Research article

The C-terminal domain of the Bloom syndrome DNA helicase is essential for genomic stability

Victor Yankiwski[1,2], James P Noonan[1,3] and Norma F Neff*[1]

Address: [1]Laboratory of Molecular Genetics, New York Blood Center, 310 East 67th Street, New York, NY 10021 USA, [2]Graduate School of Biomedical Sciences, University of Medicine and Dentistry of New Jersey, Newark, NJ 07107 USA and [3] Department of Genetics, Stanford School of Medicine, Stanford, CA 94305 USA

E-mail: Victor Yankiwski - Yankiwski@aol.com; James P Noonan - jnoonan@stanford.edu; Norma F Neff* - nneff@nybc.org
*Corresponding author

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Abstract

Background: Bloom syndrome is a rare cancer-prone disorder in which the cells of affected persons have a high frequency of somatic mutation and genomic instability. Bloom syndrome cells have a distinctive high frequency of sister chromatid exchange and quadriradial formation. BLM, the protein altered in BS, is a member of the RecQ DNA helicase family, whose members share an average of 40% identity in the helicase domain and have divergent N-terminal and C-terminal flanking regions of variable lengths. The BLM DNA helicase has been shown to localize to the ND10 (nuclear domain 10) or PML (promyelocytic leukemia) nuclear bodies, where it associates with TOPIIIα, and to the nucleolus.

Results: This report demonstrates that the N-terminal domain of BLM is responsible for localization of the protein to the nuclear bodies, while the C-terminal domain directs the protein to the nucleolus. Deletions of the N-terminal domain of BLM have little effect on sister chromatid exchange frequency and chromosome stability as compared to helicase and C-terminal mutations which can increase SCE frequency and chromosome abnormalities.

Conclusion: The helicase activity and the C-terminal domain of BLM are critical for maintaining genomic stability as measured by the sister chromatid exchange assay. The localization of BLM into the nucleolus by the C-terminal domain appears to be more important to genomic stability than localization in the nuclear bodies.

Background

BLM is a member of the RecQ family of DNA helicases

Bloom syndrome (BS) is a rare cancer-prone autosomal recessive disorder characterized by genomic instability, immunodeficiency, infertility and small stature [1,2]. BS cells have a distinctive genomic instability: a high frequency of sister chromatid exchange (SCEs) and quadriradial formation. *BLM,* the gene mutated in BS, encodes a DNA helicase (BLM) of the RecQ family [3]. BLM shares the most identity in the helicase domain to the mouse and Xenopus orthologs [4,5,6], to a predicted *C. elegans* protein CAB05609 [7], and to *D. melanogaster* dmBLM [8]. BLM can partially complement phenotypes of mutations in the *S. cerevisiae SGS1* gene [9,10]. There are two published reports of *BLM* knock-out mice: one strategy used a single deletion allele and found that the homozygous null mutants are embryonic lethals [4]; the second strategy used two different deletion alleles and

recovered full sized, fertile compound heterozygote mice with an elevated incidence of cancer [5]. The second mouse model was made with ES cells that have a high frequency of SCEs before injection and recapitulates the BS phenotypes more accurately.

There are four other human genes in the RecQ family: *RecQL / RecQl, WRN, RecQ4, RecQ5* [11,12]. *WRN* is the gene mutated in Werner syndrome, a premature aging disorder; WS cells also show features of genomic instability [13]. WRN encodes an exonuclease activity and shares many similar in vitro helicase activities with BLM [14,15]. Mutations in the *RECQ4* gene have been found in persons with Rothmund-Thomson syndrome, a rare premature aging and cancer-prone disorder [16]. Previous work from this laboratory and others [10,17,18,19] demonstrated the DNA helicase activity of BLM in vitro on a variety of DNA substrates. Transfection of the normal BLM cDNA (but not missense alleles lacking helicase activity) into BS cells reduces the frequency of SCEs [10], indicating that the DNA helicase activity of BLM is essential for genomic stability.

BLM is localized in nuclear bodies and the nucleolus

The BLM DNA helicase is found in two distinct nuclear structures in normal human cells, ND10 or PML nuclear bodies (NBs) and the nucleolus [20]. The NBs are dynamic PML-dependent depots of multiple proteins disrupted upon viral infection and in certain human malignancies [21,22,23]. BS cells have NBs of normal morphology [21,22], and cells lacking PML destabilize the NBs and have a two fold increase in the frequency of SCEs [22]. NBs have been implicated in the regulation of apoptosis [21,24,25] although their precise function is still unknown. BLM expression is cell-cycle regulated, showing a marked increase in S phase and peaking in G2 [26,27]. The increase in BLM mRNA and protein expression coincides with its appearance in the nucleolus [20]. This report uses a series of inducible cell lines containing deletion alleles of BLM to investigate the role of the N-terminal and C-terminal domains of BLM in nuclear localization and in the maintenance of genomic stability. We find that the N-terminal domain directs BLM for packaging in NBs, while the C-terminal domain is required for efficient nucleolar localization. Compared to the normal BLM protein, deletions of the C-terminus and mutation of the helicase domain have a strong negative effect on genomic stability whereas deletions of the N-terminus have less effect.

Results

GFP-BLM has in vitro DNA helicase activity

Epitope-tagged genes facilitate the analysis of transfected cell lines and protein recovery. The green fluorescent protein (GFP) is especially useful as a reporter and epitope tag due to its ease in detection and apparent independent folding that does not interfere with the activity of its fusion partner [28]. A GFP-BLM expressing recombinant baculovirus was produced in Sf9 cells. Single virus isolates expressing GFP-BLM were identified using FACS and PCR analysis [29]. Sf9 cells infected with the purified primary stocks were screened by immunofluorescent microscopy to verify accumulation of GFP-BLM in the nucleus (Figure 1A). This screening verified full length expression as GFP is fused to the N-terminus of BLM and the nuclear localization signal is in the last 100 amino acids of the 1417 amino acid BLM coding region [30]. GFP-BLM accumulates in the nucleus of infected Sf9 cells as large aggregates (Figure 1A). This observation is consistent with a report of BLM oligomerization [31]. This property is an obstacle to purification as only about 10% of the expressed GFP-BLM is soluble. GFP-BLM was purified using ion-exchange and metal chelate resin chromatography (Figure 1B). The purified protein has DNA-dependent ATPase activity and DNA unwinding activity (Figure 1C & 1D) equivalent to previous preparations without GFP expressed in *S. cerevisiae* cells [10]. GFP-BLM has 5 units/ng unwinding activity and 10 units/ng DNA-dependent ATPase activity as compared to 6 units/ng and 7 units/ng for BLM purified from yeast. These data demonstrate that GFP-BLM has the same enzymatic activity as normal BLM and GFP-tagged variant alleles can be used to discover the functional domains of BLM.

Selection and analysis of transfected cell lines expressing normal and mutant BLM alleles under doxycycline control

An SV40-transformed BS cell line (GM0855B) was transfected with a plasmid containing an expression unit for the tetracycline or doxycycline (Tet) responsive transcriptional transactivator [32]. Only one cell line (HG2522.1a) out of 17 showed good responsiveness (15 fold) and low uninduced background expression levels (0.25% green cells). A series of deletion alleles of the normal BLM cDNA (B3) [3] were constructed using restriction sites and oligonucleotide adapters (Figure 2). These genes were made as in frame fusion genes at the C-terminus of EGFP and cloned behind the Tet-responsive promoter in plasmid pTRE. HG25221a cells were transfected with the normal and mutant GFP-BLM genes and stable hygromycin-resistant cell lines were selected. Clones were picked and screened for nuclear localized green fluorescence after induction. Cell lines expressing each construction were chosen for further study when the background level of green cells was less than 10% and the percentage of green cells after induction was reproducibly elevated (Table 1).

