[CANCER RESEARCH 64, 1102–1109, February 1, 2004]

A Genome-Wide Screen in *Saccharomyces cerevisiae* Reveals Altered Transport As a Mechanism of Resistance to the Anticancer Drug Bleomycin

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ABSTRACT

The potent DNA damaging agent bleomycin (BLM) is highly effective for treating various cancers, although, in certain individuals, the development of cellular resistance to the drug can severely diminish its antineoplastic properties. We performed two independent genome-wide screens using a *Saccharomyces cerevisiae* mutant collection to isolate variants exhibiting either sensitivity or resistance to BLM. This procedure reproducibly identified a relatively large collection of 231 BLM-hypersensitive mutants, representing genes belonging to diverse functional groups. In contrast, only five BLM-resistant mutants could be recovered by our screens. Among these latter mutants, three were deleted for genes involved in plasma membrane transport, including the L-carnitine transporter Agp2, as well as the kinases Ptk2 and Sky1, which are involved in regulating polyamine transport. We further showed that Agp2 acts as a transporter of BLM and that overexpression of this transporter significantly enhances BLM-induced cell killing. Our data strongly implicate membrane transport as a key determinant in BLM resistance in yeast. This finding is critical, given that very little is known about BLM transport in human cells. Indeed, characterization of analogous mechanisms in humans may ultimately lead to enhancement of the antitumor properties of BLM.

INTRODUCTION

Bleomycin (BLM) is used to treat various cancers, including lymphoma, squamous cell carcinoma of the cervix, head, and neck, and Hodgkin's disease (1–4). In the case of testicular cancer, BLM in combination with cisplatin and etoposide is particularly effective, yielding a striking cure rate of 70–80% (4, 5). However, the remainder of patients eventually develop resistance to BLM therapy and relapse (4, 6). Furthermore, there is clear evidence that individuals afflicted with various other human cancers (*e.g.*, Daudi lymphoma and colon carcinoma) are highly resistant to BLM therapy at the outset (7, 8). Although the mechanism of BLM-induced cytotoxicity has been well studied (see below and Refs. 3, 9–12), much less attention has been devoted to establishing the determinants that regulate tumor resistance to the drug.

BLM directly attacks DNA via a free radical-driven process to generate a narrow set of lesions similar to those induced by ionizing radiation (*e.g.*, apurinic/apyrimidinic sites, single-strand breaks containing 3′-blocking groups that inhibit the progression of DNA polymerase) and double-strand breaks (10, 13, 14). Such DNA lesions are known to be highly genotoxic, and, furthermore, it is clear that they account for the potent antitumor effects of the drug (15–18). Thus, increased levels of DNA-repair enzymes are likely to contribute to BLM tumor resistance. Indeed, a recent study demonstrated that overproduction of hApe/ref-1, which belongs to a family of enzymes that can directly repair BLM-induced DNA lesions (19–22), engenders a 2–3-fold increase in protection against BLM in testicular cancer cells (23). Whether hApe/ref-1 plays a role in BLM-resistant tumors awaits further investigation.

Previous studies have indicated that processes other than direct removal of BLM-induced DNA damage are required to protect the budding yeast *Saccharomyces cerevisiae* from the lethal effects of BLM (see review in Ref. 24). To better understand such processes, we performed two genome-wide screens using an entire collection of haploid yeast mutants to systematically identify variants that were hypersensitive to BLM. Analysis of these hypersensitive mutants revealed many interesting genes corresponding to various diverse functional groups (see below). However, of particular interest here, the screens were also designed to isolate BLM-resistant strains, a class that had not been sought previously. Only five resistant mutants could be reproducibly isolated by the screens. Among these mutants was one defective in the L-carnitine transporter Agp2, which we demonstrated to regulate the entry of BLM into the cell and, as a consequence, the cytotoxic potential of the drug. Two of the other BLM-resistant mutants were deficient in either of the kinases Ptk2 or Sky1, which are both involved in polyamine transport. Our data clearly indicate a critical role for membrane transport in mediating BLM resistance in yeast.

MATERIALS AND METHODS

Yeast Strains and Media. The wild-type strains used were BY4741 and SEY6210 (25, 26). The collection of nonessential haploid *MATa* deletion strains, derived from the parent BY4741, was obtained from EUROSCARF (Frankfurt, Germany; Ref. 27). Standard YPD (yeast-peptone-dextrose) and selective growth media were used as described previously (28).

High-Throughput BLM Screen and Drug Sensitivity Analysis. The strains were arrayed in quadruplet, in a clockwise manner to create a dilution in a given square. A total of 96 colonies were arrayed per solid YPD plate containing either no drug or 2.0 or 7.5 μg/ml BLM A5 (BLM, ICN Pharmaceuticals), using a 96-floating pin replicator operated by a Biomek 2000 (Beckman). Plates were incubated for 48 h at 30°C and photographed with a digital camera (Gel Doc 2000, Bio-Rad) to visually compare the growth of every mutant in the presence or absence of BLM (see Fig. 1). Putative BLM-hypersensitive or -resistant strains were further analyzed by spot-test analysis (26). Briefly, exponentially growing cultures in YPD were adjusted to an A_{600} of 0.6, and 5 μl of a set of serial dilutions, as indicated, were spotted onto YPD supplemented with 1.0 or 4.0 μg/ml BLM to test for BLM hypersensitivity and resistance, respectively.

Gradient Plate and Survival Curve Assays. These assays were performed as described previously (26). In the case of gradient plate assays, the bottom layer contained either 0.5 μg/ml 4-nitroquinoline-1-oxide (4-NQO) or 0.03% methyl methane sulfonate (MMS). For the survival curves, exponentially growing cultures were treated at an A_{600} of 1.0 for 1 h with 4-NQO (0 to 6 μg/ml) or MMS (0 to 0.3%) or cisplatin (0 to 10 μg/ml) or camptothecin (0 to 10 μg/ml), and or γ-rays (0 to 40-Krad) in either YDP or selective media. Cells were washed, serially diluted in 20 mM potassium phosphate buffer (pH 7.0), and plated onto solid YPD to score for survivors after 48 h growth at 30°C.

