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The Bloom's Syndrome Gene Product Is a 3′-5′ DNA Helicase*

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Bloom's syndrome (BS) is an autosomal recessive condition characterized by short stature, immunodeficiency, and a greatly elevated frequency of many types of cancer. The gene mutated in BS, *BLM*, encodes a protein containing seven "signature" motifs conserved in a wide range of DNA and RNA helicases. BLM is most closely related to the subfamily of DEXH box-containing DNA helicases of which the prototypical member is *Escherichia coli* RecQ. To analyze its biochemical properties, we have overexpressed an oligohistidine-tagged version of the *BLM* gene product in *Saccharomyces cerevisiae* and purified the protein to apparent homogeneity using nickel chelate affinity chromatography. The recombinant BLM protein possesses an ATPase activity that is strongly stimulated by either single- or double-stranded DNA. Moreover, BLM exhibits ATP- and Mg^{2+}-dependent DNA helicase activity that displays 3′-5′ directionality. Because many of the mutations in BS individuals are predicted to truncate the BLM protein and thus eliminate the "helicase" motifs or map to conserved positions within these motifs, our data strongly suggest that these mutations will disable the 3′-5′ helicase function of the BLM protein.

Bloom's syndrome (BS)[1] is a rare, autosomal recessive disorder associated with a variety of phenotypic characteristics, of which the most prominent are pre- and post-natal growth retardation, immunodeficiency, and a sun-sensitive facial erythema. Individuals with BS are strongly predisposed to develop malignancies, including both solid tumors and leukemias. Cells from BS individuals exhibit a number of abnormalities *in vitro*, the most striking of which is an increase in genomic instability. This is manifested as an elevated frequency of chromosome breaks and exchanges, as well as a characteristic increase in the level of sister-chromatid exchanges (reviewed in Ref. 1).

The gene defective in BS, *BLM*, was identified recently (2) and is located on chromosome 15 at 15q26.1, adjacent to the *FES* proto-oncogene. The mRNA specific for the *BLM* gene is approximately 4.5 kb in length and encodes a protein with a predicted M_r of 159,000. The primary sequence of the BLM protein shows strong similarity to sequences of a subfamily of DEXH box-containing DNA helicases, including RecQ, a 3′-5′ helicase that participates in the RecF pathway of genetic recombination in *Escherichia coli* (reviewed in Ref. 3). RecQ has been shown recently to act as a suppressor of illegitimate recombination (4). Other members of the RecQ subfamily are *Saccharomyces cerevisiae* Sgs1 (5, 6), *Schizosaccharomyces pombe* Rqh1 (7), and the human RECQL (8, 9) and WRN (10) proteins, all of which include a highly conserved RecQ homology domain containing the seven "signature" motifs of DNA and RNA helicases. However, the Sgs1, Rqh1, BLM, and WRN proteins are distinct from RecQ and RECQL in that they are much larger proteins (~160 *versus* ~70 kDa) due to the presence of additional domains flanking the central RecQ homology domain (11, 12). WRN protein is defective in individuals with Werner's syndrome, a condition with many features of premature aging (reviewed in Ref. 13). Cells from Werner's syndrome individuals are genetically unstable and, in particular, display an increased frequency of gene deletions. Recently, it has been shown that the WRN protein is a DNA helicase, although the direction of the unwinding reaction was not determined (14).

To understand more fully the role of the BLM protein, it will be necessary to characterize its biochemical properties. To achieve this goal, we have developed a yeast-based expression system that permits the preparation of homogeneous BLM protein in reasonable yield. We demonstrate that BLM protein is a DNA-stimulated ATPase and an ATP-dependent DNA helicase. Further, we show that BLM unwinds DNA in the 3′-5′ direction with respect to the strand to which the enzyme is bound.

