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Bombyx mori β-tubulin Promoter for High-level Expression of Heterologous Genes

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Abstract

We previously isolated 9 clones that show stronger signal compared to *Bombyx mori* cytoplasmic actin gene (BmA3) by using a dot blot hybridization. In this study, we focused on one clone among these clones which has high amino acid similarity with β-tubulin gene of *B. mori*. This clone was ubiquitously expressed in all tissues and developmental stage of *B. mori*. As result of promoter assay using dual luciferase assay system, we found the highest transcription activity region (-750/-1) in the 5'-flanking region of β-tubulin gene, which has about 47 fold more intensive promoter activity than BmA3 promoter. Moreover, the β-tubulin promoter was normally regulated in Bm5, Sf9, and S2 cells. Therefore, we suggest that β-tubulin promoter may be used more powerful and effectively for transgene expression in various insects containing *B. mori* as a universal promoter.

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Introduction

Advance in biotechnology has made it possible to express heterologous genes in organisms. Numerous therapeutic proteins of human have been synthesized by expressing their genes in various host systems. The domestic silkworm, *Bombyx mori* as a bioreactor is one of the most attractive host systems for the production of recombinant proteins (Wurm, 2003). *B. mori* is uniquely suited for recombinant protein expression because of the following reasons: transgenesis was achieved using transposon *piggyBac* and minos (Tamura *et al*., 2000; Uchino *et al*., 2007); aseptic mass rearing system is available all the year round; its cells have mimic the complex post-translational modification processes of mammalian cells, such as glycosylation, disulfide bond formation, and proteolytic processing (Ailor and Betenbaugh, 1999; Jacobs and Callewaert, 2009; Kato *et al*., 2010). Silkworm transgenesis is now a routine method leading to satisfactory yield of transformed animals and to a reliable expression of transgenes during multiple successive generations (Tamura *et al*., 2000; Uchino *et al*., 2007; Zhao *et al*., 2010; Wang *et al*., 2015; Qian *et al*., 2018). For the expression of transgenes, *piggyBac* vectors have already been incorporated with seven promoters: the transposon promoter itself (Handler *et al*., 1998), the artificial 3XP3 (Thomas *et al*., 2002), the *Drosophila melanogaster* hsp70 (Uhlírová *et al*., 2002), the *B. mori* cytoplasmic actin A3 (Tamura *et al*., 2000), and *B. mori* silk gland-specific promoters such as the heavy and light chain of fibroin (Tomita *et al*., 2003), the sericin 1 (Tomita

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et al., 2007), and the fibrohexamerin/P25 (Royer *et al*., 2005). Among five promoters originated from *B. mori*, only BmA3 promoter can express m arker genes and heterologous genes in most developmental stages and tissues (Mangé *et al*., 1997). In most case, for stable germline transformation of *B. mori*, BmA3 promoter was used to drive the enhanced green fluorescent protein (EGFP) in *piggyBac* vector. The BmA3-EGFP marker was useful for the screening of G1 transgenic individuals at the larval stage, but not detectable in G0 embryonic tissues or in the eggs of the later generations (Thomas *et al*., 2002). Therefore, it is impossible to select transgenic individuals before the G1 larval stage using BmA3 promoter. Furthermore, the BmA3 promoter is predominantly active in the midgut, which makes it difficult to reliably identify transformants since autofluorescence of many insect foods can mask low-level fluorescence (Horn *et al*., 2002). Recently, we isolated 9 clones that show stronger signal compared to BmA3 by using a dot blot hybridization, and demonstrated ubiquitous and powerful promoter activity for two clones which encodes a heat shock protein 70 (bHsp70) and hypothetical protein 32 (bHp32), respectively (Goo *et al*., 2010; Goo *et al*., 2011). In order to develop promoters stronger than BmA3 promoter, we selected one clone from previously isolated 9 positive clones, which has high amino acid sequence similarity with β-tubulin gene of *B. mori*. In addition, its promoter was successfully isolated, and its potential and characteristics as constitutive promoter were investigated and compared with those of BmA3 promoter in this study.