Received 8/29/03; revised 11/24/03; accepted 11/26/03.

Grant support: This research was supported by funds from the National Cancer Institute of Canada (with funds from the Canadian Cancer Society) to D. Ramotar and to M. Peter by the Swiss National Science Foundation and the ETH/Zurich. D. Ramotar is a senior fellow of the Fonds de la Recherche en Santé du Québec. N. Pagé holds a fellowship from Roche Research Foundation.

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Notes: M. Aouida and N. Pagé contributed equally to this work. Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org).

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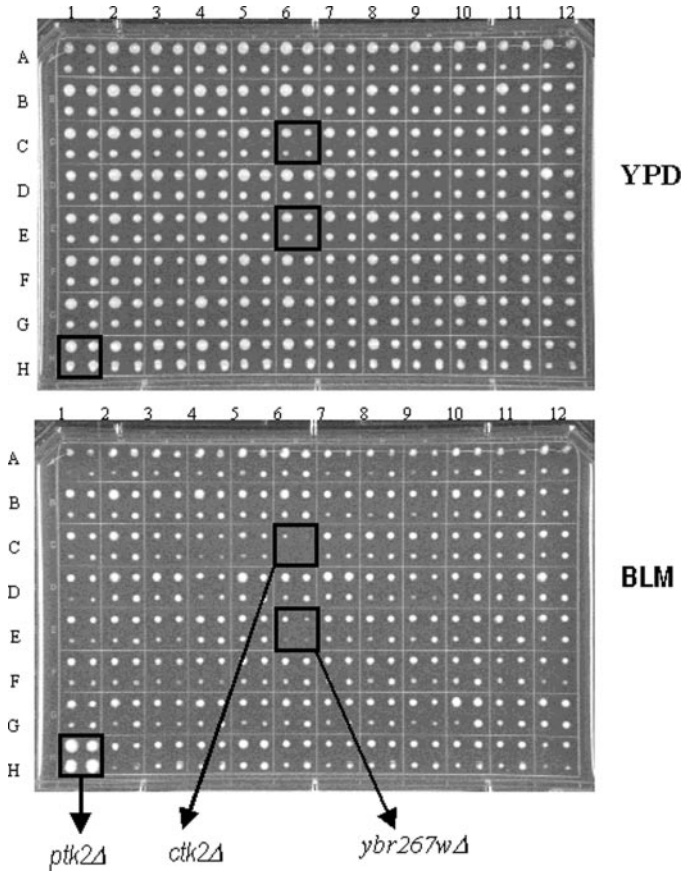

Fig. 1. Representative plate generated from the high-throughput bleomycin (*BLM*) screen. The complete haploid mutant collection was pinned onto solid yeast-peptone-dextrose (*YPD*) agar with 0, 2.0, and 7.5 μg/ml BLM (see "Materials and Methods"). The photograph depicts a set of 96 mutants, each pinned four times to create a dilution onto solid YPD without and with 2.0 μg/ml BLM. The *small colonies* represent putative BLM-hypersensitive mutants, whereas the *large colonies* represent a BLM-resistant mutant. Plates were photographed after 48 h at 30°C. The entire collection was screened twice.

Primer Extension Assay. This assay was performed as described previously (29). Briefly, chromosomal DNA isolated from BLM-untreated and -treated (10 μg/ml for 2 h) cells was assessed for the ability to permit incorporation of [*methyl*-^{3}H]dTMP by purified *E. coli* DNA polymerase 1 (Promega). Apn1 used for pretreating the chromosomal DNA was purified in this laboratory (30). Specific activity of the labeled [*methyl*-^{3}H]dTTP (NEN Life Sciences Products) was 1230 cpm/pmol.

Construction of pAGP2-Green Fluorescent Protein and Yeast Transformation. The primers AGP2-F1: 5′-CCCTGCCTCTGTGAGCTCTGCATTGTATACTATATAC-3′ and AGP2-GFPR1: 5′-CATTATGCTTTGGTACCATATTGAAATTTTTCGAAGGAGC-3′ (restriction sites *Sac*I and *Kpn*I underlined) were used to amplify the entire *AGP2* gene (*i.e.*, coding region and 430-bp of the putative promoter region) from yeast genomic DNA using PCR. The amplified product, digested with *Sac*I and *Kpn*I, was cloned into the multi-copy plasmid pYEp-GFP lacking a promoter (31) to produce the expression plasmid pAGP2-GFP. In a similar manner, the *AGP2* gene with 200 nucleotides downstream was cloned into the single and multi-copy vectors YEplac33 and YEplac195 to generate the plasmids pAGP2-1 and pAGP2-2, respectively. pYEp-GFP, pAGP2-GFP, pAGP2-1, and pAGP2-2 were separately introduced into the indicated yeast strains by the lithium acetate method (32).

Immunoblot Analysis. Total protein extracts were prepared from cells carrying either pYEp-GFP or pAGP2-GFP and probe by Western analysis using anti-GFP monoclonal antibody (Clontech) as described previously (31).

Coupling of FITC to BLM and Uptake Analysis. A 100-μl aliquot of 2.1 mM of the fluorescent molecule 5-(and-6)-carboxyl fluorescein [5(6)-FAM] succinimidyl ester (SE; succinimidyl-FITC, Molecular Probes) in 0.2 M $NaHCO_3$ (pH 9.0) was added to 300 μl of 0.6 mM BLM (prepared in 0.2 M $NaHCO_3$, pH 8.3), and the mixture was incubated for 2 h at room temperature. The reaction was stopped by the addition of 10 μl of 1.5 M hydroxylamine (pH 8.5). A control reaction was done in parallel, except for the addition of BLM. The reaction products were resolved on 1% agarose gel for 2 h at 100 V using 40 mM 2-(*N*-morpholino) ethanesulfonic acid monohydrate (USB) buffer (pH 6.0). The active product (slower migrating band toward the cathode) containing FITC coupled to BLM was visualized by a hand-held long wavelength UV lamp, excised, placed at −80°C until frozen, centrifuged at 9,000 × *g*/5 min, lyophilized, and resuspended in 300 μl of sterile water. A fluorescently labeled for of BLM (F-BLM) was aliquoted and stored at −20°C. The concentration of F-BLM was assessed against natural BLM by absorbance at 292 nm and by DNA fragmentation of the purified plasmid pBluescript (data not shown). The