EXPERIMENTAL PROCEDURES

Construction of pJK1—A cDNA comprising the complete open reading frame (ORF) of the *BLM* gene was generated from a human B-cell library (kindly donated by D. Simmons, Imperial Cancer Research Fund, Oxford) using the polymerase chain reaction. This cDNA was cloned downstream of the *GAL1* gene promoter between the *Bam*HI and *Not*I sites of the yeast expression vector pYES2 (Invitrogen),to give pRKC15. To generate pJK1 for expression of BLM in yeast, the following modifications were made to pRKC15: a C-terminal hexahistidine tag was added, a yeast Kozak consensus sequence was included upstream of the coding sequence, and the first five codons of *S. cerevisiae TOP2* gene replaced the initiation codon of the *BLM* cDNA. To generate the C-terminal modification, the 5′ primer S9 (5′-CTCAAGAAGCTTGCAGAATC-3′) and the 3′ primer JK4 (5′-AGAGAGCTCGAGTCA*GTGGTGGTGGTGGTGGTGTGAGAATGCATATGAAGGCTT-3′) were used to generate a 0.5-kb fragment of the 3′ end of the BLM ORF. Primer JK4 incorporates a *Xho*I site (underlined), a stop codon (asterisk), and 18 nucleotides (bold) encoding the hexahistidine tag. A *Xho*I-*Hin*dIII fragment of the above product was used to replace the corresponding section of pRKC15. To generate the N-terminal modification with the inclusion of a yeast Kozak consensus sequence, the 5′ primer JK3 (5′-AGAGAGGGATCCCTAACCATGTCAACTGAACCGGCTGCTGTTCCTCAAAATAAT-3′) and the 3′ primer R2 (5′-TCCTAGGGTGGTAGTCAGTAAACA-3′) were used to generate a 1.4-kb 5′ section of the BLM gene. Primer JK3 has a *Bam*HI site (underlined), a yeast Kozak sequence (TAAAC), and the first five codons of the yeast *TOP2* ORF (bold). A *Bam*HI-*Eco*RI fragment of this product was used to replace the corresponding region in pRKC15. The final construct, pJK1, was sequenced (ABI™ Dye Terminator Cycle Sequencing, Perkin-Elmer Corporation) to confirm that the predicted BLM amino acid sequence contained no polymerase chain reaction-induced changes from the BLM sequence published previously (2).

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[1] The abbreviations used are: BS, Bloom's syndrome; kb, kilobase pair(s); ORF, open reading frame; rBLM, recominant BLM; PAGE, polyacrylamide gel electrophoresis; bp, base pair.

Expression of Recombinant BLM in S. cerevisiae—Strain JEL1 (MATα *leu2 trp1 ura3–52 prb1–1122 pep4–3 Δhis3::PGAL10-GAL4*) (15) was transformed with pJK1. 10 liters of cells were grown in complete medium containing 2% raffinose at 30 °C to an optical density of 0.7 at 600 nm. For induction of BLM protein, galactose was added to a final concentration of 2%, and the cells were cultured for another 24 h at 20 °C. Cells were harvested by centrifugation, washed in 50 mM sodium phosphate pH 7.0 buffer, and stored at −70 °C until required.

Purification of Recombinant BLM Protein—Cells were resuspended in an equal volume of 50 mM sodium phosphate, pH 7.0, 500 mM KCl, and 10% glycerol in the presence of an EDTA-free protease inhibitor mixture (Boehringer Mannheim) at 4 °C. Glass beads (425–600 μm, Sigma) were then added to 50% of the volume, and the cells were lysed by vortexing for 10 × 30 s with incubations of 30 s on ice after each burst. To remove solid particles, the lysate was centrifuged at 30,000 × *g* for 30 min at 4 °C. The supernatant was subjected to nickel chelate affinity chromatography under the following conditions: after charging a 1.7-ml Poros 20 MC column (PerSeptive Biosystems) with 50 ml 100 mM $NiSO_4$, it was saturated with 5 bed volumes of 1500 mM imidazole in 50 mM phosphate, pH 7.0, 250 mM NaCl and then equilibrated with 5 bed volumes of 15 mM imidazole in the same buffer. The yeast cell extract was loaded onto the column using a BioCAD workstation (Perseptive Biosystems), and the column was washed with 15 bed volumes of the same buffer containing 50 mM imidazole. Elution was performed with an imidazole gradient of 50–1500 mM in the same buffer applied over 8 bed volumes. Fractions of 1 ml were collected, to which EDTA was added to a final concentration of 10 mM. Fractions containing recombinant BLM (rBLM), as determined by SDS-PAGE, were dialyzed for 16 h against buffer containing 60 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1 mM 2-mercaptoethanol and were stored in 25% glycerol at −70 °C.

