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Transfection of *BLM* into Cultured Bloom Syndrome Cells Reduces the Sister-Chromatid Exchange Rate toward Normal

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Summary

The gene *BLM*, mutated in Bloom syndrome (BS), encodes the nuclear protein BLM, which when absent, as it is from most BS cells, results in genomic instability. A manifestation of this instability is an excessive rate of sister-chromatid exchange (SCE). Here we describe the effects on this abnormal cellular phenotype of stable transfection of normal *BLM* cDNAs into two types of BS cells, SV40-transformed fibroblasts and Epstein-Barr virus (EBV)–transformed lymphoblastoid cells. Clones of *BLM*-transfected fibroblasts produced normal amounts of BLM by western blot analysis and displayed a normal nuclear localization of the protein by immunofluorescence microscopy. They had a mean of 24 SCEs/46 chromosomes, in contrast to the mean of 69 SCEs in controls transfected only with the vector. *BLM*-transfected fibroblast clones that expressed highest levels of the BLM protein had lowest levels of SCE. The lymphoblastoid cells transfected with *BLM* had SCE frequencies of 22 and 42 in two separate experiments in which two different selectable markers were used, in contrast to 57 and 58 in vector-transfected cells; in this type cell, however, the BLM protein was below the level detectable by western blot analysis. These experiments prove that *BLM* cDNA encodes a functional protein capable of restoring to or toward normal the uniquely characteristic high-SCE phenotype of BS cells.

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Introduction

In a recessively transmitted disorder, the abnormal cellular phenotype that is the consequence of the absence of a normal allele should be corrected by the experimental introduction of the normal allele into mutant cells. This will be the case provided the normal allele is expressed and the protein it encodes is produced in adequate amounts.

In recessively transmitted Bloom syndrome (BS [MIM 210900]) (German 1993), a feature of the abnormal somatic-cellular phenotype is genomic instability of a specific type. An important manifestation of the genomic instability in BS is hyperrecombinability, and an elevation in the number of sister-chromatid exchanges (SCEs) is an easily demonstrable—and uniquely diagnostic—cellular phenotype. *BLM*, the gene mutated in BS, encodes a 1,417-amino-acid protein that contains a domain homologous to the RecQ subfamily of DNA helicases, and RecQ genes have been implicated in cellular mechanisms that maintain genomic stability. Most of the ~60 mutations that we have identified in the *BLM* gene in individuals with BS result in premature translation termination of the protein (Ellis et al. 1995; J. German and N. A. Ellis, unpublished data). Thus, the cellular phenotype of BS is considered to be the consequence of the absence of a functional BLM protein. The clinical, genetic, cytogenetic, and molecular genetic aspects of BS are reviewed elsewhere (German and Ellis [in press]).

When the *BLM* gene was isolated (Ellis et al. 1995), we demonstrated that stable transfection of BLM into cultured BS cells brought their high-SCE phenotype toward normal (German et al. 1996). Giesler et al. (1997) have confirmed this finding. Here, we present the cytogenetic data more fully. We also show by use of western blot analysis that the stable transfection of *BLM* into SV40-transformed BS fibroblasts results in expression of BLM protein and by use of immunofluorescence microscopy that BLM localizes to the nucleus with a distribution resembling that seen in normal cells. Reduction of SCE was also accomplished in Epstein-Barr virus (EBV)–transformed BS lymphoblastoid cells, and in some cells it reached a completely

normal level; however, for as-yet-unexplained reasons, in that type of cell the BLM protein was not synthesized to a level that was detected by either western blot analysis or immunofluorescence.

Material and Methods

Cell Culture

The SV40-transformed fibroblast cell line (FCL) GM08505 was established at the Coriell Institute for Medical Research (Camden, NJ) as a permanently proliferating cell line. This highly aneuploid line, which maintains the high-SCE phenotype of BS, was derived from a diploid FCL developed from a skin biopsy sample obtained from individual 42(RaFr), an Ashkenazi Jewish woman with BS. 42(RaFr) was homozygous for the 6-bp deletion and 7-bp insertion (BLMc.2207-2212delATCTGAinsTAGATTC), a mutation referred to as "blm^{Ash}." Homozygosity for blm^{Ash} results in an 80% reduction in the steady-state levels of *BLM* mRNA, as determined by means of both northern analysis and clonal analysis of reverse transcriptase–PCR products (N. A. Ellis and T.-Z. Ye, unpublished data), and premature translation termination at codon 740 in the *BLM* cDNA (Ellis et al. 1995). For comparison with GM08505 we chose the permanently proliferating and aneuploid SV40-transformed FCL GM00637, which also was established at the Coriell Institute but derived from a diploid FCL developed from a normal adult skin sample. GM01492 (obtained from the Coriell Institute) is an FCL developed from a skin biopsy sample of 44(AbRu), a person with BS homozygous for blm^{Ash}. HG2619 is a diploid FCL developed here from a skin sample obtained from a normal individual.

