Rapid Communication *J. Biochem.* **129**, 671–674 (2001)

Use of Bleomycin- and Heat Shock–Induced Calreticulin Promoter for Construction of a Mammalian Expression Vector

Hashim Elmileik,* Takanori Kumagai,† Manuel Berengena,† Kazuhiro Ueda,* and Masanori Sugiyama†,[1]

**Department of Pediatrics, School of Medicine, and †Institute of Pharmaceutical Sciences, Faculty of Medicine, Hiroshima University, Kasumi 1-2-3, Minami-ku, Hiroshima 734-8551*

Received December 18, 2000; accepted March 8, 2001

Addition of bleomycin (Bm) to an NIH/3T3 cell culture induced the overproduction of four cellular proteins [Kumagai and Sugiyama (1998) *J. Biochem.* 124, 835–841]. The two proteins were identified on N-terminal amino acid sequence analysis as calreticulin and mitochondrial matrix protein P1, which are known as heat shock proteins, respectively. In this study, we cloned the calreticulin promoter region from the genomic DNA of NIH/3T3 cells and observed that heat shock treatment at 42°C or the addition of Bm to the cell culture caused overexpression of the luciferase gene controlled by the cloned calreticulin promoter. This suggests that Bm induces the transcriptional activation of stress-heat shock genes. We constructed an expression vector for mammalian cells, which is controlled by the calreticulin promoter.

Key words: bleomycin, bleomycin resistance, calreticulin, heat shock protein, stress response.

An antibiotic, bleomycin (Bm), causes nucleotide sequence–specific DNA cleavage, and inhibits the growth of both bacterial and mammalian cells (*1*). Since Bm effectively inhibits the growth of malignant cells, it is frequently used to treat human malignancies. However, Bm is known to induce lung disease, which occurs as a result of cell injury followed by fibroblast proliferation (*2*), as a side-effect.

Cells respond to injury by synthesizing a family of heat shock proteins. The heat shock response comprises rapid but transient reprogramming of cellular activities to ensure survival during the stress period, to protect essential cell components against heat damage and to allow the resumption of normal cellular activities during the recovery period.

We have found that when exposed to Bm, NIH/3T3 cells exhibit malformed spindle bodies and overexpress at least 4 kinds of protein including calreticulin and mitochondrial matrix protein P1 (Hsp60) (*3*). Stress-induced up-regulation of calreticulin has been suggested to contribute to the mechanisms by which the endothelium and lung tissue, and possibly other organ systems, maintain homeostasis when exposed to pathophysiological conditions (*4*). Although calreticulin is ubiquitous Ca^{2+}-binding protein of the sarcoplasmic and endoplasmic reticulum, its precise function is unknown in detail (*5*).

We have cloned two independent genes, designated as *blmA* and *blmB*, encoding Bm-resistance determinants from Bm-producing *Streptomyces verticillus* (*6*). The former gene has been shown to encode a Bm-binding protein (*6*). NIH/3T3 cells carrying *blmA* could grow without morphological changes and expressed no stress proteins even in the presence of Bm (*3*).

Based on the phenomenon that a DNA-cleaving antibiotic, Bm, acts as a pathophysiological stress agent, we cloned, in the present study, a Bm-induced promoter region from NIH/3T3 cells and utilized it for construction of a mammalian expression vector.

The genomic DNA was isolated from the NIH/3T3 cells to clone the calreticulin promoter regions. Based on the nucleotide sequence of the calreticulin promoter from a mouse liver genomic DNA (*7*), sense (5′-TATAACCGGTTAATTTTTATTTTATTTGTATAGATGTTTTGCCTGCACGTATGTCTCTGT-3′) and anti-sense (5′-CTAGAATTCCGAGGGGGCGGCG-3′) oligonucleotide primers were designed, synthesized and used for PCR together with the NIH/3T3 genomic DNA as a template. The nucleotide sequence of the amplified gene was identical with that of the calreticulin promoter from a mouse liver (*7*). The amplified DNA containing the calreticulin promoter was double-digested with *Hin*dIII and *Eco*RI, and then blunt-ended with T4 DNA polymerase. The resulting 1.79-kb fragment was inserted just upstream of the luciferase gene in the pGL3-enhancer vector (Promega product), dephosphorylated with bacterial alkaline phosphatase (TaKaRa, Osaka) after digestion with *Sma*I. The resulting plasmid, designated as pGL3-E/CP (Fig. 1), carries the calreticulin promoter–controlled luciferase gene, poly A signal and the SV40-derived enhancer.