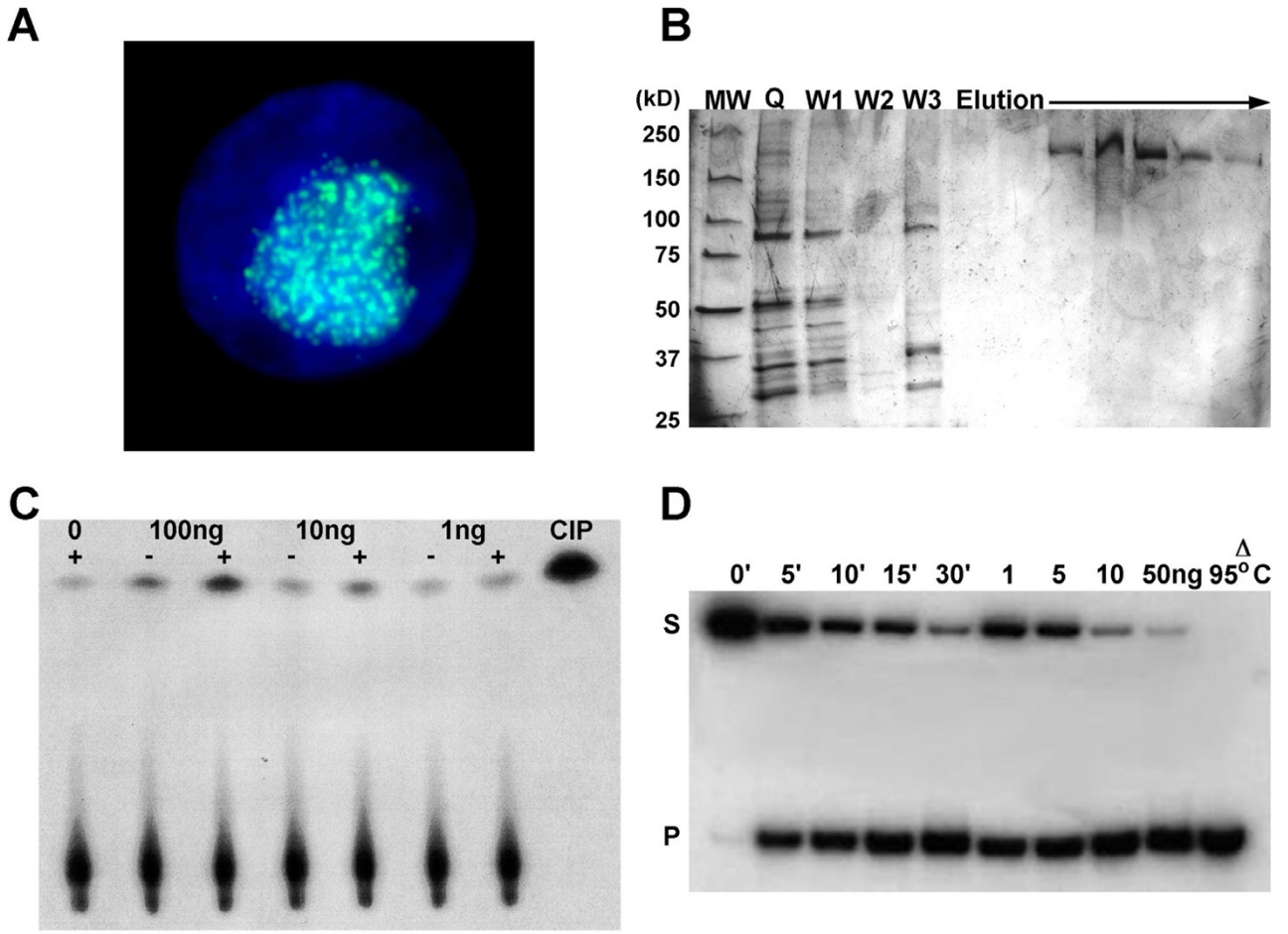

Figure 1

Purification and Assay of GFP-BLM. A. Fluorescent microscope image of GFP-BLM in the DAPI-stained nucleus of an Sf9 cell 72 hours after infection with a recombinant baculovirus. GFP-BLM forms large green aggregates. B. Silver-stained SDS 4-12% polyacrylamide gel of the purification of GFP-BLM using Macroprep Q ion exchange chromatography followed by metal chelate resin chromatography. Lane Q shows the ion exchange column pool of the green GFP-BLM fractions and lanes W1-W3 are proteins eluted from the metal resin with increasing imidazole washes (wash1 = 10 mM, wash2 = 50 mM, wash3 = 100 mM). The elution lanes show purified GFP-BLM eluted with a final step of 200 mM imidazole. The molecular weights (MW) of the standard markers (BioRad Precision) are indicated. C. Autoradiogram of a thin-layer plate ATPase assay of GFP-BLM. The amount of protein in the assay is shown. The addition of single-stranded Mpl8 DNA is indicated. Calf intestinal phosphatase (CIP) is used as a positive control for [γ-^{32}P]ATP hydrolysis. D. Unwinding of a ^{32}P-labeled 54 base oligonucleotide annealed to Mpl8 DNA by GFP-BLM. The position of the substrate (S) and the product (P) on the autoradiogram of a 10% polyacrylamide gel are indicated. The gel shows reactions containing 25 ng of GFP-BLM incubated for increasing time at 27°C and unwinding by increasing amounts of GFP-BLM incubated at 27°C for 30 minutes.

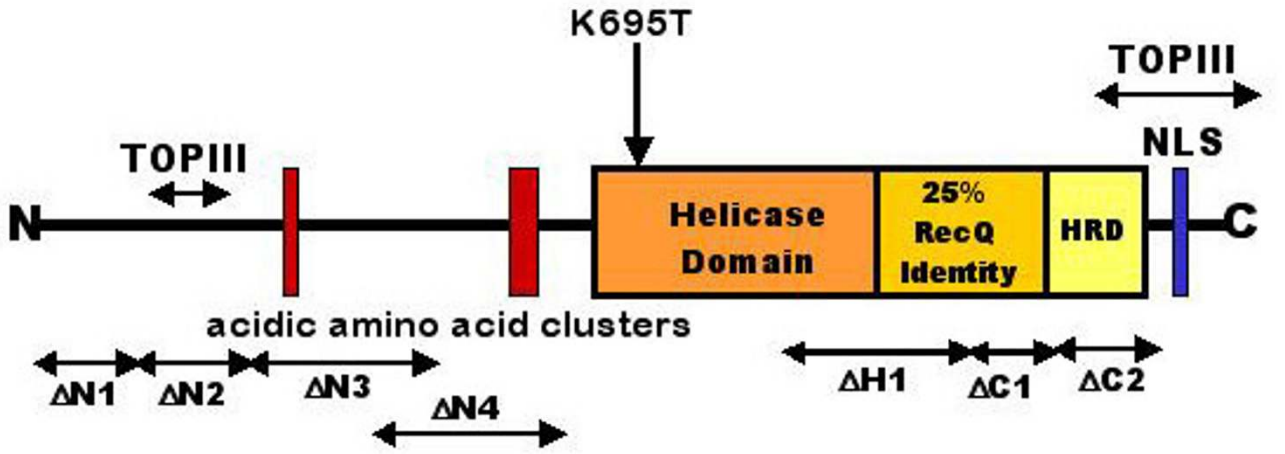

Figure 2
BLM Mutation Map. A diagram of the of the deletion and missense alleles of BLM used in this study. The amino acids removed by each deletion are: ΔN1 (2-128), ΔN2 (135-235), ΔN3 (241-469), ΔN4 (402-600), ΔH1 (903-1115), ΔC1 (1118-1164) and ΔC2 (1166-1331). The location of the missense allele K695T is shown. The red boxes indicate positions of regions of the N-terminus of BLM that have a high density of aspartic and glutamatic acid residues: region 1 (261-310 = 42% DE) and region 2 (541-570 = 50% DE). Deletion N3 removes a serine-rich region (310-320 = 64%S). The blue box indicates the location of the nuclear localization signal [29] (NLS = 1331-1350). The two regions of reported in vitro interaction of BLM with TopIIIα (143-213 & 1266-1417) are marked [34]. The helicase domain consists of residues 672-985 and the HRD (Helicase and RNaseD identity domain) [41] is indicated (1212-1293).

Table 1: Phenotypes of transfected cell lines expressing inducible GFP-BLM alleles.