Materials and Methods

Experimental insects and cell lines

The silkworm, *B. mori* (Jam 124) were reared on an artificial diet at 25°C, 65±5% relative humidity, and 12 h light : 12 h dark photoperiod. Bm5 cell lines derived from *B. mori* and Sf9 cell lines derived from *Spodoptera frugiperda* were maintained as adherent cell culture in TC-100 insect medium (Sigma, USA) supplemented with 10% (v/v) heat inactivated fetal bovine serum (Invitrogen, USA), as described previously (Summers and Smith, 1987). Schneider S2 cell lines derived from *D. melanogaster* were maintained as adherent cell culture in Drosophila-SFM medium with 90ml/l of 200 mM L-glutamine and 5 ml/l of penicillin-streptomycin (Invitrogen, USA). Bm5 cells were subcultured with interval of 5-7 days and Sf9 and S2 cells subcultured with interval of 2-3 days.

Screening of bTub genomic DNA

We screened genomic library constructed from *B. mori* larvae using the λ DASH Vector Kit (Stratagene, USA). To prepare a probe, a *B. mori* β-tubulin (bTub) cDNA was labeled with [α-^{32}P] dATP using a Random Primer Labeling Kit (Stratagene, USA). We obtained a positive clone in the 3rd screening. Phage DNA was extracted and digested with *Eco*R I, *Xba* I and *Eco*R I/*Xba* I enzymes. The digested DNA fragments were subcloned into pUC18 vector. The nucleotide sequences of subcloned DNA fragments were determined using ABI 377 automatic sequencer (Perkin-Elmer, USA).

Relative Quantification analysis of mRNA by RT-PCR

Total RNAs were prepared using RNA STAT-60 (Tel-Test, USA) according to the manufacturer's instructions and reverse transcribed with oligo (dT) primer using High Capacity cDNA Archive Kit (Applied Biosystems, USA). After cDNA synthesis, PCR was performed with a PCR Thermal Cycler Dice (TaKaRa, Japan) for 4 min at 95°C, followed by 25 cycles of 20 s at 95°C, 30 s at 55°C, and 25 s at 72°C, followed by a final extension at 72°C for 10 min.

Prediction for transcription start site

To identify the transcription start site of bTub mRNA, 5'-RACE PCR was carried out with poly (A^+) RNA using CapFishingTM Kit (Seegene, Korea) according to manufacturer's instructions. The adapter sequence attached to the ends of the cDNA enabled it to be used in 5'- RACE. Three gene-specific primers were designed using the sequences of the bTub fragment. These primers were used in 5'-RACE in conjunction with the anchor primer (5'-RACE primer: 5'-AGTGAAGGCCACGTTATGAAGGTGTGATG CG-3') to amplify the 5'-ends of cDNA. The PCR conditions were as followed: initial denaturation at 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 40 s, annealing at 68°C for 40 s and elongation at 72°C for 60 s, and a final elongation step at 72°C for 5 min.

Table 1. Primers used to promoter constructs. The *Mlu* I sites in forward primers and *Xho* I sites in the reverse primers are underlined. The position of the primers is related to the transcription start site in *B. mori* β-tubulin (bTub) gene

Name	Position	Sequences	Forward/reverse	Construct name
D1	-1,103/-1,083	5'-ACGCGTTTCATAAAAA AATGTGAGAG-3'	F	pGL3-bTub-D1
D2	-950/ -930	5'-ACGCGTGTCCAAA GCCTGCCTTA TAA-3'	F	pGL3- bTub-D2
D3	-750/-730	5'-ACGCGTTCTC AACCCCCTTT TTGGTCA-3'	F	pGL3- bTub-D3
D4	-500/-480	5'-ACGCGTAATA TTTTATCTAG TAAGTC-3'	F	pGL3- bTub-D4
D5	-250/-230	5'-ACGCGTCCGAC CAACTTAAAT TTACT-3'	F	pGL3- bTub-D5
R1	-21/-1	5'-CTCGAGGGCGCGGTGGGGAATTTAGA-3'	R	

