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Cloning and Analysis of cDNA Encoding Rat Bleomycin Hydrolase, a DNA-Binding Cysteine Protease

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We isolated and characterized almost the entire cDNA encoding BLM hydrolase from rat spleen cDNA libraries. The cDNA encoded a polypeptide composed of 454 amino acids, that had a slightly larger molecular mass than that was previously estimated by SDS-PAGE for purified BLM hydrolase subunit. Amino acid sequence alignments showed that rat BLM hydrolase is homologous to that of rabbit (94% identity of partial amino acid sequence), yeast cysteine protease (39% identity), and the *pepC* gene products of three bacteria (34-40% identity). In addition, it contained the three regions that are conserved in other cysteine proteases and thought to constitute the catalytic center. These results indicated that rat BLM hydrolase is a member of the papain superfamily of cysteine proteases. Sequencing revealed several putative sites phosphorylated by different types of protein kinases, but no signal sequence, transmembrane domain, *N*-linked glycosylation site or DNA-binding motif. The yeast homolog is a DNA-binding cysteine protease [Xu *et al.* (1994) *J. Biol. Chem.* 269, 21177-21183]. We demonstrated that rat BLM hydrolase also binds the single-stranded form of the Gal4 DNA-binding site oligonucleotide with high affinity compared with that of the double-stranded form. Northern blots revealed that the level of BLM hydrolase mRNA expression was very low in the rat skin, lung, and skeletal muscle. Furthermore, BLM hydrolase mRNA was ubiquitously expressed in the human cell lines, HeLa, SKG-IIIa, FL, KB, HEp-2, U373 GM, P3HR-1, Raji, THP-1, Jurkat, and Molt-4. These results suggested that BLM hydrolase plays important physiological roles, including the metabolism of antibiotics.

Key words: amino acid sequence, BLM hydrolase, cDNA cloning, cysteine protease, DNA-binding protein.

Bleomycin (BLM) and its derivatives are antitumor glycopeptide antibiotics that are used extensively to several types of human cancers (*1, 2*). They bind to single- and double-stranded DNA and mediate the oxidative cleavage of DNA, leading to cell death (*3, 4*). However, the use of BLMs is limited by the development of resistance in some tumor cells (*5, 6*), and by pulmonary (*7, 8*) or skin toxicity (*9*). The susceptibility of both normal and malignant tissues to BLM-induced toxicity depends on the level of BLM hydrolase that inactivates BLM by hydrolyzing the carboxyamide bond of the β-aminoalanine moiety (*6*). BLM hydrolase is ubiquitous in mammals, birds, and reptiles in all tissues tested (*10, 11*) and it is classified into the papain superfamily of cysteine proteases (*12*). Homologs of the enzyme have been identified in yeast (*13-15*) and bacteria (*16-18*), in which the crystal structure of the yeast enzyme revealed a hexamer with a prominent central channel, whithin which the papain-like active sites are situated (*19*). Recently, we purified to homogeneity and characterized the enzyme from rat skin (*20*). The enzyme has a native molecular mass of 280 kDa and it consists of six identical subunits of 48 kDa. It exhibited peptidase activities with broad substrate specificity that are strongly inhibited by cysteine protease inhibitor E-64 but not by cystatins. However, there is no information about the complete amino acid and cDNA sequences of mammalian enzyme and the relationship between the enzyme and other cysteine proteases, although a partial cDNA of BLM hydrolase has been isolated from the rabbit (*11*). Here, we cloned and sequenced a cDNA encoding BLM hydrolase from rat spleen cDNA libraries and also examined the expression of BLM hydrolase mRNA in rat tissues as well as various human cell lines. We discuss some properties of the predicted amino acid sequence and possible biological functions of the enzyme.

MATERIALS AND METHODS

Materials—Restriction and modifying enzymes were purchased from Takara Shuzo (Ohtsu) and Gibco BRL (USA). [α-^{32}P]dCTP (3,000 Ci/mmol) and [γ-^{32}P]ATP were obtained from New England Nuclear (USA) and

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Abbreviations: BLM, bleomycin; bp, base pair(s); kbp, kilobase pair(s); PCR, polymerase chain reaction; RT, reverse transcription.

