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ORIGINAL PAPERS

Bloom helicase is involved in DNA surveillance in early S phase in vertebrate cells

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Bloom syndrome (BS) is a recessive human genetic disorder characterized by short stature, immunodeficiency and an elevated risk of malignancy. The gene mutated in BS, *BLM*, encodes a *RecQ*-type DNA helicase. BS cells have mutator phenotypes such as hyper-recombination, chromosome instability and an increased frequency of sister chromatid exchange (SCE). To define the primary role of BLM, we generated *BLM*$^{-/-}$ mutants of the chicken B-cell line DT40. In addition to characteristics of *BLM*$^{-/-}$ cells reported previously by the other group, they are hypersensitive to genotoxic agents such as etoposide, bleomycin and 4-nitroquinoline-1-oxide and irradiation with the short wave length of UV (UVC) light, whereas they exhibit normal sensitivity to X-ray irradiation and hydroxyurea. UVC irradiation to *BLM*$^{-/-}$ cells during G_1 to early S phase caused chromosomal instability such as chromatid breaks and chromosomal quadriradials, leading to eventual cell death. These results suggest that BLM is involved in surveillance of base abnormalities in genomic DNA that may be encountered by replication forks in early S phase. Such surveillance would maintain genomic stability in vertebrate cells, resulting in the prevention of cellular tumorigenesis. *Oncogene* (2001) **20,** 1143–1151.

Keywords: Bloom helicase; Bloom syndrome; DT40; chromosomal instability

Introduction

Bloom syndrome (BS) is a rare autosomal recessive disorder characterized by proportional dwarfism, immunodeficiency, sun-sensitive facial erythema, genomic instability and the early development of a wide variety of cancers (German, 1993). Cultured cells from BS patients are hypermutable and show chromosomal aberrations such as an excessive number of locus-specific mutations and a high frequency of microscopically visible chromatid gaps, breaks, and rearrangements (Rosin and German, 1985). As compared with normal cells, an abnormally high incidence of sister chromatid exchanges (SCEs) are seen in BS cells (Ray and German, 1984).

The gene for BS, *BLM*, has been identified. It encodes a protein (BLM) containing seven signature motifs that are conserved in a wide range of DNA and RNA helicases (Ellis *et al*., 1995). BLM belongs to a subfamily of RecQ-type DNA helicases that includes the *Escherichia coli* protein RecQ (Nakayama *et al*., 1985), *Saccharomyces cerevisiae* Sgs1 (Lu *et al*., 1996), *Schizosaccharomyces pombe* Rqh1/Hus2/Rad12, (Murray *et al*., 1997; Stewart *et al*., 1997) and the human proteins RecQL (Puranam and Blackshear, 1994; Seki *et al*., 1994), WRN, the protein mutated in Werner syndrome (Yu *et al*., 1996), and RecQL4, the protein mutated in Rothmund-Thomson syndrome (Kitao *et al*., 1999) and RecQL5 (Shimamoto *et al*., 2000). Recent biochemical studies have shown that BLM has 3′ to 5′ DNA-unwinding activity, also possessed by WRN and Sgs1 (Karow *et al*., 1997; Lu *et al*., 1996; Suzuki *et al*., 1997). Among the human RecQ homologues, BLM is most similar to two yeast RecQ-type helicases, Sgs1 and Rqh1 (Stewart *et al*., 1997; Watt *et al*., 1996). These three proteins contain a helicase domain of similar size at a similar position as well as two acidic amino acid clusters in their amino termini (Ellis *et al*., 1995; Stewart *et al*., 1997; Watt *et al*., 1996). In *S. pombe*, *Rqh1* mutants show arrested DNA replication and normal cell division in response to hydroxyurea (HU) as well as significant defects in chromosome segregation during subsequent mitosis (Stewart *et al*., 1997). In *S. cerevisiae*, *Sgs1* mutants are hypersensitive to HU (Yamagata *et al*., 1998), and they exhibit mitotic hyper-recombination, resulting in increased frequencies of ectopic, interchromosomal homologous and intrachromosomal excision as well as poor sporulation (Davey *et al*., 1998; Watt *et al*., 1996). Thus, during interrupted S phase, these yeast RecQ homologues are required to prevent inappropriate recombination, thus regulating genetic exchange and maintaining genomic stability (Davey *et al*., 1998; Stewart *et al*., 1997; Watt *et al*., 1996). In the *S. cerevisiae Sgs1* mutant, BLM can suppress increased

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homologous and illegitimate recombination, restore the increased sensitivity to HU (Yamagata *et al*., 1998) and prevent premature aging and increased homologous recombination (HR) and the rDNA loci (Heo *et al*., 1999). In BS cells, genetic exchange, particularly between sister chromatids and homologs, is markedly elevated and further facilitated by exposure to UV light (Kurihara *et al*., 1987) and DNA damaging agents such as ethyl methanesulfonate, N-ethyl-N-nitrosourea and 5-bromodeoxyuridine (BrdU) (Heartlein *et al*., 1987; Krepinsky *et al*., 1980).

Reently, Wang *et al*. (2000b) developed a $BLM^{-/-}$ mutant in the chicken B cell line DT40 and reported that the $BLM^{-/-}$ DT40 cells showed higher sensitivity to methyl methanesulfonate (MMS) and elevated levels of SCE and targeted integration of exogenous DNAs. We describe here further advance over their previous report (Wang *et al*., 2000b), concerning the effect of inactivation of BLM in the vertebrate using our established $BLM^{-/-}$ DT40 cells. We demonstrate increased sensitivities to DNA damages induced by UVC irradiation and several genotoxic agents other than MMS in $BLM^{-/-}$ cells. Karyotypic analysis demonstrates that UVC irradiation during G_1 to early S phase causes chromosomal aberration prior to cell death in $BLM^{-/-}$ cells. These results suggest that BLM is involved in early S phase-specific surveillance of damaged adducts of DNA in vertebrate cells and that $BLM^{-/-}$ DT40 cells are a useful system to decipher the biological functions of BLM in human cells.