ATPase Assays—ATPase activity was determined by measuring the release of inorganic phosphate by a colorimetric assay according to Chifflet *et al.* (16). The assay mixture (50 μl) contained 50 mM Tris-HCl, pH 7.5, 4 mM $MgCl_2$, 2 mM ATP, 25 μg/ml sheared salmon sperm DNA, 50 μg/ml bovine serum albumin, 50 mM NaCl, 1 mM dithiothreitol, and unless stated otherwise 50 ng of rBLM protein. After incubation for 30 min at 37 °C, 50 μl of distilled H_2O were added, and the reaction was stopped by the addition of 100 μl of 12% SDS. Following this, 200 μl of 0.5% ammonium molybdate/3% ascorbic acid in 6% SDS/0.5 M HCl were added, and the samples were incubated for 5 min at 20 °C. 300 μl of sodium metarsenite, sodium citrate, and acetic acid (all at 2% (w/v)) were added, and after a 10-min incubation at 37 °C, the absorbance of the solution was measured at 850 nm.

Preparation of Helicase Substrates—A substrate with a 91-bp duplex region was created by annealing a 90-mer oligonucleotide to single-stranded M13mp18 DNA and extending the 3′ end by 1 nucleotide using 2 units of Klenow polymerase and 20 μCi of [α-^{32}P]dCTP (3000 Ci/mmol; Amersham) for 30 min at 37 °C. Samples were then passed through a Nuctrap probe purification column (Stratagene) to remove unincorporated nucleotides. A substrate with a 38-bp region at the 5′ end and a 55-bp region at the 3′ end was constructed by annealing the 90-mer to single-stranded M13mp18 and digesting it with *Sal*I. All available 3′ ends were labeled using Klenow polymerase, [α-^{32}P]dCTP, and dTTP as described above.

Helicase Assays—For this, the release of a 3′-labeled oligonucleotide annealed to single-stranded M13mp18 was measured. The reaction mixture (20 μl) contained 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 5 mM ATP, 100 μg/ml bovine serum albumin, 50 mM NaCl, approximately 50 ng of labeled DNA substrate, and 50 ng of rBLM protein. After incubation at 37 °C for 30 min, the samples were run on a 12 or 15% nondenaturing polyacrylamide gel at 20 °C. The gel was dried and exposed to x-ray film (Kodak X-Omat). The amount of substrate displaced was determined by scanning the autoradiograms using a PhosphorImager 425 (Molecular Dynamics) and ImageQuant software.

Immunoblotting—The protein extracts were subjected to SDS-PAGE on 8 or 10% gels followed by electrotransfer of the proteins to nitrocellulose (Hybond-C super, Amersham). rBLM protein was detected by a mouse monoclonal anti-histidine tag antibody (Dianova). Immunoreactive proteins were visualized using ECL reagents (Amersham).

RESULTS

Construction of a BLM Expression Clone—To overexpress rBLM protein in *S. cerevisiae*, a full-length *BLM* cDNA was generated using the polymerase chain reaction (see "Experimental Procedures"). The predicted amino acid sequence of the BLM protein was shown to be identical to that published by Ellis *et al.* (2), although the nucleotide sequence contained six silent changes, four of which probably represent polymorphisms, because they were found in an independently generated cDNA (data not shown). Two modifications were made to the cDNA to facilitate the subsequent purification of the *BLM* gene product. First, a hexahistidine affinity tag was engineered at the 3′ end of the coding sequence to permit the expressed protein both to be detected by means of specific anti-histidine tag antibodies and to be purified on nickel-chelate resin. Second, the first 5 codons of the yeast *TOP2* gene were placed immediately upstream of the complete *BLM* open reading frame. In previous studies it was shown that the presence of these yeast Top2p-derived amino acids improved the yield of expressed recombinant human proteins (15, 17). The modified *BLM* cDNA was cloned downstream of the *GAL1* promoter in vector pYES2 by insertion between the vector's *Bam*HI and *Xho*I sites to generate pJK1.