Amersham (UK), respectively. The random prime-labeling reagents were from Amersham (UK). Various human cells (HeLa, SKG-IIIa, FL, KB, HEp-2, U373 GM, P3HR-1, Raji, THP-1, Jurkat, and Molt-4) were seeded into 75 cm^2 flasks for RNA preparation. All cell lines were supplied from Japanese Cancer Research Resources Bank (JCRB) or American Type Culture Collection. Total RNAs were extracted from various tissues of 8-week-old Wistar male rats and cultured cells with guanidine thiocyanate (*21*) and by means of ISOGEN (Nippongene), respectively. BLM hydrolase was purified from the skin of 2-day-old Sprague-Dawley newborn rats (Japan Clea) as described (*20*). Other reagents were of analytical grade.

cDNA Cloning and Sequencing—Routine molecular cloning techniques were used (*22*). Oligonucleotide primers designed based on a partial cDNA of rabbit BLM hydrolase (*11*) were as follows. Primer 1 (forward), 5′-ATGCTTCCC-TGAATCACATAC-3′; Primer 2 (reverse), 5′-CATATTG-CTTAGGTAGTCTAC-3′. The directions and locations in rat BLM hydrolase cDNA corresponding to both primers are shown in Fig. 1. Total RNA from rat spleen was reverse-transcribed using random hexanucleotide primers and *Moloney Murine Leukemia Virus* reverse transcriptase (Promega, USA). The reverse transcription (RT) product was amplified by the polymerase chain reaction (PCR) using a pair of primers for 40 cycles of 1 min at 94°C, 1 min at 62°C, and 2 min at 72°C. After electrophoresis, the predicted PCR product was cut out from the 1.8% agarose gel and purified with MERmaid™ kit (BIO 101, USA). The fragment was subcloned into the pUC18 vector and was sequenced by dideoxynucleotide chain termination (*23*) using a *Bca*BEST™ kit (Takara).

Double-stranded cDNA was synthesized with poly(A)$^+$-RNA isolated from the rat spleen and ligated into λgt10 to construct of a cDNA libraries using a commercially available system (Amersham). About 1.5×10^5 recombinant phage plaques were screened with the ^{32}P-labeled fragment. Following double rounds of plaque purification and screening, seven independent positive clones with a relatively high intensity were plaque-purified. All clones with 2.2 kbp DNA insert were amplified by PCR using λgt10 forward and reverse primers. The DNA inserts were subcloned into the pUC118 vector and a series of deletions were produced from the 5′- and 3′-ends using a kilo-sequencing deletion kit (Takara). The nucleotide sequences were determined as above. The nucleotides and predicted amino acid sequences were analyzed by means of a computer search of GenBank and EMBL databases.

Northern Blots of BLM Hydrolase mRNA of Rat Tissues—Twenty micrograms of total RNA was resolved by electrophoresis on a 1% agarose gel containing 1% formaldehyde. The RNA species were then transferred to nylon membranes in 20×SSC and UV cross-linked. Blots were hybridized with ^{32}P-labeled fragment overnight at 45°C in 50% formamide containing 6×SSC, 5×Denhardt's solution, and denatured salmon sperm DNA (50 μg/ml). The membranes were then washed twice in 2×SSC and 1% SDS and for another 15 min in 1×SSC and 1% SDS at 60°C. Washed membranes were exposed at −80°C overnight to X-ray film RXO-G (Fuji Photo Film, Tokyo).

RT-PCR of BLM Hydrolase mRNA of Human Cell Lines—RT-PCR proceeded as described above using 5 μg of total RNA from various human cells. The sequences of the forward (5′-ATGCTTCCCTGAATCGCATAC-3′) and reverse (5′-TTACCCAAACATATGCACAC-3′) primers were based on the rat BLM hydrolase cDNA sequence. The predicted 189 bp amplification product composed nucleotides 489 to 677 of rat BLM hydrolase cDNA. PCR products were resolved by electrophoresis on a 1.8% agarose gel containing ethidium bromide and visualized by UV-induced fluorescence.