The NIH/3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Sigma) at 37°C with 5% CO_2 in a humidified incubator. The cells were plated at a density of 2×10^5 per dish (35 mm in diameter) and grown until they reached 70% confluency for transfection.

Transfection of the NIH/3T3 cells with 2 μg of pGL3-E/CP was carried out by the lipofection method using lipo-

[1] To whom correspondence should be addressed. Tel: +81-82-257-5280, Fax: +81-82-257-5284, E-mail, sugi@hiroshima-u.ac.jp

Abbreviations: Bm, bleomycin; *blmA*, a gene which confers bleomycin resistance from bleomycin-producing *Streptomyces verticillus*; PBS, phosphate-buffered saline.

fectamine (Gibco BRL, USA) according to the supplier's instructions. For normalization of the transfection efficiencies, 0.5 μg of the pRL-TK vector (Promega product) was included in the transfection as an internal standard for transfection efficiency, according to the supplier's instruction manual. The cells were incubated with the transfection mixture for 5 h and then transferred to fresh DMEM. The transfectants were incubated for 48 h in the presence of 25 μg/ml of Bm. After washing with cold PBS, the cells were exposed to a passive lysis buffer (Promega) for 15 min to obtain a cell lysate with gentle shaking.

For the heat shock experiment, NIH/3T3 cells harboring pGL3-E/CP were grown at 42°C for 2 or 6 h, but control cells were maintained at 37°C.

Twenty microliters of the cell lysate expressing luciferase activity was mixed with 100 μl of a luciferase substrate solution in a Dual-Luciferase Reporter Assay System (Promega). The resulting luminescence was measured for 10 s using Lumi-counter 700 (NITI-ON, Chiba). The values are presented as the means for three independent experiments.

Calreticulin is a heat shock protein. Therefore a reporter gene, that is under the control of the calreticulin promoter, must be inducibly expressed on heat shock treatment. To confirm the heat shock response, we constructed a vector, designated as pGL3-E/CP, harboring the luciferase structural gene controlled by the calreticulin promoter and then transfected NIH/3T3 cells with it (Fig. 1). After growth at 37°C for 48 h, the transfectant was incubated at 42°C for 2 or 6 h. As expected, when incubated at 42°C, the cells harboring pGL3-E/CP expressed 2-fold higher luciferase activity than the same cells maintained at 37°C (Fig. 2). In detail, the enzyme activity increased dramatically after 2 h-incubation at 42°C, and then decreased gradually during additional incubation for 4 h, indicating that the calreticulin promoter is responsible to the heat shock. During continuous heat treatment at 42°C, the survival curve of cells was shown to exhibit a biphasic response. The cells are known to be more heat resistant after a few hours of hyperthermia (*8*). The heat shock protein productivity in Chinese hamster fibroblasts has been reported to be correlated with thermo-tolerance (*9*). Therefore, the decrease in luciferase activity in our experiment could have been a response to the heat challenge.

The NIH/3T3 cells harboring pGL3-E/CP exposed to Bm showed 1.5-fold higher luciferase activity than those not exposed to it (Fig. 3). The NIH/3T3 cells transfected with a vector, designated as the pGL3-Control vector (Promega product), which carries the luciferase structural gene controlled by the SV40-derived promoter, scarcely expressed luciferase activity (Fig. 3).

A lethal concentration of Bm causes destruction of the cell membrane, which is mediated by free radicals generated by the Bm-Fe(II) complex. The radicals react rapidly with lipids and nucleic acids, and mediate the cytotoxicity of Bm (*10*).

Our group has found that a protein, designated as BLMT, encoded by *ble* from transposon Tn*5* is almost the same Bm-binding protein as the *blmA* gene product (*11–13*). This protein has been shown to repress the formation of Bm-Fe(II) complex–induced free radicals, as a result of binding of Bm to BLMT (*11*). This suggests that the *blmA* gene product also prevents the formation of Bm-induced free radicals. When transfected with pEF-BOS carrying *blmA*, designated as pEF-BOS/*blmA*, the NIH/3T3 cells were resistant to Bm (*3*).

The previous study showed that a stable transformant of *blmA*, obtained by culturing NIH/3T3 cells transfected with pEF-BOS/*blmA*, is resistant to Bm and does not overexpress stress proteins like Hsp60 and calreticulin (*3*).