Results

BLM targeting constructs and generation of BLM-deficient DT40 clones

A full length cDNA fragment encoding a chicken BLM homologue (DDBJ/EMBL/GenBank accession No. AB032092) was isolated from a cDNA library prepared from the chicken DT40 cell line. With primers designed from the chicken *BLM* cDNA, we isolated genomic clones of the *BLM* locus by long-range PCR amplification. These were partially sequenced to determine the positions of exons and introns. To generate *BLM* deletion constructs, approximately 1 kb of genomic sequence in the series of helicase motifs of the *BLM* locus was replaced with either the histidiol- or blasticidin-resistance gene (His^R or Bsr^R) as shown in Figure 1a. Targeted integration of these constructs was expected to delete amino acids 758–832. Targeting events were defined by the presence of an 8.0-kb band

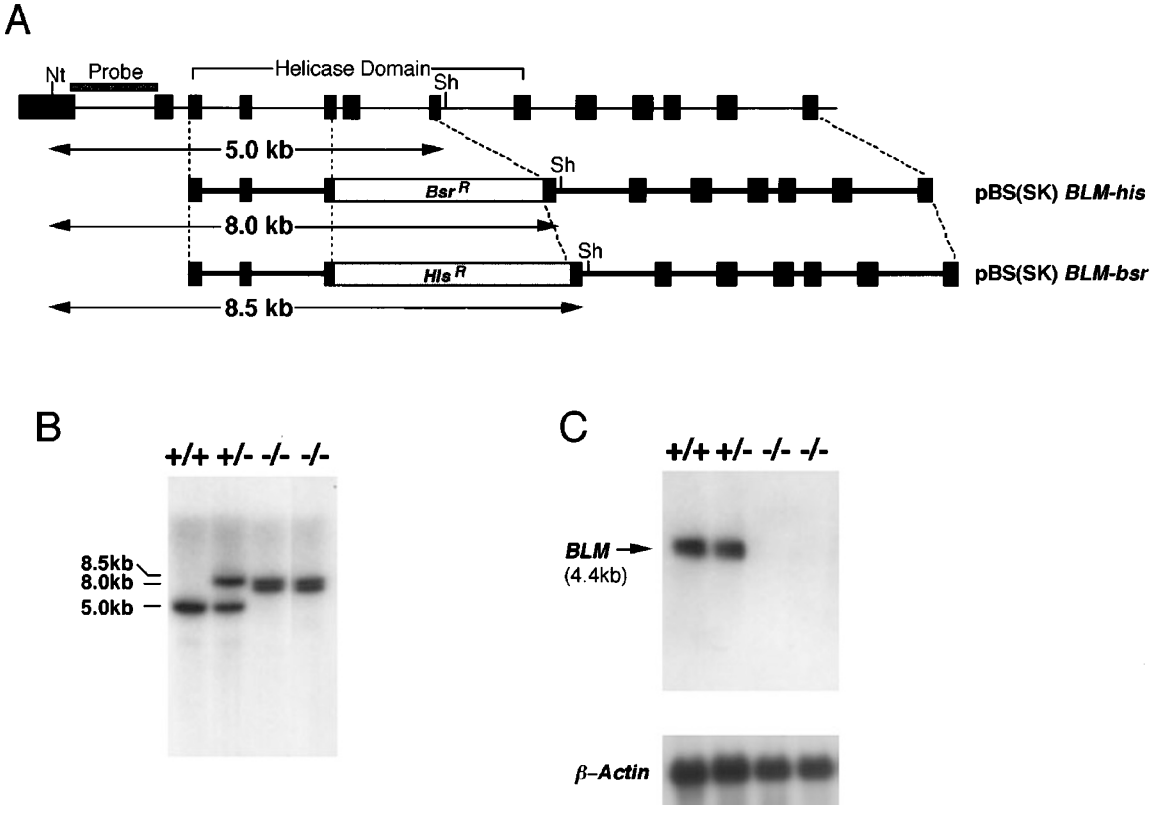

Figure 1 Generation of $BLM^{-/-}$ clones. (**a**) Schematic representation of partial restriction map of the chicken *BLM* locus, the two gene disruption constructs and the configuration of the targeted loci. Black boxes indicate the positions of exons that were disrupted. Relevant restriction enzyme sites are shown as follows: Nt, *Not*I; Sh, *Sph*I. (**b**) Southern blot analysis of wild-type (+/+), heterozygous mutant (+/−) and homozygous mutant (−/−) clones. Genomic DNA digested with *Not*I and *Sph*I was hybridized with the probe DNA shown in (**a**). (**c**) Northern blot analysis of total RNA of the indicated genotype after hybridization with a chicken *BLM* cDNA probe and chicken β-actin probe

after Southern blot analysis of *Not*I/*Sph*I-digested genomic DNA hybridized to an external probe (Figure 1a,b).

To isolate heterozygous $BLM^{+/-}$ mutant clones, the *BLM*-his construct was transfected into wild-type DT40 cells and drug resistant clones were examined by Southern blot analysis. One of the $BLM^{+/-}$ mutant clones was then transfected with the *BLM*-bsr construct to isolate homozygous $BLM^{-/-}$ mutant clones. The disruption of the *BLM* gene was verified by Northern blot analysis (Figure 1c).

$BLM^{-/-}$ cells showed delayed proliferative properties

The proliferative properties of $BLM^{-/-}$ mutant clones were monitored by growth curves and cell cycle analysis. The proliferative rates of $BLM^{-/-}$ cells, clones #17 and #29 which divided every 13–14 h respectively, were slightly lower than that of wild-type DT40 cells which divided every 10.5 h (Figure 2a). On the other hand, the proliferative rate of $BLM^{+/-}$ cells was similar to that of wild-type DT40 cells. Flow cytometric analysis showed that asynchronously cultured $BLM^{-/-}$ cells slightly accumulated in G_1 to S phases without an obvious increase in the number of apoptotic cells when compared with the wild-type cells (Figure 2b). The reduced proliferative rates of $BLM^{-/-}$ cells might be due to a slight cell cycle arrest in G_1 to S phases.

