A Strong Transcription Activity of the *Bombyx mori* Elongation Factor 1 α Promoter

Tae-Won Goo, Sung-Wan Kim, Seong-Ryul Kim, Seung-Won Park, Seok-Woo Kang, Kwang-Ho Choi, and Eun-Young Yun*

Department of Agricultural Biology, National Academy of Agricultural Science, RDA, Suwon, 441-100, Republic of Korea

(Received 15 May 2012; Accepted 28 June 2012)

We previously isolated 9 clones that show stronger signal compared to *B. 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 homology with elongation factor α gene of *B. mori*. This clone, named bEF1 α (*B. mori* elongation factor α) 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 (-702/+38) in the 5'-flanking region of bEF1 α gene, which has about 20 fold more intensive promoter activity than BmA3 promoter. Moreover, the bEF1 α promoter was normally regulated in Bm5, Sf9, and S2 cells. Therefore, we suggest that bEF1 α promoter may be used more powerful and effectively for transgene expression in various insects containing *B. mori* as a universal promoter.

Key words: *Bombyx mori*, Elongation factor 1 α , Luciferase assay, Promoter

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, *B. mori* as a bioreactor is one of the most attractive

host system for the production of recombinant proteins (Wurm, 2003). *B. mori* is uniquely suited for recombinant protein expression because of the following reasons: transgenesis was recently 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 protolytic 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.*, 2009). 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 (Uhlířová *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 *et al.*, 2007), and the fibrohexamerin/P25 (Royer *et al.*, 2005). Among five promoters originated from *B. mori*, only BmA3 promoter can express marker 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 choose 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 pro-

*To whom the correspondence addressed

Dr. Eun-Young Yun, Department of Agricultural Biology, National Academy of Agricultural Science, RDA, Suwon 441-100, South Korea.

Tel: +82-31-290-8576; Fax: +82-31-290-8543;

E-mail: yuney@korea.kr

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 bEF1 α gene

Name	Position	Sequences	Forward/ reverse	Construct name
D1	-905/-885	5'- <u>CTCGAG</u> CTGTCAACT CTCAAAATTT G-3'	F	pGL3-bEF1 α -D1
D2	-702/-682	5'- <u>CTCGAG</u> AGAATTTATTTTCCTAATTTG-3'	F	pGL3-bEF1 α -D2
D3	-504/-484	5'- <u>CTCGAG</u> TGGGCTAATCAGCTAATGCTA-3'	F	pGL3-bEF1 α -D3
D4	-244/-222	5'- <u>CTCGAG</u> GATTTTATAGTAAGATTTGTA-3'	F	pGL3-bEF1 α -D4
D5	-98/-78	5'- <u>CTCGAG</u> CAGAAACAATTTGAAGGTTTTT-3'	F	pGL3-bEF1 α -D5
R1	+1/+38	5'- <u>AGATCT</u> CACGGATTACCAACTGCGTGT-3'	R	

motor 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 more strong promoters as compared to BmA3 promoter, we selected one clone among previously isolated 9 positive clones, which has high amino acid homology with elongation factor 1 α gene of *B. mori*. In addition, its promoter was successfully isolated, and its potential and characteristics as constitutive promoter were investigated and compared to 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 $\pm$ 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) with 10% (v/v) heat-inactivated fetal bovine serum (Invitrogen, USA), as described previously [13]. Schneider S2 cell lines derived from *D. melanogaster* were maintained as adherent cell culture in Drosophila-SFM medium with 90 ml/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 bEF1 α 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* heat shock protein 70 (bEF1 α) cDNA was labeled with [α -³²P] 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 instruments and reverse transcribed with oligo (dT) primer using cDNA using High Capacity cDNA Archive Kit (Applied Biosystems, USA). After cDNA synthesis, RT-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 b bEF1 α 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 b bEF1 α fragment. These primers were used in 5'-RACE in conjunction with the anchor primer (5'-RACE primer: 5'-AGTGAAGGCCACGTTATGAAGGTGTGATGCG-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, and a final elongation step at 72°C for 5 min.