Cell Line	% green cells no inducer	% green cells +Tet	Localization of GFP-BLM fusion proteins	Relative Expression no inducer	Relative Expression +Tet
GFP	8	33	diffuse (NM1)	nd	nd
GFP-BLM	1	94	NBs + NM2	0.16(0.03)	1.0(0.15)
GFP-ΔN1	1	75	NM2 (3% NBs)	0.17(0.05)	0.96 (0.3)
GFP-ΔN2	9	90	NM2	0.30(0.08)	2.28 (0.35)
GFP-ΔN3	2	74	NM2 + BBs	0.05(0.02)	0.87(0.12)
GFP-ΔN4	1	77	NM1&2	0.09(0.01)	0.81 (0.09)
GFP-K695T	3	30	NBs + NM2 + BBs	0.12(0.01)	0.45 (0.08)
GFP-ΔH1	3	80	NBs +NM3	0.02(0.01)	0.39 (0.08)
GFP-ΔC1	1	77	NBs + BBs	0.04(0)	0.83 (0.03)
GFP-ΔC2	5	82	NBs + BBs	0.01(0)	0.74 (0.06)

At least 200 DAPI-stained cells were counted and scored for green fluorescence to determine the percentages of induced and uninduced cells expressing the GFP-BLM fusion proteins. All GFP-fusion proteins show diffuse nuclear staining. The nucleolar morphologies (NM) of each fusion protein are reported as shown in Figure 4. NM1 is a diffuse overall nucleolar localization (Figure 4A & 4H). NM2 is overall nucleolar fluorescence with exclusion from central holes " TOPIIIα. BBs are numerous spherical BLM-containing nuclear bodies that do not co-localize with either antigen. At least 300 cells were examined for determination of nuclear morphologies. Expression levels of the fusion proteins without Tet and with 1 μg/ml Tet relative to normal BLM are reported as determined in Figures 3 & 5. The values reported are the average of two experiments. The standard deviation for these data points is shown in parentheses beside each value.

GFP-BLM expression can be regulated by inducer concentration

The level of GFP-BLM produced with increasing amounts of Tet after a 24 hour induction period was determined (Figure 3). The expression of GFP-BLM was calculated relative to the amount of BLM present in a normal SV40-transformed fibroblast line (GM0637B). Tet concentrations from 0.25-2 μg/ml produced levels of stable GFP-BLM expression in this SV40-transformed BS cell line similar to the amount found in the control cell line. Based on these data 1 μg/ml and 24 hours were used as the standard induction conditions to evaluate localization and SCE frequency of the transfected cell lines.

Stable cell lines containing the mutant proteins are inducible with Tet and show altered localization

The cell lines chosen for study contain some constitutive expression of the BLM alleles but show good reproducible induction (Table 1). The localization of the normal and mutant GFP-BLM proteins was analyzed by immunofluorescent microscopy (Figure 4). Antibodies against PML and topoisomerase IIIα [33] were used as marker proteins for the NBs. A human autoimmune serum against a nucleolar antigen ANA-N was used for nucleolar co-localization (data not shown). The cell line expressing GFP alone showed diffuse nuclear and cytoplasmic green fluorescence (Figure 4A). The normal GFP-BLM fusion protein localizes to NBs and the nucleolus (Figure 4B) as previously reported for BLM in normal human fibroblasts [20]. The fusion protein co-localizes with PML and TOPIIIα (Figure 4B & 4C) as does normal BLM [20,21,22,33,34]. GFP-BLM is distributed throughout the nucleolus except for several central regions that appear as holes in the images. The N-terminal deletions of BLM show little NB localization and ΔN1 through ΔN3 retain the nucleolar localization pattern (Figure 4D,E,F,G). The ΔN4 fusion protein shows mostly diffuse localization but nucleolar localization is still evident (Figure 4H). In 95% or more of the cells in the induced populations there is little co-localization with PML or TOPIIIα (Figures 4D,E,F,G, Table 1). These results demonstrate that the normal GFP-BLM fusion protein restores the BLM nuclear localization pattern to this BS cell line and deletions of the N-terminus affect the packaging of BLM into the NBs.

The N2-N4 regions of BLM are required for NB localization

By visual inspection each induced cell line contains cells expressing different levels of the fusion proteins. The ΔN1 expressing cells which exhibit the most intense green fluorescence comprise 3% (10 cells out of 300 counted) of the population and show co-localization of the GFP signal with α-PML and α-TopIIIa. This small number of cells is identical to cells expressing normal GFP-BLM (Figure 4B & 4C). The cell lines expressing ΔN2, ΔN3 and ΔN4 do not show NB localization (0 out of 300 cells). Since the ΔN1 protein shows partial co-localization with NBs the N1 region is not absolutely required for BLM to be localized there. The other N-terminal domains are required for NB localization.

The helicase and C-terminal mutations of BLM have very different effects compared to the N-terminal mutations

The two GFP-fusion proteins with mutations in the helicase domain have different localization patterns. The K695T missense protein localizes to NBs, but less efficiently than normal BLM, and to the nucleolus with an altered morphology (Figure 4I). The K695T fusion protein is not evenly distributed throughout the outer nucleolar region but shows areas of concentration and larger central holes. The ΔH1 fusion protein has a diffuse localization pattern throughout the nucleus and has a distinct altered nucleolar localization (Figure 4J). This fusion protein appears in the central holes of the nucleolus where the normal fusion protein is not found. Co-localization of the H1 fusion protein and α-PML and TOPHI11α is seen in most cells, and fewer NBs are present. The GFP-fusion proteins with helicase mutations are packaged into the NBs and have altered nucleolar localization.

The two GFP-fusion proteins with C-terminal mutations have different localization patterns than the N-terminal mutations. The ΔC 1 and ΔC2 fusion proteins co-localize efficiently with α-PML and α-TOPIII (Figure 4K & 4L) and have little nucleolar localization. In addition the C-terminal deletion proteins form numerous small green nuclear bodies that do not co-localize with either NB antibody (BLM bodies, BBs). These BBs are found in cells expressing the ΔN3 and the K695T proteins (Figure 4H & 4I). The BBs may be due to formation of large oligomers or aggregates of less soluble fusion proteins. The significance of these aggregates is unknown. These observations demonstrate that the N-terminal domain of BLM is required for NB localization and the C-terminal domain is required for accumulation of BLM in the nucleolus.

The mutant fusion proteins are made as stable full-length proteins

The differences in localization pattern between the normal and mutant alleles of BLM is not due to vastly different levels of stable fusion protein expression. Western analysis showed that all of the GFP fusion genes are inducible and are made as full-length proteins (Figure 5). The N-terminal deletion fusion proteins do not fail to go to NBs because of reduced fusion protein expression in these cell lines. They are expressed as stable full length fusion proteins at levels close to those of the normal