Plasmid construction and luciferase assay

The Dual Luciferase Report Assay System (Promega, USA) was used to measure promoter activity. For this assay, 5 serial deleted fragments of the 5'-flanking region of bTub were generated by PCR amplification with primers listed in Table 1, and the PCR products were inserted into pGL3-Basic vector digested with *Mlu* I and *Xho* I (Promega, USA). The resulting experimental plasmids, pGL3-bTub-D1 (-1,103/-1), pGL3-bTub-D2 (-950/-1), pGL3-bTub-D3 (-750/-1), pGL3-bTub-D4 (-500/-1), and pGL3-bTub-D5 (-250/-1) were generated. For normalization of transfection efficiency, the pRL-dHsp70-Rluc plasmid expressing *Renilla* luciferase under the regulation of the *D. melanogaste*r Hsp70 (dHsp70) promoter was constructed. Approximately 5×10^5 cells per well were seeded in 24 well plate 1 day before transfection. Confluence of 50-80% was achieved at 1 day after incubation, which is suitable to obtain high transfection efficiency. For transfection, 400 ng of the experimental plasmid and 40 ng of the pRL-dHsp70-Rluc control plasmid per well were mixed with 2 μl of FuGENE HD Transfection Reagent (Roche, Germany) in TC-100 insect medium (Sigma, USA) for 15 min at room temperature. The mixture of DNA plasmids and FuGENE reagent was then applied to the appropriate well in triplicate. The transfected cells were incubated at 27°C for 48 h, and harvested in 100 μl of 1 × passive lysis buffer (Promega, USA) per well. The transcriptional activities of firefly and Renilla luciferase were determined on a luminometer (TECAN, Switzerland) using Dual Luciferase Report Assay System (Promega, USA) according to the manufacturer's instructions. The luciferase activity of each experimental plasmid was normalized to *Renilla* activity.

Data analysis

All data are shown as the mean ± standard deviation (SD). Comparisons between two groups were performed using the Student's t-test. Comparisons between three or more groups were carried out using one-way analysis of variance (ANOVA) followed by Dunnett's test. SPSS version 18.0K (SPSS Inc., Chicago, IL, USA) was used for analyses, and significant differences were analyzed by an unpaired t test (*P< 0.05; **P< 0.01; ***P< 0.001).

Results and Discussion

Selection of highly expressed cDNA clones compared to BmA3

We previously isolated 9 clones that show stronger signal compared to *B. mori* cytoplasmoc actin gene (BmA3) by using a dot blot hybridization, and partially sequenced 9 positive cDNA clones and produced expressed sequence tags (ESTs) (Goo *et al*., 2011). In this study, we focused on the clone, which has high homology (99%) with β-tubulin of *B. mori*.

Structure analysis of full-length bTub cDNA and bTub genomic DNA

The complete nucleotide sequence (Genbank accession number: AB003287.1) of the identified clone had a 5'-untranslational region of 115 nucleotides followed by an initiating ATG codon. The TAA termination codon is at nucleotide 1,458, a consensus polyadenylation signal is present at 1,934-1,939, and the poly (A) tail is 21 nucleotides downstream