DNA Binding—A gel shift assay was performed according to Xu and Johnston (*24*) with slight modification. Briefly, the binding reaction proceeded using 0.9 or 1.8 μg of rat epidermal BLM hydrolase and 0.5 μg of a single-stranded Gal4 DNA-binding site oligonucleotide (5′-AGC-TTAGCGGAAATTTGTGGTCCGAGC-3′) or its complementary sequence (5′-TCGAGCTCGGACCACAAATTTC-CGCTA-3′) as well as double-stranded oligonucleotide prepared from them. The reaction solution consisted of a 16-fold excess of rat enzyme and a 1,000-fold excess of oligonucleotide compared with the published ratio (*24*), that corresponded to a molar excess of the latter against the former. The protein-DNA complexes were resolved in 4% polyacrylamide gels in 0.5×TBE (45 mM Tris-borate, 1 mM EDTA, pH 8.0). The gels were dried and autoradiographed on Fuji Photo film RXO-G. Both native and denatured DNA-cellulose columns (0.7×5 cm) (Pharmacia, Sweden) were washed with 10 volumes of binding buffer (12 mM HEPES pH 7.8, 60 mM KCl, 12% glycerol, 0.12 mM EDTA, 5 mM $MgCl_2$, 5 mM DTT) at 4°C. The purified enzyme was dialyzed against 100 volumes of binding buffer, then applied to the columns at a flow rate of 30 ml/h at 4°C. The flow-through fraction, which did not bind to the columns, was collected. The DNA affinity protein fraction was then eluted with binding buffer containing 1 M KCl (*25*).

Amino Acid Sequence Analysis of Tryptic Peptides—Dipyridylated BLM hydrolase was digested with trypsin as described (*20*). The digested peptides were separated by reversed phase HPLC on ODS-120T (TOSOH, Tokyo), using a linear gradient elution with 0 to 60% acetonitrile in 0.1% trifluoroacetic acid for 60 min at a flow rate of 5 ml/min. Peak fractions detected by measuring the absorbance 215 nm were collected manually in test tubes and lyophilized. The amino acid sequence was determined using an Applied Biosystem Protein Sequencer linked with an Applied Biosystem model 120A PTH analyzer.

RESULTS

Cloning and Analysis of cDNA Coding for Rat BLM Hydrolase—The cDNA from rat spleen RNA was amplified by means of PCR using primers corresponding to the partial sequence of rabbit cDNA (*11*). The amplified product of 388 bp comprised nucleotides 489 to 876 of the rat BLM hydrolase sequence (thin underline in Fig. 1). The nucleotide sequence had 89% identity with that of the partial rabbit cDNA. The ^{32}P-labeled fragment hybridized to a 2.5 kb mRNA from various rat tissues (see below). This fragment was used as a probe for screening rat spleen cDNA libraries. We isolated at least seven individual clones with a DNA insert of about 2.1 kbp. Analysis of the insert revealed that the 2,149 bp sequence contained an open reading frame with a start (ATG) and a stop codon (nucleotides 1363-1365) (Fig. 1). The DNA insert was slightly shorter than the mRNA detected by Northern