HG1525 and HG2703 are near-diploid lymphoblastoid cell lines (LCLs) established here by EBV transformation of blood lymphocytes from two individuals with BS: 81(MaGrou), a French Canadian, and NR2(CrSpe), an Ashkenazi Jew. HG1525 is homozygous for mutation BLMc.1784C>A, which causes a reduction in steady-state *BLM* mRNA levels as determined by northern blot analysis and premature translation termination at codon 595 (authors' unpublished data); HG2703 is homozygous for blm^{Ash}. HG2703 was used only in indirect immunofluorescence experiments (see the Immunochemical Detection of BLM subsection, below). HG2162 and HG1943 are diploid or near-diploid control LCLs that were established here from blood drawn from normal individuals.

The FCLs were cultured in D-MEM medium supplemented with 4 mM L-glutamine (Life Technologies) and 10% (v/v) fetal bovine serum (Hyclone). For posttransfection selection, 0.2 mg geneticin/ml (Life Technologies) was added to the medium. The LCLs were cultured in RPMI 1640 medium supplemented with 4 mM L-glutamine, 15% (v/v) fetal bovine serum, and 10 μg ciprofloxacin/ml (Miles). For posttransfection selection, either 0.2 mg hygromycin/ml (Boehringer Mannheim) or 0.3 mg geneticin/ml was added, depending on the selectable marker in the vector being used for the transfection (see the Plasmids subsection, below).

Plasmids

The three cDNAs used for transfection were B3 (the H1–5′ sequence; GenBank accession number U39817), R9, and R12 (Ellis et al. 1995). During the present study R9 and R12 were sequenced by use of dideoxynucleotide-determination reactions.

For transfection of the GM08505 fibroblasts, the integration/expression vector pOPRSVI-CAT (Stratagene) was used. DNA from R12 was subcloned by standard procedures, with *BLM* being substituted for the *CAT* gene in the vector; this construct was named "pOPRSVI-BLM." For transfection of the HG1525 lymphoblastoid cells, the episomal expression vectors pREP4 and pREP9 were used. pREP4 carries the hyg^r gene, which confers resistance to hygromycin, and pREP9 carries the neo^r gene, which confers resistance to geneticin. R9 and R12 were isolated from a library in which pREP4 is the vector (Strathdee et al. 1992). DNA from B3 was subcloned into pREP9; this construct was named "B3R51." Plasmid DNAs were purified by use of Qiagen columns.

Transfection

For fibroblasts, DNAs of the test vector pOPRSVI-BLM and of the control vector pOPRSVI-CAT were transfected into GM08505 cells with the use of lipofectamine (Life Technologies) according to the manufacturer's instructions. Thirty-three neo^r pOPRSVI-BLM clones and 11 neo^r pOPRSVI-CAT clones were isolated and expanded; 8 and 6 of these clones, respectively, were chosen for further analysis. For lymphoblastoid cells, each of the two *BLM* expression constructs R9 and B3R51 and the vectors pREP4 and pREP9, which were used as controls, were transfected into HG1525 cells by electroporation (Ausubel et al. 1999). In brief, a 0.8-ml suspension of 8×10^6 cells was loaded into an ice-cold 0.4-cm Gene Pulser (Bio-Rad) cuvette; 10 μg of DNA were then added. The cells and the DNA were held on ice for 10 min and then electroporated at 330 V and a capacitance of 960 μF. After another 10 min on ice, the cells were transferred to a 25-cm^2 flask containing 5 ml nonselective medium and incubated for 4–5 d. The cells were then transferred to selective medium and cultured for several weeks. Antibiotic-resistant cultures were expanded for analysis.