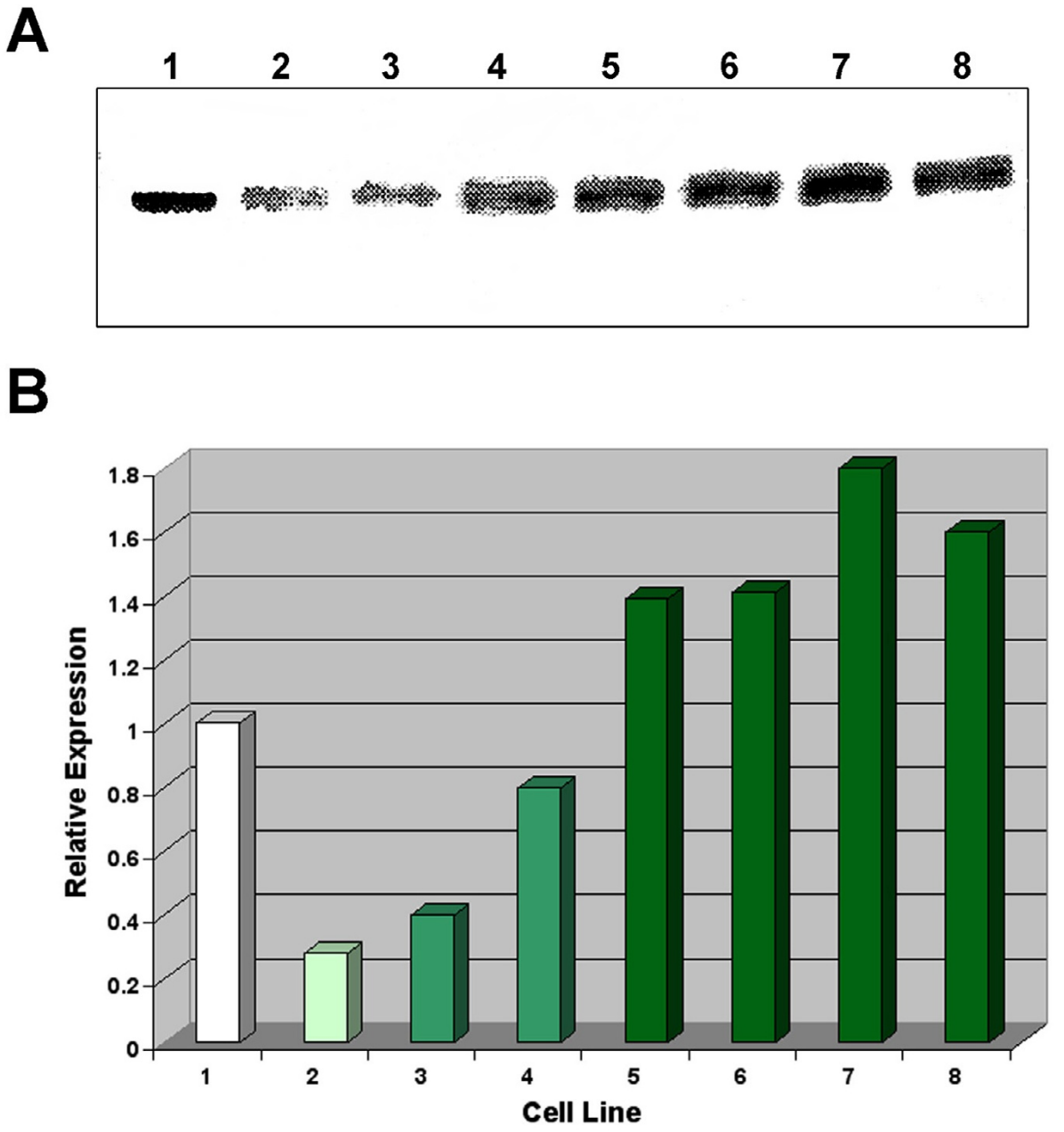

Figure 3
The expression of GFP-BLM is inducible in stable transfected BS cells. A. Equal amounts of total protein (10 μg) from whole cell lysates were resolved on a SDS-polyacrylamide gel and transferred to a membrane. The bound primary α-BLM and goat α-rabbit alkaline phosphatase conjugated secondary antibody complexes were quantitated using a ECF fluorescent alkaline phosphatase substrate. 1) GM0637G normal SV40-transformed fibroblast cells (0.17), 2) HG2522-1a cells + GFP-BLM gene no inducer (0.03), 3) +0.0625 μg/ml Tet (0.05), 4) +0.125 μg/ml Tet (0.11), 5) +0.25 μg/ml Tet (0.16), 6) +0.5 μg/ml Tet (0.3), 7) +1 μg/ml Tet (0.3), 8) +2 μg/ml Tet (0.14). The ECF fluorescent product was visualized directly with a Molecular Dynamics Storm PhosphorImager. B. The fluorescent image was quantitated using Imagequant. The amount of BLM in the lysates was normalized to the amount in equivalent total protein in the GM0637G cells. The data points are the average of two experiments. The standard deviations for each concentration are reported in parentheses above.

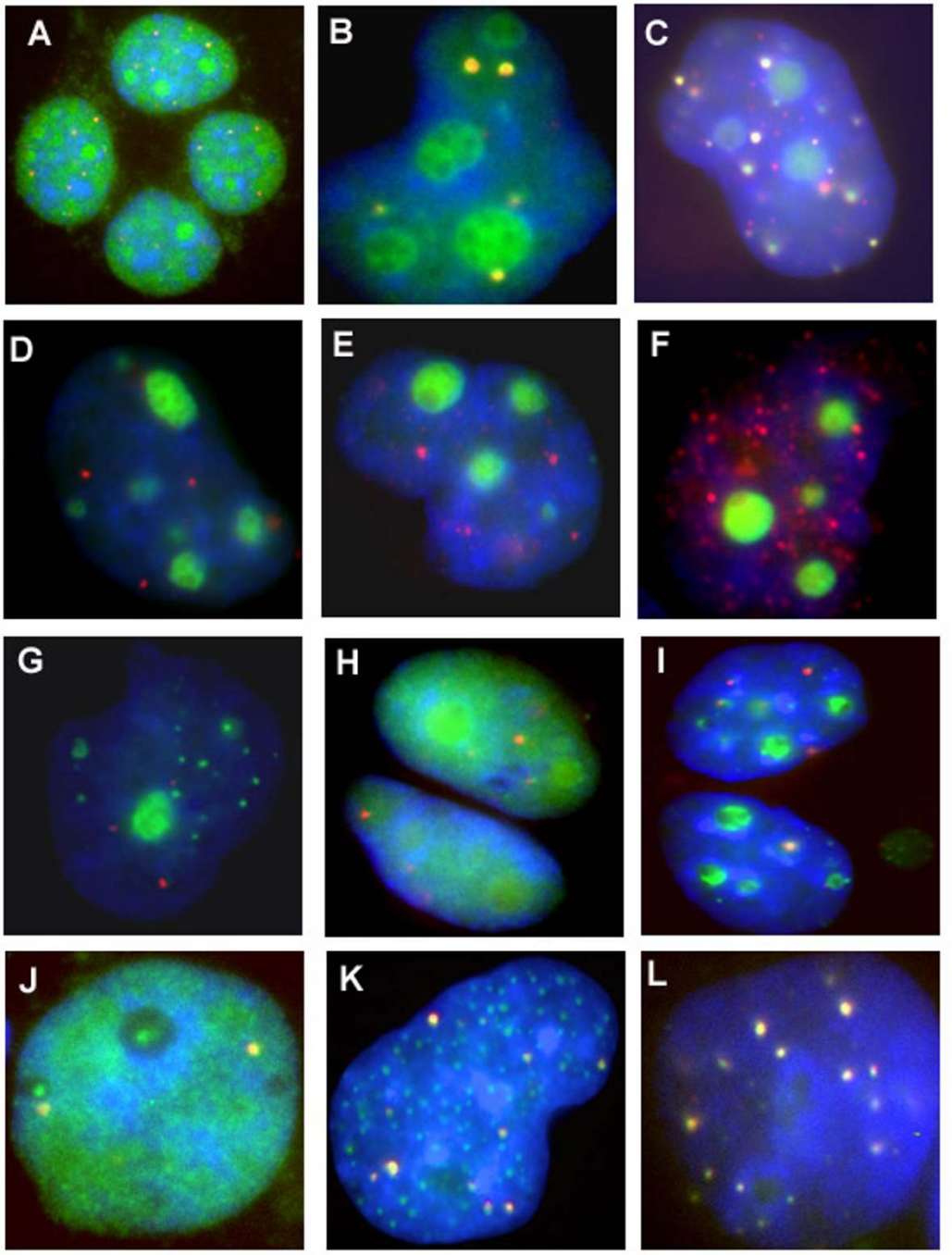

Figure 4
Localization of GFP-BLM fusion proteins. The immunofluorescent images show representative cells from each transfected cell line. Localization of GFP or GFP-BLM fusion proteins and PML (α-PML and Donkey α-mouse Texas Red secondary) or TOPIIIα (α-TOPIIIα and Donkey α-rabbit Texas Red secondary) in DAPI-stained nuclei are shown. A. EGFP + α-PML. B. GFP-BLM + α-PML. C. GFP-BLM + α-TOPIII. D. GFP-ΔN1 + α-PML. E. GFP-ΔN2 + α-PML. F. GFP-ΔN2 + α-TOPIII. G. GFP-ΔN3 + α-PML. H. GFP-ΔN4 + α-PML. L GFP-K695T + α-PML. J.GFP-ΔH1 + α-PML K. GFP-ΔC1 + α-PML. L. GFP-ΔC2 + α-TOPIII. The parental SV40-transformed BS cell line lacks detectable BLM [10].