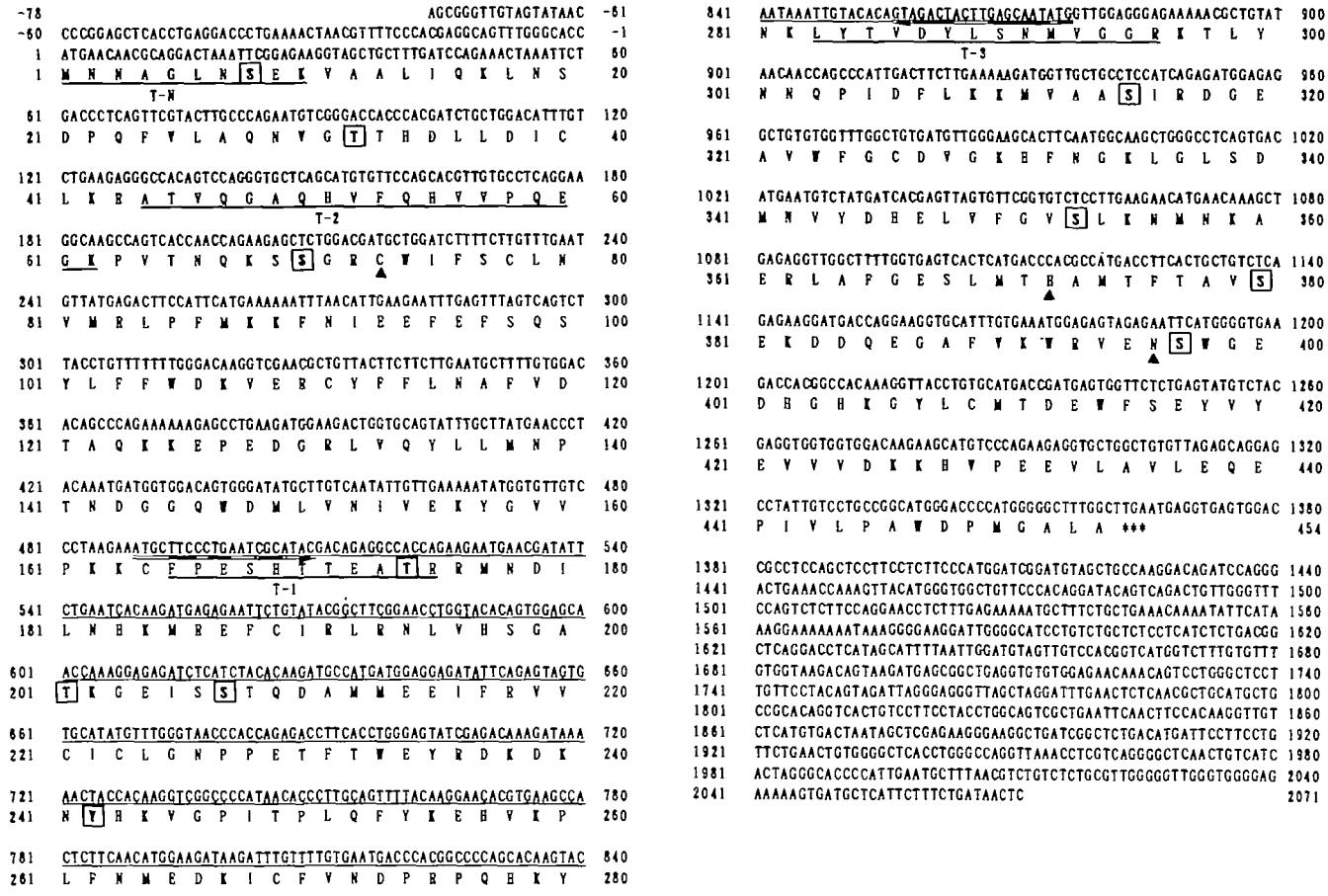

Fig. 1. **Nucleotide and predicted amino acid sequences of rat BLM hydrolase.** The nucleotide sequence is numbered starting at the initiation codon ATG. The predicted amino acid sequence is numbered from the NH_2-terminal amino acid residue of the enzyme. Arrows indicate the directions and locations in rat enzyme cDNA corresponding to PCR primers designed from the partial cDNA of rabbit enzyme. The nucleotide sequence of the PCR product used as a probe for screening cDNA libraries is indicated by a thin line. The stop codon is indicated by asterisks. Arrow heads and squares indicate cysteine protease catalytic triad residues and putative phosphorylation sites, respectively. Bold underlines indicate the amino acid sequences determined with trypsin-digested peptides (T-N, T-1, T-2, and T-3).

blotting. Thus, a consensus polyadenylated signal and a poly(A) tail was not found in these clones. When the first in-frame ATG was assigned to the initiation codon, the predicted NH_2-terminal amino acid sequence (MNNAGLNSEK) was consistent with that determined by directly sequencing the purified epidermal enzyme (*20*). This Met may be an authentic translation initiation site.