Based on the present results, it is difficult to explain reasonably that Bm, causing single-strand scission of cellular DNA, induces expression of the calreticulin promoter–con-

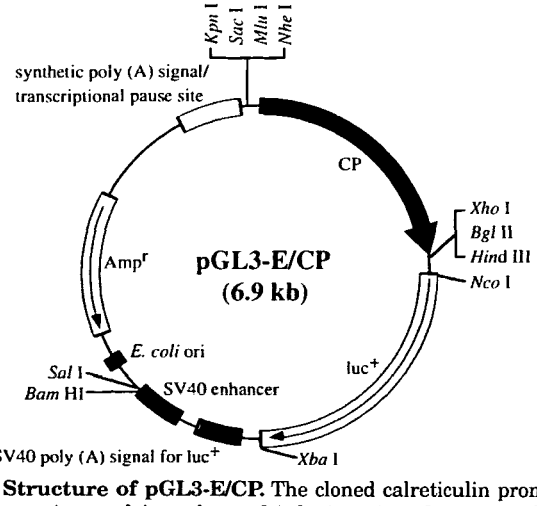

Fig. 1. **Structure of pGL3-E/CP.** The cloned calreticulin promoter region was inserted into the multi-cloning site of a vector, designated as the pGL3-Enhancer vector (Promega product), to generate pGL3-E/CP. Luc+, luciferase structural gene; Amp^r, a gene which confers resistance to ampicillin; CP, calreticulin promoter.

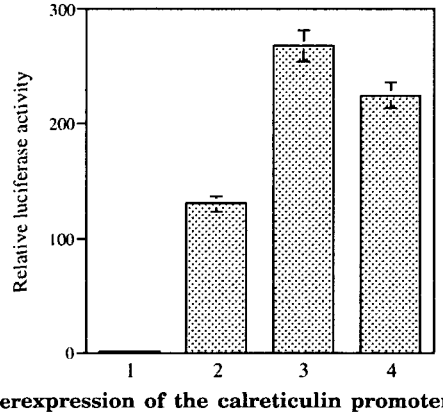

Fig. 2. **Overexpression of the calreticulin promoter-controlled luciferase gene on heat shock treatment.** NIH/3T3 cells transfected with pGL3-E/CP were grown at 37°C for 48 h, followed by incubation at 42°C for 2 or 6 h. The luciferase activity present in cell lysates was measured according to the method described in the text. 1, cells transfected with the pGL3-Enhancer vector (Promega product), which lacks the calreticulin promoter, as a control; 2, pGL3-E/CP transfected cells grown without incubation at 42°C; 3 and 4, pGL3-E/CP–transfected cells incubated at 42°C for 2 and 6 h, respectively. Relative luciferase activity was expressed as the ratio of the enzyme activity of the *Photinus pyralis* luciferase from the above vectors to that of the *Renilla reniformis* luciferase from the pRL-TK vector (Promega product) as an internal standard, according to the supplier's instruction manual (Promega). Data represent the means for three separate experiments and bars show the standard deviation.

trolled gene. Bm has been shown to alter DNA transcription through its interaction with different transcription factors, as well as the modification of a repressor protein, allowing transcription to take place (*14*). Although there is a possibility that the Bm action may involve a direct effect on the promoter or may be related to a DNA/protein interaction, it might be difficult to assume that a DNA-cleaving agent, Bm, functions directly to the calreticulin promoter.