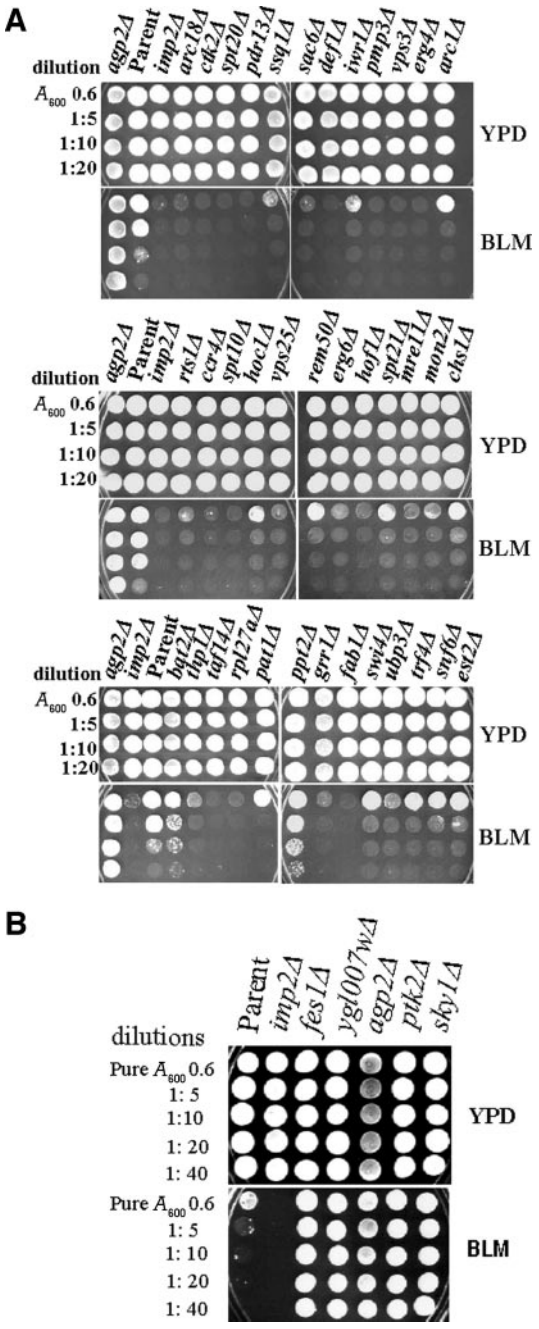

Fig. 2. Sensitivity and resistance of yeast mutants to bleomycin (BLM). Exponentially growing cells were diluted to an A_{600} of 0.6. Cells were then serially diluted as indicated and 5 μl spotted onto yeast-peptone-dextrose (YPD) agar containing 1.0 μg/ml BLM for the sensitive (*A*) or 4.0 μg/ml of BLM for the resistant strains (*B*). The parent strain is BY4741. The hypersensitive and resistant mutants *imp2*Δ and *agp2*Δ, respectively, were included as controls. Plates were photographed after 48 h of incubation at 30°C.

Table 1 *Genes that confer bleomycin sensitivity or resistance when deleted*

DNA repair chromatin structure	Transcription	Cell cycle	RNA metabolism	Protein synthesis	Protein degradation	Lipid metabolism	Mitochondria and ATP metabolism
CTF18[*a]*	*ASF1*[*a+b]*	*CDC50*[*a]*	*ARC1**	*ASC1*	*GRR1*[*a+b]*	*ARV1**	*ADK1*[*b]*
CTF4[*a+b]*	*BUR2*[*a+b]*	*CTK2*	*BRE5**	*EAP1*	*UBP3*	*ERG2*	*AFG3*
CTF8[*a+b]*	*CCR4*[*b]*	*DOC1*[*b]*	*BRF1*	*EGD2*	*UMP1**	*ERG4*	*ATP11*
*EST2**	*CTK1*	*HTL1*[*a+b]*	*CDC40*[*a+b]*	*PDR13*[*b]*		*ERG6*	*ATP12*
*FAB1**	*CTK3*	*PHO85**	*DBP7*	*PFD1*[*b]*			*ATP14*
IWR1[*b]*	*DHH1*[*b]*	*RTS1**	*DIA4*[*b]*	*RPL13B*			*ATP15*
MRE11[*a]*	*GAL11*	*SFP1*[*b]*	*KEM1**	*RPL1B*			*CAT5**
NAT3[*a+b]*	*IMP2*		*LOC1*[*b]*	*RPL27A*			*ILM1*
RAD27[*a]*	*KCS1*		*PAT1*[*b]*	*RPL35A*			*ISA1**
RAD54[*a]*	*POP2*		*SAC3**	*RPL39*			*MDJ1*
RAD57[*a+b]*	*ROX3*		*SNT309*	*RPS0B**			*MRPL51**
RAD6[*a+b]*	*RPB4*		*YER087W**	*RPP1A*			*MRPS8*
RAI1[*b]*	*RPB9*[*a+b]*			*TIF3*			*MSF1*
REM50[*a]*	*RRN10*[*a]*			*TIF4631*			*MSY1**
RNR1	*SIN4*			*ZUO1*[*b]*			*NHX1*
*RNR4**	*SPT21*			*YIF2*[*b]*			*OCT1*
SNF6	*SPT7**						*PDA1*
SPT10[*b]*	*SRB2*[*a]*						*PFK2*[*b]*
SPT20	*SRB5*[*a]*						*PPA2*
*THP1**	*SRB8**						*RML2*
*TRF4**	*SSN8*						*RSM19*
VID31[*a+b]*	*SWI4*						*RSM7*[*b]*
XRS2[*a+b]*	*SWI6*[*a]*						*RSM22*
	*TAF14**						*SNF1*
							*SPF1**
							SSQ1
							SWF3
							SWS2
							TOM5
							*TUF1**
							*UGO1**
							YDJ1
							YHM1
							YME1