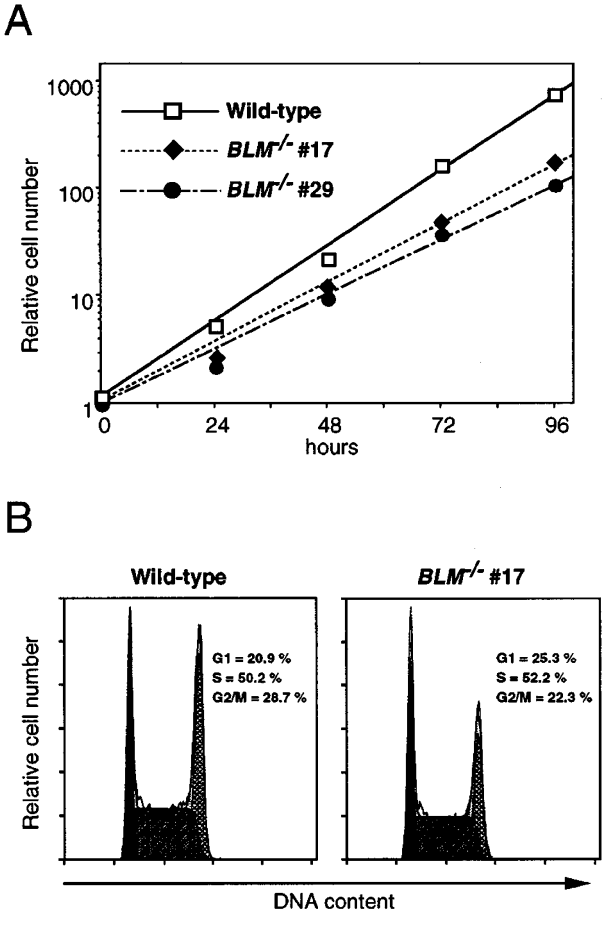

Figure 2 Proliferative characteristics of wild-type and $BLM^{-/-}$ cells. (**a**) Growth curves corresponding to the indicated cell cultures in the absence of histidiol and blasticidin. Data shown are the mean value for three experiments. (**b**) Cell cycle profiles of the wild-type and $BLM^{-/-}$ mutant clone #17 cells cultured asynchronously. DNA contents and relative cell numbers were analysed by flow cytometry

Increases in sensitivity of $BLM^{-/-}$ *cells to genotoxic agents and UVC irradiation and restored sensitivity to a genotoxic agent by human BLM*

The repair capacity of $BLM^{-/-}$ cells compared to wild-type cells was analysed in an MTT assay. The sensitivity of $BLM^{-/-}$ cells to bleomycin, etoposide, 4-nitroquinoline 1-oxide (4-NQO), MMS, HU and UVC irradiation was analysed in the present study. Figure 3 shows proliferation data for chronic cultures of $BLM^{-/-}$ cells in the presence of various concentrations of genotoxic agents. $BLM^{-/-}$ clones showed an increased sensitivity to bleomycin with clones #17 and #29 showing IC_{50} respectively of 50 and 300 ng/ml, compared to 900 ng/ml for wild-type cells. The sensitivity of $BLM^{-/-}$ cells to the topoisomerase II inhibitor, etoposide, also increased in the order of 3–6-fold in comparison with wild-type cells. 4-NQO has been used as a model of environmental carcinogen that produces both DNA strand breaks (like ionizing radiation) and alkali-stable bulky DNA lesions (like UVC light). The sensitivity of both clones of $BLM^{-/-}$ cells to 4-NQO increased by approximately 10-fold when compared to wild-type cells.

Kusano *et al*. (1999) reported that *Dmblm*, a drosophila homolog of *BLM*, partially rescued the sensitivity of the *S. cerevisiae Sgs1* mutant to the alkylating agent, MMS. As previously reported (Wang *et al*., 2000b), both clones of $BLM^{-/-}$ cells showed a 5–6-fold increase in sensitivity to MMS when compared to wild-type cells (data not shown). Although *Sgs1* mutant yeast cells showed increased sensitivity to HU (Onoda *et al*., 2000), which inhibits ribonucleotide reductase, an enzyme essential to DNA synthesis, $BLM^{-/-}$ cells did not show any increase in a sensitivity to this agent (Figure 3).

UVC sensitivity is known as one of the characteristics of BS cells (Henson *et al*., 1981). To investigate the UVC sensitivity of $BLM^{-/-}$ cells, we irradiated them with 1–50 J/m^2 of UVC light and measured the percentage of cells surviving at 4 days post-irradiation. As shown in Figure 4a, the number of proliferating cells of both $BLM^{-/-}$ clones decreased significantly in proportion to the dose of UVC irradiation when compared to wild-type cells. However, the sensitivity of cells from either clone to X-ray irradiation in the range of 3–9 Gray was unchanged in comparison to that of wild-type cells (Figure 4b).

To determine whether the increased sensitivity of $BLM^{-/-}$ cells to these genotoxic agents was the direct effect of the defect in *BLM*, we designed a comple-