Immunochemical Detection of BLM

Rabbit polyclonal antibodies to an amino-terminal segment of BLM were raised in a conventional way (Neff et al. 1999). For western blotting, whole-cell protein extracts were prepared from pellets of 10^6–10^7 cultured cells that were washed once in PBS and frozen at −70°C. Pellets were resuspended in 100 μl sample buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 0.7 M β-mercaptoethanol, 10% glycerol, and 0.005% [w/v] bromophenol blue), the DNA was sheared by passage through a 26-gauge needle, and the extracts were boiled for 5 min. Protein concentration was determined by use of the Bradford method. Thirty micrograms of protein per lane were loaded onto an SDS-polyacrylamide gel, and after electrophoresis immunoblots were prepared by standard methods (Harlow and Lane 1988). After membranes were blocked with 5% nonfat dry milk in PBST (PBS plus 1% Tween-20) for 1 h, the filters were incubated with unpurified rabbit polyclonal anti-BLM diluted 1:1,000 in PBST containing 2% milk for 1 h. After the filters were washed in PBST, horseradish peroxidase–conjugated goat anti–rabbit IgG (Vector Laboratories) diluted 1:5,000 was used as a secondary antibody. For visualization of the conjugated anti-IgG, ECL reagents (Amersham) were used.

For indirect immunofluorescence, FCLs were cultured on microscope PLUS slides (Fisher), and LCLs were attached to the slides by use of a Cytospin. Cells were fixed with equal parts of methanol and acetone for 2 min at room temperature. Preparation of cells for immunochemical detection of BLM was by standard procedures (Harlow and Lane 1988). Normal goat serum was used to block nonspecific binding. Unpurified rabbit anti-BLM diluted 1:500, biotin-conjugated goat anti–rabbit IgG diluted 1:500, and avidin-conjugated Texas Red diluted 1:500 in 5 μg 4'6'-diamidino-2-phenylindole (DAPI)/ml were used in succession with washing to detect BLM. Normal goat serum, goat anti–rabbit IgG, and avidin-conjugated Texas Red were obtained from Vector Laboratories.

SCE Analysis

The differential staining of sister chromatids in cells that had replicated their DNA for two cell cycles in bromodeoxyuridine employed Hoechst 33258, exposure to light, and finally Giemsa staining, as described elsewhere (German et al. 1977). The SCEs in intact metaphases were enumerated by light microscopy with a 100× objective. The numbers of SCEs per 46 chromosomes were determined for both diploid/near-diploid and aneuploid cell lines.

Results

Western Blot Analysis of Transfected Cells

Two types of BS cells were transfected in these studies, the SV40-transformed FCL GM08505 and the LCL HG1525. Both GM08505 and HG1525 were derived from individuals with BS who were homozygous for protein-truncating mutations, making these cells effectively null for *BLM* and therefore appropriate test recipients for transfection of normal *BLM* cDNAs.

The cDNA expression construct used for transfection of the GM08505 cell line was derived from the R12 cDNA. Its sequence was found to be identical to the published sequence of the B3 cDNA (Ellis et al. 1995). Of the two cDNA expression constructs used for transfection of the HG1525 cell line, one was derived from the B3 cDNA and the other was the R9 cDNA. The sequences of the B3 and R9 cDNAs contain two amino acid differences, namely, P868L and V1321L. On the basis of the data presented here, there is no reason to suspect that the two proteins produced by these two cDNAs are not functionally equivalent.

In the normal control SV40-transformed FCL GM00637, the results of western blot analysis of whole cell extracts revealed a protein with an apparent molecular weight of 180 kD that was absent from the SV40-transformed BS GM08505 cells (fig. 1*A*, lanes 1 and 2), a finding that proves that it is normal BLM. Thus, pOPRSVI-BLM encodes a protein with the same electrophoretic mobility as normal BLM. This 180-kD protein was expressed at varying levels in the seven clones that received pOPRSVI-BLM (fig. 1*A*, lanes 3–9).