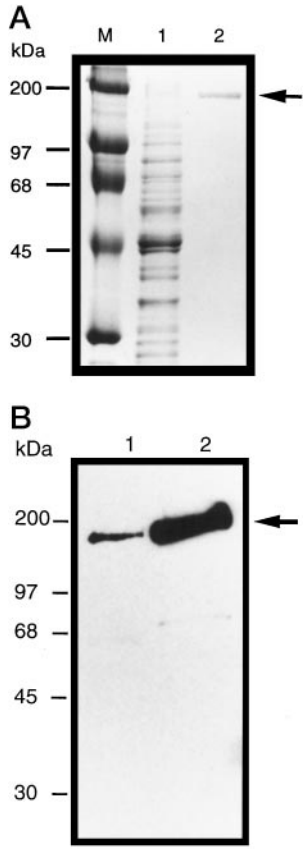

FIG. 1. **Purification of rBLM protein.** *A*, a Coomassie Blue-stained 10% SDS-polyacrylamide gel of a crude cell extract from galactose-induced JEL1/pJK1 (*lane 1*) and fraction 19 from the nickel-chelate chromatography (*lane 2*). The position of the purified rBLM protein is indicated on the *right* by an *arrow*, and the molecular mass standards (*lane M*) (in kDa) are shown on the *left*. *B*, Western blot of the protein samples from *A* using an anti-histidine tag antibody. The position of the immunoreactive protein is shown on the *right* by an *arrow*.

Purification of rBLM Protein—The pJK1 construct was transformed into the Gal^+, protease-deficient *S. cerevisiae* host strain JEL1, and expression of the rBLM protein was induced through activating the *GAL1* promoter by the addition of galactose to the growth medium. The overexpressed rBLM protein was then purified to apparent homogeneity by nickel-chelate affinity chromatography. Peak fractions represent an imidazole concentration of 100–350 mM. The purified protein had an apparent M_r on SDS-PAGE of 180,000 (Fig. 1*A*). In a typical preparation, approximately 50 μg of pure rBLM protein were obtained per liter of yeast culture.

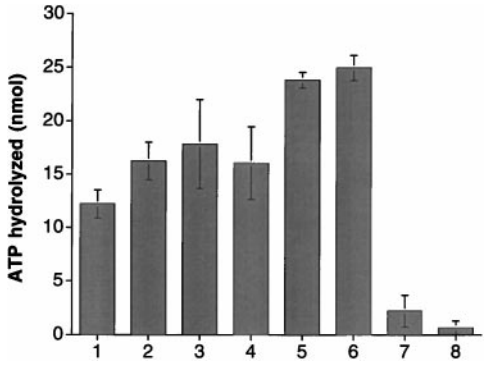

FIG. 2. **rBLM protein is a DNA-stimulated ATPase.** DNA dependence of the ATPase activity of rBLM. In each case DNA was present at 25 μg/ml, and the incubation time was 30 min. The DNA co-factors used were 17-mer oligonucleotide (*bar 1*), 90-mer oligonucleotide (*bar 2*), M13 single-stranded circular DNA (*bar 3*), supercoiled plasmid DNA (*bar 4*), native salmon-sperm DNA (*bar 5*), denatured salmon-sperm DNA (*bar 6*), no DNA (*bar 7*), and heat-inactivated rBLM (*bar 8*). Each value represents the mean of three independent determinations. *Error bars*, S.E.