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 bBEF1 α 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 sites (Promega, USA). The resulting experimental plasmids, pGL3-bEF1 α -D1 (-905/+38), pGL3-bEF1 α -D2 (-702/+38), pGL3-bEF1 α -D3 (-504/+38), pGL3-bEF1 α -D4 (-244/+38), and pGL3-bEF1 α -D5 (-98/+38) were generated. For normalization of transfection efficiency, the pRL-dHsp70-*Rluc* plasmid expressing *Renilla* luciferase under the regulation of the *D. melanogaster* Hsp70 (dHsp70) promoter was constructed.

Approximately 5×10^5 cells per well were plated in 24 well plates 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 μ l 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 μ l of 1 $\times$ 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 instruments. The luciferase activity of each experimental plasmid was normalized to *Renilla* activity.

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* cytoplasmic 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 elongation factor 1 α of *B. mori*.

Structure analysis of full-length bEF1 α cDNA

The complete nucleotide sequence (Genbank accession number: JQ638952) of the identified clone had a 5'-untranslational region of 49 nucleotides followed by an initiating ATG codon. The TAA termination codon is at nucleotide 2,027, a consensus polyadenylation signal is

Fig. 1. Expression pattern according to tissue and development stage of bEF1 α 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 tubules; 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.

present at 2,140-2,145, and the poly (A) tail is 18 nucleotides downstream of polyadenylation signal sequence (data not shown). Translation of the sequence from nucleotide 50 to 2,026 produces a 463-residue protein with a calculated Mw of 50.4 kDa (Fig. 2). We named this clone as bEF1 α (*B. mori* elongation factor 1 α) because it has high homology (99%) with elongation factor 1 α of *B. mori*. EF-1 α promotes the GTP dependent binding of aminoacyl-tRNA to A site of ribosome (Hershey, 1991). The amino acid sequence of EF-1 α proteins is highly conserved between different species, a conservation which is particularly striking in N-terminal half, where the GTP binding site is located (Dever *et al.*, 1987). The analysis of domain organization using SMART program (Schultz *et al.*, 1998) showed that the consensus sequences GHVDH-GKT (18-25), DCPG (80-83), and NKCD (135-138) involved in three GDP binding domains of *E. coli* EF-Tu (Kjeldgaard and Nybrog, 1992) are conserved in bEF1 α at amino acid residues 14-22, 91-94, and 153-156, respectively (Fig. 2).

Characterization of bEF1 α mRNA expression

To confirm whether bEF1 α mRNA was ubiquitously expressed or not in *B. mori*, RT-PCR was performed. There was a little difference in expression level of the bEF1 α mRNA according to each tissue and developmen-

CTGTCACTA CTCAAAATTT GGTGAAGCGT GAGTTTCTCT GCTAAACCAC AACGAGGACG ACCGTTTACG TAGATAGTTT TTCAGCATAT GGTCAATTCTA	-806
TAGCTGCTTT ATGTATTATA GATTAAATATT CTCTCTACCT CATAGATGTT GATTGAACAA AAATAAAAAAT ACTATTATAG TTTTCATTTA CTGTAAGAAT	-706
TTATTTTCTT AATTGATTT TAAATGAAGT GTGAATTCCT AAACAATATT ACTTTAATAT TTTTACATAT TATTAGTCTA TTTGCAACAT TTAITTTAAG	-606
TGACCAATAA AATAAGTACA TGAACAATAA ATTTCAATCA AACGATTGGC ATCTTAAGTC TCCCTTAGAT CTITTCATAA TTGATTATTT TCTGTAATGG	-506
GCTAATCAGC TAATGCTATG ATAATTTCCG AACGTTTCGC AGCGATGGTG CGTCATTTCG GTGCCAATTA AATAATTTTA ATGTTACAAG AAAAGAGGAA	-406
CTTCCCAGC TTTATGAAGA AACCTCAAAA ATGTTTCAAT CAGATTTTGA GTAAGATTGG TATAGTTCAA TACACGAAAA ACACAAAATC GAATAAATTT	-306
GCAGTACATA AATAATAAAA TAAATTTCTA GGTAAAAAGA TTGCATTGGG AATGTCACAT CACATTGAAT GTTAAATAC CACGTAAAC CGCTTTCCAA	-206
AAACCAAAAA CCAAAATTC AACAATTTCTA ACAATCAITTA AATAAGAAT TGATATGTTT CTGAACCTGA AAAAGACCGA TCAATTTTTA ACAGAAACAA	-106
TTTGAAGGTT TTTTAGAGTA ATTTCTAGC AACATTTTCAG AACGGAAGTG ACTGTTTGAG CACTGTTTCAG CTGGAAGAAA GAGCATTTCCT CGTCCATTCT	-6
+1	
GTGTGAGTCT ATCTCGGATA TTACACGCAG TTGGTAATCC GTGGTGAGTG TCAAAAAAAT ATTGTATTGT GAAGTGA AAA AGTGAATCA ATATACCCAT	+95
AACTCCGTAA GCATAACTTC TTTTAGATT AATCTTCTTG AAACATAATTA TTGTGTGAAA TAGTAAGATT AGCCGAACAC ATTTTCAGATC ACGTTTATTA	+195
CTGAACGTAT TAATGAGTGT CCACGTTTGC ATTTAACAAT TATTTCAATT GTTTAATAAA TTCTCTTATT ATTTTAAATG TAGAATGC AA TATGTTTATG	+295
CTTACGCGTC ACACATAGTG CCAACCGGCT TAACCCACCA CGTGGTTGAC ATTCGATTAT TTCTTTTAA TCTCTGCAAA GTTATCGAAG TTTATATTG	+395
CATCATTATT GTAGTTCTCT ATTTGTAATT TCTCGACAAT CGCAACGTTG CTGCAATTTT TTTAATGAAA TGGCCGACAT CTITTTGTAA ATGTCAAGAT	+495
AGGGCGAGGC GGCCATGCGG CAGCCATATT GCCGGTCTCT TAGTACACGA AGACGACCTT GCGAACGCCG GTTTCATCT CAATCATGCG ATTTTGTGCT	+595