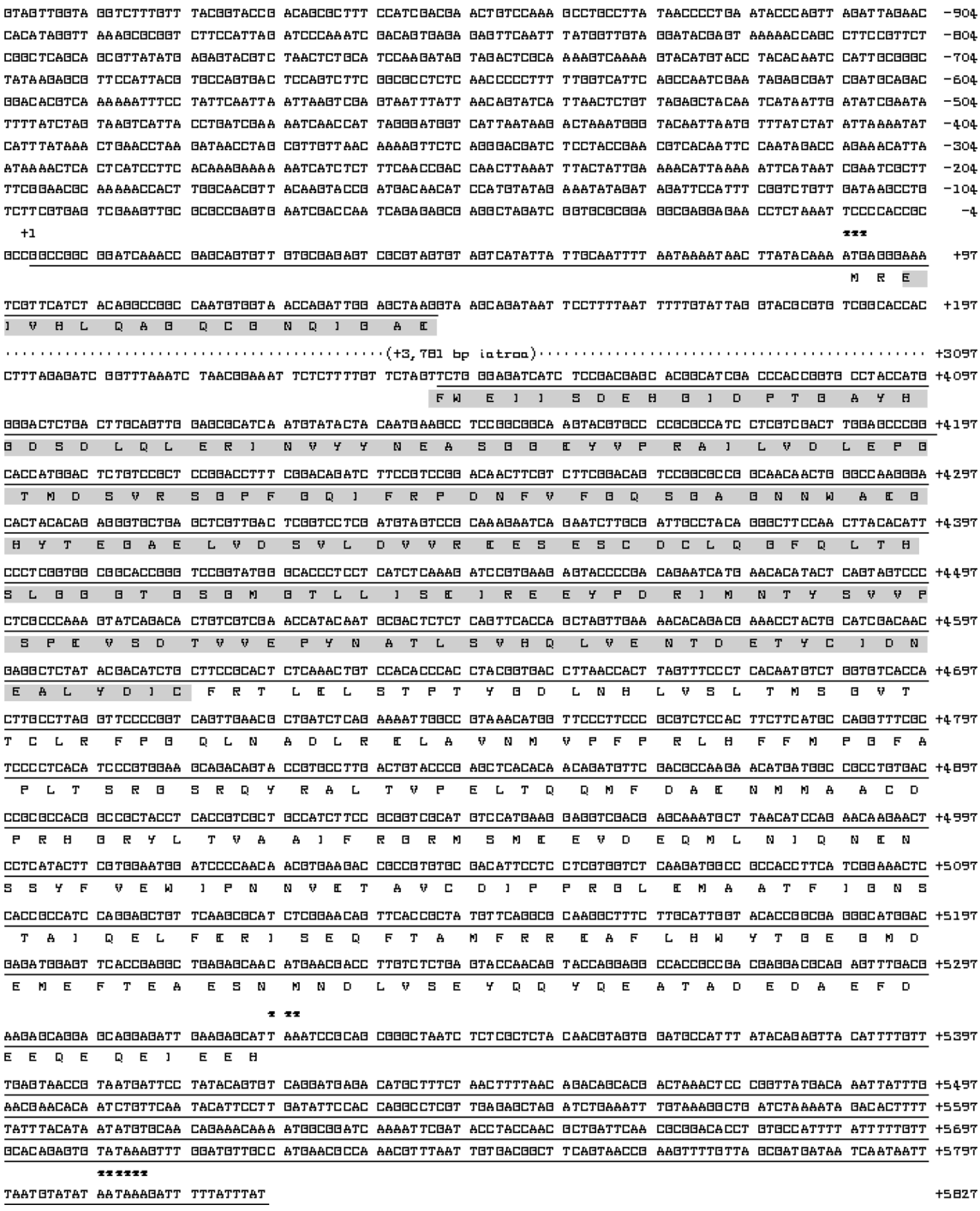

Fig. 1. The genomic nucleotide and deduced amino acis sequences of *B. mori* β-tubulin (bTub) gene. Exons are indicated by underlines. The transcription start site was defined as position +1. The shaded areas indicate consensus sequence of Tubulin/FtsZ family, GTPase domain GDP binding domains. The predicted amino acid sequences (single-letter abbreviation) are shown below the nucleotide sequences. The asterisks indicate translation start and stop codon, and putative polyadenylation signal, respectively.

of polyadenylation signal sequence (data not shown). Translation of the sequence from nucleotide 115 to 1,438 produces a 447-residue protein with a calculated Mw of 50.3 kDa (Fig. 1). We named this clone as bTub (B. mori β-tubulin) because it has high similarity (98.5%) with β-tubulin of *B. mori*. The amino acid sequence of β-tubulin protein is highly conserved between different species, a conservation which is particularly striking in N-terminal half, where the tubulin/FtsZ family, GTPase

domain (3-211 amino acids) is located (Nogalels *et al*., 1998) (Fig. 1). Tubulin/FtsZ family, GTPase domain is an evolutionary conserved protein domain. This domain is found in all tubulin chains (Nogalels *et al*., 1998), as well as the bacterial FtsZ family of proteins (Lowe and Amos, 1998). These proteins are involved in polymer formation. Tubulin is the major component of microtubules, while FtsZ is the polymer-forming protein of bacterial cell division, it is part of a ring in the middle of the dividing cell that is required for constriction of cell membrane and cell envelope to yield two daughter cells. FtsZ and tubulin are GTPases, this entry is the GTPase domain (Nogales *et al*., 1998).

