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The DNA Helicase Activity of BLM Is Necessary for the Correction of the Genomic Instability of Bloom Syndrome Cells

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Bloom syndrome (BS) is a rare autosomal recessive disorder characterized by growth deficiency, immunodeficiency, genomic instability, and the early development of cancers of many types. BLM, the protein encoded by *BLM*, the gene mutated in BS, is localized in nuclear foci and absent from BS cells. *BLM* encodes a DNA helicase, and proteins from three missense alleles lack displacement activity. *BLM* transfected into BS cells reduces the frequency of sister chromatid exchanges and restores BLM in the nucleus. Missense alleles fail to reduce the sister chromatid exchanges in transfected BS cells or restore the normal nuclear pattern. *BLM* complements a phenotype of a *Saccharomyces cerevisiae sgs1 top3* strain, and the missense alleles do not. This work demonstrates the importance of the enzymatic activity of BLM for its function and nuclear localization pattern.

INTRODUCTION

Bloom syndrome (BS) is a rare autosomal recessive trait (German, 1993; German and Ellis, 1997). The major clinical manifestations are small stature, sun-sensitive redness of the face, immunodeficiency, male infertility, a predisposition to diabetes, and the development of early cancers of many types. Cells derived from persons with BS exhibit increased numbers of chromatid gaps, breaks, and sister chromatid exchanges (SCEs). Somatic mutations of many types have been documented at multiple loci (reviewed in German, 1993). Biochemical studies have demonstrated a slow replication-fork progression and an abnormal distribution of DNA replication intermediates. Some BS cell lines exhibit increased sensitivity to DNA-damaging agents such as mitomycin C, *N*-nitroso-*N*-ethylurea, and ethyl methanesulfonate. Alterations in several enzymes involved in DNA replication and repair have been identified in some but not all BS cell lines (see references in Ellis *et al.*, 1995a).

Despite this accumulation of biochemical evidence of disturbances in DNA metabolism, no consistent defect or candidate gene product could be identified by these approaches.

The Bloom syndrome gene was cloned using molecular haplotype analysis of affected families and positional cloning methodologies (Ellis *et al.*, 1995a). The mapping of the gene was facilitated by the observation that lymphocytes from affected compound heterozygotes can revert to a normal low SCE frequency phenotype by virtue of recombination within the two copies of the *BLM* gene itself (Ellis *et al.*, 1995b). These normal circulating cells arise because of a rare somatic recombination event between the maternal and paternal chromosome 15s and generate cells containing a wild-type gene. Molecular haplotype analysis of low-SCE cells and high-SCE cells from several affected individuals narrowed the *BLM* locus to a 250-kilobase region at 15q 26.1. Expressed DNA sequences from this region were selected, and a cDNA clone was found encoding a 1417 amino acid protein with strong amino acid sequence homology with the RecQ family of DNA helicases. DNA sequence analysis of *BLM*

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cDNAs from persons with BS is consistent with recessive, loss of function mutations (Ellis *et al.*, 1995a).

The RecQ family members (Umezū *et al.*, 1984; Gangloff *et al.*, 1994; Puranam and Blackshear, 1994; Ellis *et al.*, 1995a; Watt *et al.*, 1995; Lu *et al.*, 1996; Yu *et al.*, 1996; Stewart *et al.*, 1997; Davey *et al.*, 1998) have seven conserved helicase motifs, inserted between unique N-terminal and C-terminal domains of variable size. The highest percentage of amino acid identities (~40%) among these gene products is in the 350 amino acid helicase region. Recent database searches predict a small nucleic acid binding domain in the C-terminal regions of all of the members of the RecQ family (Morozov *et al.*, 1997), suggesting common DNA binding/recognition features in the two helicases as well as a region of limited identity (25%) C-terminal to the helicase domain in all the family members. A predicted 3' to 5' exonuclease domain has been identified in the N-terminal region of WRN (Mushegian, *et al.*, 1997) that is not present in the other large members of the family.

The BLM structure is similar overall in amino acid charge distribution and in size to Sgs1p from *Saccharomyces cerevisiae* (Gangloff *et al.*, 1994; Watt *et al.*, 1995; Lu *et al.*, 1996), the *Schizosaccharomyces pombe* Rqh1 gene product (Stewart *et al.*, 1997; Davey *et al.*, 1998), and the human WRN gene product (Yu *et al.*, 1996). The *S. cerevisiae* *SGS1* gene was identified by its physical and genetic interaction with three different topoisomerase genes (Gangloff *et al.*, 1994; Watt *et al.*, 1995; Lu *et al.*, 1996). Yeast cells containing *sgs1* mutations are viable but somewhat slow growing and hyper-recombinogenic (Gangloff *et al.*, 1994; Watt *et al.*, 1996). Significant numbers of spores are inviable, and an increase in mitotic nondisjunction is found (Watt *et al.*, 1996), suggesting a defect in the maintenance of genomic integrity. New data suggest a role for Sgs1p in maintaining the stability of rDNA repeats because the nucleolar structure appears to fragment prematurely in aging yeast cells containing *sgs1* mutations (Sinclair and Guarente, 1997; Sinclair, *et al.*, 1997). The *S. pombe* gene *rqh1+* was identified independently by two different genetic approaches. Cells containing *rqh1* mutations show a hyper-recombination phenotype and hydroxyurea (HU)-dependent cell cycle checkpoint defects or are UV-sensitive (Stewart *et al.*, 1997; Davey *et al.*, 1998). Persons with Werner syndrome are generally normal until the second decade of life, when they begin to show symptoms of a premature aging-like disorder (Epstein *et al.*, 1966). Cells from persons with Werner syndrome contain loss of function alleles of the WRN gene and show chromosome instability and an elevated frequency of somatic mutation (Hoehn *et al.*, 1975; Fukuchi *et al.*, 1989).

Research into the pathways of DNA repair and recombination in bacteria has led to the proposal by Galitski and Roth (1997) that the RecF pathway of

which RecQ is a member is responsible for the recognition and repair of single-stranded gaps in the chromosome. A study of recombination events in bacteriophage lambda demonstrates a role for RecQ in reducing illegitimate recombinational events (Hanada *et al.*, 1997). The RecQ protein and most of the other members of the RecQ family of DNA helicases have the demonstrated ability to recognize and bind to single-stranded gaps in vitro and displace an oligonucleotide in the 3' to 5' manner (Umezū *et al.*, 1984; Puranam and Blackshear, 1994; Lu *et al.*, 1996; Gray *et al.*, 1997; Karow *et al.*, 1997; Bennett *et al.*, 1998). Studies of the yeast genes suggest that the larger members of the family may function during S phase to prevent recombinational events between repeated sequence elements in the genome that would lead to chromosomal entanglements. Therefore cells containing mutations in the largest members of the RecQ family have phenotypes consistent with the loss of function of an enzyme required for genomic stability. None of these larger gene products nor RecQ encodes an essential gene, and most cells lacking these gene products show alterations in recombination and genomic stability, suggesting that the DNA helicase activity of these proteins is important in maintaining chromosomal integrity.

MATERIALS AND METHODS

Plasmid Constructions

Escherichia coli Expression Vectors. The BLM cDNA B3 (Ellis *et al.*, 1995a) was cloned into the *XhoI* site of the T7 RNA polymerase-dependent expression vector pET14b (Novagen, Madison, WI) to create plasmid A3ET. This construction contains a six-histidine epitope tag at the amino terminus of BLM. A deletion of the C terminus of the BLM cDNA was made from plasmid A3ET by digesting with *StuI* (nucleotide 1367) and *BamHI*, followed by reclosure of the plasmid. This construction (pA3NET4) was used for antigen production (see below). Plasmid vectors were constructed using standard DNA technology (Sambrook *et al.*, 1989).

