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A Mammalian Vector Carrying the Bleomycin *N*-Acetyltransferase Gene from Bleomycin-producing *Streptomyces verticillus* as a Selective Marker

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A gene, *blmB*, encodes a bleomycin (Bm) *N*-acetyltransferase, designated BAT, from Bm-producing *Streptomyces verticillus* and confers resistance to Bm in *Streptomyces* and *Escherichia coli*.

COS-1 cells transfected with a plasmid designated pEF-BOS/*blmB*, in which *blmB* is under the control of a strong promoter from the human polypeptide chain elongation factor 1α, transiently produced BAT. Immuno-cytochemical analysis using an anti-BAT monoclonal antibody revealed that BAT was localized in the nucleus of the *blmB*-carrying COS-1 cells. NIH/3T3 cells, transfected with pEF-BOS/*blmB*, stably expressed BAT at least for one month. The stable transformants of *blmB* showed specific resistance to the Bm family of antibiotics, suggesting that *blmB* has potential as a selective marker in gene transfer studies with mammalian cells.

Bleomycin (Bm), produced by *Streptomyces verticillus*, inhibits the growth of bacterial and tumor cells and is used as a potent anti-tumor agent due to its DNA cleaving ability[1].

The Bm-producing microorganism must be protected from the lethal effects of its own product[2]. We have cloned and sequenced two Bm-resistance genes, designated *blmA* and *blmB*, from Bm-producing *Streptomyces verticillus* ATCC15003[2]. The gene *blmA* encodes a binding protein, designated BLMA, with a strong affinity for Bm[2,3]. Bm bound to BLMA no longer functions as an antibiotic. The other gene *blmB* encodes a bleomycin *N*-acetyltransferase (BAT) which catalyzes acetyl transfer from acetyl coenzyme A to Bm, the acetylated product no longer cleaves DNA and is devoid of antibiotic activity[2,4]. We have shown that the site of acetylation by BAT is the α-amino residue of the β-aminoalanine moiety in the Bm molecule[4] and have overproduced BAT as a fusion protein to the maltose-binding protein (MBP)[5,6] in *E. coli* and purified it homogeneity. The enzymatic properties of BAT have been characterized[5] and using the purified BAT as an antigen, we have generated a mouse monoclonal antibody specific to BAT[5].

The aim of this study is to construct a new vector that can be used in mammalian cells using *blmB* as a selective marker.

Materials and Methods

Host Cells and Vectors

COS-1 and NIH/3T3 cells were kindly provided by N. NAKAMURA, Mochida Pharmaceutical Co., Ltd. and E. MORITA, Hiroshima University School of Medicine, respectively. *E. coli* DH5α was used for the DNA manipulation.

An expression vector for mammalian genes, pEF-BOS[7], which contains a strong promoter from the human polypeptide chain elongation factor 1α and the SV40 replication origin, was kindly provided by S. NAGATA, Osaka Bioscience Institute, Japan.

Transfection

pEF-BOS/*blmB* was transfected into mammalian cells by the lipofection method using lipofectamine (Gibco BRL, USA) according to the supplier's instructions.

pEF-BOS/*blmB*(+) (10 μg) was transfected into NIH/3T3 (2.2×10^6 cells), which was suspended into 500 μl K-PBS buffer [30.8 mM NaCl, 120.7 mM KCl, 8.1 mM Na_2HPO_4, 1.46 mM KH_2PO_4 and 10 mM $MgCl_2$], by

electroporation under the condition of 1,000 μFD, 330 Volts and $\infty\Omega$.

