesized DNA were detected in the cells from BS patients (Waters *et al.*, 1978). The BLM homolog of *S.cerevisiae*, Sgs1, has been shown to possess an activity to disrupt Holliday junctions (Bennett *et al.*, 1999). If Holliday junctions are formed by the above mechanism, and the physiological function of BLM is to disrupt Holliday junctions, the defect of BLM will cause the formation of more Holliday junctions and more DSBs. In fact, induction of DSBs during DNA replication was indicated by the increase in chromosome-type DNA breaks/gaps in BLM^{-/-}/RAD54^{-/-} cells. It is therefore likely that the majority of DSBs formed during DNA replication due to the defect of BLM function are repaired by HR, resulting in an increase in SCE in Rad54-proficient cells.

The induction of a DSB in the genome by the expression of a restriction enzyme increased the allelic recombination frequency by a few hundred-fold, suggesting that induced DSBs stimulate allelic recombination in mammalian cells (Moynahan and Jasin, 1997). In this context, it is interesting that the allelic recombination frequency is increased in BS cells (Ellis *et al.*, 1995). In addition, artificially introduced DSBs in the chromosomal locus of mammalian cells highly stimulate targeted integration (Smih *et al.*, 1995). Thus, it is likely that DSBs transiently formed in the chromosomal locus in BLM^{-/-} cells stimulate targeted integration.

Materials and methods

Construction of targeting vectors

A partial chicken *BLM* cDNA was obtained by RT-PCR of chicken testis RNA using primers synthesized based on the sequences in motifs I and VI of mouse *BLM* helicase domains (Seki *et al.*, 1998). A partial N-terminal region and the C-terminal region of chicken *BLM* were obtained by 5'-RACE and 3'-RACE, respectively. We obtained an ~3.5 kb chicken *BLM* cDNA fragment including the helicase domain. The sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession No. AB040747. A partial genomic DNA segment corresponding to the helicase domain was amplified with the genomic DNA of DT40 cells as a template by long-range PCR. Chicken *BLM* targeting constructs, BLM-his and BLM-hyg and BLM-bsr, were made by replacing the helicase motif III with histidinol (his)-, hygromycin (hyg)-, or blasticidin (bsr)-selection marker cassettes. Chicken *RAD54* targeting constructs were made as described previously using hyg and puromycin as selection markers (Bezzubova *et al.*, 1997).

Cell culture and DNA transfection

Cells were cultured in RPMI 1640 supplemented with 100 µg/ml kanamycin, 10% fetal bovine serum and 1% chicken serum (Sigma, St Louis, MO) at 39.5°C. For gene targeting, 10⁷ cells were electroporated with 30 µg of linearized *BLM* targeting constructs using a Gene Pulser

apparatus (Bio-Rad, Hercules, CA) at 550 V and 25 μ F. Drug-resistant colonies were selected in 96-well plates with medium containing 1 mg/ml his, 2 mg/ml hyg or 20 μ g/ml bsr. Genomic DNA was isolated from drug-resistant clones. Gene disruption was confirmed by Southern and northern blot analysis.

Northern blotting

Northern blotting was performed according to the manual of Multiple Tissue Northern Blot (Clontech). Total RNA from wild-type or *BLM*^{-/-} cells was hybridized with the chicken N- or C-terminal *BLM* cDNA probe synthesized by PCR. The primers used were forward primer 5'-ACC-AGCGTGTGCTCTGCTG-3' and reverse primer 5'-GGATAACAT-AGCGTACGTCAG-3' for the N-terminal probe, and forward primer 5'-CTATCATGCTGGCCTCACTG-3' and reverse primer 5'-TGCTCA-CCATCATATTCAGTGTG-3' for the C-terminal probe.

Measurements of MMS, VP16 and CAM sensitivity

To determine sensitivity to MMS, 3×10^2 cells were inoculated into dishes containing various concentrations of MMS in a medium supplemented with 1.5% (w/v) methylcellulose, 1.5% chicken serum and 15% fetal bovine serum. To determine sensitivity to VP16 and CAM, cells were treated with various concentrations of VP16 or CAM for 4 h, washed twice with PBS and then inoculated (3×10^2 cells) into dishes containing the growth medium supplemented with 1.5% methylcellulose. The colonies were enumerated 14 and 9 days after inoculation for *BLM*^{-/-}/*RAD54*^{-/-} cells and other cells, respectively. Survival was determined by comparing the number of colonies of untreated cells.

SCE analysis

Cells (5×10^5) were cultured for approximately two cycle periods with culture medium containing 10 μ M bromodeoxyuridine (BrdU) and pulsed with 0.1 μ g/ml colcemid for the last 2–3 h. The cells were harvested and treated with 75 mM KCl for 20 min at room temperature and then fixed with methanol–acetic acid (3:1) for 30 min. The cells were washed once with the fixative and suspended in a small volume of the fixative. The cell suspension was dropped onto ice-cold wet glass slides and air-dried. The cells on the slides were incubated with 10 μ g/ml Hoechst 33258 in phosphate buffer pH 6.8 for 20 min and rinsed with MacIlvaine solution (164 mM Na₂HPO₄, 16 mM citric acid pH 7.0). The cells were exposed to black light ($\lambda = 352$ nm) at a distance of 1 cm for 30 min and incubated in $2 \times$ SSC (0.3 M NaCl plus 0.03 M sodium citrate) solution at 62°C for 30 min and then stained with 3% Giemsa solution at pH 6.8 for 10 min.

Chromosome aberration analysis

Cells were treated with 0.1 μ g/ml colcemid for 3 h and harvested. The cells were treated with 75 mM KCl for 20 min at room temperature and fixed with methanol–acetic acid (3:1) for 30 min. The cell suspension was dropped onto ice-cold wet glass slides and air-dried. The cells on the slides were stained with 3% Giemsa solution at pH 6.8 for 10 min and examined with a light microscope.

Measurement of targeted integration frequency

To analyze targeted integration events at the chicken *RECQL1* locus, targeting constructs (chicken *RECQL1*-puromycin or *RECQL1*-bleomycin) were transfected into cells, and cells were selected with 0.5 μ g/ml puromycin or 1.5 mg/ml zeocin, a derivative of bleomycin. Genomic DNA of drug-resistant clones was isolated and targeted integration was confirmed by Southern blot analysis and PCR.

Analysis of cell-cycle phase distribution

Cells were prepared with CycleTEST™ PLUS DNA Reagent Kit (Becton Dickinson, CA, USA). Subsequent flow-cytometric analysis was performed with a FACScan (Becton Dickinson). Data were analyzed using the CellFIT software (Becton Dickinson).

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