genotoxicity measurement was determined by clonogenic assays of a parent strain YW465 and the DNA repair deficient mutant YW778 (Ref. 30; data not shown). For F-BLM uptake studies, exponentially growing cultures were washed twice in water and resuspended in 50 mM citrate acetate buffer (pH 5.5) containing 2% glucose and 0.05% Tween 20 at a density of 2×10^8 cells/ml. F-BLM (0.1 μg) was added to 100 μl of the cells, incubated at 30°C in the dark with mild shaking for the indicated times, and uptake was stopped by adding 1 ml of stop buffer [50 mM citrate acetate buffer (pH 5.5), 2 mM sodium azide]. Cells were washed three times with PBS buffer, resuspended in 500 μl of PBS, sonicated at 30% for 15 s, and the extent of F-BLM uptake was measured using a fluorescent spectrophotometer at 495/525 nm (excitation/emission).

Fluorescence Microscopy. Cells were grown to a density of 2×10^8 cells/ml, washed two times in water, and resuspended in uptake buffer. An aliquot (100 μl) of cells was incubated with either F-BLM (0.72 μg/ml) in the absence or presence of L-carnitine (at the concentrations indicated in the text) at 30°C in the dark with mild shaking for 1 h. Cells were washed three times with 1 ml of PBS, resuspended in 100 μl of PBS, and 3 μl were mounted on microscope slides for fluorescent microscopy. For the negative and positive controls, cells were incubated with FITC (0.72 μg/ml for 1 h) and FM4–64 (30 μM for 15 min, Molecular Probes), respectively. Cells were photographed at ×100 magnification by imaging camera (Retiga GX 32–002TB-303) attached to a Leica DMRE immunofluorescent microscope, and images were processed by the Macintosh OpenLab program.

RESULTS

Isolation and Verification of BLM-Hypersensitive and -Resistant Mutants. Two independent robot-aided screens of a haploid *S. cerevisiae* mutant collection were performed to identify BLM-hypersensitive and -resistant mutants. Briefly, a robot was used to pin each mutant four times onto solid YPD medium containing 0, 2.0, and 7.5 μg/ml of BLM. The lower concentration of BLM allowed ~70% growth of the parent and <20% for hypersensitive mutants. In contrast, the higher concentration of BLM significantly reduced growth of the parent and completely prevented growth of all hypersensitive mutants, but permitted normal proliferation of resistant mutants. Although this type of screen has the limitation of potentially overlooking mutants with unstable or weak phenotypes and/or possessing a redundant function, we have nonetheless successfully identified over 200 mutants.

The two screens reproducibly identified 260 BLM-hypersensitive and, remarkably, only 5 BLM-resistant mutants (Fig. 1, representative plate depicting two BLM-hypersensitive and one-resistant mutant). A total of 67 BLM-hypersensitive mutants could not be reproduced between screens and were, therefore, eliminated from further analysis. To confirm the results of the initial screen, the 260 BLM-hypersensitive and 5 BLM-resistant mutants were subjected to spot-test analysis on solid YPD plates containing BLM (Fig. 2*A* shows randomly tested strains). The data revealed 29 strains that were not actually hypersensitive to BLM, leaving a total of 231 displaying 4–20-fold higher levels of sensitivity to the drug relative to the parental counterpart (Table 1). Thus, the two independent high-throughput screens generated a relatively low fraction (~12%) of false positives, analogous to other screens performed with growth assessment on plates containing drugs, such as hydroxyurea, to select for DNA-replication

Table 1 *Continued*

Polarity and polarized growth	Vacuoles and vesicular transport	Cytoskeleton	Cell wall	Miscellaneous	Unknown function	BLM resistant
BEM2	AKR1[b]	ARC18	ANP1	ADE12	APQ13	AGP2*
BUD16*	APG17	CDC10*	CAX4[b]	ADH1	BAT2	FES1*
BUD20*	APL2	CNM67[b]	CHS1	APM1	GON7	PTK2*
BUD23*	CHC1*	GCS1	CWH36[b]	ARP5*	SWF5	SKY1*
BUD25[a]	DID4*	HOF1*[a+b]	FKS1	CYS4	YBR168W	YGL007W
BUD27	END3*	SPC72	FYV6	DIA2	YBR267W*	
BUD31*	GLO3		GAS1	GLY1	YDR049W*	
BUD32*	IES6		HOC1	GON1*	YDR532C	
SAC2	INP53*		LAG2	GPH1	YGL072C	
SAC6[b]	LCB4*		MNN9	GUP1	YGR237C	
	LUV1		MNN10	MET22*	YGR272C	
	MON2*		OST4	NPL6[a]	YLR374C	
	PEP5*		RMD7	OPT2	YMR031W-A[a]	
	PEP12*		ROT2*	PLC1	YOR342C	
	RIC1		SLG1*	PMP3	YPR044C	
	RCY1*			PRO1		
	RVS161*[b]			REG1		
	RVS167[b]			RIB4		
	SHE4			SHP1		
	SWF1*			SLX8*		
	VAM6			SPS4		
	VPH2[b]			TPS1		
	VPS1					
	VPS3					
	VPS4					
	VPS8					
	VPS9					
	VPS15					
	VPS16*					
	VPS20					
	VPS24					
	VPS25*					
	VPS27					
	VPS45*					
	VPS66					
	VPS67					
	VPS69					
	YAF9*					
	YPT6					
	YPT7					

[a] Methyl methane sulfonate sensitive.
[b] Ionizing radiation sensitive.
* Conserved in human.

defective mutants (33).[3] The spot-test analysis also clearly confirmed that all five putative BLM-resistant mutants (Fig. 2*B*) indeed manifested extremely high resistance to BLM (see below).