In the EBV-transformed LCLs, western blot analyses demonstrated that the 180-kD protein is present in normal control lines HG2162 and HG1943 but is absent from BS line HG1525 (fig. 1*B*, lanes 1–3). In contrast to its clear demonstrability in the transfected fibroblasts, the 180-kD protein was not readily detectable in BS lymphoblastoid cells transfected with either R9 or B3 (fig. 1*B*, lanes 4–7; a faint band appeared at the same position as BLM in *BLM*-transfected cells but not in vector-transfected or untransfected cells). We do note that the LCLs analyzed had not been subcloned, and variability in the levels of expression of BLM in different cells in the culture is a possibility. However, according to the results of immunofluorescence analysis (see the Nuclear Localization of BLM subsection, below), few, if any, individual cells exhibited detectable levels of *BLM*.

Nuclear Localization of BLM

When BLM antibody is used in indirect immunofluorescence microscopy, most normal control cells contain

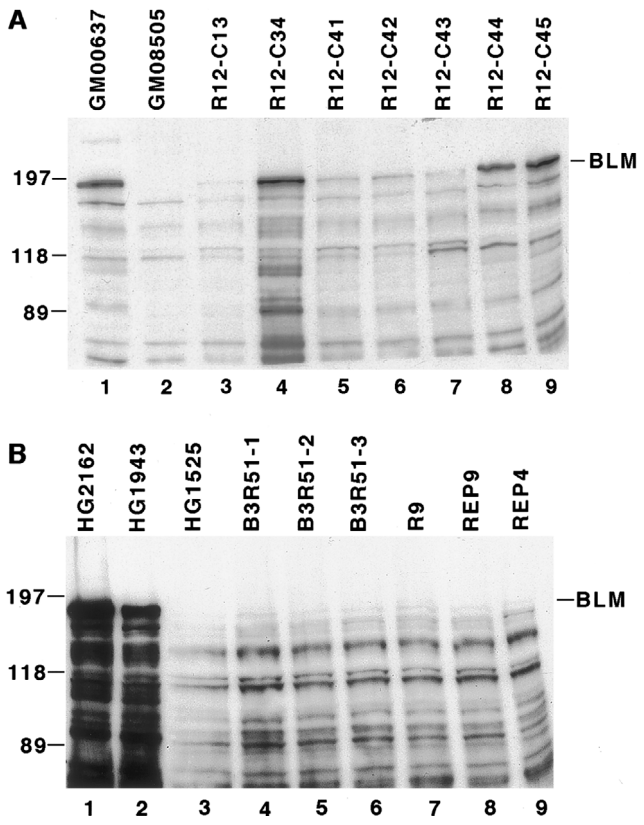

Figure 1 Western blot analysis using rabbit anti-BLM. Whole-cell protein extract (30 μg) was separated by electrophoresis through 6% SDS polyacrylamide gels. Western blots were prepared with polyvinylidene fluoride membranes, which were probed with rabbit anti-BLM; immune complexes were visualized with goat anti–rabbit IgG and the ECL reagent kit (Amersham). *A,* Analysis of normal, BS, and *BLM*-transfected SV40-transformed fibroblasts. Lane 1 represents normal control GM00637 cells; lane 2, BS GM08505 cells; and lanes 3–9, pOPRSVI-BLM-transfected clones 13, 34, 41, 42, 43, 44, and 45, respectively (clone 50 not having been analyzed). *B,* Analysis of normal, BS, and *BLM*-transfected lymphoblastoid cells: Lane 1 represents normal control HG2162; lane 2, normal control HG1943; lane 3, BS LCL HG1525; lanes 4–6, B3R51-transfected cells; lane 7, R9-transfected cells; lane 8, control pREP9-transfected cells; and lane 9, control pREP4-transfected cells. Apparent molecular mass (in kD) is shown.

discrete nuclear foci as well as a diffuse distribution throughout the nucleus of BLM protein (fig. 2*A*, *C*, and *E*). Neither the foci nor the diffuse distribution of BLM is present in cells from most individuals with BS (fig. 2*B*, *D*, and *F*). Transfection of SV40-transformed BS fibroblasts with pOPRSVI-BLM restored both the nuclear bodies and the diffuse distribution of BLM to the cells (fig. 2*G*). As mentioned previously, the *BLM*-transfected BS lymphoblastoid cells probed with anti-BLM were indistinguishable from untransfected BS lymphoblastoid cells. We conclude that when *BLM* expression reaches a detectable level in transfected cells, the protein assumes a nuclear organization resembling that in normal cells.