A positive phage clone was isolated from the screening of a B. mori genomic DNA library with bTub cDNA probes labeled with [α-32P] dATP. It was including the complete genomic sequence containing 1,103 bp of the 5'-flanking region (GenBank accession number; AB003287.1). bTub genomic DNA was composed of two exons of 144 and 1,785 bp, separated by one introns of 3,899 bp, respectively. The canonical GT/AG splice sites were present at the extreme ends of the intron (Fig. 1). The transcription start site was identified with 5'-RACE PCR, because insect specific transcription start site, ATCAT(G) (Tryselius *et al*., 1992) and ATCATAC (Sun *et al*., 1990) was not found in bTub gene. Insect specific transcription start site, ATCAT(G) and ATCATAC was not completely conserved for each protein.

The putative transcription start site was located 87 bp upstream from the ATG, the translation start codon and defined as position +1 (see Prediction for transcription start site in Materials and Methods) (Fig. 1). A putative TATA and CAAT box were not found in bTub gene.

Characterization of bTub mRNA expression

To confirm whether bTub mRNA was ubiquitously expressed or not in *B. mori*, RT-PCR was performed. There were a little difference in transcription level of the bTub mRNA according to each tissue and developmental stage of 5th instar larvae, but the bTub was ubiquitously transcribed in all tissues and developmental stages tested (Fig. 2). As these results, we supposed that the 5' flanking region of bTub has potential of development as a valuable promoter for ubiquitous and powerful expression of heterologous proteins.

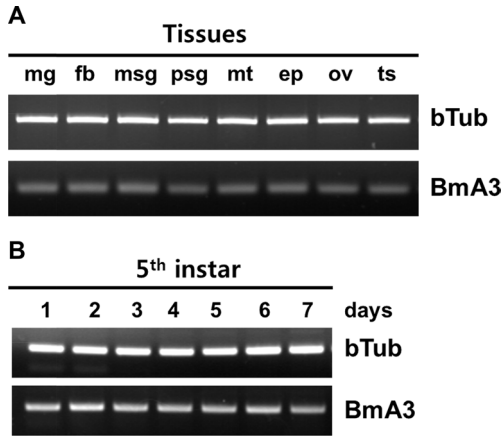

Fig. 2. Expression pattern according to tissue and development stage of *B. mori* β-tubulin (bTub) mRNA. For analysis of mRNA expression on tissue (panel A), 1st-strand cDNA was synthesized from organs as follows: mg, midgut; fb, fatbody; msg, middle silk gland; psg, posterior silk gland; mt, Malpighian tubles; ep, epidermis; ov, ovary; ts, testis. For analysis of mRNA expression on developmental stage (panel B), 1st-strand cDNA was synthesized from day 1, 2, 3, 4, 5, 6, and 7 of 5th instar *B. mori* larvae. RT-PCR was carried out using 1st-strand cDNAs synthesized from panel A and B.

Activity of bTub promoter

To investigate the activity of bTub promoter, a 1,103 bp bTub genomic DNA between -1,103 and -1 bp containing exon 1 was cloned into promoterless pGL3-basic vector (Promega, USA). A series of deletion fragments were made from this plasmid using some primers (Table 1), and cloned into *Mlu* I and *Xho* I sites of pGL3-Basic vector. The resulting deletion constructs, pGL3-bTub-D1 (-1,103/-1), pGL3-bTub-D2 (-950/-1), pGL3-bTub-D3 (-750/-1), pGL3-bTub-D4 (-500/-1), and pGL3-bTub-D5 (-250/-1) were co-transfected with control plasmid into Bm5 cells. The promoter activity of each construct was evaluated by measuring luciferase activity in comparison with a co-transfected, pRL-dHsp70-Rluc control plasmid. When the luciferase activity of the longest construct, pGL3-bTub-D1 was designated as 100%, the relative promoter activity (%) of other deletion constructs to pGL3-bTub-D1 was shown in Fig. 3A. Among these 5 deletion constructs, the third construct, pGL3-bTub-D3 had the highest effect on transcriptional activity, with approximately 141% activity compared to that of pGL3-bTub-D1. The transcriptional activities of other deletion constructs from pGL3-bTub-D4 to pGL3-bTub-D5 were gradually decreased in a size-dependent manner. The pGL3-bTub-D5 had no promoter activity with similarity to pGL3-Basic vector (Fig. 3A). Accordingly, we