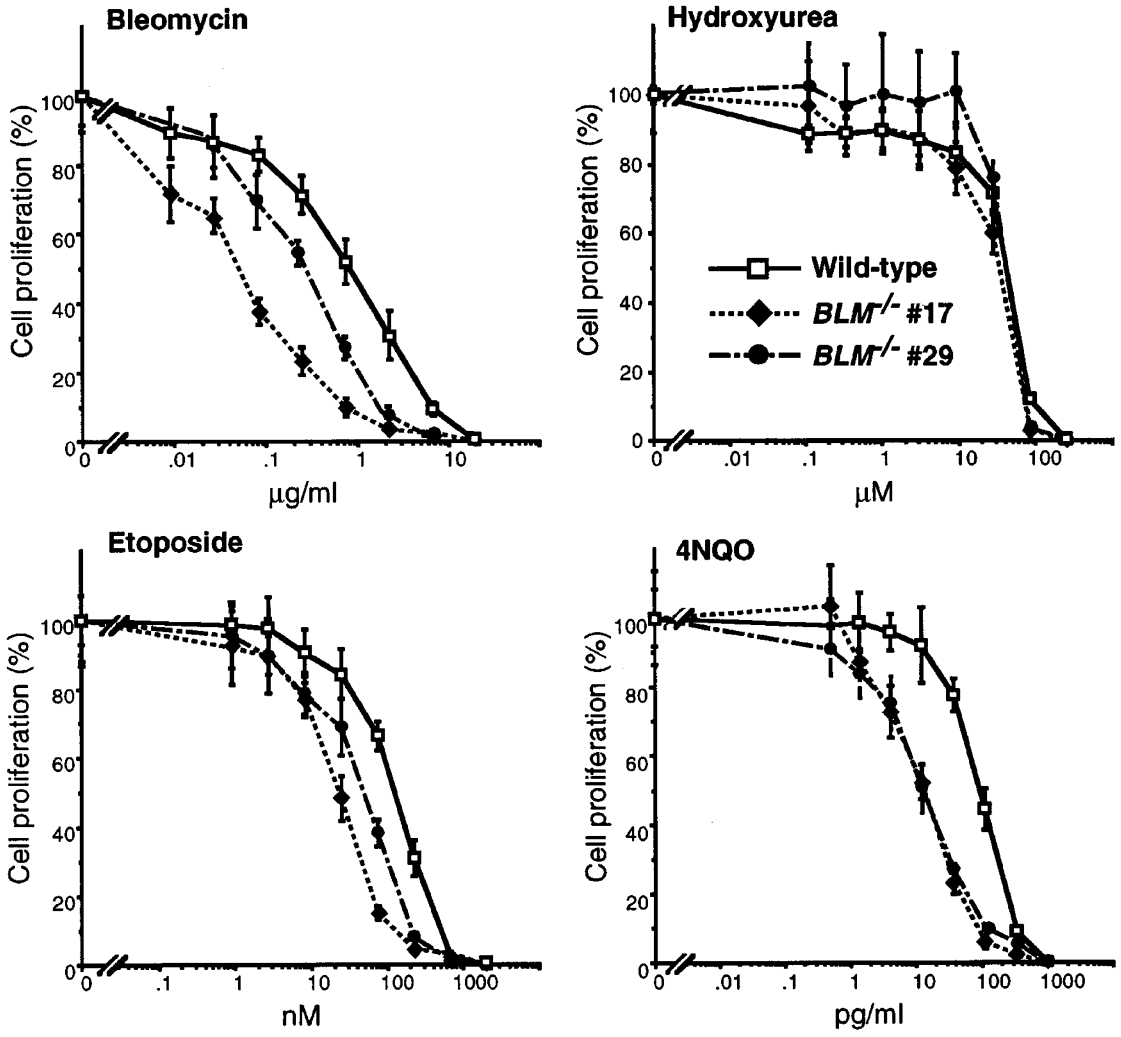

Figure 3 Dose-dependent growth inhibition curves displaying the sensitivities of asynchronous cells to various genotoxic chemicals. Rates of the surviving cells and the concentrations of each genotoxic chemical are displayed on the *y* and *x* axes, respectively. The genotypes of cells are indicated at the right bottom panel. The genotoxic chemical used is indicated at the top of each panel. Each value represents the mean of survival rates from cells cultured on eight wells. Error bars show the standard deviation of the mean

mentation experiment involving the transfection of human *BLM* cDNA into $BLM^{-/-}$ cells. As shown in Figure 5, stable transfection of normal human *BLM* cDNA induced obvious expression of HsBLM protein (molecular weight of 180 kD) and restored the sensitivity to bleomycin to the level obtained with wild-type cells. This result indicates that the sensitivity of the mutated cells to the genotoxic agent really is the consequence of disruption of the *BLM* gene. BLM exhibits ATP-dependent DNA helicase activity that displays 3′ to 5′ directionality (Karow *et al*., 1997). Substitution of the lysine residue in the ATP-binding site of HsBLM with threonine disables the 3′ to 5′ helicase function (Ellis *et al*., 1999). In the present study, we transfected $BLM^{-/-}$ cells with the missense human *BLM* gene encoding an ATPase-deficient HsBLM protein. The expression of similar amounts of ATPase-deficient HsBLM protein failed to restore the sensitivity of $BLM^{-/-}$ cells to bleomycin (Figure 5). These experiments demonstrate that the enzymatic activity of BLM is important for its function.

Chromosomal aberrations are associated with DNA damage during progression from G_1 to early S phase in $BLM^{-/-}$ *cells*

DT40 cells display a stable karyotype with a modal chromosome number of 80 and do not show obvious abnormalities except for a trisomy of chromosome 2 and one additional microchromosome (Sonoda *et al*., 1998). Chromosomal aberrations were hardly detectable in asynchronous wild-type cells, whereas they were a little more frequently observed in $BLM^{-/-}$ cells (Table 1). When asynchronous cultures of wild-type cells were treated with 1 J/m^2 of UVC-irradiation, cells in late S to G_2 phase were expected to enter mitosis within 3–6 h. Chromatid-type gaps and breaks were frequently observed in these cells (Table 1). These may

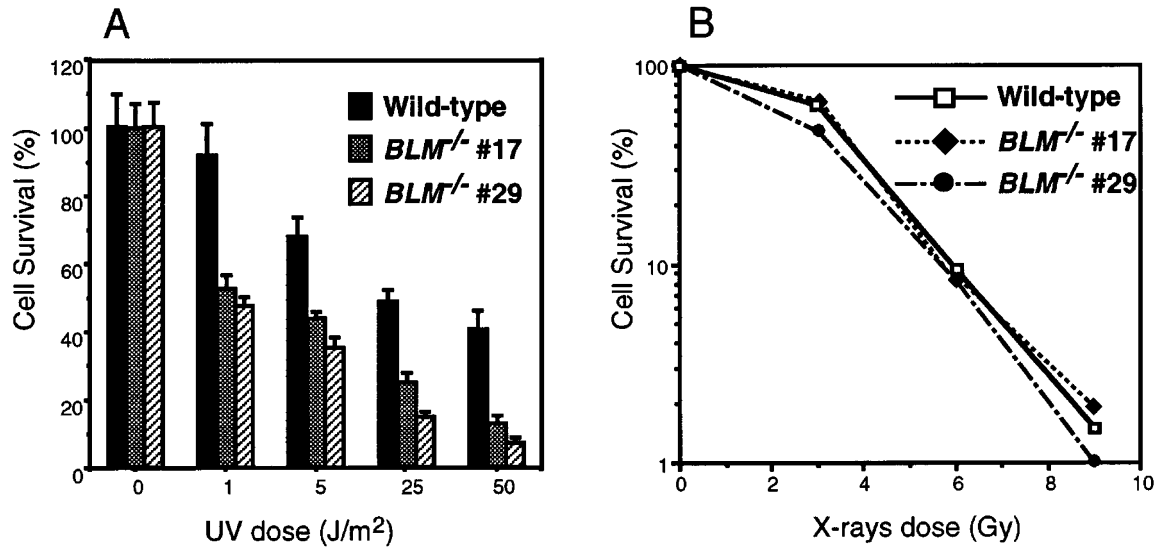

Figure 4 Graphs displaying the sensitivities of asynchronous cells to UVC and X-ray irradiation. (**a**) The relationship between rates of the surviving cells and UVC irradiation doses are displayed as histograms. The genotypes of the cells used are indicated at right top. Others are the same as in Figure 3. (**b**) The fraction of surviving colonies after X-ray irradiation. The genotypes of the cells used are indicated at right top. Each value represents the mean of results from two separate experiments