Mammalian Expression Vectors. The BLM cDNA R12 (Ellis *et al.*, 1995a) was cloned into the *NotI* site of mammalian expression vector pOPRSVI-CAT (Stratagene, La Jolla, CA), replacing the CAT gene. Three missense mutations were constructed in this normal BLM cDNA using a site-directed mutagenesis kit (Clontech, Palo Alto, CA). Position 672 (glutamate) was altered to arginine (A2089G) [5'-GCATAATTTTGAAGACTAATCGGCTAGAGGCG-3'], position 695 (lysine) was changed to threonine (AG2157CC) [5'-CTGGAGGTGGTACCAGTTTGTGTTACC-3'], and position 1055 (cysteine) was changed to serine (G3238C) [5'-CCTGATTTTCTAAGAAA-CACCC-3'].

Yeast Expression Vectors. The normal BLM cDNA B3 was inserted into the yeast expression vector pYES2 (Stratagene) behind the GAL1 promoter (pB3YES3). This BLM gene was modified at the 3' end to contain a six-histidine epitope tag (pC4YES3). Plasmids were introduced into yeast cells by LiOAc transformation (Golemis *et al.*, 1998). The missense mutation-containing genes, originally made in the mammalian expression vector (above), were moved into the yeast vector in a one-step procedure using plasmid gap-repair (Kunes *et al.*, 1987). Plasmid pC4YES3 DNA was digested with *BglIII*

and *Sall* to create a gap of ~2 kilobases within the *BLM* cDNA. The missense mutation constructions were digested with *NotI* to release the *BLM* cDNA from the vector sequences. Approximately equal amounts (1 μ g) of digested yeast expression construction DNAs were mixed together and transformed into AMR61 cells. Ura⁺ colonies were screened by PCR analysis and DNA sequencing to confirm the introduction of the missense alleles. This method was highly efficient because 20 of 20 clones analyzed by PCR and restriction mapping contained the new *KpnI* site introduced by the K to T mutation, 2 of 2 analyzed by DNA sequencing picked up the Q to R mutation, and 3 of 4 contained the C to S mutation. Standard yeast media containing glucose or galactose was used (Sherman, 1991). HU was purchased from Sigma (St. Louis, MO). The *S. cerevisiae* strains used in this study are W3031a = *a ura3-1 trp1-1 can1-100 ade2-1 leu2-3, 112 his3-11,15* (Thomas and Rothstein, 1989); AMR59 = *a top1::LEU2 sgs1-3::TRP1 ura3-1 trp1-1 can1-100 ade2-1 leu2-3,112 his3-11,15*; and AMR61 = *a top3::HIS3 sgs1-3::TRP1 ura3-1 trp1-1 can1-100 ade2-1 leu2-3,112 his3-11,15* (Lu *et al.*, 1996). Growth rates were measured from 36 h cultures of transformed colonies in yeast minimal medium with 2% raffinose as the carbon source. The cultures of cells were diluted into yeast minimal medium containing 2% glucose or 2% galactose, supplemented with needed amino acids and adenine, at an A_{600 nm} of ~0.1 to begin the experiment. When the optical density of the cultures began to increase (usually 2–3 h after dilution), time points for growth rates at 30°C were measured every 1–3 h up to 9 h.

Fibroblast Cell Transfection and SCE Analysis

Human fibroblast cell lines HG2855 (GM00637) SV40-transformed normal and HG2522 (GM08505) SV40-transformed Bloom syndrome were obtained from the Coriell Institute for Medical Research (Camden, NJ). HG2619 was generated in this laboratory from a piece of skin from a normal male using standard techniques (Korf, 1997). HG2940 is a fibroblast cell line generated from a piece of skin from a 3-y-old female with Bloom syndrome [(JePa) Bloom Syndrome Registry designation]. These cells were cultured in DMEM and 4 mM L-glutamine (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT).

HG2522 cells (10⁵) were seeded into 35-mm tissue culture wells. After 48 h the cells were transfected with 5 μ g of plasmid DNA and 7.5 μ g of LipofectAMINE (Life Technologies) according to the supplier's recommendations. The cells were grown for 3 d in DMEM containing 10% FBS and then in the same medium plus 200 μ g/ml Geneticin (Life Technologies) until clones formed. Eight clones containing the normal *BLM* cDNA, seven clones with the control CAT-containing plasmid, and three of each of the *BLM* missense mutation genes were chosen for SCE analysis.

Differential staining of sister chromatids was modified from Goto *et al.* (1975). Cells were cultured in the presence of 10 μ M BrdU (Sigma) in the dark at 37°C in 5% CO₂ for 48 h and subsequently were harvested by standard techniques. Slides made from the cell suspensions fixed in methanol:acetic acid (3:1) were allowed to air-dry overnight protected from light. Cells were stained with 50 μ g/ml Hoechst 33258 (Sigma) for 10 min and rinsed in distilled water. The preparations were mounted under a coverslip in citric acid-phosphate buffer at pH 7.0 and were exposed to a 150-W plant light (Duro-lite; Sylvania, St. Mary's, PA) for 1–2 h at a distance of 20–25 cm. Slides were rinsed in distilled water and stained in 2% Giemsa (Harleco, Wright Giemsa; EM Diagnostics, Gibbstown, NY) diluted in Gurr's buffer, pH 6.8, for 10 min. The slides were rinsed with water and allowed to air dry. Preparations were mounted in Permount (Fisher, Pittsburgh, PA).

Expression and Purification of BLM

Yeast cells (AMR61) transformed with plasmids containing the normal *BLM* cDNA and the missense alleles were grown at 30°C in yeast minimal medium (1 \times Yeast Nitrogen Base, Difco, Detroit, MI

+ 2% raffinose (Sigma) + 50 μ g/ml adenine, tryptophan, histidine, and leucine (Sherman, 1991). When the A_{600 nm} of the culture reached 0.3–0.6, the expression of BLM was induced by adding 2% galactose (Sigma). After 5 h, the cells were harvested by centrifugation and stored frozen at –70°C.

All steps in the purification of BLM use Buffer A (50 mM HEPES, pH 7.5, 100 mM KCl, 10% glycerol, and 0.01% NP40) and are carried out at 4°C. Breaking buffer is Buffer A + 5 mM β -mercaptoethanol + 1 mM PMSF + 1 \times "Complete" protease inhibitors (Boehringer Mannheim, Indianapolis, IN) added just before use. Cells were broken according to standard methods using acid-washed glass beads (Golemis *et al.*, 1998). The cleared crude lysate (10 ml of 5 mg/ml total protein) is applied to a small phosphocellulose column (P11, Whatman) (6 \times 1.5 cm diameter) equilibrated in breaking buffer. The column is washed with breaking buffer, followed by two washes of increasing NaCl steps (Buffer A + 150 mM NaCl; Buffer A + 300 mM NaCl). After initial load and wash with breaking buffer, 1 μ g/ml each leupeptin and pepstatin, and 5 mM benzamide are substituted for the protease inhibitor mixture. The BLM elutes in Buffer A + 700 mM NaCl. The high salt fractions containing BLM by silver staining or Western analysis (4 ml of 1–2 mg/ml total protein) are pooled and diluted with an equal volume of Buffer A. This fraction is bound to 0.2–0.5 ml of the Talon metal chelate chromatography resin (Clontech) by batch incubation. The resin is washed with wash buffer A + 400 mM NaCl and buffer A + 1 M NaCl. The metal resin is then washed with wash buffer A + 5 mM imidazole and packed into a small column. The column is washed with five column volumes of buffer A + 10 mM imidazole followed by a 20 mM imidazole wash. Elution buffer is A + 50 mM imidazole (yield is ~1–5 μ g/l). Protease inhibitors were purchased from Boehringer Mannheim, protein reagents and apparatus were from Bio-Rad (Hercules, CA), and other reagents were from Sigma. Protein concentrations of final fractions are determined by comparison to a bovine serum albumin standard (1–100 ng/lane) on an 8% SDS polyacrylamide gel stained with silver (Bio-Rad).