SCE Analysis

In the eight clones of GM08505 BS fibroblasts that had received pOPRSVI-BLM the mean number of SCEs per 46 chromosomes was 24, whereas in the six GM08505 clones that had received pOPRSVI-CAT the mean number of SCEs per 46 chromosomes was 69 (fig. 3*A*). The SDs of *BLM*-transfected GM08505 clones increased with the mean numbers of SCEs, as did the differences in the high and low values of the ranges, whereas the SDs and ranges for control *CAT*-transfected GM08505 clones were roughly the same as those for untransfected cells. The three clones that exhibited the greatest reduction in SCEs were the three that expressed the highest levels of BLM (compare fig. 1*A* and fig. 3*A*).

The mean number of SCEs in the LCL transfected with R9 cDNA was 22, and the mean number of SCEs in the LCL transfected with B3R51 cDNA was 40 (mean of three transfection experiments). The means in the control cultures transfected with the corresponding vectors were 57 and 58 (fig. 3*B*). In the R9-transfected cultures, 8 of the 30 cells examined had $\leq$10 SCEs per metaphase (i.e., the number of SCEs in diploid cells from normal individuals), whereas no completely normal–SCE cell was detected in the B3R51-transfected cultures. The SDs of *BLM*-transfected and vector-transfected cultures were roughly the same; as expected, the high and low ranges in the *BLM*-transfected cultures are reduced compared with those of the vector-transfected cultures. The reduction in SCEs was greater with the *hyg*^{r}-containing vector (R9) than with the *neo*^{r}-containing vector (B3R51); however, because the levels of protein in *BLM*-transfected HG1525 cells were below the levels that could be detected by means of either western blot or immunofluorescence analysis (see the Nuclear Localization of BLM subsection, above), we could not determine whether the SCE-reduction difference between vectors was related to a difference in the levels of *BLM* expression.

Discussion

In the present study, our primary goal was the correction of a particularly distinctive abnormal phenotype of BS cells, namely, their striking SCE elevation. Transfection of the cDNA of the gene *BLM* into each of two types of BS cells did decrease the mean SCE frequencies. Furthermore, BLM protein became demonstrable by western blot analysis in one of the two cell types transfected, the fibroblasts, and by immunofluorescence microscopy BLM was found to have assumed a nuclear localization and distribution similar to that of normal (non-BS) fibroblasts. These experiments constitute proof, independent of earlier