Western Blotting

Mammalian cells were grown in DMEM medium [10 g DMEM (Gibco BRL, USA), 3.7 g $NaHCO_3$, 2.38 g HEPES, pH 7.2, per liter] supplemented with 10% calf serum. Cultured host cells or pEF-BOS/*blmB*-transfected cells were washed twice with PBS(−) [9.6 g/liter of Dulbecco's phosphate-buffered saline(−), Nissui, Japan]. The cell mass was suspended in 100 μl of PBS(−), and sonicated (Bioruptor, Cosmo Bio, Japan). After centrifugation of the cell-extracts for 10 minutes at 4°C at 10,000×*g*, the resulting supernatant was subjected to Tricine-SDS-PAGE[8], which is employed for the separation of the proteins in the range from 1 to 100 kDa. The proteins were electrophoretically transblotted at 150 mA for 1 hour onto a nitrocellulose membrane (Hybond-C super, Amersham, England) and immunologically detected using an anti-BAT mouse monoclonal antibody (0.5 μg/ml), as described previously[5].

Genomic Southern Analysis

The mammalian cells were washed with PBS(−) and suspended in HMW solution [10 mM Tris-HCl (pH 8.0), 150 mM NaCl and 10 mM EDTA·Na_2] supplemented with 100 μg/ml proteinase K (Merck). The cell suspension was incubated in 0.1% SDS for 90 minutes at 55°C and mixed with HMW-saturated phenol. After a low speed centrifugation, the resulting aqueous layer was collected and added with ethanol. The precipitated DNA was washed with 70% ethanol, suspended in 0.1×TE buffer [1 mM Tris-HCl (pH 8.0) and 0.1 mM EDTA·Na_2] and used as genomic DNA. The genomic DNA was digested with *Hin*dIII, subjected to agarose gel electrophoresis and transblotted onto a nylon membrane (Hybond N^+, Amersham). A PCR-amplified *blmB* structural gene as a probe DNA was labelled with AlkPhos Direct Kit (Amersham). After hybridization, the labelling signal on genomic DNA was detected using Gene Images CDP-Star Detection Kit (Amersham) according to the supplier's instructions.

Enzyme Linked Immunosorbent Assay

The cell-extracts from the host and the pEF-BOS/*blmB*-transfected cells were prepared by the same method as described above, diluted to 200 μg protein/ml with PBS(−) and used for enzyme-linked immunosorbent assay (ELISA). ELISA was carried out as described previously[3].

Immuno-cytochemical Localization Analysis of BAT

The host and the pEF-BOS/*blmB*-transfected cells were grown on a micro coverglass in Bm-free medium, washed with PBS(−) and fixed with 4% paraformaldehyde contained in PBS(−) at room temperature. After 10 minutes, the cells were washed twice with PBS(−) and permeabilized with PBS(−) containing 0.2% Triton X-100 for 2 minutes at room temperature. After additional washing with PBS(−) four times, the cells were used for immunostaining using 1 μg/ml of the anti-BAT mouse monoclonal antibody. Detection was performed with the *Elite* ABC Kit (Vectastain, USA) according to the supplier's instructions.

Drug-resistance Assay

To determine the susceptibility of cells to various antibiotics, a cell survival assay, based on the measurement of the absorbance at 450 nm, was carried out as described previously[9]. NIH/3T3 or its pEF-BOS/*blmB*-transfected cells (2×10^3 cells), were grown into 0.6 cm^2 in 96-well plates, in DMEM supplemented with 1% calf serum. After incubation for 12 hours at 37°C in a humidified atmosphere of 5% CO_2 and 95% air, the medium was removed and replaced with medium supplemented with various drugs at a given concentration. After incubation for 7 days, the surviving cells were counted using a Cell Counting Kit (Dojindo Laboratories Ltd., Japan) with an automatic microplate reader (Model 3550, BIO-RAD, USA).

Results and Discussion

Transient Expression of BAT in the COS-1 Cells

In the preliminary experiment, the original GTG initiation codon in *blmB* was not recognized as a translation start signal in mammalian cells. Therefore, PCR primers to amplify an artificial *blmB* structural gene (906 bp) having an ATG codon instead of the GTG, together with *Xba*I at the 5′- and 3′-adjacent regions were prepared. For amplification, we used the 3 kb-*Xba*I segment from plasmid pKBAT as a template DNA and sense 5′-CACTCTAGAATGACCGAACACCCGCGGGC-3′ and antisense 5′-CACTCTAGATCAGACAGGAGCGGACACGG-3′ oligonucleotide PCR primers. To overproduce the gene product, BAT, a chimeric plasmid designated pEF-BOS/*blmB* was constructed, which carries the modified *blmB* under the control of a strong promoter from the human polypeptide chain elongation factor 1α which has a high transcriptional efficiency[7]. Since COS-1 cells and a vector containing a SV40 replication origin are