Helicase Assays

Oligonucleotide Displacement Assay. The substrate for the reactions consists of a [³²P]-labeled oligonucleotide of 17–54 bases in length annealed to Mp18 ssDNA. The substrate was separated from unincorporated nucleotides and unannealed oligonucleotides by use of a spin column (Bio-Rad 30 or Clontech 1000). Assays were performed at 37°C using published conditions (Seo and Hurwitz, 1993). The activity of the helicase was calculated as percentage displacement relative to 100% displacement, determined by heating one reaction tube to 95°C before running the 12% polyacrylamide gel (19:1) in 1 \times Tris borate–EDTA. The helicase displacement activity was quantitated using a Molecular Dynamics (Sunnyvale, CA) Storm PhosphorImager and Imagequant software. One unit of activity is that amount of enzyme that will displace 10% of the labeled oligonucleotide in 30 min at 37°C. Single-stranded Mp18 DNA was purchased from US Biologicals (Cleveland, OH) or New England Biolabs (Beverly, MA). Oligonucleotides were made by Life Technologies. The oligonucleotides used for the helicase substrates were HS1 (5'-GTAAAACGACGGCCAGT-3'); HS2 (5'-CGACG-GCCAGTGCCAAGCTTGCATGCCTGCAGGTCGACTCTAGGAT-3'); HS3 [(dT)₁₅HS1]; HS4[(dT)₁₅HS2]; HS5[HS1(dT)₁₅]; and HS6[(dT)₁₅HS1(dT)₁₅]. The polarity of the BLM helicase activity was determined using oligonucleotide HS2 labeled at the 5' end with T4 polynucleotide kinase (Boehringer Mannheim) and [γ -³²P]ATP, and/or at the 3' end using Klenow polymerase (New England Biolabs) and [α -³²P]dCTP and dGTP to extend the length to 54 bases. This oligonucleotide spans the multicloning site of Mp18 DNA. Digestion with *PstI* yields two end-labeled oligonucleotides of 26 and 28 bases situated at opposite ends. Reactions are quantitated as described above.

DNA-dependent ATPase Activity. This assay measures the DNA-dependent hydrolysis of [³²P] P_i from [γ -³²P] ATP. One enzyme unit

is that amount of BLM that hydrolyzes 1 pmol of ATP per minute at 37°C with 1 mM ATP and 25 ng/ μ l poly dA:poly dT₁₂. Reactions (25–100 μ l) were stopped by the addition of 5–20 μ l of 0.1 M EDTA, pH 8, + 1% SDS. Aliquots (1 μ l) of the reaction were spotted on polyethyleneimine (Fisher) cellulose thin layer plates and developed in LiCl and formic acid as described (Seo and Hurwitz, 1993). Substrates were purchased from Sigma (herring sperm DNA) or Pharmacia (Piscataway, NJ) (poly dA:poly dT₁₂). ATPase activity was quantitated using a Molecular Dynamics PhosphorImager. Calf intestinal phosphatase (Boehringer Mannheim) was used as a positive control for ATP hydrolysis.

Antibody Production and Purification

Plasmid A3NET3 contains the 5' end of the *BLM* cDNA from translation start to the *StuI* site at nucleotide 1367. This T7 RNA polymerase-dependent construction (pET14b, Novagen) was expressed in *E. coli* BL21 (DE3) cells by induction with isopropyl- β -D-thiogalactoside (Studier *et al.*, 1990). The six histidine-tagged N-terminal region of BLM (~45 kDa) was extracted under denaturing conditions and bound to Ni-NTA resin (Qiagen, Hilden, Germany) according to the supplier's recommendations. The antigen preparation was eluted in pH 4.5 buffer containing 8 M urea, neutralized with 1 M Tris-base, and stored frozen at -70°C. Each rabbit received 50–100 μ g of the antigen preparation once every 4–6 wk, and after 3 mo they began to produce detectable antibodies against BLM. The immune sera was pooled at 4°C and mixed with an equal volume of saturated ammonium sulfate, pH 7.0. The 50% ammonium sulfate precipitate was collected by centrifugation, dissolved in a minimal volume of cold 1 \times PBS, and dialyzed overnight at 4°C against 1 \times PBS. The dialysate was collected and passed at 4°C over a Sepharose 4B column (~1 ml) that contained 500 μ g/ml of bound antigen. The column was prepared from CNBr-activated Sepharose 4B (Pharmacia) activated and washed according to the manufacturer's recommendations. The column was washed with 10 column volumes of 1 \times PBS and eluted with 0.2 M glycine pH 3.5 buffer. The protein containing fractions were neutralized with 1 M Tris-base and stored at 4°C for daily use and at -20°C for long-term storage (Harlow and Lane, 1988).

Western Analysis and Immunofluorescence

Cultured cells (10⁶–10⁷) were harvested and stored as frozen pellets at -70°C. Whole-cell protein extracts were prepared by lysing the cells in 5 vol of RIPA buffer (Harlow and Lane, 1987) and 1 \times Complete protease inhibitors (Boehringer Mannheim) and 1 mM PMSF on ice. Protein concentrations of the cleared lysates were determined by the method of Bradford (1976) (Bio-Rad). Equal amounts of protein (20 μ g) were loaded onto each lane of a 5% SDS polyacrylamide mini-gel (Bio-Rad). After electrophoresis the proteins were transferred electrophoretically to Immobilon PVDF membranes (Millipore, Bedford, MA) overnight at 15 V in a Tris-glycine buffer (Harlow and Lane, 1987). The dried membranes were blocked in 1 \times PBS plus 5% nonfat dried milk and incubated with affinity-purified BLM antisera diluted in the same solution plus 0.1% Tween 20 (Sigma). The membrane was developed with the ECL detection system (Amersham) according to the manufacturer's instructions and exposed to BioMax x-ray film (Kodak, Rochester, NY).

Indirect immunofluorescence detection of BLM was performed on fibroblast cells grown on Fisher Superfrost Plus glass microscope slides (Fisher). The cells were fixed in methanol:acetone (1:1) at room temperature for 2 min. Slides were blocked with cold 1 \times PBS + 0.5% BSA (Sigma) + 0.2% cold-water fish gelatin (Sigma) for 1 h and stained with affinity-purified anti-BLM, followed by Texas Red-conjugated donkey anti-rabbit secondary antibodies (Jackson ImmunoResearch, West Grove, PA). The slides were washed with 1 \times PBS + 0.4% Tween 20 and stained with DAPI using a published protocol (Harlow and Lane, 1987).

RESULTS

Expression of Transfected Normal and Mutant *BLM* cDNAs in Bloom Syndrome Cells

Bloom syndrome cells have a high frequency of SCEs (German, 1993). This cytogenetic assay is diagnostic for BS and was used to determine whether the normal *BLM* cDNA was sufficient to reduce the frequency of SCEs in BS cells. Plasmid constructions containing a normal *BLM*, a control chloramphenicol acetyltransferase (CAT) gene, or missense mutant cDNAs were transfected into Bloom syndrome cells (HG2522). HG2522 is an SV40-transformed fibroblast cell line and is used for the present experiments because these cells have a high mitotic index for SCE assays and they transfect reproducibly. Stable cell lines were cloned from primary transfectant pools using limiting dilution and selection for resistance to G418 (Geneticin). The cell lines were evaluated for SCE and for the presence of BLM by immunofluorescence and Western analysis.