TCATACTACA ATTTAATTTA TTTTGTITTA GACTAACCAA AAATGGGCAA GGAAAAAGCT CACATTAACA TTGTCGTCAT CGGACACGTC GACTCCGGCA	+695
M G K E K T H I N I V V I G H V D S G	
AGTCCACCAC CACTGGTCAC TTGATCTACA AATGTGGTGG TATTGACAAA CGTACCATCG AGAAGTTCCA GAAGGAGGCC CAGGAAATGG GTAAAGGATC	+795
<u>K S T T T G H L I Y K C G G I D K R T I E K F E K E A Q E M G K G S</u>	
CTTCAATAT GCTTGGTAT TGGACAACT AAAGGCTGAG CGTGAGCGTG GTATCACAAT CGATATTGCT CTCTGGAAGT TCGAACTAG CAAGTACTAT	+895
F K Y A W V L D K L K A E R E R G I T I D I A L W K F E T S K Y Y	
GTATACATCA TTGATGCTCC TGGACACAGA GATTTCATCA AGAATGATG CACAGGAACC TCTCAGGCTG ATTCGCTGT GTCATCGTA GCTGCCGTA	+995
V T I I D A P G H R D F I K N M I T G T S Q A D C A V L I V A A G	
CCGTGAATT CGAGCTGGT ATCTTAAGA ACGGTCAAAC CGTGAGCAT GCCTGTCTCG CTITCACCT CGGTGCAAA CAGCTCATG TAGGATGAAA	+1095
T G E F E A G I S K N G Q T R E H A L L A F T L G V K Q L I V G V N	
CAAAATGGAT TCCACTGAAC CACCATACAG TGAGCCGAGA TTTGAGGAAA TCAAGAAGGA AGTATCCTCA TACATCAAGA AGATTGGCTA CAACCCAGCT	+1195
<u>K M D S T E P P Y S E P R F E E I K K E V S S Y I K K I G Y N P A</u>	
GCTGTCTCT TCGTGGCCAT TTCTGGATGG CACGGAGACA ACATGTTGGA GCCTTCAACC AAAATGCCTT GGTTCAGGG ATGGCAGTG GAGCGTAAGG	+1295
A V A F V P I S G W H G D N M L E P S T K M P W F K G W Q V E R K	
AAGGCAAGC TGACGAAAA TGCCCTCATG AAGCTCTCGA TGCCATCTG CCACCTGCCG GCCCCACTGA CAAGCCCTG CGTCTTCCCC TGCAAGACGT	+1395
E G K A D G K C L I E A L D A I L P P A R P T D K P L R L P L Q D V	
ATACAAATC GGTGGTATTG GTACCGTCCG CGTCGCGAGA GTTGAATGT GTGTGTGAA ACCAGGTACC ATTTGTTGCT TTGCCCCCG CAACATCACT	+1495
Y K I G G I G T V P V G R V E T G V L K P G T I V V F A P A N I T	
ACTGAAGTCA AGTCTGTGGA GATGACCCAC GAAGCTCTCC AAGAAGCTGT ACCTGGAGAG AATGTAGTT TCAACGTAAA GAACGTGTCC GTCAAGGAAT	+1595
T E V K S V E M H H E A L Q E A V P G D N V G F N V K N V S V K E	
TGCGTCGTGG TTATGTTGGT GGTGACTCCA AAAACAACCC ACCTAAGGGT GCTGCAGATT TTACAGCTCA AGTCATTGTG CTAAACATC CTGGTCAAAAT	+1695
L R R G Y V A G D S K N N P P K G A A D F T A Q V I V L N H P G Q I	
CTCAACCGT TACACACAG TCTTGGATTG CCACACTGCC CACATTGCCT GCAAAITTCG AGAAATCAAA GAAAAAGTTG ACCGTCGTAC TGGTAAATCT	+1795
S N G Y T P V L D C H T A H I A C K F A E I K E K V D R R T G K S	
ACTGAAGTCA ACCCAAAATC CATCAAGTCT GGAGATGACG CCAITGTCAA CTGTGACTCT TCCAGCCTC TATGTGTAGA GTCCCTCCAG GAATTCACAC	+1895
T E V N P K S I K S G D A A I V N L V P S K P L C V E S F Q E F P	
CCCTCGTGC TTTTGTGTC CGTGACATGA GGCAGACAGT TGCTGTGCGA GTCATCAAGG CTGTCAACT CAAGGAGGCT GGTGGTGCA AGTCACTAA	+1995
P L G R F A V R D M R Q T V A V G V I K A V N F K E A G G G K V T K	