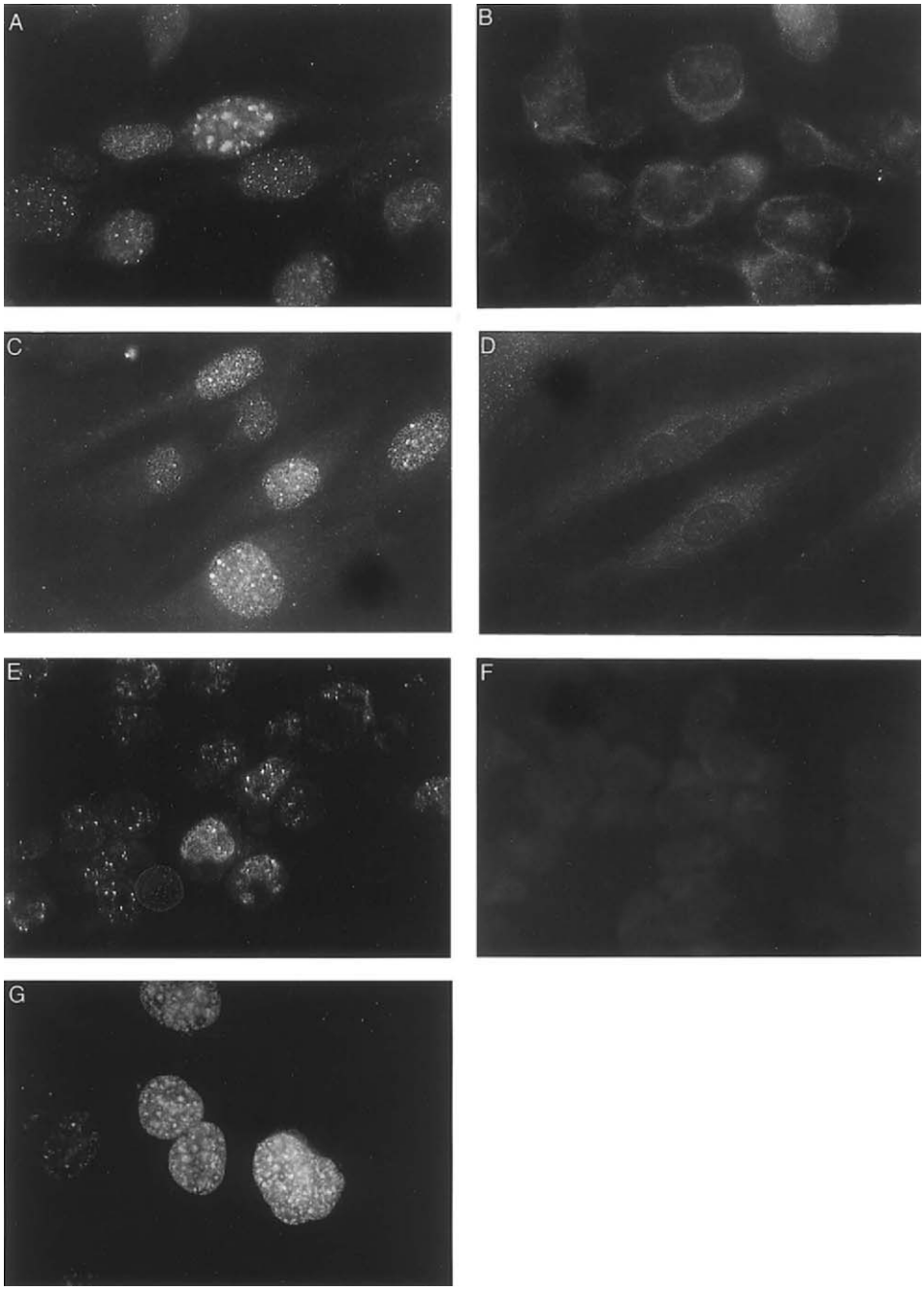

Figure 2 Indirect immunofluorescence of normal and BS cells stained with polyclonal rabbit anti-BLM antibodies to demonstrate focal nuclear localization. *A,* Normal control SV40-transformed FCL GM00637. *B,* BS SV40-transformed FCL GM08505. *C,* Normal control FCL HG2619. *D,* BS FCL GM01492. *E,* Normal control LCL HG1943. *F,* BS LCL HG2703. *G,* GM08505 clone 34 transfected with pOPRSVI-BLM.

mutational evidence, that the gene that we have isolated and referred to as "*BLM,*" is the gene that when mutated is responsible for BS.

Cells from individuals who are heterozygous for BS-associated mutations at *BLM,* that is, the parents of individuals with BS, have normal SCE rates. Therefore, successful transfection and expression of at least one nonmutant *BLM* allele into a BS cell, even if aneuploid, was predicted to reduce the SCE rate to the normal range. Although the mean numbers of SCEs in fibro-