To verify that the purified protein was rBLM, Western blotting studies were performed. Fig. 1*B* shows that a specific anti-histidine tag antibody reacted both with a single 180-kDa species in crude lysates of galactose-treated JEL1/pJK1 cells and with the purified rBLM protein. This protein was not detected in JEL1/pJK1 cells prior to activation of the *GAL1* promoter (data not shown). Moreover, affinity purified anti-BLM antibodies raised in rabbits to a small portion of the N-terminal domain of BLM expressed in *E. coli* (to be described elsewhere) recognized the same 180-kDa species (data not shown).

rBLM Protein Is a DNA-stimulated ATPase—The presence of the characteristic "Walker Box" motifs in the primary sequence of BLM (2) predicts that the protein would be an ATPase. The ability of the rBLM protein to hydrolyze ATP was therefore examined. Fig. 2 shows that the BLM protein was associated with an ATPase activity that was strongly stimulated by the presence of DNA. The ATPase reaction catalyzed by rBLM was linear during a 60-min incubation period (data not shown), and the specific activity of the rBLM protein as an ATPase using native salmon sperm DNA as a co-factor was calculated to be 10,000 units/mg (where 1 unit hydrolyzes 1 nmol ATP/min). All forms of single- and double-stranded DNA tested gave a broadly similar level of stimulation (Fig. 2). Nevertheless, a short (17-mer) single-stranded oligomer was consistently less efficient as a co-factor than was a longer (90-mer) oligonucleotide (compare *bars 1* and *2* on Fig. 2).

To confirm that the ATPase activity was associated directly with the rBLM protein, individual fractions from a nickel-chelate chromatography elution were both assayed for ATPase activity and subjected to SDS-PAGE to quantify the level of BLM protein in each fraction. Fig. 3 shows that there was a strong concordance between the level of ATPase activity and the quantity of rBLM protein in each fraction, confirming that the activity was an intrinsic property of the BLM protein.

rBLM Is a DNA Helicase—The *E. coli* RecQ protein is a 3′-5′ DNA helicase (18). BLM, like RecQ, contains the seven consensus amino acid motifs conserved in the DEXH box-containing subfamily of helicases, suggesting that BLM would also be a DNA helicase. To analyze whether the purified rBLM protein possesses helicase activity, a partially double-stranded substrate was prepared comprising single-stranded M13 viral form DNA annealed to a radiolabeled 91-mer oligonucleotide (Fig. 4*A*). This substrate was incubated with the purified rBLM protein, and the reaction products were separated on a polyacrylamide gel. The labeled oligonucleotide was then detected by autoradiography. Fig. 4*B* shows that rBLM possesses a DNA unwinding activity that is dependent upon the presence of both

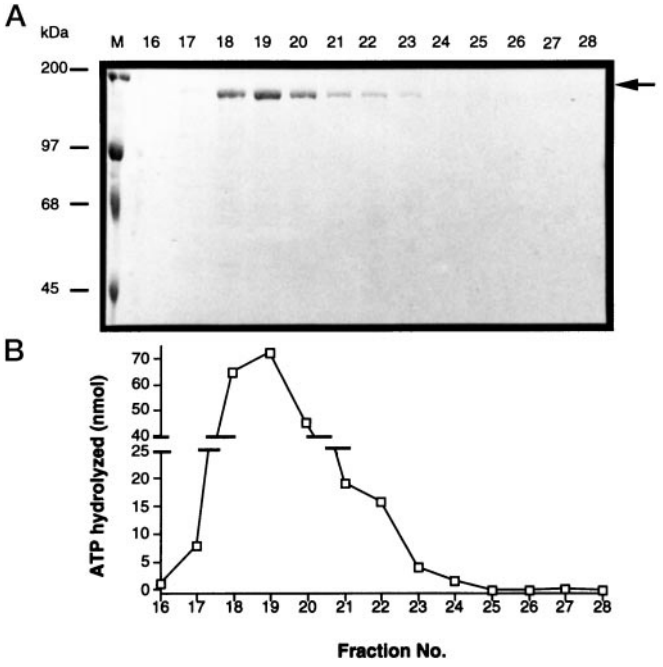

FIG. 3. **The ATPase activity is associated with the rBLM protein.** Fractions from the nickel-chelate chromatography were analyzed by SDS-PAGE followed by Coomassie Blue staining (*A*) and for ATPase activity (*B*). Fraction numbers are indicated above each lane in *A*. Note that the *y* axis is broken in *B*.