Stable transfection of the normal *BLM* cDNA reduced the mean number and range of SCEs in HG2522 cells (Figure 1). A report of the complete cytogenetic analysis of the transfection of the normal *BLM* cDNAs into SV40-transformed BS fibroblasts and BS lymphoblastoid cell lines will be published elsewhere (our unpublished results). Cells transfected with the control CAT gene or any of the three missense *BLM* genes failed to show reduction (Figure 1C). Two of the missense genes (Q672R and C1055S) were identified in affected individuals (Ellis *et al.*, 1995a), whereas the third (K695T) is a constructed mutation in the ATP-binding site (GKT/S) present in the first conserved helicase motif. These data demonstrate that stable transfection of the normal *BLM* cDNA lowers the high SCE phenotype of BS cells, and the three missense genes tested fail to alter the frequency of SCEs.

Normal human fibroblasts and BS fibroblasts were evaluated for BLM expression by indirect immunofluorescence and Western analysis (Figures 2 and 3). Normal human fibroblasts have variable amounts of nuclear localized BLM that is organized in both small foci and more diffuse patches (Figure 2A). SV40-transformed normal fibroblasts have more BLM than untransformed fibroblasts per microgram of total cell protein (Figure 3, lanes 1 and 2), and the nuclear staining pattern shows small foci and more of the patches (Figure 2B). BS cells, either fibroblasts (HG2940) or SV40-transformed fibroblasts (HG2522), lack nuclear staining with BLM antibody (Figure 2, C and D), and lack BLM by Western analysis (Figure 3, lane 5). The HG2522 cell line is derived from an Ashkenazi Jewish individual [42(RaFr) Bloom Syndrome Registry designation] and is homozygous for the *blm*^{Ash} frameshift mutation (our unpublished results) (Ellis *et al.*, 1995a).

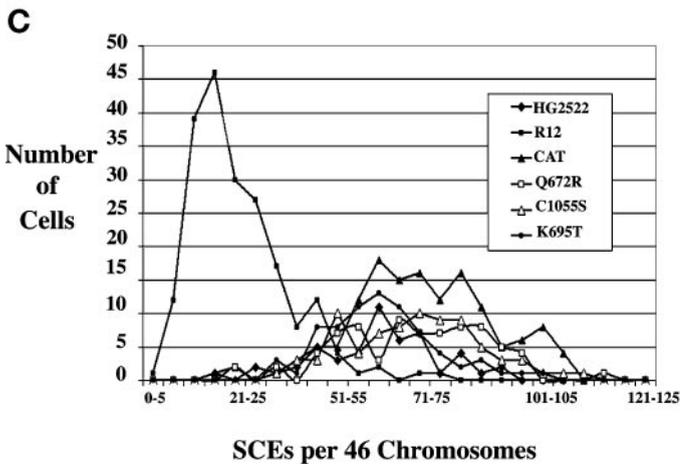
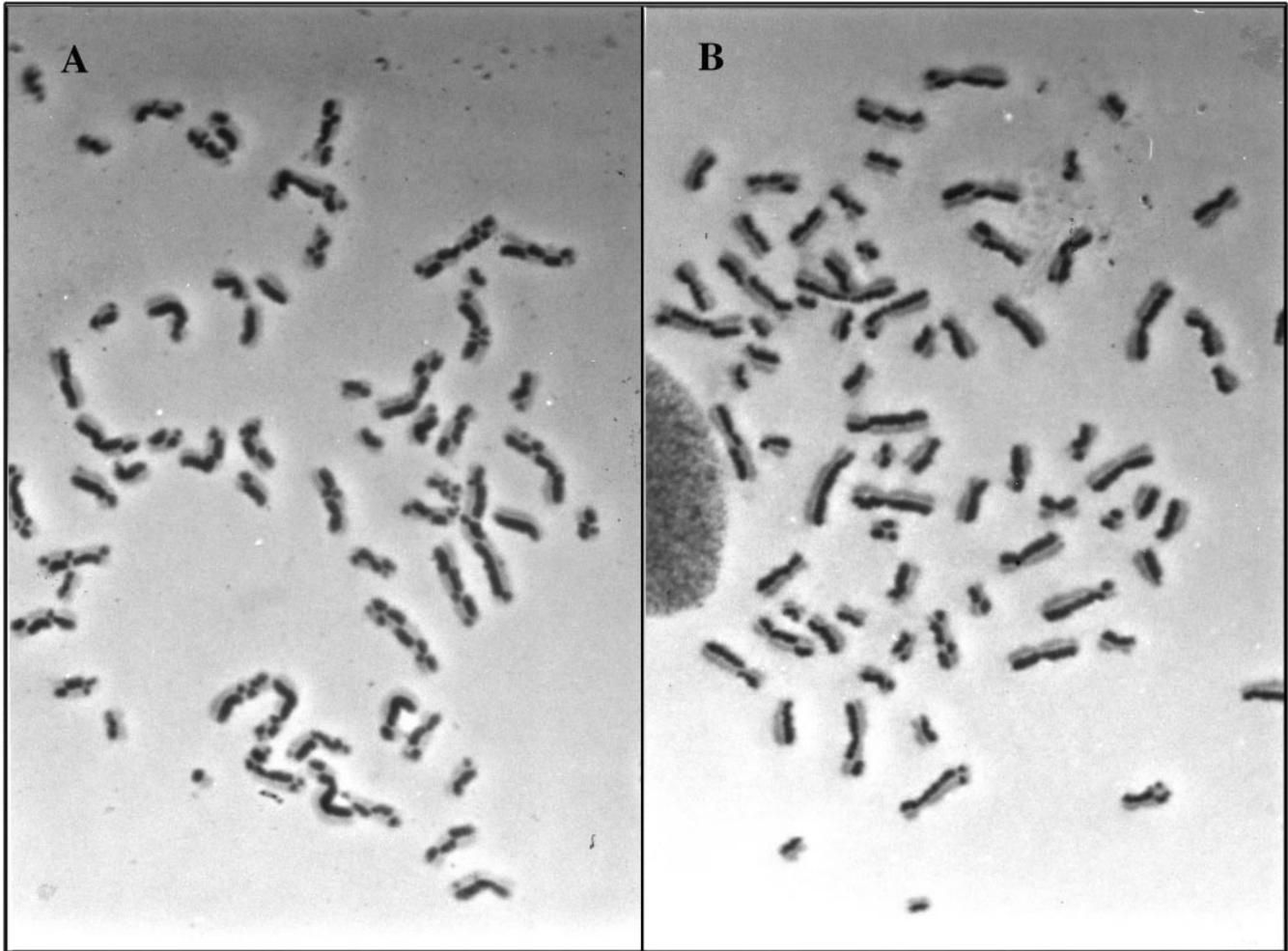


Figure 1. SCE assay. (A) Metaphase chromosomes from SV40-transformed BS fibroblast cell line HG2522 differentially stained to show the high frequency of SCEs. (B) Metaphase chromosomes from SV40-transformed BS fibroblast cell line HG2522 transfected with the normal BLM cDNA. (C) A graphic display of the range and distribution of SCEs in the fibroblast cell lines transfected with cDNAs encoding normal and missense BLM proteins. The mean number of SCEs per 46 chromosomes and range are as follows: HG2522 (55 and 16–88); CAT (55 and 18–105); R12 [WT] (24 and 5–74); Q672R (71 and 38–104); K695T (59 and 33–98); and C1055S (67 and 39–100). The values reported for cell line HG2522 and for the R12-transfected line represent experimental baseline numbers for these cell lines. At least 25 metaphases were counted for each transfected cell line.