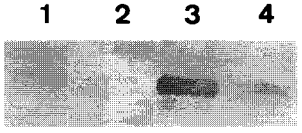
commonly used for transient expression system, this plasmid was introduced into COS-1 cells and analyzed the expression of BAT in the cell-extracts from the pEF-BOS/*blmB*-transfected cells, grown for 72 hours. Western blot analysis shows that BAT was transiently produced (Fig. 1).

Immuno-cytochemical Localization of BAT

We determined the subcellular localization of BAT in COS-1 cells transfected with pEF-BOS/*blmB*, which was incubated for 48 hours after its transfection, by immunostaining using an anti-BAT monoclonal antibody. After permeabilization with Triton X-100, the cells were incubated sequentially with the anti-BAT antibody, biotinylated secondary antibody and horse-radish peroxidase-conjugated avidin-biotine complex. Fig. 2 shows that the signal for BAT was stronger in the nucleus of COS-1 cells than in the cytoplasm. No signals were detected in the COS-1 host cells. We have recently shown that the *blmA* gene product, BLMA, is also localized in the nucleus of the mammalian cells carrying *blmA*[10], suggesting that these Bm-resistant determinants are localized in the nucleus of the mammalian cells, so protecting DNA from the activity of Bm.

Fig. 1. Western blot analysis of BAT expressed in COS-1 cells transfected with pEF-BOS/*blmB*.

Lanes: 1 & 4, the purified BAT (100 ng); 2, the cell-free extract from COS-1 cells; 3, the cell-free extract from COS-1 cells transfected with pEF-BOS/*blmB*.



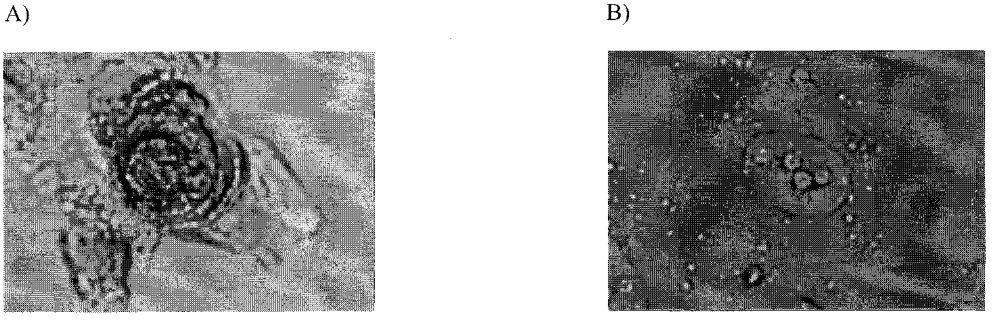
Stable Expression of BAT and Drug-resistance in the NIH/3T3 Cells Carrying *blmB*

Stable transformants were obtained by culturing the NIH/3T3 cells transfected with pEF-BOS/*blmB* for 2 weeks in DMEM supplemented with 20 μg/ml of Bm (as bleomycin A_2 sulfate), the Bm-resistant clones were designated MT-1~MT-3 cells. ELISA analysis using an anti-BAT monoclonal antibody showed that BAT was produced in MT-3 cells, which were cultured for 72 hours (data not shown). Western blot analysis showed that the resulting Bm-resistant cells continuously produced BAT and the production lasted for one month without Bm (data not shown). Southern hybridization analysis of the *Hin*dIII digested-genomic DNA from MT-1, MT-2 or MT-3 cells using the *blmB* structural gene as a probe (Fig. 3) suggested that one copy of *blmB* had integrated into the genome at different sites for each transformant. This finding was confirmed by similar experiments using *Bam*HI, *Sph*I and *Pst*I digestion (data not shown).