Analysis of BLM-Hypersensitive Mutants Reveals Genes That May Specifically Mediate BLM Protection. We compared the 231 BLM-hypersensitivity genes identified here (Table 1) with those reported previously to protect yeast against other DNA damaging agents, *i.e.,* either the alkylating agent methyl methane sulfonate (MMS), or γ-rays (34, 35). We observed that 27 BLM-hypersensitive genes were also involved in sensitivity to MMS (Table 1, denoted by superscripted *a*). There were also 38 genes in common that protected cells from γ-ray exposure (Table 1, superscripted *b*), and 14 of which overlapped with those identified in the MMS screen (Table 1, superscripted $a + b$). In summary, among BLM-hypersensitive genes, 52 exhibited cross-sensitivity to other DNA-damaging agents; thus, the remaining 179 could have been expected to play various roles in protecting yeast against BLM-induced lethality. To test this notion further, we randomly checked 84 of the 179 mutants mediating BLM-specific protection for cross-sensitivity to MMS, as well as to 4-nitroquinoline-1-oxide (4-NQO), which induces bulky DNA adducts. None of the 84 mutants showed any significant sensitivity to either MMS or 4-NQO, as assessed by gradient plate assay (data not shown).

[3] Unpublished observations.

Functional Assignment of the Defective Genes Causing BLM Hypersensitivity. Searches performed with the Saccharomyces Genome Database (SGD) and YPD databases revealed a diverse collection of BLM-hypersensitive genes (Table 1 and Supplementary Table 2, showing a brief summary of the function encoded by each gene). Loci implicated in vacuolar function represented the largest number of BLM-hypersensitive mutants (40 genes), followed by 34, 24, 23, 16, and 15 genes involved in mitochondrial function, transcription machinery, the DNA damage response, protein synthesis, and cell wall biosynthesis, respectively. At least 22 genes were relegated to "miscellaneous" pathways, and a further 15 had no previously assigned biological function. The database search also revealed that 79 of the BLM hypersensitivity genes encode proteins that share between 22 and 68% homology with potential human functional counterparts (Table 1, conserved genes are indicated by an asterisk). Thus, it is apparent that human cells may use some similar cellular processes as yeast to mitigate BLM-induced lethality.

BLM-Resistant Mutants. The five BLM-resistant mutants identified by our genome-wide screens are deleted for the following genes: *AGP2*, *SKY1*, *PTK2*, *FES1*, and *YGL007W* (Table 1). Remarkably, three among the 5 BLM-resistant genes, *i.e.*, *AGP2*, *PTK2*, and *SKY1*, encode proteins that execute a role in plasma membrane transport. Agp2 has been shown previously to transport L-carnitine, whereas Ptk2 and Sky1 were shown to be members of two different kinase families that act independently to positively influence polyamine