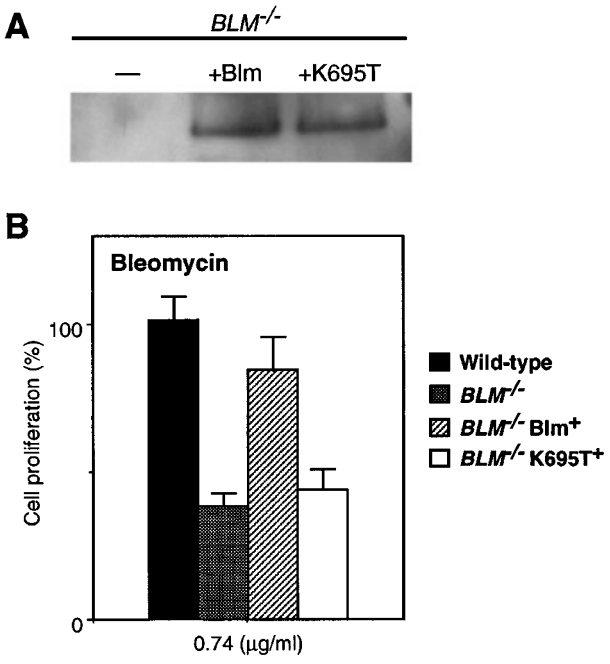

Figure 5 Human BLM can restore the hypersensitivity of $BLM^{-/-}$ cells to bleomycin. (**a**) Immunoblot analysis of HsBLM expression in cell extracts using anti-HsBLM antibody. The following samples were loaded on each lane of a SDS-polyacrylamide gel; $BLM^{-/-}$ clone #17 cells (left lane), $BLM^{-/-}$ clone #17 cells carrying the HsBLM transgene (middle lane), $BLM^{-/-}$ clone #17 cells carrying ATPase-defective HsBLM (K695T) transgene (right lane). (**b**) Histograms displaying the mean of relative ratio of a survival rate of $BLM^{-/-}$ cells clone #17 in the presence of wild-type HsBLM or mutant-type of HsBLM (K695T) in comparison with that of wild-type cells as 100%. The concentration of bleomycin in the culture medium was 0.74 μg/ml. Each value represents the mean and the standard deviation of results obtained from cells cultured on eight wells

reflect the generation of UVC- or γ-ray-induced (Sonoda *et al*., 1998) double strand breaks of DNA after DNA replication. At 9–12 h post-irradiation with γ-rays mostly isochromatid-type structural aberrations were detected in addition to chromatid exchanges indicating that cells had been in G_1 to early S phase at the time of γ-irradiation (Sonoda *et al*., 1998). However, at 9–12 h post-irradiation with UVC light, wild-type cells which had been in G_1 to early S phase at the time of UVC-irradiation still showed increased frequencies of chromatid-type aberrations in addition to increased frequency of chromatid exchanges named as chromosomal quadriradials (Table 1). $BLM^{-/-}$ cells which had been in G_1 to early S phase at 9–12 h post-irradiation also showed increased frequencies of chromatid-type breaks and a more remarkable increase in frequency of chromosomal quadriradials (Table 1). UVC irradiation has been known to cause formation of DNA adducts such as cyclobutane pyrimidine dimers (Tornaletti and Pfeifer, 1996). Holliday junctions are generated during S phase at sites of replication forks stalled due to such DNA adducts (Karow *et al*., 2000). Our results may implicate that, in the absence of BLM, Holliday junctions that persist during S phase are likely to allow junction-resolution to occur, leading to the initiation of double-strand breaks.

Discussion

We report here novel observations in the phenotype caused by the disruption of the *BLM* gene in the chicken B-cell line DT40. As previously reported (Wang *et al*., 2000b), *BLM*-defective cells showed

hyper-recombination indicated by increases in the frequencies of both SCEs and targeted genome integration (data not shown). In addition, they exhibited hypersensitivity to genotoxic agents such as bleomycin, etoposide, MMS and 4-NQO as well as to UVC irradiation, showing chromosomal aberrations that lead to cell death. The present observation that phenotypes characterized in $BLM^{-/-}$ DT40 cells are very similar to those reported with cells derived from BS individuals and that expression of HsBLM in $BLM^{-/-}$ DT40 cells can restore normal drug sensitivity are supportive of a conserved function for BLM in vertebrates. It has been difficult to handle BS cells for investigation because of their severely retarded growth due to genomic instability. $BLM^{-/-}$ DT40 cells exhibit an appropriate cellular proliferation despite their characteristic of genomic instability being similar to BS cells. Since DT40 cell lines, like most transformed chicken cell lines, do not express p53 (Takao *et al*., 1999), $BLM^{-/-}$ DT40 cells may be able to proliferate well throughout the cell cycle without checkpoint control downstream of p53. Therefore, $BLM^{-/-}$ DT40 cells are a useful model to study the biological functions of BLM in human cells.

DNA modification with genotoxic agents may cause cell death in $BLM^{-/-}$ *cells*

Although BLM is believed not to be involved in DNA repair, various reports have demonstrated that cultured cells from BS patients show varied and abnormal responses to DNA damaging agents such as N-ethyl-N-nitrosourea, ethyl methanesulfonate, MMS and 4-NQO, as well as to irradiation with UVC (Krepinsky *et al*., 1979; Kurihara *et al*., 1987; Shiraishi, 1985). In the present study, bleomycin, etoposide, MMS, 4-NQO and UVC irradiation caused more severe, dose-dependent growth inhibition of $BLM^{-/-}$ cells compared to wild-type cells. Hypersensitivities of $BLM^{-/-}$ cells to these DNA damaging agents could be reproduced by analysis of inactivation of colony formation (data not shown), whereas Wang *et al*. (2000b) demonstrated by colony formation assay that $BLM^{-/-}$ DT40 cells showed no significant difference in etoposide sensitivity compared with wild-type cells. This discrepancy in etoposide sensitivity might be explained with transient (4 h) exposure of cells before colony formation assay in their experiments in contrast to continuous exposure during both assays in our experiments. Except for etoposide, the above chemical genotoxins are known to contribute to the formation of certain types of mutagenic adducts in DNA (Dedon *et al*., 1998; Smith and Grisham, 1983; Galiegue-Zouitina *et al*., 1984). UVC irradiation also causes formation of DNA adducts such as cyclobutane pyrimidine dimers as well as other lesions including DNA nicks (Tornaletti and Pfeifer, 1996). X-ray irradiation causes both DNA nicks and double strand breaks, a process that bleomycin is thought to mimic. Because $BLM^{-/-}$ cells showed hypersensitivity to bleomycin but not to X-ray irradiation, the defect in *BLM* may induce hypersensitivity to mutated adducts of DNA without increasing the sensitivity to DNA strand breaks. Previous work has shown that G-quadruplex DNA (G4 DNA) junctions are a preferred substrate of BLM, as measured by the efficiency of unwinding (Sun *et al*., 1998). G4 DNA is assumed to be formed in a single stranded G-rich region exposed by recombination or replication. It is speculated that BLM has an ability to recognize DNA adducts or DNA structures associated with stalled replication forks, such as Holliday junctions, in addition to G4 DNA junctions.