Analysis of the Predicted Amino Acid Sequence of Rat BLM Hydrolase—The predicted amino acid sequence of rat BLM hydrolase contains 454 amino acid residues. Amino acid sequences of the three tryptic peptides (T1-T3) from rat epidermal BLM hydrolase were determined as shown in Fig. 1 (bold underlines) and perfectly matched the predicted amino acid sequences. The calculated molecular mass from the predicted amino acid sequence was 52.3 kDa, being slightly larger than that of the enzyme subunit estimated by SDS-PAGE (*20*). A hydropathy analysis of the predicted amino acid sequence indicated that there was no long stretch of hydrophobic residues that could serve as a putative signal sequence for protein secretion or a transmembrane domain. Furthermore, the sequence lacked an *N*-linked glycosylation site and DNA binding motifs, but there were putative sites of phosphorylation by various protein kinases at residues 8, 32, 70, 174, 201, 207, 242, 315, 353, 380, and 397 (squares in Fig. 1).

Comparison of Rat BLM Hydrolase Amino Acid Sequence with That of Other Cysteine Proteases—The complete amino acid sequence of rat BLM hydrolase was aligned with the published sequences of a BLM hydrolase group in the papain superfamily of cysteine proteases (Fig. 2A). The rat enzyme is most homologous to that of rabbit. Within 277 amino acid residues of rabbit BLM hydrolase (*11*), rat and rabbit enzymes were identical with respect to 261 amino acids (94%) and gaps were not necessary for optimal alignment. The rat BLM hydrolase is less homologous with the yeast enzyme (*13-15*) with 174/441 matches (39%) requiring 9 gaps, as well as with *pepC* gene products (cysteine aminopeptidase) from *Lactococcus lactis* subsp. *cermoris* AM2 (*16*), *Streptococcus thermophilus* (*17*), and *Lactobacillus helveticus* CNRZ32 (*18*) with 171/423 (40%), 171/429 (40%), and 148/429 matches (34%) requiring 3 gaps, respectively. A comparison of the predicted amino acid sequence of rat BLM hydrolase with that of other cysteine proteases in the databases showed that the catalytic triad residues (Cys^{73}, His^{372}, and Asn^{396}; arrow heads in Figs. 1 and 2) of these enzymes were well