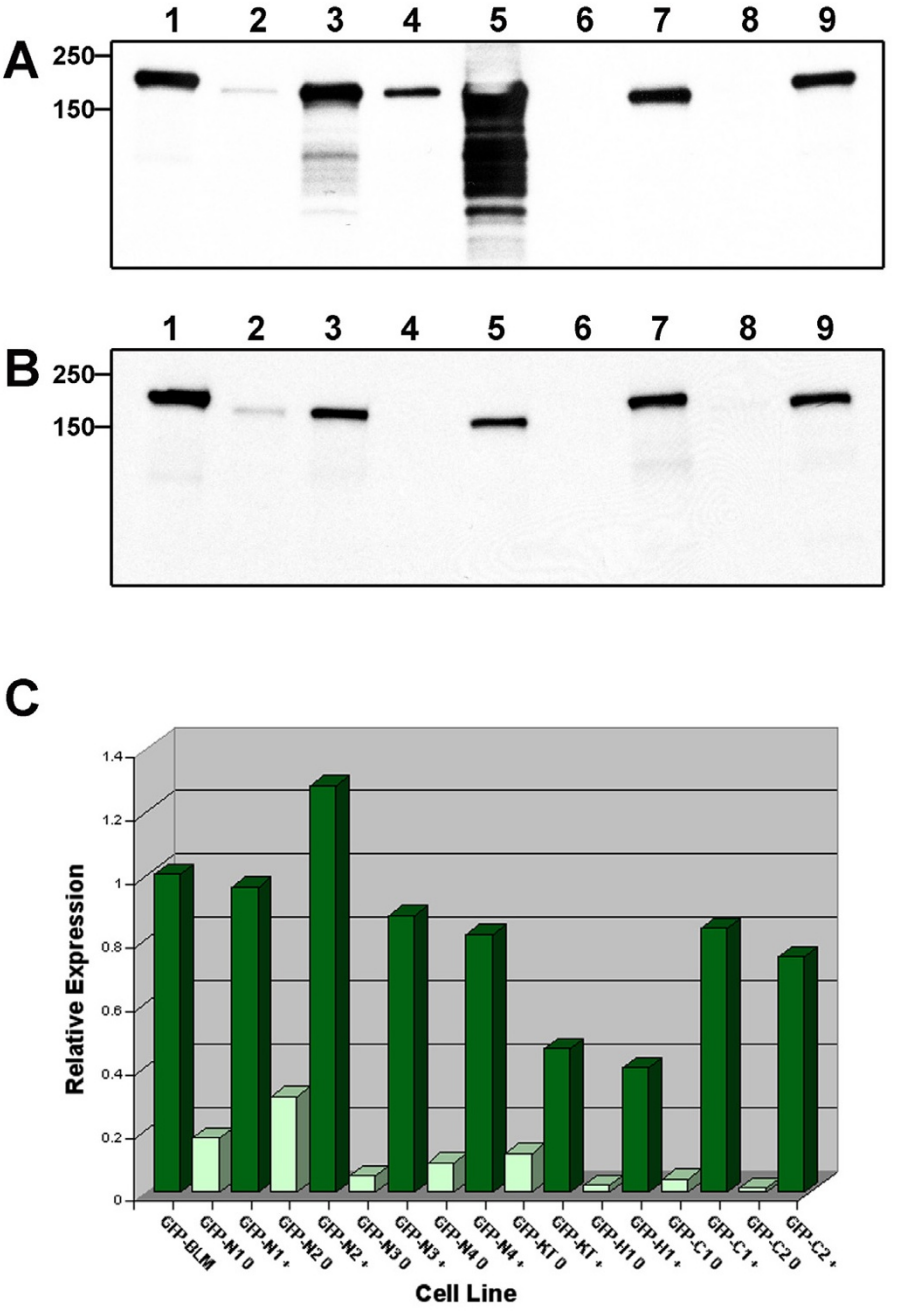

Figure 5
Western analysis of stable transfected cell lines expressing mutant BLM alleles. A. Whole cell lysates (15 μg total protein) from uninduced and induced cells were resolved on 4-12% SDS polyacrylamide gels and transferred to PVDF membranes. The bound complexes were detected with ECL reagents and exposed to x-ray film: 1) GFP-BLM+Tet, 2) GFP-ΔN1 no Tet, 3) GFP-ΔN1+Tet, 4) GFP-ΔN2 no Tet, 5) GFP-ΔN2+Tet. 6) GFP-ΔN3 no Tet, 7) GFP-ΔN3+Tet, 8) GFP-ΔN4 no Tet, 9) GFP-ΔN4+Tet. B. ECL western analysis of whole cell lysates: 1) GFP-BLM+Tet, 2) GFP-K695T no Tet, 3) GFP-K695T+Tet, 4) GFP-ΔH1 no Tet, 5) GFP-ΔH1+Tet, 6) GFP-ΔC1 no Tet, 7) GFP-ΔC1+Tet, 8) GFP-ΔC2 no Tet, 9) GFP-ΔC2+Tet. The positions of molecular weight standards are indicated to the left. Filters were reacted with α-GFP and goat α-mouse secondary conjugated with horseradish peroxidase. The bound complexes were detected with ECL reagents and exposure to x-ray film. C. Identical filters were reacted with α-GFP and goat α-mouse secondary conjugated with alkaline phosphatase. The fluorescent ECF product was visualized with a Molecular Dynamics Storm. The fluorescent image was quantitated using Imagequant. The values were normalized to normal GFP-BLM expression.

Table 2: Sister Chromatid Assay of Transfected Cell Lines: exchange data

Cell Line	Mean # of SCEs per 46Cs-Tet	Mean # of SCEs per 46Cs +Tet	Range of SCEs -Tet	Range of SCEs +Tet	% Relative Correction
GFP	70	65	58-96	55-75	7
GFP-BLM	40	12	30-49	10-19	70
GFP-ΔN1	56	20	32-73	12-29	64
GFP-ΔN2	47	17	25-63	9-27	64
GFP-ΔN3	58	23	40-85	11-35	60
GFP-ΔN4	66	45	46-89	20-68	30
GFP-K695T	84	106	63-118	79-131	-26
GFP-ΔH1	108	95	84-159	87-142	12
GFP-ΔC1	63	89	42-101	44-205	-41
GFP-ΔC2	68	78	51-82	59-108	-15

Frequency of SCEs are shown as mean number of exchanges per 46 chromosomes (Cs) for at least 20 metaphases for each cell line without inducer and with inducer. The range of the SCEs is shown. The relative percentage correction is calculated from the ratio of the mean number of exchanges per 46 chromosomes +Tet / the mean number of exchanges per 46 chromosomes - Tet. The cell line expressing GFP alone is 0.93 and 7% correction. The GFP-BLM cell line ratio is 0.3 and 70% correction. Cell lines were induced with 1 μg/ml Tet.

GFP-BLM fusion protein (Table 1, Figure 5). The ΔN2 protein is relatively over-expressed under the induction conditions used, while the ΔN3 and ΔN4 proteins are expressed at near normal levels under the same induction conditions. The N-terminal deletion alleles have higher levels of constitutive expression relative to the C-terminal mutations and have overall higher levels of protein expression; one explanation is that the C-terminal mutations are deleterious.

Most N-terminal mutations have little effect on genomic stability as measured by SCE frequency

BS cells have an elevated frequency of SCEs [1]. This increase in homologous recombination leads to an increased somatic mutation frequency and genomic instability. The SCE frequency was assayed in the inducible cell lines (Figure 6, Tables 2 & 3). The cell line expressing GFP alone had a nearly identical frequency of SCEs with and without induction (65 vs. 70 exchanges per 46 chromosomes). The GFP-BLM cell line had an elevated baseline level (40 exchanges) but showed significant reduction upon addition of inducer (40 to 12 exchanges). The ΔN1, ΔN2 and ΔN3 cell lines showed less reduction (17-23 exchanges) and slightly higher baseline levels (47-58 exchanges). These cell lines also have a distribution of SCEs slightly elevated relative to normal (Table 2, Figure 6A). The ΔN4 cell line has a different distribution and shows less correction of the SCE frequency (Table 2, Figure 6A). The N-terminal deletion proteins show a gradual increase in the range of the SCEs as the deletions approach the helicase domain (Table 2).