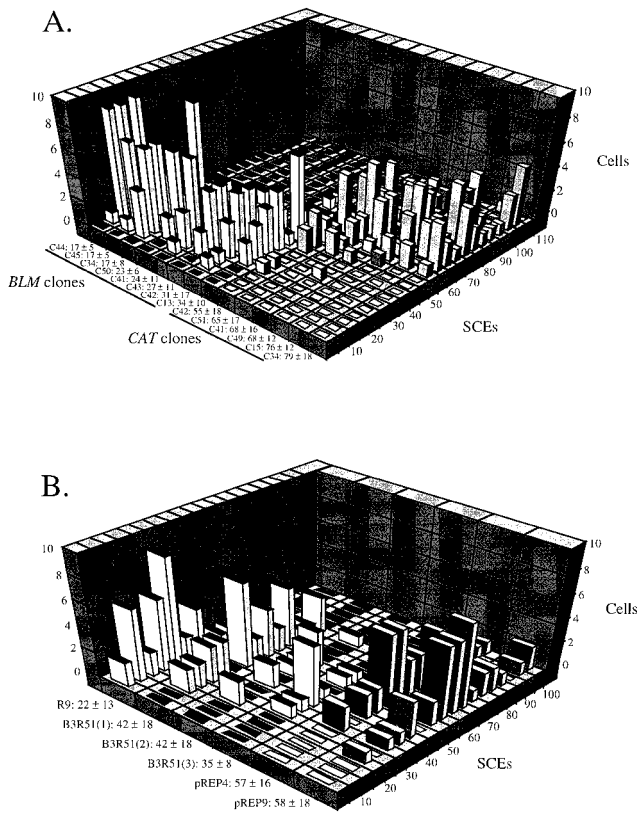

Figure 3 *A*, Distribution of SCEs in clones of SV40-transformed BS fibroblasts (GM08505) transfected with pOPRSVI-BLM or with the control pOPRSVI-CAT. Normal control GM00637 cells have 7 ± 2 SCEs/46 chromosomes, whereas BS GM08505 cells have 58 ± 16 SCEs/46 chromosomes. (The average number of chromosomes in GM00637 cells was 81, and the average number in GM08505 cells was 82.) Twenty-five metaphases were examined for all lines, with the exception of CAT clones 49 and 51, in which 20 cells were analyzed. *B*, Distribution of SCEs in cultures of BS lymphoblastoid cells (HG1525) transfected either with *BLM* (R9 and B3R51) or with control expression constructs (pREP4 and pREP9). Normal control HG1943 cells have 7 ± 3 SCEs per metaphase, whereas BS HG1525 cells have 69 ± 16 SCEs per metaphase. (These cells had 46 chromosomes.) At least 35 metaphases were examined for all lines, with the exception of B3R51(2) and B3R51(3), in which only 10 cells each were analyzed.

blasts transfected with *BLM* were not as low as in the normal control, they did approach the levels observed previously by others who had introduced into the same cells (GM08505) a normal *BLM* gene in situ in chromosome 15 (McDaniel and Schultz 1992). In our experiments, those clones of *BLM*-transfected fibroblasts that expressed the highest levels of protein exhibited the lowest mean SCE rate (fig. 1*A* and fig. 3*A*). These observations from different laboratories suggest that, for currently obscure reasons, the SCE reduction is as low as can be expected in experiments in which the aneuploid SV40-transformed GM08505 cells are the test recipient. By this argument, the SCE reduction observed in our experiments represents full correction of the high-SCE phenotype of GM08505 cells.

With respect to near-diploid EBV-transformed lymphoblastoid cells, transfection of the HG1525 cells with the different *BLM* cDNAs, R9 and B3R51, resulted in different mean numbers of SCEs (fig. 3*B*). Some fully corrected cells (<10 SCEs per metaphase) were found in the R9-transfected but not in the B3R51-transfected cultures; however, cultures transfected with both these cDNAs contained cells with either an intermediate- or a high-SCE phenotype. Sequence differences between R9 and B3R51 fail to explain the difference in SCE reduction, for they encode identical proteins. In addition, the low level of BLM expression in the lymphoblastoid transfectants determined by western blot analysis is puzzling (fig. 1*B*). To explain the cells with normal numbers of SCEs, we postulate that *BLM* expression in some of the R9-transfected lymphoblastoid cells is sufficient to provide full correction but that in others it falls short, and there an intermediate- or even high-SCE phenotype persists. By this interpretation, the cells in the R9-transfected cultures (*hyg*^{r}) in aggregate would be expressing more BLM than those in the B3R51-transfected cultures (*neo*^{r}).

The experiments reported fulfill the prediction made in the first paragraph of our Introduction. Now, with the successful transfection of *BLM* into BS cells and correction of the marker-phenotype SCE elevation, the way lies open for structure-function experiments employing variously mutated *BLM* genes. Transfection of BLM cDNA constructs experimentally altered to encode proteins with specific amino acid substitutions and deletions will help determine the portions of the protein that are required both for BLM's characteristic nuclear distribution and for its colocalization with other nuclear proteins at certain periods of the cell cycle (observations to be reported), thence an understanding of the function(s) of BLM in chromosome mechanics and DNA metabolism. The work possibly deserves extension to untransformed diploid BS cells with a view toward examination of the feasibility of gene therapy for BS.

Finally, the experience gained in this work indicates that the SCE assay remains the most reliable test available for the presence of a normal allele at the BS locus, specifically an allele that is being transcribed and from which a functional BLM protein is produced.

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

GenBank, http://www.ncbi.nlm.nih.gov/ (for BS [U39817])
Online Mendelian Inheritance in Man (OMIM): http://www.ncbi.nlm.nih.gov/Omim (for BS [210900])

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