When the normal *BLM* cDNA is transfected into HG2522 cells, the nuclear staining with BLM antibody is restored (Figure 2E; Figure 3, lanes 3 and 4). The missense proteins are expressed in the stable cell lines

to a lesser extent than in the cells transfected with the normal *BLM* cDNA (Figure 3, lanes 6–8). The cells expressing the missense BLM proteins show a nuclear staining pattern with little or no focal concentration of

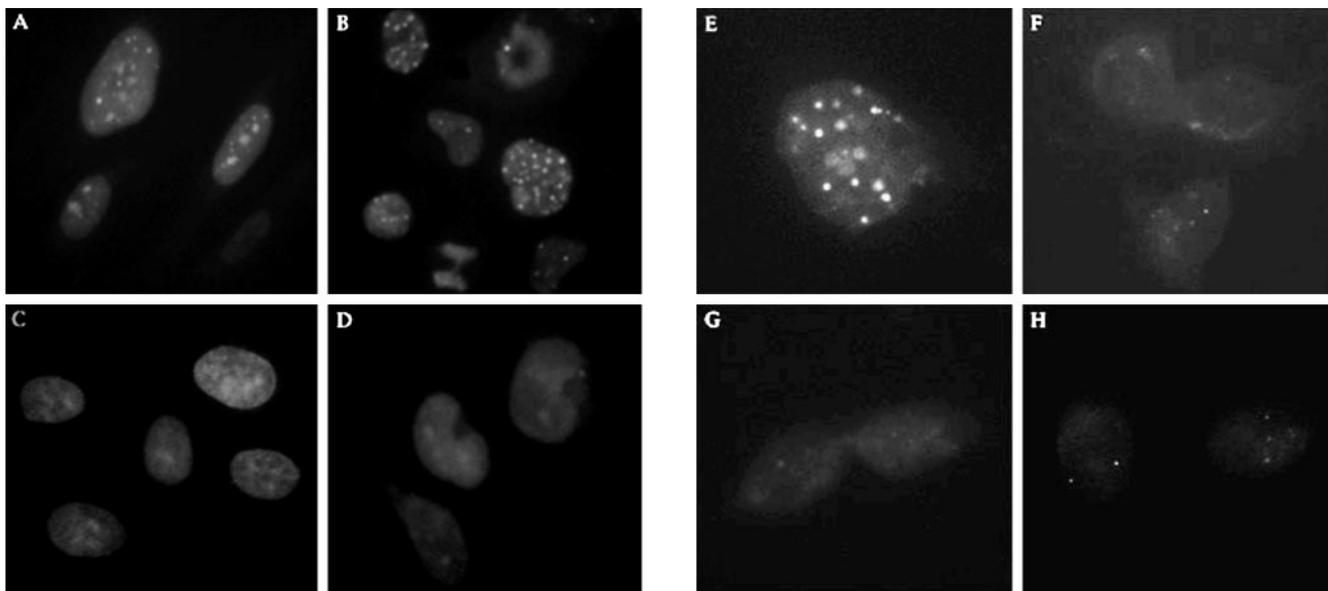


Figure 2. Indirect immunofluorescence study of BLM in the nucleus of normal, Bloom syndrome, and SV40-transformed human fibroblast cell lines. All cells are fixed and stained with BLM antibodies, followed by donkey anti-rabbit secondary antibodies conjugated to Texas Red. Cells in A–E are stained with DAPI. Cells in F–H are not stained with DAPI to show diffuse BLM staining. (A) Normal human fibroblasts (HG2619). (B) SV40-transformed normal human fibroblasts (HG2855). (C) BS fibroblasts (HG2940). (D) SV40-transformed BS fibroblasts (HG2522). (E) HG2522 transfected with the normal *BLM* cDNA. (F) HG2522 transfected with the Q672R cDNA. (G) HG2522 transfected with the C1055S cDNA. (H) HG2522 transfected with the K695T cDNA.

the antigen (Figure 2, F and G). The only foci seen in the nucleus of the cells transfected with the missense cDNAs are a few small dots. The cells expressing the C1055S allele have a diffuse nuclear distribution of this missense BLM protein. The missense proteins fail to reduce the SCEs and fail to localize in the normal pattern.

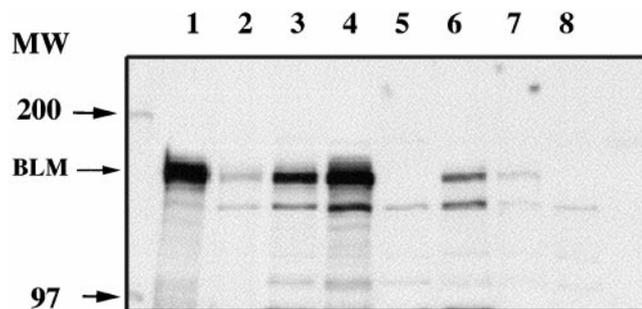


Figure 3. Western transfer analysis of BLM proteins in cell lines and transfected clones. Twenty micrograms of total cell protein were loaded on each lane, and proteins were displayed on a 5% SDS polyacrylamide gel and transferred to a PVDF membrane. Positions of molecular weight markers and BLM are indicated by arrows. Lane 1: SV40-transformed normal fibroblast cell line (HG2855); lane 2: normal fibroblast cell line (HG2619); lane 3: SV40-transformed BS fibroblast cell line (HG2522) transfected with the normal *BLM* cDNA (R12c41); lane 4: HG2522 transfected with the normal *BLM* cDNA (R12c45); lane 5: HG2522; lane 6: HG2522 transfected with the Q672R allele; lane 7: HG2522 transfected with the C1055S allele; lane 8: HG2522 transfected with the K695T allele.

Expression and Purification of Normal and Mutant BLM Proteins

The normal *BLM* cDNA was cloned into the T7 RNA polymerase-dependent *E. coli* expression vector pET14b (Studier *et al.*, 1990). No evidence of production of BLM could be found in several *E. coli* strains tested. This construction was truncated to make a 45-kDa N-terminal fragment of BLM for antigen production.

The normal *BLM* cDNA was cloned into the yeast expression vector pYES2 (Stratagene) under control of the *GAL1* promoter. This high copy-number plasmid construction was transformed into *S. cerevisiae* AMR61 cells by complementation of the *ura3-1* mutation in the cells by the *URA3* gene on the plasmid. These cells contain *sgs1* and *top3* mutations as well (Lu *et al.*, 1996). The polyclonal rabbit BLM antibody was used to monitor the purification (Figure 4A). Small amounts of highly purified BLM could be recovered for assays (Figure 4, A and B).