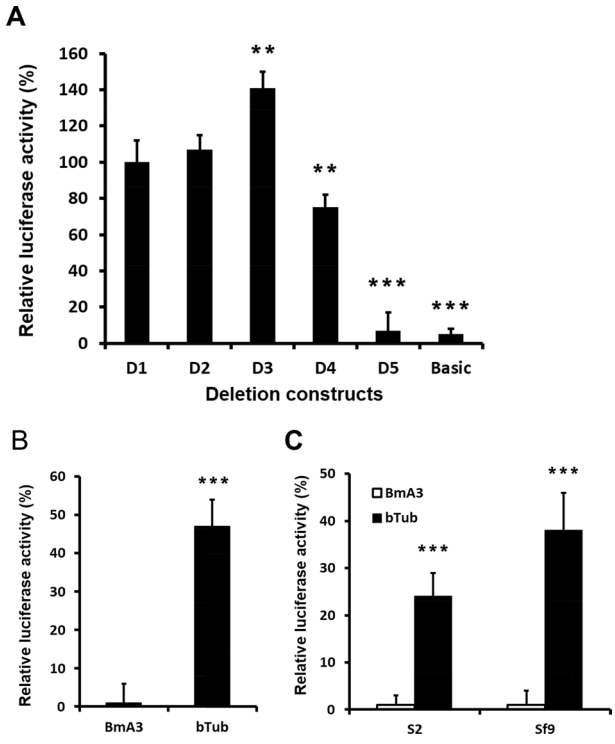

Fig. 3. Analysis of *B. mori* β-tubulin (bTub) deletion constructs by dual luciferase reporter gene activity. (A) Various lengths of 5'-DNA sequences of bTub were linked to a promoterless luciferase reporter plasmid, pGL3-Basic. These deletion constructs were used for the transient transfection into Bm5 culture cells. Luciferase activity by pGL3-bTub-D1 (-1,103/-1) was defined as 100%. (B) Comparison of luciferase activity with BmA3 promter. Luciferase activity by pGL3-BmA3 was defined as 1 fold. (C) Promoter activity of bTub gene in insect lines, Sf9 and S2 cells. Luciferase activity by pGL3-BmA3 was defined as 1 fold. The mean luciferase activity of deletion constructs was based on at least three independent experiments performed in triplicate, each normalized to an internal control. The bars represent standard deviation of each mean. The results of densitometric analysis are the means ± SDs (n=3). *P value (*P< 0.05; **P< 0.01; ***P< 0.001) indicate statistical significances compared to those of deletion constructs.

selected the D3 region between -750 and -1 as the most suitable promoter region for expression of heterologous proteins.

The promoter activity of pGL3-bTub-D3 was evaluated in comparison with BmA3 promoter which have used for ubiquitous expression of foreign protein in transgenic silkworm. The promoter activity of pGL3- bTub-D3 was approximately 47 fold higher than that of BmA3 promoter (Fig. 3B).

To confirm whether the bTub promoter also is normally regulated at other insect cell lines, the pGL3-bTub-D3 and pGL3-BmA3 plasmids were transfected into Bm5, Sf9 and S2 insect cells, and then their promoter activity were measured, respectively. The bTub promoter normally worked in Bm5, Sf9 and S2 cells, and had higher transcriptional activity than BmA3 promoter in all insect cells tested (Fig. 3B and 3C). From the above results, we suggest that bTub promoter is more powerful and effective for construction of the transgenic silkworm or cell expression system due to higher transcription activity than that of BmA3 promoter. Furthermore, it can be used for heterologous protein expression in various host insect as a universal promoter.

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