The helicase and C-terminal mutations have a destabilizing effect

The helicase and C-terminal mutations have a greater effect on genomic stability than do the N-terminal mutations (Table 2 & 3, Figure 6A). There is a polarity of loss of function seen across the gene from the N-terminus to the C-terminus (Figure 6A & 6B). Most of the C-terminal mutations appear to have a dominant negative effect, as the frequency of SCEs is higher with Tet than without. The most dramatic effects are seen in the ΔC1 and to a lesser degree in the ΔC2 induced cultures, as evidenced by an elevated frequency of ring chromosomes [35] (Table 3, Figure 6C). This may indicate breakage and rejoining events or tangled telomeres. The cell line containing the helicase deletion, ΔH1, has an elevated basal frequency of SCEs and a higher chromosome number than the GFP control cell line (Table 3) yet does not appear to be as deleterious as the K695T and C-terminal deletions are when comparing relative SCE events. The SCE analysis of this cell line was complicated by difficulty in finding chromosome spreads and by the small number of metaphases present in the cultures. Many of the metaphases could not be counted, as the chromosomes did not separate under the optimized spreading conditions that worked efficiently for the other cell lines. The SCE assay is therefore biased towards resolved or more corrected metaphases and it is likely that the induced ΔH1 SCE frequency is actually higher than reported here. The SCE assay was successful for all cell lines indicating that the doubling times are close to 24 hours. This indicates that the overall cell cycle timing is similar in all of the cell lines but does not rule out variations in stage-specific progression.

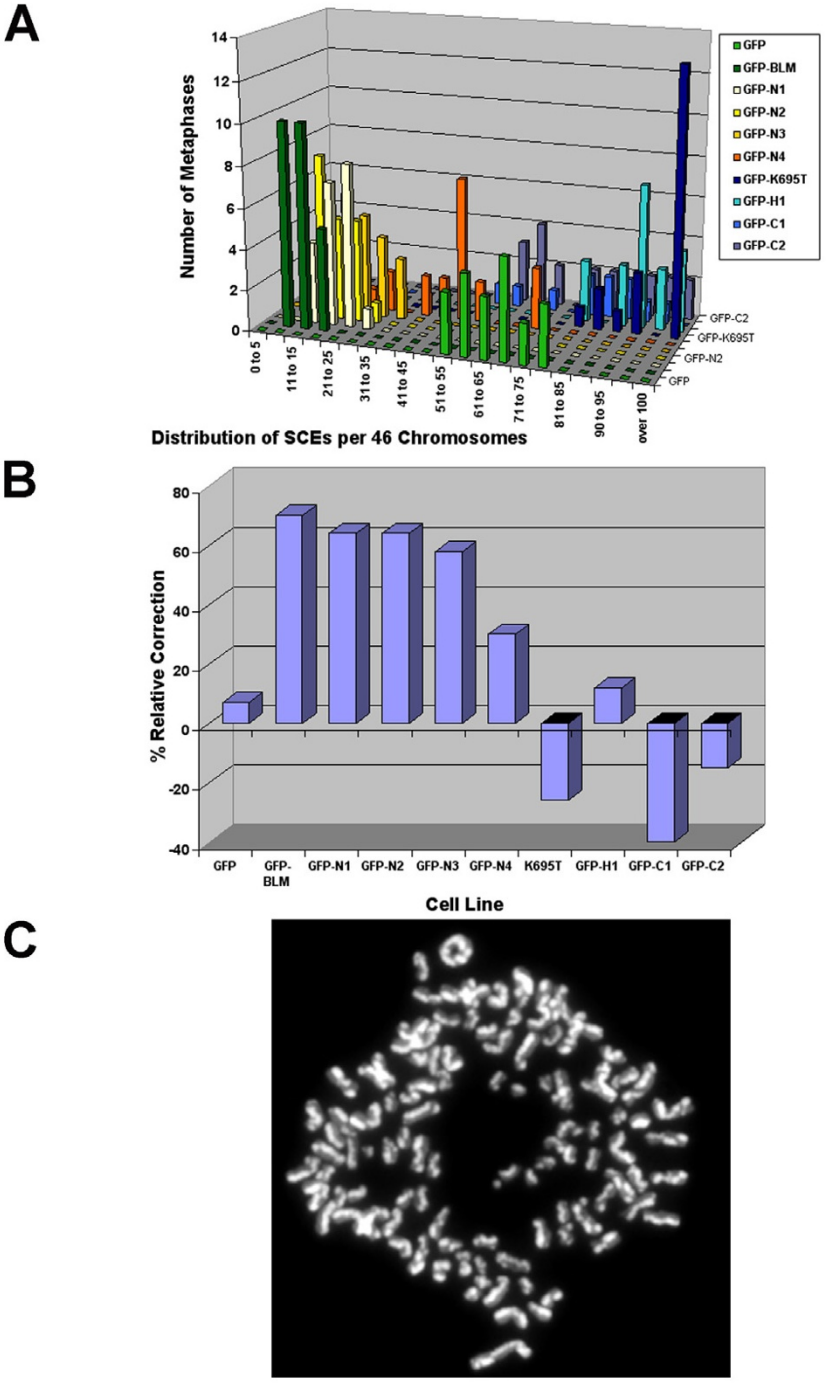

Figure 6
SCE Assay on cell lines with and without inducer. A. Histogram of the distribution of SCEs from induced cell lines. The number of SCEs per 46 chromosomes is plotted vs. the number of cells or metaphases. B. Graphical representation of the relative correction for each GFP-BLM allele (Table 2 & 3). C. Metaphase chromosome spread from an induced cell transfected with GFP-ΔC1 BLM. A large ring chromosome is shown at the top of the spread. The SCEs are visualized with acridine orange staining.

Table 3: Sister Chromatid Assay of Transfected Cell Lines: chromosome data

Cell Line	Mean # of Cs-Tet	Mean # of Cs+Tet	Range of # of Cs-Tet	Range of # of Cs+Tet	Ring Cs to Total Cs -Tet	Ring Cs to Total Cs +Tet
GFP	57	58	40-112	48-69	0/1116	0/1140
GFP-BLM	67	67	60-75	61-70	0/1340	0/1340
GFP-ΔN1	78	74	61-103	58-112	0/1560	0/1480
GFP-ΔN2	58	63	50-70	58-67	0/1116	0/1260
GFP-ΔN3	62	60	48-72	56-68	0/1240	0/1200
GFP-ΔN4	55	57	48-71	50-103	0/1100	0/1114
GFP-K695T	60	62	48-68	56-70	0/1200	0/1240
GFP-ΔH1	84	82	52-102	54-102	0/1680	0/1640
GFP-ΔC1	105	95	77-203	88-150	1/2100	7/1900
GFP-ΔC2	88	88	58-102	76-100	0/1760	3/1760

The mean number of chromosomes (Cs) per metaphase is shown for each cell line. The range of the chromosome number is shown. The number of ring chromosomes found is reported as number per total chromosomes examined for each cell line. Cell lines were induced with 1 μg/ml Tet.

The expression of the C-terminal mutations is deleterious relative to the N-terminal mutations

The C-terminal deletion containing cell lines have a higher mean chromosome number than the control and N-terminal deletion cell lines, except for the ΔN1 cell line which is somewhat elevated (Table 3). This increase in chromosome number suggests that the C-terminal mutations lead to an increase in the aneuploidy of these SV40-transformed cells by inhibiting proper chromosome separation. The increase in SCE frequency correlates with an overall lower level of fusion proteins in the C-terminal mutation cell lines and higher levels of protein expression in the cell lines with N-terminal deletions. These data demonstrate that mutation of the helicase and C-terminal domains of BLM have a greater effect on the SCE frequency than does mutation of the N-terminal domain.

Discussion

The Tet-on system has strengths and weaknesses

The Tet inducible expression system has experimental weaknesses in that the activity of the Tet-responsive activator is not tightly regulated and some cells do not produce inducible cell lines. All of the GFP-BLM fusion proteins were produced at a low level in the absence of inducer. Cell lines with a favored reproductive potential grew well and multiple useful stable transfectants were obtained (normal BLM and N-terminal deletion genes) as compared to those containing deleterious mutant alleles (helicase and C-terminal deletions). The BS cell line used here lacks BLM [10] and all cell lines obtained from transfection of the normal GFP-BLM gene had a background level of constitutive expression leading to a partially corrected SCE frequency relative to the parental line (40 vs. 58) [10]. EGFP itself is not an innocuous protein. The cell lines expressing EGFP alone grew the most slowly of all of the clones in the collection and had a slightly elevated SCE frequency relative to the parental cell line (65-70 vs. 58 exchanges) [10]. When co-transfections were performed with one plasmid containing the Tet-responsive GFP-BLM gene and another containing the selection unit, all of the cell lines tested had low SCEs without induction (4 of 4). Only when both the GFP-BLM and hygromycin-resistance expression units were combined on the same plasmid were useful inducible clones recovered. Despite the time and effort required to screen multiple clones, some with constitutive levels of expression, experimentally useful cell lines were obtained that allowed investigation of the function of the N-terminus and C-terminus of BLM.