Chromatid-type breaks caused by UVC irradiation prior to DNA replication are enhanced in $BLM^{-/-}$ *cells*

Chemical modifications, such as cyclobutane pyrimidine dimers, and base damage to genomic DNA caused by hydrolysis, oxidation and nonenzymatic methylation, occur at significant rates *in vivo* (Lindahl, 1990). Chemical and base damage in genomic DNA is usually repaired by the base-excision repair pathway (Wood, 1999). When a replication fork is stalled at such a damaged site, it may produce a single-strand gap between the damaged base and a new Okazaki fragment being synthesized downstream, possibly resulting in a chromatid break (Sonoda *et al*., 1999; Yamaguchi-Iwai *et al*., 1999). Such strand discontinuities can be repaired post-replicationally by homologous recombination (HR) with the sister chromatid, as is the case for recombinational repair in yeast cells (Kadyk and Hartwell, 1993). In the present study, chromatid breaks occurred with a high frequency in both wild-type and $BLM^{-/-}$ cells that were irradiated with UVC light during G_1 to S phase. Large populations of thymine dimers as well as nicks on single stranded DNA formed by UVC irradiation prior to replication may be overlooked by the nucleotide-excision repair (NER) pathway and subsequently cause chromatid breaks. BS cells have been shown to have normal NER pathways and most BS cell lines are not radiation-sensitive, indicating that most DNA repair functions are within the normal range (German, 1993). However, repair efficiency of such DNA damage in $BLM^{-/-}$ DT40 cells must be decreased, especially in early S phase. Recently, Wang *et al*. (2000a) identified BLM as a member of a group of proteins that associate with BRCA1 to form a large complex that includes tumor suppressors and DNA damage repair proteins MSH2, MSH6, MLH1, ATM, and the Rad50-Mre11-Nbs1 protein complex. On the other hand, Karow *et al*. (2000) showed that BLM possesses the ability to selectively recognize Holliday junctions and efficiently promotes ATP-dependent branch migration of Holliday junctions during S phase *in vitro*, resulting in prevention from promiscuous recombination events and stabilization of the replication fork in human cells. Therefore, a BLM defect would lead to the initiation of double-strand break repair by HR, resulting in an increase in SCE. BS cells has been characterized with an increased rate of SCEs (Langlois *et al*., 1989).

Table 1 Frequencies of chromosomal aberrations in wild-type and *BLM*-deficient DT40 cells after UVC irradiation

Cells	*Time after irradiation*[a] (h)	*No. of cells analysed*	*Chromatid*		*Chromosome*		*Chromatid exchanges*	*No. of aberrations* (per cell)
			Gaps	*Breaks*	*Gaps*	*Breaks*		
Wild-type	Spontaneous	100	2	0	0	0	0	0.02
	3	200	40	27	6	7	1	0.41
	6	200	37	24	3	3	0	0.34
	9	200	12	5	5	2	7	0.16
	12	200	15	7	5	1	8	0.18
$BLM^{-/-}$	Spontaneous	200	4	0	1	2	0	0.04
	3	200	44	29	7	3	1	0.42
	6	200	35	19	5	7	4	0.35
	9	200	16	28	1	3	22	0.35
	12	200	5	14	0	3	36	0.29

[a]Chromosome aberrations were scored at every 3 h following irradiation with 1 J/m^2 of UVC light. All samples were treated with colcemid for the last 3 h

Wang *et al*. (2000b) demonstrated that level of SCE in $BLM^{-/-}$ DT40 cells was considerably reduced in the absence of *Rad54*, indicating that a large proportion of the SCE in $BLM^{-/-}$ cells occurs *via* HR. BLM may play a role in early S phase-specific DNA surveillance mechanisms resulting in the disruption of recombinogenic molecules that arise at sites of stalled replication forks and guidance of the above-mentioned large repair complex to a damaged base on a DNA strand.

Chromosomal quadriradials are well described as one of the representative phenotypes seen in BS cells (Werner-Favre *et al*., 1984). DNA crosslinking within GC-rich region in mammalian chromosomes can create quadriradials (Matsumoto *et al*., 1999). Previous work has shown that BLM is a general helicase with preference for G4 DNA formed in a single stranded G-rich region exposed by recombination (Sun *et al*., 1998). As shown in Table 1, UVC irradiation in G_1 to early S phase caused chromosomal aberrations and a defect in *BLM* resulted in a remarkable enhancement of quadriradial formation in DT40 cells. These results suggest that adducts of DNA formed prior to replication might be repaired by post-replicational HR between the sister chromatid and its homologous chromatid and that BLM may play a role in disrupting DNA secondary structures leading to recombination repair.

In summary, our results suggest that BLM surveys damaged bases in genomic DNA that may be encountered by a replication fork in early S phase in order to maintain genomic stability, resulting in the prevention of tumorigenesis. Our idea is supported by recent work which shows that after DNA damage caused by UV irradiation the RecQ helicase and RecJ nuclease in *E. coli* process the nascent lagging DNA strand at stalled replication forks prior to the resumption of replication (Courcelle and Hanawalt, 1999). Since BLM has not been demonstrated to have nuclease activity, it will be interesting to identify whether there is a factor in vertebrate cells like RecJ that acts as a specific nuclease in concert with BLM helicase activity.

Materials and methods

Cloning of the chicken BLM gene

The degenerate primers corresponding to amino acid residues contained in motifs I (TGGGKSLC) and V (ATIAFGMG) of human BLM (HsBLM), were used to clone a cDNA fragment coding for a portion of the helicase domain of chicken BLM (GdBLM) by PCR. The reaction product was subcloned into a pGEM-T vector (Promega, Madison, WI, USA). The cloned cDNA fragment was used to screen the cDNA library of avian leukosis virus (ALV)-induced B cell line DT40 (Buerstedde *et al*., 1990) to obtain full-length chicken *BLM* cDNA clones. The sequence of the assembled cDNA was verified by DNA sequencing.

Plasmid constructs

The 9-kb genomic chicken *BLM* locus was cloned from DT40 genomic DNA by long-range PCR with primers designed for the chicken *BLM* cDNA sequence. The positions of the exons and introns were determined by sequencing. Chicken *BLM* disruption constructs, *BLM*-his and *BLM*-bsr, were made by replacing approximately 1 kb of *Bam*HI/*Bam*HI-linked genomic sequence with His^R or Bsr^R-selection marker cassettes under the control of the *β-actin* promoter (Bezzubova *et al*., 1997). The *BLM* disruption constructs were made linear prior to electroporation.