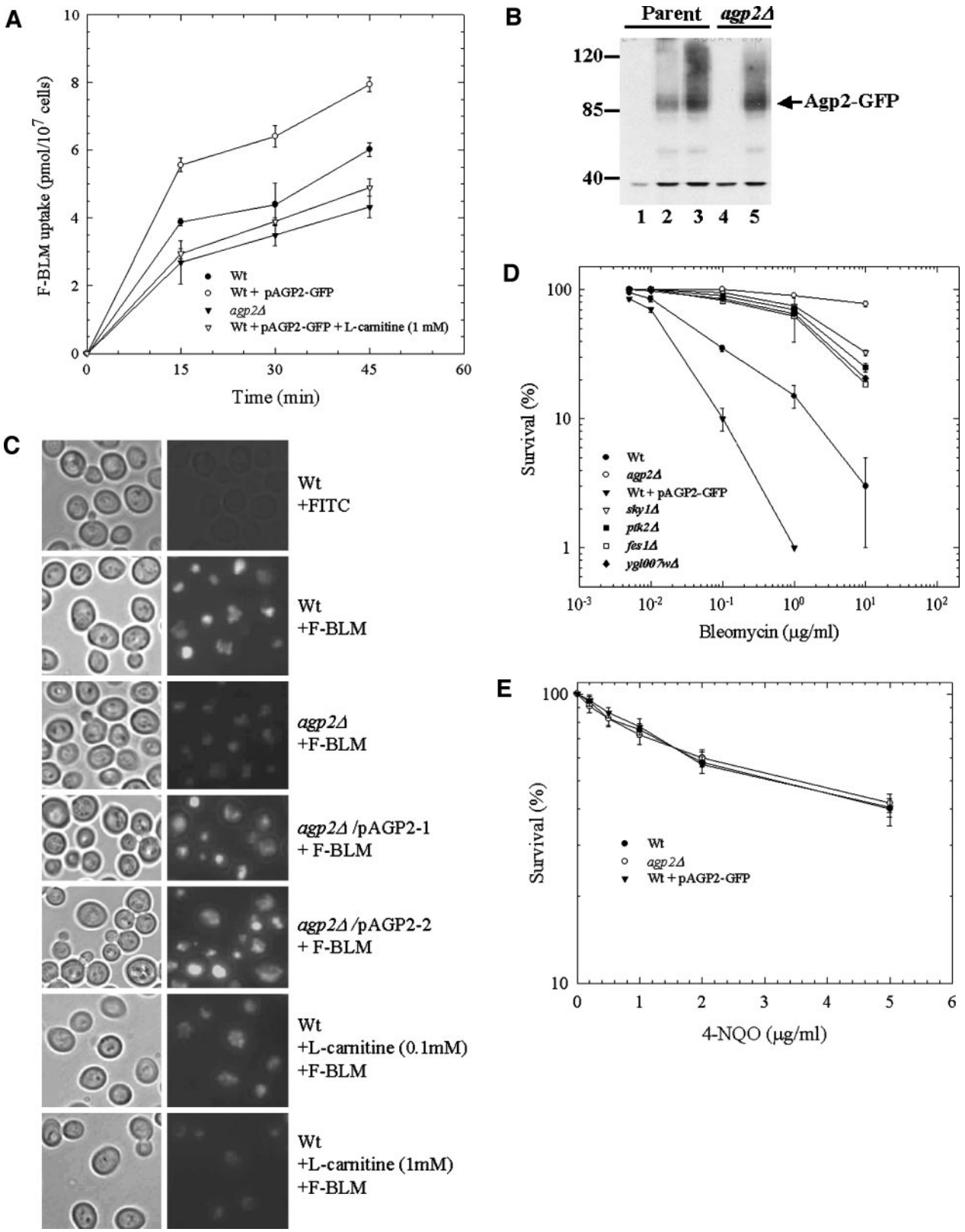

Fig. 3. Effects of alteration of Agp2 level on the uptake, cellular distribution, and cell survival of a fluorescently labeled form of bleomycin (F-BLM). *A*, F-BLM uptake into the parent and *agp2*Δ mutant carrying either pYEp-Green Fluorescent Protein (GFP) or pAGP2-GFP. Exponentially growing cells were incubated with F-BLM in the absence or presence of L-carnitine and processed as in "Materials and Methods." *B*, overexpression of Agp2-GFP fusion protein in the parent and *agp2*Δ mutant. Total extracts were prepared from strains containing either pYEp-GFP (*Lanes 1* and *4*) or the pAGP2-GFP (*Lanes 2*, *3*, and *5*) and probed with anti-GFP. pYEp-GFP cannot express GFP. Molecular weight standards are indicated on the *left* (*120*, *85*, and *40*: M_r 120,000, M_r 85,000, and M_r 40,000, respectively). *C*, immunofluorescent analysis of F-BLM cellular distribution in the parent and *agp2*Δ mutant. Exponentially growing cells were incubated with either fluorescein (FITC) or F-BLM in the absence or presence of L-carnitine and processed for immunofluorescent microscopy ("Materials and Methods"). *Left*, cells in bright field; *right*, cells incubated with the indicated fluorescent molecule. pAGP2-1 and pAGP2-2 are single- and multi-copy plasmids, respectively, carrying the native *AGP2* gene. *D* and *E*, fractional survivors of various strains after drug exposure. Exponentially growing cultures were treated with the indicated concentrations of BLM or 4-nitroquinoline-1-oxide (4-NQO) for 1 h, serially diluted, and plated onto solid yeast-peptone-dextrose agar to score for survivors. F-BLM uptake and survival curve experiments were repeated two and three times, respectively. *Wt*, wild type.

transport (36–38). The two other BLM-resistant genes, *FES1* and *YGL007W*, have no documented role in transport function. The protein encoded by *FES1* is implicated in a complex that regulates protein translation (39). In the case of *YGL007W*, there are currently no clues regarding its function.

Clonogenic survival analysis revealed that all five mutants (*agp2*Δ, *ptk2*Δ, *sky1*Δ, *fes1*Δ, and *ygl007w*Δ) were extremely resistant to BLM, as compared with the parent (see Fig. 3*D* and below). The most resistant to BLM was *agp2*Δ, followed by *ptk2*Δ, *sky1*Δ, *fes1*Δ, and *ygl007w*Δ, all of which showed nearly equal levels of resistance to the drug (see Fig. 3*D* and below). A similar result was obtained when each of these five genes was independently deleted in a different parental background (SEY6210), suggesting that the BLM-resistant phenotype is not strain specific (data not shown). On the basis of the above-mentioned information, it is reasonable to postulate that Agp2 may act as a transporter of BLM.

Agp2 Transports BLM Into the Cell and Mediates Cellular Sensitivity to BLM-Induced Cell Killing. To examine whether Agp2 could actually mediate the transport of BLM into cells, F-BLM was prepared by coupling activated FITC to the drug. This new molecule retained nearly full capacity to damage DNA *in vivo* and to decrease cell survival (data not shown). As shown in Fig. 3*A*, F-BLM uptake was significantly reduced in the *agp2*Δ mutant, as compared with the parent. The fractional uptake of F-BLM into the *agp2*Δ mutant was attributable to fluid endocytosis into vacuoles (data not shown). It is noteworthy that the other 4 BLM-resistant mutants also showed similar reduction in F-BLM uptake as the *agp2*Δ mutant (data not shown).

We next tested the effect of Agp2 overexpression on F-BLM uptake. Introduction of a multi-copy plasmid pAGP2-GFP into either the parent or the *agp2*Δ mutant expressed the expected M_r 93,000 Agp2-GFP fusion protein, which appeared as a broad band perhaps attributable to posttranslational modifications, *e.g.*, glycosylation or phosphorylation (Fig. 3*B*). Agp2-GFP overproduction greatly enhanced the uptake of F-BLM into the cells (Fig. 3*A*; for simplicity, shown only for the parent). Because the only documented function for the Agp2 protein is to transport L-carnitine into the cell, we tested whether this natural substrate of the transporter could compete with and prevent F-BLM entry into the parent strain overexpressing Agp2-GFP. As shown in Fig. 3*A*, L-carnitine clearly diminished F-BLM uptake into the cells to the residual level observed in the *agp2*Δ mutant. These data strongly indicate that the L-carnitine transporter Agp2 is also required to efficiently transport BLM into the cell. To further assess this, the intracellular distribution of F-BLM was examined in the parent and *agp2*Δ mutant using fluorescent microscopy. F-BLM intensely stained the vacuoles of the parent strain, but only weakly those of the *agp2*Δ mutant (Fig. 3*C*). In control experiments, fluorescein (FITC) showed no detectable staining of the vacuoles (Fig. 3*C*), whereas the vacuolar marker FM4–64 was localized to this organelle of both strains (data not shown). Introduction of a single-copy plasmid pAGP2-1, bearing an untagged form of the entire *AGP2* gene, into the *agp2*Δ mutant restored parental level of F-BLM in the vacuoles (Fig. 3*C*). Likewise, a multi-copy plasmid pAGP2-2 carrying the *AGP2* gene caused accumulation of a higher level of F-BLM in the vacuoles of the *agp2*Δ mutant, although this was difficult to document because of the increased brightness (Fig. 3*C*). As expected from the uptake studies (Fig. 3*A*), the addition of L-carnitine (1 mM) blocked the accumulation of F-BLM in the vacuoles of either the parent or *agp2*Δ mutant carrying pAGP2-2 (Fig. 3*C*, shown for the parent). Taken together, these findings confirm that the *agp2*Δ mutant is indeed defective for the *AGP2* gene and further strengthen the notion that the encoded protein Agp2 can act as a transporter of BLM. The data also indicate that after F-BLM uptake, the drug is channeled to the vacuoles for detoxification. In fact, mutants (*e.g.*, *end3*Δ) that are defective in the endocytic pathway to the vacuoles accumulated F-BLM in the cytoplasm and displayed hypersensitivity to the drug (Table 1, Fig. 2*A*, and data not shown).