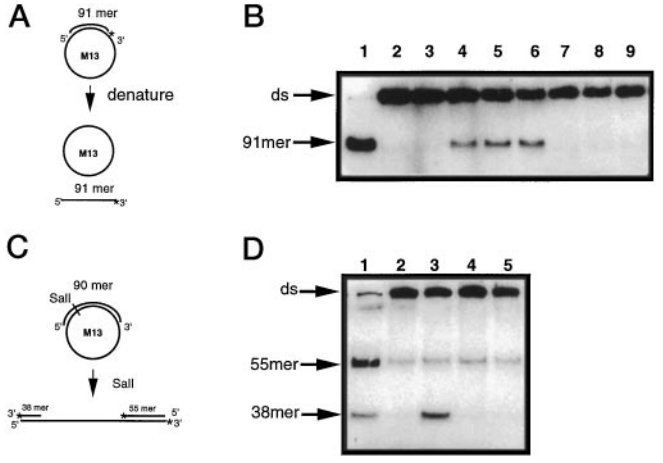

FIG. 4. **rBLM protein is an ATP-dependent DNA helicase that unwinds DNA in a 3′-5′ direction.** *A*, scheme for the helicase assay. A single-stranded 90-mer oligonucleotide was annealed to M13 viral form DNA, labeled at the 3′ end, and incubated with rBLM protein (or alternatively heat-denatured as a control). *B*, autoradiogram of a 15% polyacrylamide gel of the products from an incubation of the 91-mer substrate with BLM. *Lane 1*, substrate incubated at 98 °C for 10 min; *lane 2*, substrate alone; *lanes 3–6*, substrate plus 2.5, 5, 25, and 50 ng of rBLM protein, respectively; *lane 7*, as *lane 6*, except no Mg^{2+}; *lane 8*, as *lane 6* except no ATP; *lane 9*, as *lane 6* except heat-inactivated rBLM (65 °C for 10 min). *C*, scheme for the assay to determine the direction of unwinding. A 90-mer oligonucleotide was annealed to M13 viral form DNA and digested with *Sal*I, and all available 3′ ends were radiolabeled using Klenow polymerase to yield a partially double-stranded substrate comprising 38- and 55-mer oligonucleotides annealed to linear M13 DNA. *D*, autoradiogram of a 15% polyacrylamide gel of the products from incubating the substrate depicted in *C* with rBLM protein. *Lane 1*, substrate heated to 98 °C for 10 min; *lane 2*, substrate alone; *lane 3*, substrate plus 50 ng rBLM; *lane 4*, as *lane 3*, except no Mg^{2+}; *lane 5*, as *lane 3*, except no ATP. The positions of the double-stranded substrate (*ds*) and the 55- and 38-mer products are indicated on the *left*.

Mg^{2+} and ATP and that could be destroyed by incubating the rBLM preparation at 65 °C for 10 min.

The Helicase Activity of rBLM Shows 3′-5′ Polarity—To determine the polarity of DNA unwinding by the BLM helicase, a substrate comprising a 90-mer oligonucleotide annealed to single-stranded M13 DNA was prepared. This partial duplex DNA was digested with *Sal*I and radiolabeled at all available 3′ ends using Klenow polymerase and [α-^{32}P]dCTP to generate a linear DNA molecule with 38- and 55-mer double-stranded portions at its termini (Fig. 4*C*). In this substrate, the 55-bp oligomer was consistently preferentially labeled compared with the 38-mer, as can be seen in *lane 1* of Fig. 4*D* following heat denaturation of the double-stranded substrate. Following incubation of the substrate with the rBLM protein, no increase in the amount of unwound 55-mer oligonucleotide over background was observed (compare *lanes 2* and *3*, Fig. 4*D*). In contrast, complete release of the 38-mer oligonucleotide was evident, indicating that the direction of DNA unwinding catalyzed by the BLM protein was 3′-5′ with respect to the strand to which the enzyme was bound. Consistent with the ATPase data described above, the helicase activity of rBLM was completely dependent upon Mg^{2+} and ATP (Fig. 4*D*, *lanes 4* and *5*).