We obtained from Nathan A Ellis (Sloan-Kettering Cancer Center, New York, NY, USA) plasmids in which wild-type and ATPase-defective HsBLMs were cloned into the *Not*I site of mammalian expression vector pOPRSVI-CAT (Stratagene, La Jolla, CA, USA), replacing the CAT gene (Ellis *et al*., 1999). Position 695 (Lysine) was altered to threonine in the latter human *BLM* gene product. This missense mutation in the ATP-binding site present in the first conserved helicase motif leads to an inactivation of HsBLM ATPase activity.

Gene targeting and cell culture

The elevated HR (Bezzubova and Buerstedde, 1994; Buerstedde and Takeda, 1991) in DT40 cells allowed us to perform targeted disruption of both *BLM* alleles. Cells were maintained in RPMI#1640 medium supplemented with penicillin, streptomycin, 10% fetal bovine serum and 1% chicken serum (Sigma, St. Louis, MO, USA) at 37°C. For DNA transfection, 10^7 cells were suspended in 0.5 ml of PBS containing 30 μg of linearized plasmid and electroporated

with a GENE Pulser apparatus (BioRad, Hercules, CA, USA) at 550 V and 25 μF. Following electroporation, cells were transferred to 20 ml of fresh medium and incubated for 24 h. Cells were then resuspended in 90 ml of medium containing the appropriate drugs and divided into four 96-well microtiter plates. After 7–10 days, drug-resistant colonies were transferred to 24-well plates.

Northern blot analysis

10^7 cells were washed once with PBS and total RNA was extracted using TRIzol Reagent (Gibco–BRL, Grand Island, NY, USA). RNA (20 μg/lane) was separated in a 1.2% formaldehyde gel, transferred to a nylon membrane, and hybridized with a ^{32}P-labeled chicken *BLM* cDNA fragment.

Flow cytometric analysis

Cells were fixed at 4°C overnight with 70% ethanol, and incubated in 5 μg/ml propidium iodide in PBS. Subsequent flow cytometric analysis was performed on an EPIX Flow Cytometer (Coulter, Hialeah, FL, USA).

Measurement of sensitivity of cells to genotoxic agents and UVC irradiation

Growth inhibition experiments were carried out in 96-well flat-bottomed microplates and the amount of viable cells at the end of the incubation was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, essentially as described by Mosmann (1983). Thus, 2000 cells/well in 100 μl were plated and another 100 μl of the drug or the medium alone was added as a control. For measuring UVC sensitivity, cells were irradiated with 1 J/m^2 of UV light ($\lambda = 254$ nm) prior to plating on microplates. The cells were cultured for 3–4 days and after addition of MTT (50 μl/well, 5 mg/ml in PBS), the plates were incubated for a further 4 h. The blue dye formed was dissolved by the addition of 50 μl/well of 25% SDS. The absorbance was measured at 595 nm using a Microplate Reader Model 550 (BioRad).

Measurement of surviving colonies following X-ray irradiation

Serially diluted cells were plated in triplicate onto 6-well clusters with 5 ml/well of 1.5% (w/v) methylcellulose (Aldrich, Milwaukee, WI, USA) plates containing D-MEM/F-12 (Gibco–BRL), 15% FCS and 1.5% chicken serum. Subsequently, X-ray irradiation of the cells with 150 kVp X-rays was performed using an MBR-1520R (Hitachi Medical Corporation, Tokyo, Japan) operated at 10 mA with 2.0 mm aluminum filtration. Colonies were counted at 7 days post-irradiation. Percentage survival was determined relative to numbers of colonies from untreated cells.

Karyotype analysis

Karyotype analysis was carried out as previously described (Sonoda *et al*., 1998). Briefly, cells were treated for 3 h with medium containing 0.1 μg/ml colcemid (Gibco–BRL) prior to harvest. Harvested cells were incubated in 1 ml of 0.9% sodium citrate for 15 min at room temperature and fixed with 5 ml of a freshly prepared 3:1 mixture of methanol/acetic acid. The cell suspension was dropped onto a glass slide, wetted with 50% ethanol and immediately flame-dried. Slides were stained with 3% Giemsa solution at pH 6.4 for 10 min.

Immunoblot analysis

Cells were washed with PBS and lysed in a buffer containing 10 mM Tris-HCl (pH 8.0), 1% NP-40, 150 mM NaCl, 4 mM EDTA-2Na and a protease inhibitor cocktail, Complete™ (Boehringer Mannheim, Mannheim, Germany). After centrifugation of the cell lysate, supernatant containing 200 μg of protein was diluted and incubated with goat polyclonal anti-human BLM antibodies, sc-7790 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), conjugated with Protein G Sepharose (Pharmacia Biotech, Uppsala, Sweden) at 4°C overnight. Immunoprecipitates were solubilized in 25 μl of buffer [25 mM Tris-HCl (pH 6.5), 1% SDS, 0.24 M β-mercaptoethanol, 0.1% Bromophenol Blue, 5% glycerol] followed by boiling. Aliquots were subjected to 7.5% SDS–PAGE. After transfer to nylon membranes, proteins were detected by the above mentioned antibodies and rabbit HRP-conjugated anti-goat IgG (DAKO, Denmark) using ECL™ (Amersham Life Science, UK).

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References

Bezzubova OY and Buerstedde JM. (1994). *Experientia,* **50,** 270–276.
Bezzubova O, Silbergleit A, Yamaguchi-Iwai Y, Takeda S and Buerstedde JM. (1997). *Cell,* **89,** 185–193.
Buerstedde JM, Reynaud CA, Humphries EH, Olson W, Ewert DL and Weill JC. (1990). *EMBO J.,* **9,** 921–927.
Buerstedde JM and Takeda S. (1991). *Cell,* **67,** 179–188.
Courcelle J and Hanawalt PC. (1999). *Mol. Gen. Genet.,* **262,** 543–551.
Davey S, Han CS, Ramer SA, Klassen JC, Jacobson A, Eisenberger A, Hopkins KM, Lieberman HB and Freyer GA. (1998). *Mol. Cell. Biol.,* **18,** 2721–2728.
Dedon PC, Plastaras JP, Rouzer CA and Marnett LJ. (1998). *Proc. Natl. Acad. Sci. USA,* **95,** 11113–11116.
Ellis NA, Groden J, Ye TZ, Straughen J, Lennon DJ, Ciocci S, Proytcheva M and German J. (1995). *Cell,* **83,** 655–666.
Ellis NA, Proytcheva M, Sanz MM, Ye TZ and German J. (1999). *Am. J. Hum. Genet.,* **65,** 1368–1374.
Galiegue-Zouitina S, Bailleul B and Loucheux-Lefebvre MH. (1984). *Anal. Biochem.,* **138,** 454–457.
German J. (1993). *Medicine (Balt.),* **72,** 393–406.
Heartlein MW, Tsuji H and Latt SA. (1987). *Exp. Cell Res.,* **169,** 245–254.