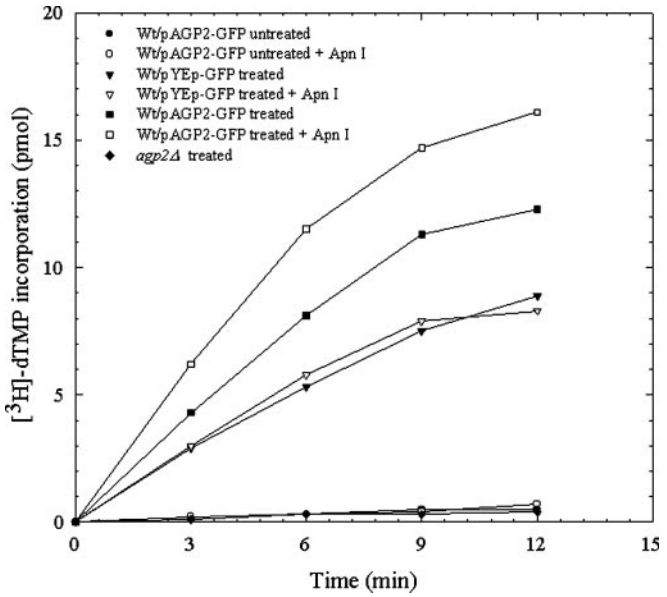

Fig. 4. Accumulation of bleomycin (BLM)-induced DNA lesions in Agp2-GFP overproducing cells as detected by *in vitro* incorporation of [*methyl*-^{3}H]dTMP. Exponentially growing cells were either untreated or treated with BLM (10 μg/ml for 2 h) and chromosomal DNA processed for [*methyl*-^{3}H]dTMP incorporation. Where indicated (*open symbols*), the chromosomal DNA was preincubated with 20 ng of purified Apn1 for 20 min before monitoring [*methyl*-^{3}H]dTMP incorporation. Wild-type (*Wt*) strain BY4741 carrying either the plasmid pYEp-GFP or pAGP2-GFP. Data are representative of two independent experiments.

We next checked whether the level of Agp2 expression correlated directly with cell survival after BLM exposure. As shown in Fig. 3*D*, the *agp2*Δ mutant was at least 1000-fold more resistant than the parent. Overproduction of the Agp2-GFP fusion caused parental, or *agp2*Δ mutant, cells to become exquisitely sensitive to BLM (Fig. 3*D*, shown for the parent). No difference in cell survival was observed between the parent and the *agp2*Δ mutant (either with or without overexpression of Agp2-GFP) after exposure to other DNA-damaging agents including MMS, cisplatin, γ-rays, and 4-NQO (Fig. 3*E*, shown for 4-NQO). Thus, the data clearly indicate that Agp2 levels mediate cellular susceptibility to BLM-induced killing.

Agp2 Overproducing Cells Accumulate High Levels of BLM-Induced DNA Lesions. Using a primer extension assay (29, 40), we next examined whether the BLM sensitization of cells overproducing Agp2-GFP was a result of increased damage to chromosomal DNA. This assay detects a major class of BLM-induced oxidative DNA lesions that are processed into 3′-hydroxyl groups *in vivo*. These 3′-hydroxyl groups can then be used as substrates to monitor the *in vitro* incorporation of [*methyl*-^{3}H]dTMP by purified *E. coli* DNA polymerase I (29, 40). Briefly, exponentially growing cultures of the parent strain (BY4741) carrying either the plasmid pYEp-GFP or pAGP2-GFP was treated with BLM and the isolated chromosomal DNA analyzed for the ability to incorporate [*methyl*-^{3}H]dTMP. BLM-damaged DNA isolated from strain BY4741/pAGP2-GFP showed a higher level of [*methyl*-^{3}H]dTMP incorporation, as compared with the damaged DNA derived from strain BY4741/pYEp-GFP (Fig. 4). Preincubation of the BLM-damaged DNA obtained from strain BY4741/pAGP2-GFP with the purified yeast DNA repair enzyme Apn1, which can remove BLM-induced DNA lesions (29), caused a stimulation of [*methyl*-^{3}H]dTMP incorporation (Fig. 4). In contrast, no Apn1-stimulated [*methyl*-^{3}H]dTMP incorporation was observed

into the BLM-damaged DNA isolated from strain BY4741/pYEp-GFP (Fig. 4). In control experiments, DNA isolated from the drug-untreated strain BY4741/pAGP2-GFP showed a very weak level of [*methyl*-^{3}H]dTMP incorporation in the absence or presence of Apn1-preincubation (Fig. 4). These data indicate that cells overexpressing Agp2-GFP are subject to increase levels of BLM-induced DNA lesions, which are not efficiently removed.

To test this further, we examined the level of [*methyl*-^{3}H]dTMP incorporation into DNA isolated from the *agp2*Δ mutant challenged with BLM. As shown in Fig. 4, DNA derived from the BLM-exposed *agp2*Δ cells showed no detectable [*methyl*-^{3}H]dTMP incorporation, as compared with the treated parent cells (Fig. 4). This finding distinctly indicates that the Agp2 transporter plays a critical role in allowing BLM to eventually attack cellular DNA.