DISCUSSION

We have purified to near homogeneity the product of the gene mutated in BS and demonstrated that the BLM protein is a DNA-stimulated ATPase and an ATP-dependent DNA helicase that unwinds DNA in a 3′-5′ direction. DNA helicases differ from other helix-destabilizing enzymes in their ability to unwind DNA substrates irrespective of their length. Consistent with BLM acting as a bona fide DNA helicase, we have shown that rBLM can unwind both an 18-mer and a 91-mer substrate with similar efficiencies (Fig. 4 and data not shown).

The purified rBLM protein exhibits an ATPase activity that is strongly stimulated by the presence of DNA. Because both circular M13 viral form DNA and plasmid DNA were effective as co-factors in this reaction, there is clearly no requirement for BLM to interact with free DNA ends in order for its ATPase activity to be stimulated. The relatively modest increase in ATPase activity seen in reactions containing high molecular weight single-stranded DNAs compared with those containing an 17-mer oligonucleotide might indicate that the optimal length of DNA for interaction with BLM is greater that 17 nucleotides. This can now be tested.

The available evidence indicates that the members of the RecQ subfamily of DE*X*H box-containing enzymes utilize a 3′-5′ DNA helicase activity to perform critical roles in DNA metabolism (11, 12, 19). Although the precise role of these enzymes has yet to be determined in any organism, in every case studied to date lack of a RecQ family helicase leads to some form of genomic instability. Moreover, emerging evidence suggests that abnormalities in recombination underly this instability. Because many of the mutations at the BLM locus that are associated with BS are predicted to eliminate the function of one or more of the helicase motifs (2), we suggest that the 3′-5′ helicase activity of the BLM protein is required for the BLM protein to suppress inappropriate recombination. It will be interesting to test whether the particular BLM gene mutations found in BS individuals, both those mapping within the helicase motifs and those lying outside the RecQ homology domain, inactivate the helicase function of the protein. This will be an important indication as to whether the N- and C-terminal domains of BLM are important for catalysis or for conferring other functions on the protein, such as an ability to interact with a unique set of cellular proteins. In this regard it is also interesting to note that the major differences between the sequences of the BLM and WRN (Werner's syndrome) proteins are found outside of the RecQ homology domain (2, 10), suggesting that the unique N- and C-terminal domains of the proteins confer at least some of the functional differences between BLM and WRN (19).

Sgs1p, the *S. cerevisiae* homolog of BLM, apparently performs a very similar role in budding yeast to that of BLM in human cells. In support of this contention, *sgs1*Δ strains show a spontaneous hyperrecombination phenotype (5, 20) reminiscent of that of *BLM*-deficient cell lines. The cellular functions of Sgs1p are intimately connected with those of the three topoisomerases expressed by budding yeast. *SGS1* interacts genetically with *TOP1* (21) and *TOP3* (5), and Sgs1p associates physically with Top2p (6) and Top3p (5). Considering the high level of sequence conservation among eukaryotes for these proteins, it seems likely that some, if not all, of these interactions will be conserved in human cells. As a result of the purification of BLM protein reported here, we are now in a position to analyze whether a physical interaction occurs between BLM and purified topoisomerases IIα, IIβ, or III, the human homologs of Top2p and Top3p. It would appear that helicases and topoisomerases might co-operate to perform many different important roles in eukaryotic DNA metabolism (reviewed in Ref. 22). Among the numerous possibilities for such co-operation are; (i) to facilitate replication fork progression and/or the segregation of newly replicated daughter DNA molecules, (ii) to manipulate chromosome functions via the modification of nucleosomal structure and/or DNA topology, (iii) to promote recombination through branch migration or to suppress recombination by actively disrupting inappropriate recombinant intermediates. The challenge is now to provide experimental evidence in favor of one or more of these putative roles.

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