To evaluate the function of BAT in mammalian cells, the susceptibility of MT cells to several antibiotics was tested. IC_{50}s for Bm in NIH/3T3, MT-1, MT-2 and MT-3 cells were 1.0, 12.0, 45.5 and 65.5 μg/ml, respectively, indicating that Bm resistance of MT-3 cells was about 65-fold higher than that of the host cells (Fig. 4A), IC_{50} of NIH/3T3 and MT-3

Fig. 2. Immuno-cytochemical localization of BAT.

(A) COS-1 cells transfected with pEF-BOS/*blmB*; (B) COS-1 cells. All micrographs were photographed at ×400.



COS-1 cells transfected with or without pEF-BOS/*blmB* were fixed with paraformaldehyde and permeabilized with Triton X-100. After blocking with goat serum, cells were incubated with an anti-BAT monoclonal antibody prepared previously[5], the immunostained using a Vectastain Elite ABC kit.

Fig. 3. Genomic Southern blot analysis of the NIH/3T3 cells transfected with pEF-BOS/*blmB*.

A, B and C indicate MT-1, MT-2 and MT-3 cells, respectively.

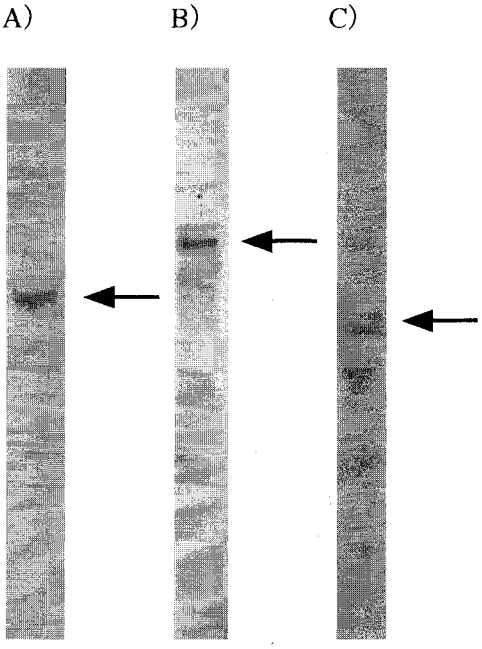
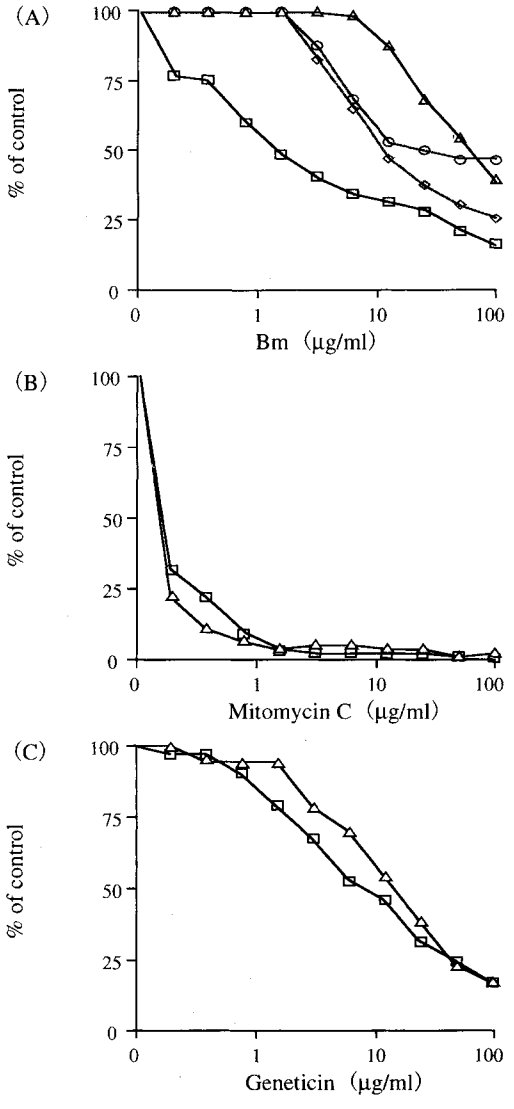


Fig. 4. Dose-dependent survival curves of NIH/3T3 and the pEF-BOS/*blmB*-transfected NIH/3T3 cells exposed to Bm (A), mitomycin C (B) or genecitin (C).