The normal BLM purified from yeast had DNA helicase displacement activity (Matson and Kaiser-Rogers, 1990; Lohman and Bjornson, 1996) (Figure 4B). The size of the oligonucleotide (17–54 bases) or the presence of a 5' or 3' poly(dT)₁₅ nonhomologous tail had no effect on the activity. All substrates tested are used equally well by the enzyme (6 U/ng). The activity is dependent on time of incubation and amount of enzyme and ATP (or dATP) and is inhibited >95% by

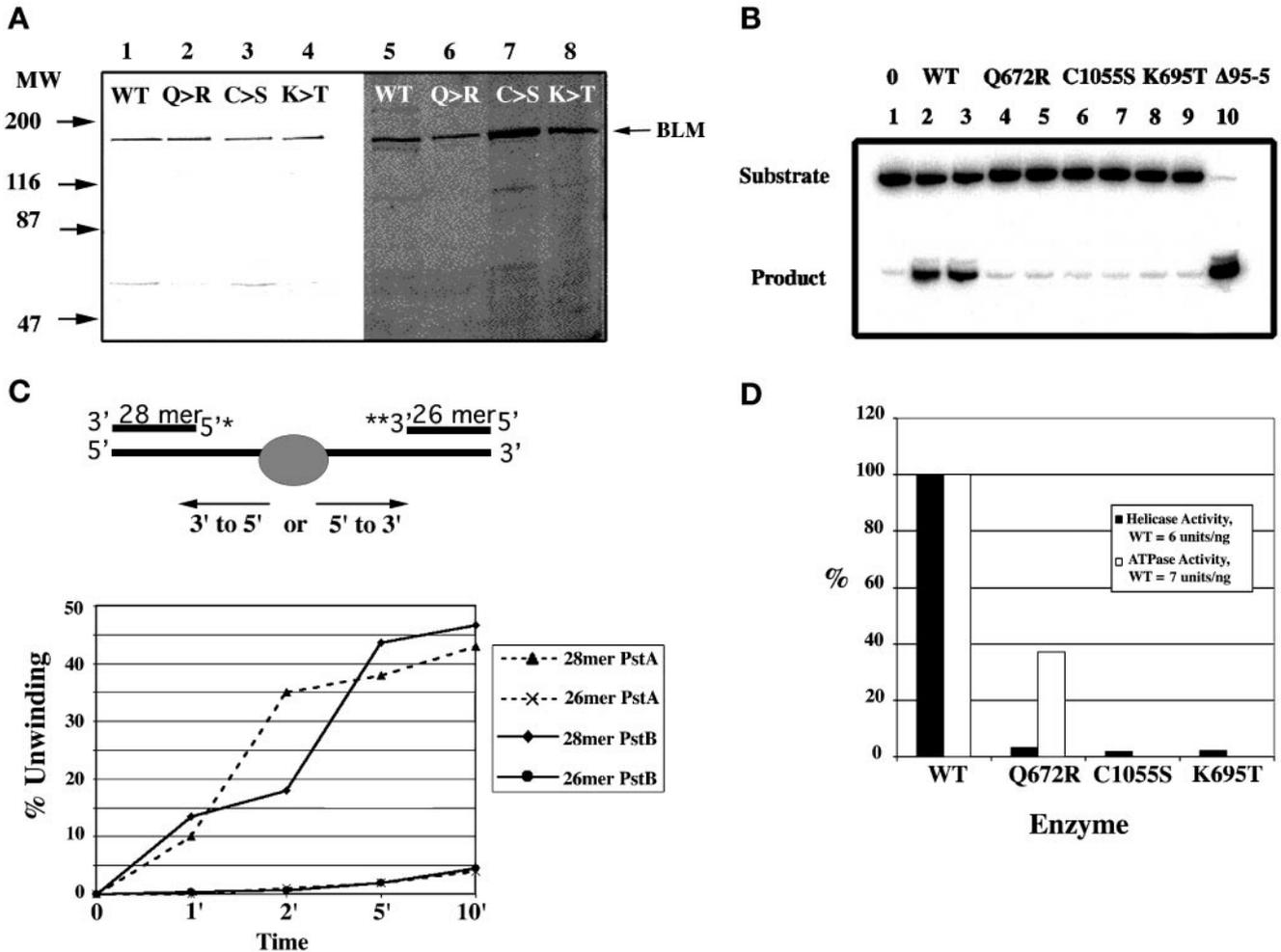


Figure 4. Purification and DNA helicase activity of BLM. (A) Isolation of normal and missense proteins of BLM. The left panel (lanes 1–4) contains isolated BLM proteins transferred to a PVDF membrane and reacted with the BLM antisera. Equal amounts of recovered protein are loaded in each lane. The right panel is a silver-stained polyacrylamide gel of the isolated proteins (lanes 5–8). Equal volumes of equivalent fractions from each preparation are loaded to show relative recoveries of the different proteins from yeast strain AMR 61. Molecular weight markers are indicated on the left. Lanes 1 and 5 contain normal BLM; lanes 2 and 6 contain Q672R missense protein; lanes 3 and 7 contain C1055S missense protein; lanes 4 and 8 contain K695T missense protein. (B) Helicase activity of the normal and missense BLM proteins. Displacement activity is measured using a 3' end-labeled oligonucleotide 54 bases long annealed to ssMp18 DNA. Lanes marked 0 and Δ95–5 are the substrate (no enzyme) and the product (substrate heated at 95°C for 5 min). Each reaction contains 1 ng of recovered protein: lanes 2 and 3, normal BLM; lanes 4 and 5, missense Q672R BLM; lanes 6 and 7 missense C1055S BLM; lanes 8 and 9 missense K695T BLM. (C) Polarity of the BLM helicase. The direction of movement of the BLM helicase was evaluated using a substrate digested with *PstI* that creates two oligonucleotides of different sizes at opposite ends of the long ssMp18 DNA. In experiment PstA, both ends of the same substrate molecule were labeled (5' and 3') before digestion with *PstI*, and in PstB two different substrate molecules were labeled either at the 5' or the 3' end, digested with *PstI* and equal amounts of each mixed together in the reaction tubes. Assays were quantitated with a Molecular Dynamics PhosphorImager. (D) Comparison of helicase activities of normal and missense BLM proteins. Displacement activity and DNA-dependent ATPase specific activity is shown as a percentage of normal protein activity.

substitution of a nonhydrolyzable analogue of ATP [adenosine-5'-O-(3-thio)triphosphate]. The BLM preparations have DNA-dependent ATPase activity (7 U/ng). The activity is stimulated equally well by 1 μg of single-stranded Mp18 DNA and poly(dA):p(dT)₁₂.

The polarity of the DNA helicase activity of BLM was evaluated using two short-labeled oligonucleotides annealed to a long linear single strand. The RecQ

DNA helicases are thought to recognize and bind to the long single-stranded gap between the two oligonucleotides and displace preferentially one of the two test oligonucleotides (Matson and Kaiser-Rogers, 1990; Lohman and Bjornson, 1996). The helicase substrate HS2 anneals to the polylinker region of Mp18 ssDNA, creating a small duplex region. The oligonucleotide is labeled on the 5' and/or 3' ends and di-

gested with restriction enzymes to create two end-labeled oligonucleotides annealed to the linearized long single-strand DNA. The BLM helicase preferentially displaces the 28 mer versus the 26 mer in the *Pst*I-digested substrate consistent with a 3' to 5' movement relative to the long single-stranded linear molecule (Figure 4D). In experiment PstA, both ends of the same substrate molecule were labeled and then digested with *Pst*I. In experiment PstB, the two ends were labeled independently, and the substrates were digested with *Pst*I and mixed together in equal amounts. Results were similar for both substrates.

The three genes encoding the missense alleles of *BLM* were expressed, and the mutant proteins were purified (Figure 4, A and B). These genes contain single amino acid substitutions constructed in the mammalian expression vector pOPRSVI using oligonucleotide-directed mutagenesis. These cDNAs were cloned directly into the yeast expression vector using yeast plasmid gap repair (Kunes *et al.*, 1987), replacing the normal *BLM* gene. Small amounts of each missense protein were purified (Figure 4, A and B), and in two independent experiments each preparation tested lacked helicase displacement activity (Figure 4C). Each preparation had ~1% the specific activity of the normal protein, indicating some potential contamination of the preparations with a yeast helicase or another DNA-binding protein (Figure 4D). The Q672R protein has DNA-dependent ATPase specific activity (2.7 U/ng). The other two missense protein preparations have less than measurable amounts of DNA-dependent activity (< 1 U/ng).