AGCTGCCGAA AAGGCCACCA AGGGCAAGAA GTAGCTAGAG CTGTTAACAG CACAATTTT CATACAAGT CGATACTTCA TTCACCGAAA GGTGTTTCGA	+2095
A A E K A T K G K K	
AGGAAAAAG GGCTACAAAC TCATTCTTT TCTATATTTT TTACAAGGCT TGTAATGTAA CATTATTTTA TAATTTATAT AAGGTTATAC ATAATATATC	+2195

TGAAACATT TGTATGACT GCAAACTAAA GTGTAAATTC ATAGAATAAA GGTAGCTCC AGTA	+2259

Fig. 2. The genomic nucleotide and deduced aa sequences of *B. mori* elongation factor 1 α (bEF1 α) gene (GenBank accession number: JQ638952). Exons are indicated by underlines. The transcription start site was defined as position +1. The shaded areas indicate consensus sequence of GDP binding domains of *E. coli*. 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.

tal stage of 5th instar larvae, but the bEF1 α mRNA was ubiquitously expressed in all tissues and developmental stages tested (Fig. 1). As these results, we suppose that bEF1 α cDNA has potential of development as a valuable promoter for ubiquitous and powerful expression of a heterologous gene.

Structural analysis of bEF1 α genomic DNA

A positive phage clone was isolated from the screening of a *B. mori* genomic DNA library with bEF1 α cDNA probes labeled with [α -³²P] dATP. It was including the complete genomic sequence containing 906 bp of the 5'-flanking region (GenBank accession number; JQ638952)

Fig. 3. Analysis of bEF1 α deletion constructs by dual luciferase reporter gene activity. (A) Various lengths of 5'-DNA sequences of bEF1 α 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-bEF1 α -D1 (-905/+38) was defined as 100%. (B) Comparison of luciferase activity with BmA3 promoter. Luciferase activity by pGL3-BmA3 was defined as 1 fold. (C) Promoter activity of bEF1 α gene in insect lines, Sf9, Bm5 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.