Henson P, Selsky CA and Little JB. (1981). *Cancer Res.,* **41,** 760 – 766.
Heo SJ, Tatebayashi K, Ohsugi I, Shimamoto A, Furuichi Y and Ikeda H. (1999). *Genes Cells,* **4,** 619 – 625.
Kadyk LC and Hartwell LH. (1993). *Genetics,* **133,** 469 – 487.
Karow JK, Chakraverty RK and Hickson ID. (1997). *J. Biol. Chem.,* **272,** 30611 – 30614.
Karow JK, Lia A, Constantinou JL, West SC and Hickson ID. (2000). *Proc. Natl. Acad. Sci. USA,* **97,** 6504 – 6508.
Kitao S, Shimamoto A, Goto M, Miller RW, Smithson WA, Lindor NM and Furuichi Y. (1999). *Nat. Genet.,* **22,** 82 – 84.
Krepinsky AB, Heddle JA and German J. (1979). *Hum. Genet.,* **50,** 151 – 156.
Krepinsky AB, Rainbow AJ and Heddle JA. (1980). *Mutat. Res.,* **69,** 357 – 368.
Kurihara T, Tatsumi K, Takahashi H and Inoue M. (1987). *Mutat. Res.,* **183,** 197 – 202.
Kusano K, Berres ME and Engels WR. (1999). *Genetics,* **151,** 1027 – 1039.
Langlois RG, Bigbee WL, Jensen RH and German J. (1989). *Proc. Natl. Acad. Sci. USA,* **86,** 670 – 674.
Lindahl T. (1990). *Mutat. Res.,* **238,** 305 – 311.
Lu J, Mullen JR, Brill SJ, Kleff S, Romeo AM and Sternglanz R. (1996). *Nature,* **383,** 678 – 679.
Matsumoto L, Kurek K, Larocque K, Gustafson G, Pires R, Zhang J, Tantravahi U and Suggs JW. (1999). *Mutat. Res.,* **426,** 79 – 87.
Mosmann T. (1983). *J. Immunol. Methods,* **65,** 55 – 63.
Murray JM, Lindsay HD, Munday CA and Carr AM. (1997). *Mol. Cell. Biol.,* **17,** 6868 – 6875.
Nakayama K, Irino N and Nakayama H. (1985). *Mol. Gen. Genet.,* **200,** 266 – 271.
Onoda F, Seki M, Miyajima A and Enomoto T. (2000). *Mutat Res.,* **459,** 203 – 209.
Puranam KL and Blackshear PJ. (1994). *J. Biol. Chem.,* **269,** 29838 – 29845.
Ray JH and German J. (1984). *Chromosoma,* **90,** 383 – 388.
Rosin MP and German J. (1985). *Hum. Genet.,* **71,** 187 – 191.
Seki M, Miyazawa H, Tada S, Yanagisawa J, Yamaoka T, Hoshino S, Ozawa K, Eki T, Nogami M, Okumura K, Taguchi H, Hanaoka F and Enomoto T. (1994). *Nucleic Acids Res.,* **22,** 4566 – 4573.
Shimamoto A, Nishikawa K, Kitao S and Furuichi Y. (2000). *Nucleic Acids Res.,* **28,** 1647 – 1655.
Shiraishi Y. (1985). *EMBO J.,* **4,** 2553 – 2560.
Smith GJ and Grisham JW. (1983). *Mutat. Res.,* **111,** 405 – 417.
Sonoda E, Sasaki MS, Buerstedde JM, Bezzubova O, Shinohara A, Ogawa H, Takata M, Yamaguchi-Iwai Y and Takeda S. (1998). *EMBO J.,* **17,** 598 – 608.
Sonoda E, Sasaki MS, Morrison C, Yamaguchi-Iwai Y, Takata M and Takeda S. (1999). *Mol. Cell. Biol.,* **19,** 5166 – 5169.
Stewart E, Chapman CR, Al-Khodairy F, Carr AM and Enoch T. (1997). *EMBO J.,* **16,** 2682 – 2692.
Sun H, Karow JK, Hickson ID and Maizels N. (1998). *J. Biol. Chem.,* **273,** 27587 – 27592.
Suzuki N, Shimamoto A, Imamura O, Kuromitsu J, Kitao S, Goto M and Furuichi Y. (1997). *Nucleic Acids Res.,* **25,** 2973 – 2978.
Takao N, Kato H, Mori R, Morrison C, Sonada E, Sun X, Shimizu H, Yoshioka K, Takeda S and Yamamoto K. (1999). *Oncogene,* **18,** 7002 – 7009.
Tornaletti S and Pfeifer GP. (1996). *Bioessays,* **18,** 221 – 228.
Wang Y, Cortez D, Yazdi P, Neff N, Elledge SJ and Qin J. (2000a). *Genes Dev.,* **14,** 927 – 939.
Wang W, Seki M, Narita Y, Sonoda E, Takeda S, Yamada K, Masuko T, Katada T and Enomoto T. (2000b). *EMBO J.,* **19,** 3428 – 3435.
Watt PM, Hickson ID, Borts RH and Louis EJ. (1996). *Genetics,* **144,** 935 – 945.
Werner-Favre C, Wyss M, Cabrol C, Felix F, Guenin R, Laufer D and Engel E. (1984). *Am. J. Med. Genet.,* **18,** 215 – 221.
Wood RD. (1999). *Biochimie,* **81,** 39 – 44.
Yamagata K, Kato J, Shimamoto A, Goto M, Furuichi Y and Ikeda H. (1998). *Proc. Natl. Acad. Sci. USA,* **95,** 8733 – 8738.
Yamaguchi-Iwai Y, Sonoda E, Sasaki MS, Morrison C, Haraguchi T, Hiraoka Y, Yamashita YM, Yagi T, Takata M, Price C, Kakazu N and Takeda S. (1999). *EMBO J.,* **18,** 6619 – 6629.
Yu CE, Oshima J, Fu YH, Wijsman EM, Hisama F, Alisch R, Matthews S, Nakura J, Miki T, Ouais S, Martin GM, Mulligan J and Schellenberg GD. (1996). *Science,* **272,** 258 – 262.