These data demonstrate that the amino acid substitutions found in the two *BLM* genes from individuals with BS are loss-of-function mutations and not amino acid polymorphisms. Alteration of the conserved ATP-binding motif at amino acid 695 (GKT to GTT) abolishes the enzymatic activity of the protein. These results demonstrate that the normal and missense proteins are expressed in AMR61 cells as stable soluble proteins, because they are recovered in approximately equal yields (Figure 4B) with a slight bias toward recovery of the inactive proteins (C1055S and K695T). The BLM proteins are isolated from a yeast strain containing *sgs1* and *top3* mutations because this strain grows well, will not form potential BLM/Sgs1p heterodimers, and allows investigation of the phenotype that *BLM* might have in yeast.

Partial Complementation of a Phenotype of an *S. cerevisiae sgs1 top3* Strain by BLM

The plasmids containing the normal *BLM* cDNA, the vector pYES2, and pSGS1 were transformed into the wild-type parental strain W3031a (Thomas and Rothstein, 1989), AMR61 (*sgs1 top3*), and AMR59 (*sgs1 top1*) cells (Lu *et al.*, 1996). AMR61 grows as well as W3031a

Table 1. Doubling times for yeast strains

Yeast strain	Carbon source	Doubling time (h)		
		+pYES2	+pSGS1	+pBLM
W3031a (WT)	Glucose	1.8	2.6	3.1
	Galactose	5.3	6.2	6.4
AMR 61 (<i>sgs1 top3</i>)	Glucose	2.4	8.2	3.3
	Galactose	5.5	12.5	19.7
AMR59 (<i>sgs1 top1</i>)	Glucose	5.1	3.3	6.2
	Galactose	10.1	6.8	14.9

(Table 1), because the *sgs1* mutation suppresses the *top3* slow-growth phenotype (Gangloff *et al.*, 1994). AMR59 grows poorly relative to W3031a (Table 1) because the loss of Top1p combined with an *sgs1* mutation may place additional demands on the essential Top2p in haploid yeast cells (Lu *et al.*, 1996). When yeast Sgs1p is restored in these strains the phenotypes reverse, i.e., AMR59 grows well (doubling time of 5 h goes to 3 h in glucose) and AMR61 grows poorly (2 h converts to 8 h). The expression of Sgs1p (under control of its own promoter on a high copy-number plasmid) does slow the growth of W3031a but not greatly (doubling time 2 vs. 3 h in glucose).

When BLM is expressed in these strains under control of a galactose-inducible promoter, the growth of W3031a in galactose is not greatly affected (Table 1). AMR59 grows in galactose more slowly (10 h without BLM and 15 h with BLM), and AMR61 grows much more slowly (5 h vs. 20 h). The expression of BLM under these conditions mimics the effect of Sgs1p expression in AMR61 cells but not in AMR59 cells. Expression of BLM in AMR59 slows growth rather than enhancing it, as does Sgs1p. BLM may have just enough structural identity with Sgs1p to interfere with yeast Top3p function and provide additional demands on the activity of Top2p in these cells in which Top1p is absent. These data show that *BLM* partially complements the phenotypes associated with *sgs1* mutations in *S. cerevisiae*.

The slow growth phenotype is difficult to work with because suppressors arise frequently (Gangloff *et al.*, 1994; Lu *et al.*, 1996). To find a more stringent phenotype for screening *BLM* alleles and potentially for selecting new mutations in *BLM*, the AMR61 strains were tested on medium containing HU, based on the observation that an HU-dependent checkpoint phenotype is seen in strains of *S. pombe* that contain mutant *hus2* alleles (Stewart *et al.*, 1997) and some *rad12* alleles (Davey *et al.*, 1998) of the *rqh1+* gene.

Strain AMR61 can grow in the presence of HU under the conditions used here (Figure 5, row A). Wild-type strains such as W3031a can grow at 100 mM HU (our unpublished results). When Sgs1p is restored, the cells fail to grow with increasing HU (Figure 5, row B).

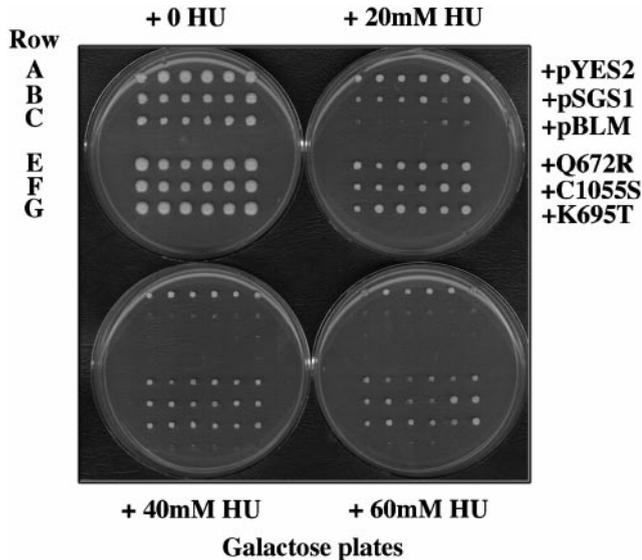


Figure 5. BLM complements a phenotype of a *sgs1 top3* *S. cerevisiae* strain. Yeast strain AMR61 (*sgs1 top3*) was transformed with plasmids pYES2, pSGS1, and pC4YES3 (normal *BLM* cDNA under *GAL1* control), as well as with missense alleles of *BLM* under galactose control. Colonies were picked into microtiter wells, replica-plated onto minimal plates with galactose as the carbon source, and incubated at 30°C for 5 d. A comparison of the growth efficiency of these strains on increasing concentrations of hydroxyurea (HU) is shown. Each row displays six colonies from the transformation plates. Row A = +pYES2; row B = +pSGS1; row C = +pC4YES; row E = +pQ672R/*BLM*; row F = +pC1055S/*BLM*; row G = +pK695T/*BLM*.

Expression of the *BLM* cDNA has a similar effect and affects the cell growth at a lower concentration of HU (Figure 5, row C). AMR61 cells expressing the missense alleles grow on HU plates as well as with the vector alone (Figure 5, rows E–G). Because the strains expressing the missense alleles of *BLM* grow on HU plates and the normal gene does not, the helicase activity of *BLM* is necessary for this phenotype in *sgs1 top3* yeast cells.

DISCUSSION

This work documents assays for the function of the normal *BLM* cDNA in human cells, in yeast cells, and in enzymatic assays. Transfection of the normal *BLM* cDNA reduces the high SCE phenotype of BS cells. Similar results have been observed previously (Giesler *et al.*, 1997). Transfection of the normal cDNA restores *BLM* to the nucleus of BS cells. *BLM* has enzymatic properties in vitro consistent with its predicted membership in the RecQ family of DNA helicases. Similar results have been reported previously (Karow *et al.*, 1997). Two missense alleles of *BLM* found in individuals with clinical BS (Ellis *et al.*, 1995a) encode full-length *BLM* protein that lacks helicase displacement

activity in vitro and fails to reduce the high-SCE phenotype of BS cells, as does a constructed missense mutation disrupting the helicase ATP-binding site. In *S. cerevisiae* the expression of the normal *BLM* cDNA from a strong inducible promoter can complement the growth phenotype of an *sgs1 top3* strain but not that of an *sgs1 top1* strain. In *sgs1 top3* cells, expression of Sgs1p or *BLM* creates an HU-sensitive growth phenotype. This phenotype is dependent on expression of a helicase-competent *BLM* gene.