The previous study suggested that the expression of *blmA* in lung fibroblasts may prevent Bm-induced pulmonary toxicity (*3*). In this case, since the constitutive expression of the *blmA* gene product is thought to be toxic to the host cells, the use of the calreticulin promoter may make it possible to express the gene inducibly. In the present study, we constructed an expression vector for mammalian cells, which is controlled by heat shock treatment or the addition of Bm, according to the following strategy: PCR primers having *Apa*I and *Xba*I sites in the 5′- and 3′-adjacent regions as to the calreticulin promoter were used to amplify the calreticulin promoter from pGL3-E/CP. The DNA fragment obtained was inserted into a vector, pcDNA3.1(−) (Invitrogen product), which was double-digested with *Apa*I and *Xba*I. After the chimeric plasmid had been digested with *Mfe*I and *Apa*I to eliminate the cytomegalovirus promoter from pcDNA3.1(−), the resulting large fragment was blunt-ended and then self-ligated to generate the pcDNA/CP vector (Fig. 4). This vector has *Xho*I, *Eco*RI, *Eco*RV, *Not*I, and *Hin*dIII sites, as typical and unique restriction sites. To express *blmA* inducibly under the control of the calreticulin promoter, the *blmA* structural gene having *Not*I and *Eco*RI, respectively, in the 5′- and 3′-adjacent regions was PCR-amplified using plasmid p181EB1 (*15*) as a template. The sense and anti-sense oligonucleotide primers for amplifying the *blmA* gene were 5′-GGGAGCGGCCG-CATGGTGAAATTCTTGGGTGC-3′ and 5′-GGAAGAATTC-TCACTCCCCCGCGGTGAAGT-3′, respectively. The *blmA* structural gene was inserted into the pcDNA/CP vector double-digested with *Not*I and *Eco*RI. The resulting vector, designated as pcDNA/CP/*blmA*, was introduced into NIH/3T3 cells. The transfectant was incubated at 37°C for 48 h in the presence or absence of 25 μg/ml Bm. The mRNA (80 ng), isolated from each cell using a QuickPrep Micro mRNA Purification Kit (Pharmacia Biotech. USA), was used for the reverse transcription-polymerase chain reaction (RT-PCR) using Superscript II (Gibco BRL, USA) and Ex *Taq* polymerase (Takara). As expected, when incubated with Bm, the cells harboring pcDNA/CP/*blmA* expressed marked *blmA* cDNA, as detected with the above oligonucleotide primers, than the same cells incubated without Bm (Fig. 5), suggesting that Bm induces the *blmA* gene expression under the control of calreticulin promoter.

Despite the high efficiency of Bm as a chemotherapeutic agent against various human malignancies, its use is sometimes limited by alveolar damage and pulmonary fibrosis. If the pcDNA/CP/*blmA* vector is introduced and expressed in lung tissues, the administer Bm will induce the expression of the calreticulin promoter–controlled *blmA*. The Bm-induced gene product may protect the lung tissues from the toxicity of Bm. Thus, the inducible expression of *blmA*, but not its constitutive expression, with the pcDNA/CP/*blmA* vector, may be useful for protecting patients from the immunological cell response to the *blmA* protein.

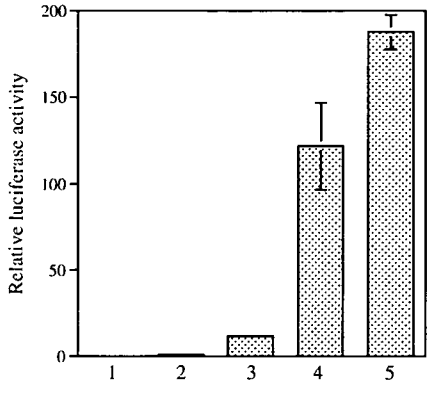

Fig. 3. **Overexpression of the calreticulin promoter–controlled luciferase gene on the addition of Bm.** NIH/3T3 cells transfected with pGL3-E/CP were grown at 37°C in the presence or absence of 25 μg/ml Bm. The luciferase activity present in cell lysates was measured according to the method described in this text. 1, untransfected host cells, as a control; 2, cells transfected with the pGL3-Enhancer vector (Promega product), which lacks the calreticulin promoter; 3, cells transfected with the pGL3-Enhancer vector carrying the luciferase gene controlled by the SV40 promoter; 4 and 5, pGL3-E/CP transfected–cells grown in the absence or presence of Bm, respectively. Data represent the means for three separate experiments and bars show the standard deviation.

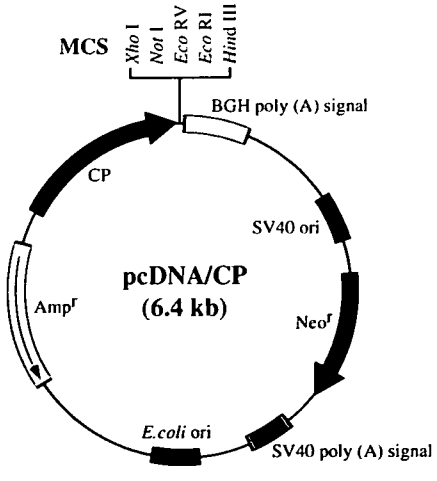

Fig. 4. **Structure of an expression vector, pcDNA/CP, controlled by heat shock treatment or the addition of Bm for mammalian cells.** MCS, multi-cloning sites; BGH, bovine growth hormone; Neo^r, a gene which confers resistance to neomycin.