The Tet-inducible system shows better SCE correction than constitutive expression

Our previous study used stable transfected cell lines derived from the same SV40-transformed BS cell line used here, except that the normal and missense alleles of BLM were expressed from a constitutive promoter [10]. The normal BLM cDNA reduced the SCE frequency from 58 to 24 exchanges per 46 chromosomes. This is partial correction as normal cell lines have from 4-10 exchanges per 46 chromosomes [1]. In addition the cells expressing the helicase-defective K695T allele grew slowly and eventually died. These results led us to use an inducible expression system. In this study the SCE frequency of the induced normal GFP-BLM cell line is closer to correction (12 exchanges). The K695T protein localizes in a pattern similar to the normal protein yet increases the SCE frequency, suggesting that the dominant negative effect of this allele caused the cell lines made in the previous study to die. Despite the limitations discussed above, the Tet-on system allowed isolation of inducible cell lines which more accurately paralleled normal than did lines

with a constitutive promoter, and allowed the isolation and evaluation of cell lines expressing deleterious alleles.

The NBs appear to be regulatory depots for BLM

All of the N-terminal deletion proteins are expressed at a stable level near normal, reduce the SCE frequency to near normal, and are not efficiently packed in the NBs. Cells which lack PML do not form NBs and have approximately a two-fold increase in SCE frequency relative to control cells [29]. This is similar to the SCE data for the N-terminal BLM deletions and demonstrate that NB localization is not a requirement for BLM function. NBs are likely to be storage locations or depots that regulate the release or recovery of BLM. All of the N-terminal deletion proteins except for ΔN4 show nucleolar morphologies and SCE frequencies close to normal. The ΔN4 protein has a lower correction efficiency and a different localization pattern than the other N-terminal deletions. This deletion removes a cluster of acidic amino acids, similar to acidic activation domains known to activate transcription in a variety of eucaryotes [36]. Clusters of acidic amino acids that function as transcriptional activation domains can also activate chromosomal DNA replication by interacting with replication protein A (RPA) [37]. BLM interacts with RPA70 in vitro [38] and associates with RPA clusters in cells [20]. Other studies have demonstrated that acidic activators can relieve nucleosomal repression and that the anti-repression function may be mediated by chromatin remodeling systems [39]. Deletion of this acidic cluster of BLM has an effect on localization and SCE frequency and may target BLM to RPA bound to single-stranded DNA, or it may allow BLM to function with chromatin remodeling complexes at sites of ectopic invasion events.

The N-terminal deletions do not efficiently associate with TOPIIIα by immunofluorescent examination. This does not rule out binding with TOPIIIα in the more diffusely stained regions. Studies in *S. cerevisiae* suggest that BLM and TOPIIIα function together as an enzymatic complex [33, 34]. Alternatively TOPIIIα could regulate BLM activity by altering its ability to bind DNA substrates. In vitro binding studies [34] identified two regions of BLM (amino acids 143-213 & 1266-1417, Figure 2) that interact with TOPIIIα. One of these regions (143-213) is removed by the ΔN2 construction. The cells express TOPIIIα in multiple sized spherical bodies but no co-localization with the GFP fusion protein is evident (Figure 4F). It is possible that TOPIIIα binds BLM only when BLM is in NBs or that the C-terminal BLM binding domain is of low affinity.

The nucleolus is likely to be an important site for BLM function

The C-terminal deletion proteins do co-localize with PML and TOPIIIα in the NBs yet elevate the SCE frequency. The helicase and C-terminal mutations that fail to localize normally in the nucleolus fail to reduce the SCEs and implicate the nucleolus as a functional location for BLM during DNA replication. One study on chromosome abnormalities in cells from persons with BS documented an elevated frequency of recombination events in the satellite stalks of acrocentric chromosomes [40]. In this report lymphocyte chromosomes from two persons with BS were compared to those from unaffected persons. The frequency of exchanges in the BS cells was estimated to be 6/1000 and less than 0.1/1000 in control cells. In addition the cells from one of the persons with BS had acrocentrics with Q-bright satellites. The lymphocytes from this individual varied greatly with regard to the number of Q-bright satellites and which acrocentric chromosomes contained them, documenting a high frequency of recombination in the rDNA loci of BS cells. These data are consistent with a model in which BLM functions to untangle invasive events in multiple rDNA loci and SCEs are used as a bypass mechanism when BLM is absent.

The localization of BLM in the nucleolus is not an artifact but rather a function specified by the C-terminal domain

The precise function of the C-terminus that directs BLM to the nucleolus is still unclear. An NMR structural analysis of the HRD domain of Sgs1p and models for the WRN and BLM domains suggests that this region is a single-strand nucleic acid binding domain that functions as an accessory DNA binding region with the helicase domain [41]. Helicase motifs are conserved in many proteins. The specific function of individual helicases may be controlled by a substrate selection mechanism in the accessory regions flanking the helicase domain. The C-terminus may confer on BLM the ability to recognize and bind abnormal DNA structures such as quadraplexes [17]. Alternatively the flanking regions may bind to recombination proteins such as RPA or RAD51 at these chromosomal sites [38]. The dominant negative nature of the C-terminal deletions suggests that these mutant proteins can be incorporated into normal BLM-containing complexes and inhibit function or regulation. The numerous BLM bodies formed by the C-terminal deletion fusion proteins may sequester repair complexes that BLM has been shown to associate with [42].

Many of the cell lines showed modest levels of over-expression of the fusion proteins relative to levels of BLM found in a normal SV40-transformed cell line. The immunofluorescent images show an apparent greater accumulation of fusion protein in the nucleolus than is found

in normal cells [20]. The stronger signals are caused by the expression levels as well as the sensitivity of the GFP reporter gene. When proteins are over-expressed they can localize non-specifically. The advantage of the system here is that the parental cell line lacks BLM and therefore the fusion protein can occupy normal positions without competing with endogenous BLM. The GFP-BLM fusion protein accumulates in the same nuclear structures as determined previously [20] and does not appear to cause aberrant structures at the levels expressed here.

BLM most likely functions in recombinational repair during DNA replication

The DNA helicases of the RecQ family are not essential for cellular viability but are important to genomic stability. Studies in model organisms suggest a role for the human orthologs in recombinational repair during DNA replication. Experimental evidence shows that RecQ and nuclease RecJ act together to restart stalled forks in *E. coli* [43]. A recent study of the *S. cerevisiae* Sgs1p gene suggests that it removes or prevents ectopic recombination events between non-homologous chromosomes [44]. Experiments with *X. laevis* BLM demonstrates its requirement in a reconstituted DNA replication system [6]. The in vitro enzymatic activities documented for BLM, unwinding G4 quadraplexes, holliday junctions and D-loops [17,18,19], as well as the phenotypes of BS cells [1] are consistent with both of these models. BLM has been localized to two GC-rich repeated regions (nucleolus and telomeres) [20] where the replication machinery is likely to need assistance to back up in order to untangle stalled forks and resolve inappropriate invasive events between repeated sequence elements found on multiple chromosomes. BLM has been found in large protein complex with BRCA1 and multiple DNA repair and replication factors [42] that may recognize and repair abnormal DNA structures that occur during DNA replication. As both the helicase activity and the C-terminus of BLM are required to maintain genomic stability it is likely that the C-terminus of BLM recognizes abnormal DNA structures and the helicase activity unwinds these multi-stranded structures to engender efficient repair.

Conclusions

The GFP-BLM fusion protein has helicase activity in vitro and functions to reduce the SCE frequency in BS cells. Deletions of BLM expressed as GFP fusion proteins demonstrate that the N-terminal domain directs BLM into nuclear bodies and the C-terminal domain is necessary for efficient nucleolar localization. The stable nucleolar localization of BLM is not due to over-expression but rather to a function encoded in the C-terminus of BLM. Mutations in the helicase domain and deletions in the C-terminal domain have a dominant negative effect on the SCE frequency and cause an increase in chromosome abnormalities, whereas N-terminal deletions have relatively little effect. These data demonstrate that the helicase activity and C-terminal domain directed nucleolar localization of BLM is essential to maintain genomic stability. The NBs appear to be storage or regulatory sites for BLM and are not required for BLM activity.