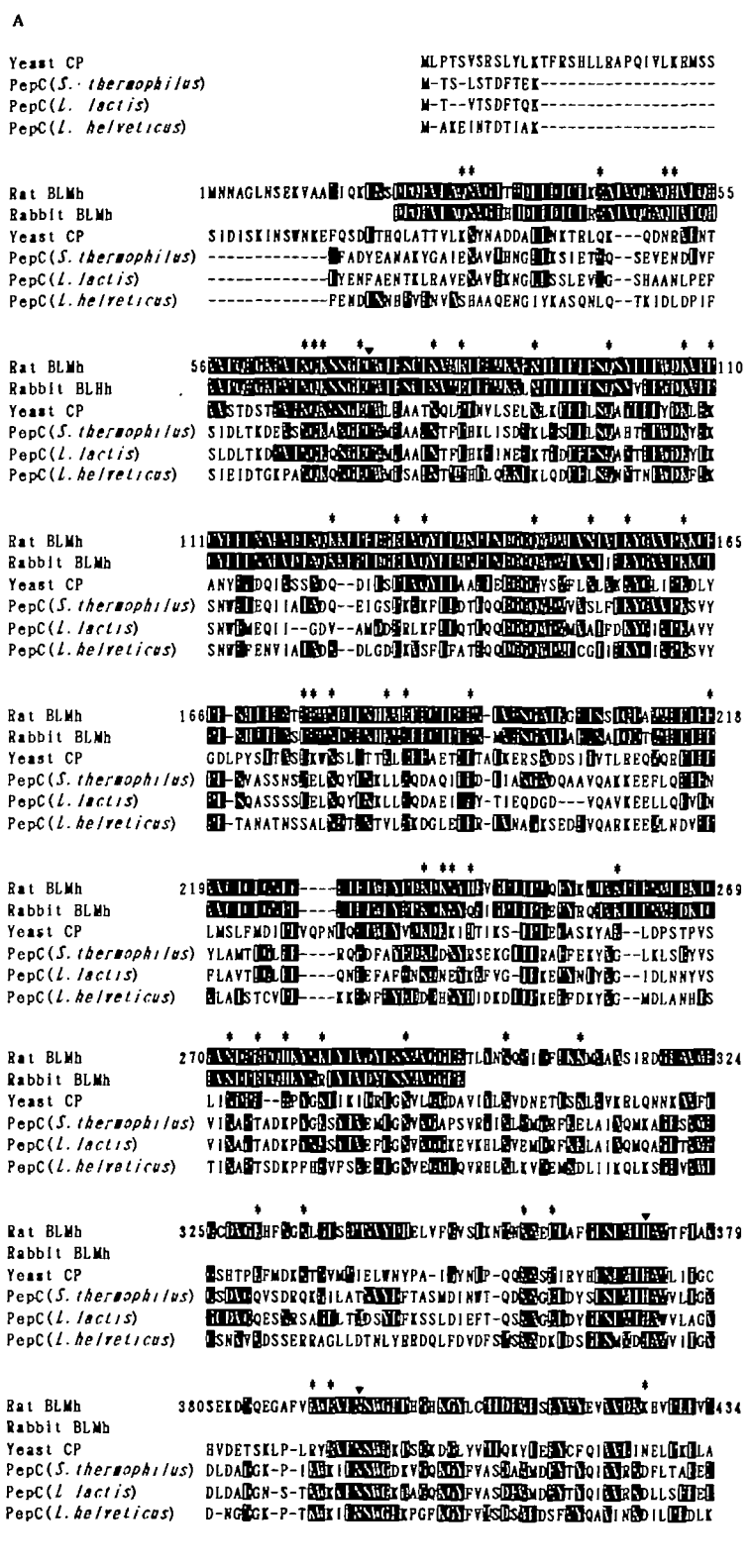

Fig. 2. **Alignment of amino acid sequences of rat BLM hydrolase and related cysteine proteases.** A: The sequence of rat BLM hydrolase was compared with those of related cysteine proteases using gaps to achieve maximal alignment. Numbers refer to the sequences of rat BLM hydrolase. Residues that are identical with those in rat BLM hydrolase are indicated by black boxes, and gaps are represented by dashes. Arrow heads indicates cysteine protease catalytic triad residues. Asterisks indicate basic amino acid residues showing identity or similarity between the rat and yeast enzymes. Rat BLMh, rat bleomycin hydrolase; rabbit BLMh, rabbit bleomycin hydrolase; yeast CP, yeast cysteine protease; pepC(*S. thermophilus*), *pepC* gene product from *S. thermophilus*; pepC(*L. lactis*), *pepC* gene product from *L. lactis* subsp. *cermoris* AM2; pepC(*L. helveticus*), *pepC* gene product from *L. helveticus*. B: Alignment of rat BLM hydrolase with other cysteine proteases in the regions containing the putative active site Cys, His, and Asn (indicated by arrow heads). Amino acids identical to rat BLM hydrolase are indicated by black boxes. Sequences were taken from Refs. *11, 14, 16-18, 37-44*.

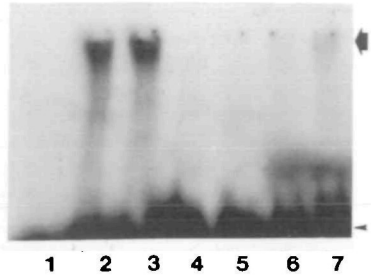

Fig. 3. **DNA-binding properties of rat BLM hydrolase.** The binding reaction, analysis of the complex and preparation of double-stranded DNA with the Gal4 DNA-binding oligonucleotide and its complementary sequence proceeded as described in "MATERIALS AND METHODS." Concentrations of rat BLM hydrolase: 0 μg in lane 1, 0.9 μg in lanes 2, 4, and 6, and 1.8 μg in lanes 3, 5, and 7. DNA: single-stranded DNA of Gal4 DNA-binding site oligonucleotide in lanes 1, 2, and 3; single-stranded DNA of the complimentary oligonucleotide in lanes 4 and 5; double-stranded DNA in lanes 6 and 7. Large and small arrows indicate the positions of the complex and free DNA, respectively.