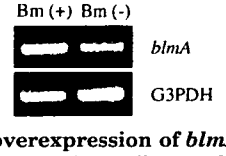

Fig. 5. **Bm-induced overexpression of *blmA* controlled by the calreticulin promoter.** NIH/3T3 cells transfected with pcDNA/CP/*blmA* were grown at 37°C in the presence or absence of 25 μg/ml Bm. The mRNA from each cell was subjected to RT-PCR to detect the *blmA* cDNA. The glyceraldehyde 3-phosphate dehydrogenese (G3-PDH) gene was used as an internal control. The PCR conditions were 24 and 30 cycles for *blmA* and G3PDH, respectively, of 45 s at 94°C, 45 s at 60°C, and 2 min at 72°C. The amplified DNAs were subjected to 1% agarose gel electrophoresis.

We wish to thank Nippon Kayaku Co. Ltd., for the gift of bleomycin A_2 sulfate.

REFERENCES

1. Kross, J., Henner, W.D., Hecht, S.M., and Haseltine, W.A. (1982) Specificity of deoxyribonucleic acid cleavage by bleomycin, phleomycin, and tallysomycin. *Biochemistry* **21**, 4310–4318
2. Moseley, P.L., Hemken, C., and Hunninghake, G.W. (1986) Augmentation of fibroblast proliferation by bleomycin. *J. Clin. Invest.*.**78**, 1150–1154
3. Kumagai, T. and Sugiyama, M. (1998) Protection of mammalian cells from the toxicity of bleomycin by expression of a bleomycin-binding protein gene from *Streptomyces verticillus*. *J. Biochem.* **124**, 835–841
4. Conway, E.M., Liu, L., Nowakowski, B., Steiner-Mosonyi, M., Rebeiro, S.P., and Michalak, M. (1995) Heat shock-sensitive expression of calreticulin. *J. Biol. Chem.* **270**, 17011–17016
5. Krause, K.-H. and Michalak, M. (1997) Calreticulin. *Cell* **88**, 439–443
6. Sugiyama, M., Thompson, C.J., Kumagai, T., Suzuki, K., Deblaere, R., Villarroel, R., and Davies, J. (1994) Characterisation by molecular cloning of two genes from *Streptomyces verticillus*. *Gene* **151**, 11–16
7. Waser, M., Mesaeli, N., Spencer, C., and Michalak, M. (1997) Regulation of calreticulin gene expression by calcium. *J. Cell Biol.* **138**, 547–557
8. Burdon, R.H. (1986) Heat shock and the heat shock proteins. *Biochem. J.* **240**, 313–324
9. Li, G.C. and Werb, Z. (1982) Correlation between synthesis of heat shock proteins and development of thermotolerance in Chinese hamster fibroblasts. *Proc. Natl. Acad. Sci. USA* **79**, 3218–3222
10. Sugiura, Y. and Kikuchi, T. (1978) Formation of superoxide and hydroxy radicals in iron (II)-bleomycin-oxygen system: electron spin resonance detection by spin trapping. *J. Antibiot.* **31**, 1310–1312
11. Kumagai, T., Nakano, T., Maruyama, M., Mochizuki, H., and Sugiyama, M. (1999) Characterization of the bleomycin resistance determinant encoded on the transposon Tn*5*. *FEBS Lett.* **442**, 34–38
12. Kumagai, T., Maruyama, M., Matoba, Y., Kawano, Y., and Sugiyama, M. (1999) Crystallization and preliminary X-ray diffraction studies of bleomycin-binding protein encoded on the transposon Tn*5*. *Acta Cryst. Sec.* **D55**, 1095–1097
13. Kawano, Y., Kumagai, T., Muta, K., Matoba, Y., Davies, J., and Sugiyama, M. (2000) The 1.5 Å crystal structure of a bleomycin resistance determinant from bleomycin-producing *Streptomyces verticillus*. *J. Mol. Biol.* **295**, 915–925
14. Vig, B.K. and Lewis, R. (1978) Genetic toxicology of bleomycin. *Mutat. Res.* **55**, 121–145
15. Yuasa, K. and Sugiyama, M. (1995) Bleomycin-induced β-lactamase overexpression in *Escherichia coli* carrying bleomycin-resistance gene from *Streptomyces verticillus* and its application to screen bleomycin analogues. *FEMS Microbiol. Lett.* **132**, 61–66