Materials and Methods

Expression and purification of GFP-BLM

The normal BLM cDNA (B3) [3] was fused to the C terminus of EGFP (Clontech). An 8x polyhistidine epitope tag was added to the N-terminus of EGFP with an oligonucleotide adapter (5'AAAACTGCAGCGGCCGCCAC-CATGCACCAC-
CACCACACCACCACCACGTGAGCAAGGGCGAGGAGC
TGTTCACCG3'). DNA sequencing confirmed the construction. A recombinant baculovirus expressing the fusion gene behind the polyhedrin promoter was made in Sf9 cells using Invitrogen's BlueBac kit according to the supplier's protocol. Sf9 cells were infected for 72 hours at a multiplicity of infection (MOI) of 1 and sorted for green cells by FACS analysis (MoFlo, Cytomation) at one and ten cells per well into 96 well microtiter plates containing uninfected Sf9 cells [29]. These primary viral stocks were assayed and amplified by infecting Sf9 cells in 24 well plates. The secondary stocks were analyzed by PCR and screened for a nuclear-localized GFP signal [30]. Viral stocks containing only the recombinant GFP-BLM construction were amplified to produce high titer stocks for expression. Maximal expression of GFP-BLM was found to be 72 hours of infection by western analysis with α-BLM [10]. Sf9 cells in Sf900-II medium (Invitrogen) were infected at a MOI of 5-10 and harvested after 72 hours. Efficiency of infection was monitored by immunofluorescent microscopy (Figure 1A). The cell pellets were stored at -70°C.

Cell lysis was monitored by fluorescent microscopy and aliquots of column fractions were assayed in the wells of 96 well microtiter plates for green fluorescence using a Molecular Dynamics Storm Phosphorlmager. The frozen cell pellets from 500 ml spinner flask culture were lysed by sonication in Buffer A (50 mM Tris-HCL pH 8, 5 mM β-mercaptoethanol, 1 mM EDTA, 1 μg/ml leupeptin, pepstatin, and E64, and 1 mM PMSF) containing 450 mM NaCL and 1% NP40. The crude lysate was diluted 1:3 with Buffer A. After centrifugation (15 min at 20,000 × g at 4°C) the soluble material was loaded onto a BioRad Macroprep High Q column (2.5 cm × 20 cm) equilibrated with Buffer A + 150 mM NaCl. The column was washed with 5 column volumes of Buffer A + 150 mM NaCl and 5 column volumes of Buffer A + 250 mM NaCl (no EDTA). GFP-BLM was eluted with Buffer B (Buffer A + 500 mM NaCl and no EDTA). Pooled Macroprep Q fractions con-

taining GFP-BLM were loaded onto a 5 ml HiTrap metal chelate column (Amersham Pharmacia) charged with $_{NiSo}4$ equilibrated in Buffer B. The metal chelate resin was washed with successive 10 column volumes of Buffer B + 10mm imidazole, Buffer B + 50 mM imidazole, and Buffer B + l00 mM imidazole. Highly purified GFP-BLM eluted in Buffer B + 200 mM imidazole and was stored at -70°C. Assays for DNA-dependent ATPase and oligonucleotide unwinding were performed as before [10].

Construction of inducible cell lines expressing GFP-BLM and mutant alleles

The SV40-transformed BS cell line GM0855B (bl-$m^{Ash[2281\Delta6ins7]}$ / $bim^{Ash\,[2281\Delta6ins7]}$) [3] was transfected with a plasmid expressing the tetracycline or doxycycline (Tet)-responsive transcriptional transactivator from a CMV promoter [32]. G418-resistant cell lines were selected in DMEM + 10% fetal bovine serum (Clontech, Tet-free) + 200 μg/ml Geneticin (Invitrogen). Seventeen G418-resistant clones were screened for transfection efficiency and Tet-responsiveness. The efficiency was measured by transient transfection of a CMV promoter-driven GFP gene (pEGFP-Cl, Clontech) and responsiveness by transfection of a Tet-regulated GFP gene (pBi EGFP, Clontech). One cell line (HG2551a) had an 8% transfection efficiency (constitutive GFP) and 0.25% green cells without Tet and 3.8% with 1 μg/ml Tet (15 fold induction and 45% inducible transfection efficiency vs. constitutive), and was chosen for transfection of GFP-BLM alleles.

The BLM alleles used here were cloned as fusions with the C-terminus of EGFP (Clontech). The K695T missense allele was described before [10]. The deletion alleles of BLM were constructed using digestion with two restriction enzymes and insertion of an oligonucleotide adapter. The sites and the sequences of the adapters were:

ΔN1 (EcoRI-SmaI, 5'AATCTGCAGCTATGAAATCCC3'/5'GACGTCGATACTTTAGGG3'),

ΔN2 (SmaI-ClaI, 5'GGGATACTGCTATTTGCAT3'/5'CCCTATGACGATAAACGTAGC3'),

ΔN3 (ClaI-AvrII, 5'CGATGATCTGACTACCACCC3'/5'TACTAGACTGATGGGTGGGATC3'),

ΔN4 (XmnI-AhdI, 5'GCTTTCCTCAGCCAAGACAGA3'/5'CGAAAGGAGTCGGTTCTGTC3'),

ΔH1 (NcoI-SalI, 5'CATGAATCTGGCTG3'/5'TT AGA CCGACAGCT3'),

ΔC1 (SalI-PvuI, 5'TCGACATTGACCAGGCGAT3'/5'GTAACTGGTCCGC3') and ΔC2 (PvuI-EcoNI, 5'CGCTAATGAAAGGAAGAGGAAAAAGATGCCAG CCT CC 3'/5'TAGCGATTACTTTCCTTCTCCTTTTTCTACG GT3').

The ΔC2 construction restores the nuclear localization signal [30]. Oligonucleotides were made by Invitrogen. The deletion junctions were confirmed by DNA sequencing.

Cell line HG2551a was transfected [10] with normal and mutant GFP-BLM genes cloned behind the Tet-responsive promoter in plasmid pTRE (Clontech). Stable transfected cell lines containing these constructions were selected by two methods: co-transfection of these plasmids with a second plasmid containing a hygromycin-resistance gene driven by a Herpes simplex thymidine kinase promoter or transfection of GFP-BLM allele plasmids that had been modified to contain the hygromycin-resistance gene expression unit. Few useful cell lines were obtained from the first method. Most transfected clones that were resistant to 200 μg/ml G418 and 200 μg/ml hygromycin did not produce GFP upon induction. In contrast transfection with the modified vector where the two expression units are on the same plasmid produced many useful cell lines. The ΔC2 cell line was from the first transfection method and all of the others were selected using the second.

Immunoflourescent and western analysis of cell lines

Cells were plated onto glass coverslips in 6 well tissue culture plates in selective medium, induced the next day with 1 μg/ml Tet and fixed with 2% paraformaldehyde 24 hours later. Immunofluorescent staining and microscopy was as performed previously [20]. Antibody against human Topoisomerase IIIα was provided by F. B. Johnson and L. Guarente [33]. Antibody against BLM was described before [10]. Antibody against GFP was from Roche Molecular Biochemicals. Western transfers were as before [10]. ECF and ECL reagents were purchased from Amersham/Pharmacia and used according to the supplier's instructions. SDS 4-12% polyacrylamide gels and Precision protein molecular weight markers were from BioRad.

Sister Chromatid Exchange Assay

Fibroblast cell cultures were divided into two T-25 tissue culture flasks and one was induced with 1 μg/ml Tet the next day. After a 24 hour period of induction 20 μM BrdU was added to both flasks. Labeling, colcemid treatment and harvest were as before [10]. Fixed preparations were dropped onto clean glass slides and stained with 0.1 mg/ml acridine orange for 5 minutes, followed by a wash in running water for 2 minutes and a 1 minute incubation

in Sorensen's buffer (67 mM KH_2PO_4 + 67 mM Na_2HPO_4). The wet slides were covered with glass coverslips and metaphases photographed immediately with a Zeiss Axioskop fluorescence microscope using the FITC filter and a 100× objective. Images were captured with a CCD camera (Kodak Micromax 1400) connected to an Apple Power Mac 9600.

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