DISCUSSION

The identification here of genes belonging to diverse functional groups, including those involved in vacuolar, mitochondrial, and cell wall functions, indicates that yeast uses multiple biological processes to defend against BLM lethality. In addition, because BLM induces cell death via the induction of highly genotoxic DNA double strand breaks, we expected to recover mutants deficient in DNA double-strand break repair. Indeed, the screens reproducibly identified several genes directly involved in this pathway including *MRE11*, *RAD54*, *RAD57*, and *XRS2* (Table 1), which underscores the validity of our experimental approach. It is noteworthy that the remaining genes *RAD50*, *RAD51*, *RAD52*, and *RAD55* of this DNA repair pathway were not recovered by the screens and may be a reflection of the genetic instability of the corresponding mutants causing an unstable phenotype (33). However, it should be noted that unlike genes of the DNA double-strand break repair pathway, the known genes (*FKS1*, *SLG1*, and *GAS1*) of the cell wall biosynthetic pathway involved in BLM-defense were recovered (26). Thus, of the 15 previously reported nonessential genes that protect yeast cells from BLM lethality, 4 were not isolated, suggesting a false-negative rate of ~26%, although this could be an overestimate (24).

As anticipated, mutants of the nucleotide excision-repair pathway were not isolated, because this pathway is ostensibly not required to repair BLM-induced DNA lesions. Interestingly, several unexpected gene products, possibly involved in DNA repair, were also recovered. Of particular interest are chromatin remodeling factors such as Snf6 (a subunit of SWI/SNF ATP-dependent chromatin assembly factor), Spt10 (a putative histone acetylase), and Spt20 (a component of the SAGA coactivator complex; Refs. 41–43). The involvement of such factors suggests a requirement for remodeling BLM-damaged DNA to promote repair. This possibility is consistent with previous studies showing that mutations in the INO80 ATPase chromatin remodeling complex of yeast, and in the TIP60 histone acetylase of mammalian cells, cause sensitivity to MMS and γ-rays, respectively (44–46).

The isolation of several BLM-hypersensitive mutants deleted for genes (*e.g.*, *IMP2*, *RPB7*, and *SLG1*) previously identified by different methods further emphasizes the utility of the current experimental approach (26, 29, 47). Although we previously showed that *IMP2* encodes a transcriptional co-activator, neither the gene(s) that it controls nor its mechanism of action is known (29). Thus, the current list of BLM-hypersensitive genes (Table 1) should facilitate the identification of Imp2 transcriptional target(s). Whether the Imp2-target gene(s) encodes a protein that either repairs BLM-induced DNA lesions, and/or detoxifies the drug will require additional investigation. In the current study, we have identified several transcriptional activators including Ccr4, Swi4, Pop2, and Spt7 (Table 1), which Imp2 could co-activate in response to BLM exposure.

In addition to the relatively large collection of 231 BLM hypersensitive genes, it is remarkable that our screen revealed only 5 that cause resistance to BLM when deleted, *i.e.*, *AGP2*, *PTK2*, *SKY1*, *FES1*, and *YGL007W*. Among these latter genes, *AGP2* was previously shown to encode a plasma membrane transporter of L-carnitine (36). Herein, we have demonstrated a novel function for Agp2 by clearly showing that yeast mutants deleted for this gene are deficient in transport of BLM. Furthermore, the overproduction of Agp2 increases drug influx, resulting in severe damage to the genome and leading to rapid cell death. It is worthy to note that although Agp2 overproduction caused only a 2-fold increase in F-BLM uptake, the cytotoxic effect was much more dramatic (Fig. 3). This apparent discrepancy could be explained if F-BLM transport is impeded as Agp2-overproducing cells are undergoing cell death from the drug influx. Alternatively, the 2-fold increase in F-BLM uptake may lead to more toxic DNA lesions. In fact, it is known that distinct DNA lesions are generated in yeast cells in a manner that depends on the BLM intracellular concentration (48). Nonetheless, the consequences of altering Agp2 levels appear to be specific for BLM, because either Agp2-overproducing or -null strains show parental resistance to several other diverse genotoxic agents including MMS, 4-NQO, cisplatin, and camptothecin (Fig. 3 and data not shown). In short, Agp2 exhibits substrate specificity toward BLM, and modulation of its activity impacts BLM resistance. On the other hand, the precise mechanism by which the other four BLM-resistance genes regulate protection awaits further experimentation. However, although the potential roles of Fes1 and Ygl007w in BLM resistance remain completely unknown, the Ptk2 and Sky1 kinases have been shown to control transporter activity (49, 50). Indeed, Ptk2 has been shown to be a regulator of the proton pump Pma1 in yeast (50). Moreover, Ptk2 and Sky1 are independently involved in polyamine transport in yeast (37, 38). As such it is distinctly possible that Ptk2 and/or Sky1 may also directly regulate Agp2 activity.

The fact that our genome-wide screen revealed altered transport as a critical mechanism of BLM resistance in yeast raises the possibility that mammalian cells may use similar genes to regulate protection against BLM. Thus far, two high-affinity L-carnitine transporters, CT2 and OCTN2, have been identified in humans (51, 52). It is, therefore, possible that CT2 and/or OCTN2 may also act as BLM transporters. Interestingly, CT2 is expressed exclusively in human testis, whereas OCTN2 is expressed strongly in kidney, skeletal muscle, heart, and prostate (51, 52). The fact that CT2 is expressed exclusively in testis and that testicular cancers have a high cure rate with BLM therapy is striking, offering strong support for the notion that CT2 could be the human transporter of BLM. Because transporter-dependent drug resistance is a frequent problem in clinical therapy, it is possible that patients who develop resistance to BLM could have defects in CT2. We note that further compelling evidence for a conserved BLM-resistance, transport-based mechanism in human cells is bolstered by a recent report showing that a dominant negative form of the mammalian Sky1 homologue (SRPK1) confers BLM-resistance in Chinese hamster lung fibroblast and HeLa cells, but not to various other DNA damaging agents (53). Thus, elucidation of homologous BLM resistance genes in human would be expected to reveal multiple novel molecular targets toward enhancing the antitumor properties of the drug.

ACKNOWLEDGMENTS

We are grateful to Andrea Shatilla and Drs. Elliot Drobetsky, Eric Milot, and Luc Gaudreau for comments on the manuscript and to Guillaume Lesage for assistance with the database searches.

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Cancer Res 2004;64:1102-1109.