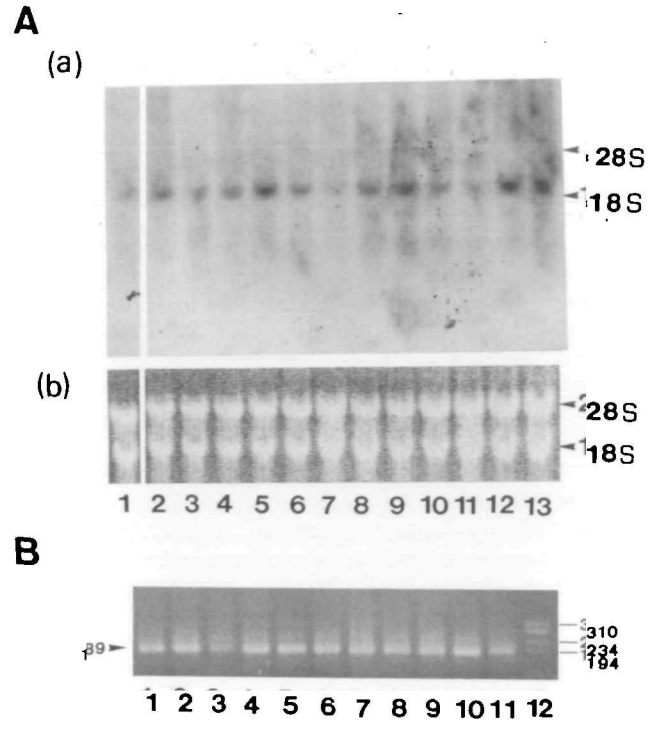

Fig. 4. **Expression of BLM hydrolase mRNA in rat tissues (A) and human cell lines (B).** A: Northern blots. (a) Total RNA (20 μg) isolated from rat tissues hybridized with ^{32}P-labeled fragment. Lanes: 1, skin; 2, brain; 3, heart; 4, liver; 5, stomach with the esophagus; 6, small intestine; 7, lung; 8, kidney; 9, spleen; 10, adrenal gland; 11, skeletal muscle; 12, thymus; 13, testis. 28S and 18S indicate the positions of eukaryotic ribosomal RNA markers. (b) Photograph of the ethidium bromide stained gel before transfer to display about 20 μg of RNA isolated in each lane. B: RT-PCR. Total RNA (5 μg) from various human cell lines were reverse-transcribed and the cDNAs were amplified by PCR as described in "MATERIALS AND METHODS." Lanes: 1, HeLa; 2, SKG-IIIa; 3, FL; 4, KB; 5, HEp-2; 6, U373 GM; 7, P3HR-1; 8, Raji; 9, THP-1; 10, Jurkat; 11, Molt-4; 12, markers (ϕX174 RF DNA/*Hae*III fragment). The numbers on the right and left indicate base pair(s).

conserved and sequence homology was detected only in the active site subset (Fig. 2B).

Characterization of DNA Binding Ability of Rat BLM Hydrolase—We analyzed the DNA-binding ability of rat epidermal BLM hydrolase by means of a gel shift assay and by chromatography on DNA-cellulose columns. The complex migrated as an intense band after binding rat epidermal enzyme with the single-stranded form of the Gal4 DNA-binding site nucleotide, but as a faint band with the double-stranded form in gel shift assays (Fig. 3). These results indicated that the binding affinity to single-stranded form was significantly higher than that to the double-stranded nucleotide, which was consistent with that of yeast enzyme (*24*). No complex was detected using 0.5 ng of both single- and double-stranded oligonucleotides after binding with rat epidermal enzyme (data not shown), indicating that the DNA binding affinity of rat enzyme is lower than that of the yeast. To verify the sequence specificity of the DNA for the enzyme-DNA binding, we performed DNA-cellulose column chromatography. The purified rat epidermal BLM hydrolase did not bind to either native or denatured DNA-cellulose columns.

Expression of BLM Hydrolase mRNA in Rat Tissues and Human Cells—We examined the expression of BLM hydrolase mRNA in rat tissues and human cells by Northern blotting and RT-PCR, respectively. BLM hydrolase mRNA was expressed in all rat tissues tested, at relatively higher levels in the stomach with esophagus, spleen, thymus, and testis, and at much lower levels in the skin, lungs, and skeletal muscle (Fig. 4A). These findings were consistent with those of immunoblotting (*20*). Furthermore, the BLM hydrolase transcript was found in all samples from of cell lines tested by RT-PCR using primers based on rat BLM hydrolase cDNA (Fig. 4B).