Normal and missense proteins are recovered from AMR61 cells as stable, soluble proteins in approximately the same yield. This suggests that the missense proteins fold close to the normal conformation because yeast cells do not degrade them differentially relative to normal or package them into an insoluble form. The Q673R missense protein has 37% of the DNA-dependent ATPase specific activity of the normal protein, demonstrating that it retains some normal function and therefore must be close to normal conformation.

Antibodies raised against the N-terminal region of the *BLM* cDNA sequence are used here to demonstrate the nuclear localization of *BLM*, its focal arrangement in the nucleus, and its absence from cells derived from persons with BS. This reagent allows the evaluation of BS cells transfected with different *BLM* alleles. The focal organization of *BLM* is restored to BS cells by transfection of the normal *BLM* cDNA. Missense alleles in *BLM* are expressed in variable amounts in the different stable transfected cell lines and fail to localize in the numerous bright, discrete nuclear foci that are seen by immunofluorescence analysis of normal human cells. The significance of this focal pattern of localization of *BLM* in the nucleus of human cells remains to be determined. The lack of extensive amino acid identities between the N- and C-terminal domains of *BLM* and *WRN*, and the profound differences between the clinical phenotypes of the affected individuals (German, 1993; Epstein *et al.*, 1996), suggest specialized roles or different cellular locations for these two helicases. Recently *WRN* has been shown to be present in the nucleolus of human cells, a location distinct from the major sites of *BLM* localization (Marciniak *et al.*, 1998), a finding consistent with this hypothesis.

Differences were seen among cloned cell lines derived from HG2522 cells transfected with the three different missense alleles. These cell lines express *BLM* genes from a strong constitutive promoter (RSV) and were selected for good growth in culture. The plasmids encoding the two missense alleles found in BS individuals, Q672R [139(ViKr)] and C1055S [113(DaDe)], transfected nearly as well as the normal *BLM* cDNA. The three missense alleles of *BLM* studied here alter amino acids that are either conserved in all family members (Q672R and K695T) or in the ex-

tended C-terminal homology domain (C1055S). The C1055S missense allele showed stable accumulation of full-length BLM, but the protein was present in a diffuse overall nuclear staining pattern. The cells transfected with the Q672R missense allele showed a diffuse pattern and few small dots. These cloned cell lines expressing the Q672R and C1055S missense proteins grew fairly well and generated multiple stable cell lines (six of six). In contrast, most of the cell lines transfected with the helicase domain knockout allele (K695T) died in culture (five of six and three of six in two independent transfection experiments) and express very little stable protein. The K695T mutation may potentially function as a dominant negative mutation. What BLM is present in these surviving cells appears to be in a few small dots per nucleus or diffusely localized. These observations suggest that the Q672R and K695T missense proteins can assemble into a small number of focal nuclear structures but fail to form as many nuclear foci as the normal BLM gene product does.

Because the missense proteins are found in lower amounts in these stable transfected cell lines relative to normal BLM, the failure to reduce the high SCE phenotype may be due simply to the lower concentration of these proteins in the selected cell lines rather than the loss of BLM function. Another factor in this analysis is the stable focal localization of the normal protein and the generally diffuse pattern seen with the missense proteins. BLM may function in these nuclear foci, and the failure of the missense proteins to localize into or form these numerous structures may be the reason for their failure to reduce the SCEs. The missense alleles may accumulate in HG2522 cells to a lesser extent than normal BLM and fail to be incorporated into nuclear foci because they are not recognized by a protein partner because they are not folded properly. Misfolding of the missense proteins would create an unstable molecule that would likely be targeted for proteolysis; however, the proteins are expressed in yeast cells as soluble proteins to approximately the same yield, and the Q672R missense protein retains some enzymatic activity *in vitro*. This indicates that the overall structure of these proteins is likely to be close to normal. They may fail to be localized focally and accumulate to the same stable concentration as normal BLM if the incorporation of BLM molecules that are inactive but of normal conformation may form poisonous complexes that are dispersed or unstable in the nucleus. The K695T missense protein is especially deleterious to the cells used in this study. This missense gene was constructed *in vitro*, whereas the other two missense alleles are found in affected individuals, consistent with the transfection efficiency *in vitro*. The stability of the mutant proteins in yeast cells, the immunofluorescent results, and the transfection data support the idea that the activity and location of the

missense BLM proteins and not simply the lower concentrations of these proteins are the essential features of the failure to reduce the SCEs in BS cells.

DNA helicases, like topoisomerases and other enzymes that manipulate DNA strands, can be disruptive if unregulated. The activity and localization of these enzymes must be controlled to prevent collisions with polymerases and alterations of DNA topology that might disrupt gene expression. Other circumstantial evidence that supports the idea that defective RecQ helicase proteins are deleterious to the cell is the fact that the nuclear localization signal of both WRN and BLM is found in the last 100 amino acids of these large proteins, suggesting a cellular safety mechanism such that mutant helicases arising from translational stop signals can never be nuclear-localized (Kaneko *et al.*, 1997; Matsumoto *et al.*, 1997).

In *sgs1 top3 S. cerevisiae* cells the HU-sensitive phenotype seen when Sgs1p or BLM is expressed may reflect an enhanced rate of ectopic recombination occurring in these cells because HU depletion of deoxynucleotide triphosphate pools causes stalled and broken replication forks (Vassilev and Russev, 1984; Kuzminov, 1995). The stalling of the replication forks can create additional single-stranded regions in cells allowing the entry of these helicases into the DNA duplex, creating additional single-stranded DNA that can invade a neighboring DNA duplex, especially a sister chromatid. The three missense alleles of BLM that lack *in vitro* helicase activity do not confer this HU-sensitive phenotype, demonstrating that the helicase activity of BLM is required. These additional recombination events may not be resolved in a timely manner such that cells enter mitosis with entanglements, as is thought to occur in the *rqh1-h2 (hus2)* mutants of *S. pombe* (Stewart *et al.*, 1997). It is possible that S and G2 cell cycle checkpoints that monitor the completion of DNA replication and block mitosis in the presence of DNA damage fail to recognize unresolved recombination junctions between sister chromatids as damage or as potentially deleterious. Failure to resolve these events in BS cells in a timely and efficient manner could lead to an elevated frequency of nondisjunction and somatic mutation by an error-prone repair mechanism.

Other models for the function of this helicase include a role during S/G2 phase to help remove single-stranded DNA created by replication slippage (Schachman *et al.*, 1960) in AT-rich repeated-sequence elements that may anneal ectopically or a role in unwinding sequence-specific DNA conformations that repeated-sequence elements may assume potentially. An interesting relationship between Sgs1p, WRN, and the nucleolus has been found recently by the Guarente laboratory (Sinclair and Guarente, 1997; Sinclair, *et al.*, 1997; Marciniak *et al.*, 1998), suggesting a role for RecQ DNA helicases in the stability of rDNA repeats. Re-

cently the telomeric regions of the chromosomes of *Ustilago maydis* were isolated and found to contain RecQ DNA helicase genes as one of the two major middle repeated subtelomeric sequences (Sanchez-Alonso and Guzman, 1998), suggesting a need for multiple RecQ helicase genes in this highly recombinogenic fungi.

One of the major characteristics of the few known disorders that feature genomic instability is the potential for somatic mutation and disintegration of the genomic complement at each cell division. Bloom syndrome is one of the most cancer-prone disorders known (German, 1993). The work reported here demonstrates that the DNA helicase activity of the BLM gene product is important for the maintenance of genomic stability and for the stable localization and function of BLM in complexes in the nucleus of human cells. Therefore the loss of this DNA helicase leads ultimately to the development of cancer in persons with BS, and the elucidation of its structure and function will lead to a new understanding of one mechanism by which neoplastic cells can arise and progress into clinical cancer in normal individuals.

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