(Fig. 2). bEF1 α genomic DNA was composed of two exons of 38 and 1,633 bp, separated by two introns of 906 and 588 bp, respectively. The canonical GT/AG splice sites were present at the extreme ends of the intron (Fig. 2). The transcription start site was identified with 5'-RACE PCR, because insect specific cap site, ATCAT(G) [19] and ATCATAC [14] was not found in bEF1 α gene. The putative transcription start site was located 49 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. 2). A putative TATA and CAAT box were not founded in bEF1 α gene.

Activity of bEF1 α promoter

To investigate the activity of bEF1 α promoter, a 944 bp bEF1 α genomic DNA between -905 and +38 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-bEF1 α -D1 (-905/+38), pGL3-bEF1 α -D2 (-702/+38), pGL3-bEF1 α -D3 (-504/+38), pGL3-bEF1 α -D3 (-244/+38), and pGL3-bEF1 α -D5 (-98/+38) 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-bEF1 α -D1 was designated as 100%, the relative promoter activity (%) of other deletion constructs to pGL3-bEF1 α -D1 was shown in Fig. 3A. Among these 5 deletion constructs, the second construct, pGL3-bEF1 α -D2 had the highest effect on transcriptional activity, with approximately 116% activity compared to pGL3-bEF1 α -D1. The transcriptional activities of other deletion constructs from pGL3-bEF1 α -D3 to pGL3-bEF1 α -D5 were gradually decreased in a size-dependent manner. The pGL3-bEF1 α -D5 had no promoter activity with similarity to pGL3-Basic vector Fig. 3A. Accordingly, we selected the D2 region between -702 and +38 as the most suitable promoter region for expression of heterologous genes.

The promoter activity of pGL3-bEF1 α -D2 was evaluated in comparison with BmA3 promoter which have used for ubiquitous expression of foreign genes in transgenic silkworm. The promoter activity of pGL3-bEF1 α -D2 was 20.1 fold higher than that of BmA3 promoter (Fig. 3B).

To confirm whether the bEF1 α promoter also is normally regulated at other insect cell lines, the pGL3-bEF1 α -D2 and pGL3-BmA3 plasmids were transfected into Bm5, Sf9 and S2 insect cells, and then their promoter activity was measured, respectively. The bEF1 α promoter normally worked in Bm5, Sf9 and S2 cells, and had higher transcriptional activity than BmA3 in all insect cells tested (Fig. 3C). From the above results, we suggest that bEF1 α 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. Furthermore, it can be used for heterologous protein expression in various host insect as a universal promoter.

Acknowledgements

This research was supported by Bio-industry Technology Development Program (grant no. 311059-4), Ministry for Food, Agriculture, Forestry and Fisheries, Republic of Korea.