DISCUSSION

In this study we isolated almost the entire cDNA clone for rat BLM hydrolase and determined its sequence. We also examined the expression of BLM hydrolase mRNA in various rat tissues and human cell lines. An open reading frame contained the identical amino acid sequences with NH_2-terminus (T-N) and tryptic peptides (T1-T3) of rat epidermal BLM hydrolase. The amino acid composition deduced from the sequence was similar to that of rat epidermal BLM hydrolase (*20*). The slight discrepancy in the amounts of Asx, Thr, Cys, and Met might have been caused by the conditions under which samples were hydrolyzed and analyzed. An oligonucleotide probe amplified by RT-PCR hybridized to a single mRNA species of 2.5 kb from various rat tissues. These data indicated that the cDNA clone corresponds to the subunit of rat BLM hydrolase. The molecular mass calculated from the predicted amino acid sequence was larger than that of the rat epidermal BLM hydrolase subunit estimated by SDS-PAGE (*20*). This may be because rat BLM hydrolase is post-translationally modified. An NH_2-terminus acetylmethionine was identified in the epidermal enzyme (*20*). In addition, the predicted amino acid sequence contained putative phosphorylation sites. The modified enzyme has increased mobility towards the anode on SDS-PAGE, so that its apparent molecular mass would become slightly smaller than that predicted. However, further studies are

necessary to resolve this issue.

Rat BLM hydrolase did not contain any DNA binding motifs such as a basic helix-turn-helix, a Zn-finger, or a leucine zipper (*26*). In addition it did not have the features typical of a basic protein, having a large amount of basic amino acid residues, a glycine-rich region and a proline cluster. Although many DNA-binding proteins have similar features, those without major DNA binding motifs have been described (*27*). The yeast homolog, Gal4, is a DNA-binding cysteine protease (*24*). Its crystal structure has revealed that a putative region of DNA binding is situated within a central channel that is lined with basic amino acid residues derived from six subunits (*19*). The sequence alignment of rat BLM hydrolase revealed significant identity or similarity of basic amino acid residues at the central channel or others predicted as the DNA binding site in the yeast enzyme (asterisks in Fig. 2). The rat epidermal enzyme also bound to single-stranded form of the Gal4 binding site nucleotide. However, it could not bind to DNA-cellulose, suggesting that some modification is necessary for binding to DNA (*28, 29*). Another possibility is that BLM hydrolase binds to DNA with a specific conformation such as β-hairpin loop structure (*30, 31*). Although there is no direct evidence that BLM hydrolase binds to DNA in the cell nucleus, the DNA binding of both BLM and its hydrolase may be physiologically relevant.

The sequence homology was remarkable at the carboxyl termini in the sequences of the BLM hydrolase group of the papain family. The carboxyl terminus of the yeast enzyme projected into the active site (*19*) and its structure resembled those of inhibitors complexed with papain (*32-35*). This structure may explain why the activity of BLM hydrolase has been limited to small peptides such as the aminoacyl-β-naphthylamide of various amino acids. Furthermore, E-64 was the most potent inhibitor of BLM hydrolase from different species (*11, 14, 17, 20, 24*). It might displace the carboxyl-terminal arm in the active site cleft. We inferred that rat BLM hydrolase also has characteristics similar to those of the yeast enzyme as well as other homologs at the structural and functional levels, and its activity could be regulated by interaction with other molecules.

The expression levels of BLM hydrolase may closely correlate with the toxic side effects of BLM (*7, 8*). Pei *et al.* (*36*) have reported that NIH3T3 cells transfected with the yeast homolog gene *ycp1* of mammalian BLM hydrolase become more resistant to BLM than control cells. We demonstrated that the tissues susceptible to BLM-induced cell toxicity such as those of the lung and skin expressed low levels of enzyme mRNA. However, the ubiquitous expression of BLM hydrolase mRNA in various rat tissues and human cell lines suggests that the enzyme plays important physiological roles, including that of antibiotic metabolism during cancer chemotherapy. Although more detailed studies are required, the results presented here will provide useful information for further characterization of BLM hydrolase.

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