□, ◇, ○ and △ indicate NIH/3T3, MT-1, MT-2 and MT-3 cells, respectively.



Cells were seeded in a 96-well plate and exposed to each drug at the given concentrations. After incubation for 5 days, the survival cells were determined using a Cell Counting kit, as described in the experimental procedure.

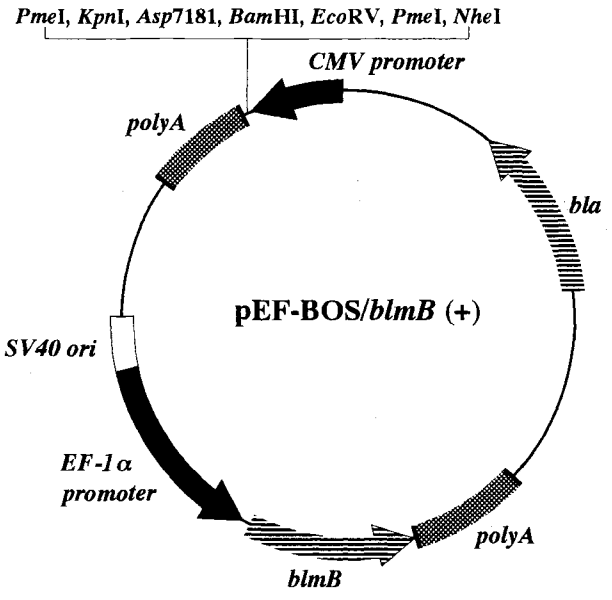
cells were also determined to Bm analogue, peplomycin. The IC_{50} value in the host NIH/3T3 cells treated with peplomycin was 6.0, but that in MT-3 cells was 75.0 μg/ml. MT-3 cells are most resistant to Bm in the three MT cells, whereas susceptible to mitomycin C (Fig. 4B) and streptonigrin as DNA inhibitors (data not shown). In addition, the growth of MT-3 cells was inhibited by an aminoglycoside antibiotic, geneticin (Fig. 4C). These results suggest that BAT acetylates the Bm family of antibiotics and exhibits the Bm-resistant phenotype in mammalian cells as well as in bacterial cells.

Construction of a New Mammalian Vector Using *blmB* as a Selective Marker

In order to test the potential of *blmB* as a selective marker gene in mammalian cells, an expression vector for mammalian cells was constructed having multiple cloning sites and the bovine growth hormone polyadenylation signal under the control of the cytomegalovirus immediate-early promoter: pEF-BOS/*blmB* digested with *Spa*I was ligated to a 1.1 kb *Nur*I-*Pvu*II DNA fragment from pcDNA 3.1(+) (Invitrogen, USA). Fig. 5 shows the map of the resulting chimeric plasmid designated pEF-BOS/*blmB*(+). When pEF-BOS/*blmB*(+) was introduced into NIH/3T3 cells by electroporation, transformants of 2400 which exhibit resistance to 20 μg Bm/ml appeared. This experiment indicates that the transfection efficiency is 1.1×10^{-4} per μg DNA.

Fig. 5. Structure of pEF-BOS/*blmB* (+).

bla, β-lactamase-encoding gene; *CMV*, cytomegalovirus.



Conclusion

We have shown that *blmB* is useful as a selective marker in gene transfer experiments. To date, the neomycin[11] and hygromycin[12] resistance genes have been reported to be useful as dominant selectable marker in mammalian cells, however, there is still a shortage of useful selective markers. The present study demonstrates that *blmB* is a valuable addition and since we have already established an assay determining the enzymatic activity of BAT[2], *blmB* could be used as a reporter assay system in mammalian cells.

Acknowledgments

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