References

- Ailor E, Betenbaugh MJ (1999) Modifying secretion and post-translational processing in insect cells. *Curr Opin Biotechnol* 10, 142-145.
- Dever TE, Glynias MJ, Merrick WC (1987) GTP-binding domain: Three consensus sequence elements with distinct spacing. *Proc Natl Acad Sci U S A* 84, 1814-1818.
- Goo TW, Yun EY, Hwang JS, Kang SW, Park S, You KH, Kwon OY (2002) Molecular characterization of a *Bombyx mori* protein disulfide isomerase (bPDI). *Cell Stress Chaperones* 7, 118-125.
- Goo TW, Kim SW, Kim SR, Park SW, Kang SW, Lee KG, Kwon OY, Yun EY (2010) Utilization of the *Bombyx mori* Hypothetical Protein 32 Promoter for Efficient Transgene Expression. *Int J Indust Entomol* 20, 107-114.
- Goo TW, Kim SW, Kim YB, Kim SR, Park SW, Kang SW, Kwon OY, Yun EY (2011) A Powerful Ubiquitous Activity of *Bombyx mori* Heat Shock Protein 70 Promoter. *Genes & Genomics* 33, 635-643.
- Handler AM, McCombs SD, Fraser MJ Jr, Saul SH (1998) The Lepidopteran transposon vector, piggyBac, mediates germline transformation in the Mediterranean fruit fly. *Proc Natl Acad Sci USA* 95, 7520-7525.
- Hershey JW (1991) Translational control in mammalian cells. *Annu Rev Biochem* 60, 717-755.
- Horn C, Schmid BGM, Pogoda FS, Wimmer EA (2002) Fluorescent transformation markers for insect transgenesis. *Insect Biochem Mol Biol* 32, 1221-1235.
- Kato T, Kajikawa M, Maenaka K, Park EY (2010) Silkworm expression system as a platform technology in life science. *Appl Microbiol Biotechnol* 85, 459-470.
- Kjeldgaard M, Nyborg J (1992) Refined structure of elongation factor ef-tu from *Escherichia coli*. *J Mol Biol* 223, 721-742.
- Mangé A, Julien E, Prudhomme JC, Couble P (1997) A strong inhibitory element down-regulates SRE-stimulated transcription of the A3 cytoplasmic actin gene of *Bombyx mori*. *J Mol Biol* 265, 266-274.
- Royer C, Jalabert A, Da Rocha M, Grenier AM, Mauchamp B, Couble P, Chavancy G (2005) Biosynthesis and cocoon-export of a recombinant globular protein in transgenic silkworms. *Transgenic Res* 14, 463-472.
- Schultz J, Milpetz F, Bork P, Ponting CP (1998) Smart, a simple modular architecture research tool: Identification of signaling domains. *Proc Natl Acad Sci USA* 95, 5857-5864.
- Summers MD, Smith GE (1987) A methods for baculovirus vector and insect cell culture procedures, Texas Agricultural Experiment Station, Bulletin No. 1555.
- Sun SC, Lindstrom I, Boman HG, Faye I, Schmidt O (1990) Hemolin: an insect-immune protein belonging to the immunoglobulin superfamily. *Science* 250, 1729-1732.
- Tamura T, Thibert C, Royer C, Kanda T, Abraham E, Kamba M, Komoto N, Thomas JL, Mauchamp B, Chavancy G, Shirk P, Fraser M, Prudhomme JC, Couble P (2000) Germ-line transformation of the silkworm *Bombyx mori* L. using a piggyBac transposon-derived vector. *Nat Biotechnol* 18, 81-84.
- Thomas JL, Da Rocha M, Besse A, Mauchamp B, Chavancy G (2002) 3xP3-EGFP marker facilitates screening for transgenic silkworm *Bombyx mori* L. from the embryonic stage

- onwards. Insect Biochem. Mol Biol 32, 247-253.
- Tomita M, Munetsuna H, Sato T, Adachi T, Hino R (2003) Transgenic silkworms produce recombinant human type III procollagen in cocoons. Na. Biotechno. 21, 52-56.
- Tomita M, Hino R, Ogawa S, Iizuka M, Adachi T, Shimizu K, Sotoshiro H, Yoshizato K (2007) A germline transgenic silkworm that secretes recombinant proteins in the sericin layer of cocoon. Transgenic Res 16, 449-465.
- Tryselius Y, Samakovlis C, Kimbrell DA, Hultmark D (1992) CecC, a cecropin gene expressed during metamorphosis in *Drosophila* pupae. Eur J Biochem 204, 395-399.
- Uchino K, Imamura M, Shimizu K, Kanda T, Tamura T (2007) Germ line transformation of the silkworm, *Bombyx mori*, using the transposable element Minos. Mol. Genet. Genomics 277, 213-220.
- Uhlířová M, Asahina M, Riddiford LM, Jindra M (2002) Heat-inducible transgenic expression in the silkworm *Bombyx mori*. Dev Genes Evol 212, 145-151.
- Wurm FM (2003) Human therapeutic proteins from silkworms. Nat. Biotechnol. 21, 34-35.
- Xue R, Chen H, Cui L, Cao G, Zhou W, Zheng X, aGong C (2012) Expression of hgm-csf in silk glands of transgenic silkworms using gene targeting vector. Transgenic Res. 21, 101-111.
- Yun EY, Goo TW, Kim SW, Choi KH, Hwang JS, Kang SW, Kwon, OY (2005) Galatosylation and sialylation of mammalian glycoproteins produced by baculovirus-mediated gene expression in insect cells. Biotechnol Lett 7, 035-7039.
- Zhao A, Zhao T, Zhang Y, Xia Q, Lu C, Zhou Z, Xiang Z, Nakagaki M (2010) New and highly efficient expression systems for expressing selectively foreign protein in the silk glands of transgenic silkworm. Transgenic Res. 19, 29-44.