A Strong Transcription Activity of the *Bombyx mori* Elongation Factor 1α 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 bEF1α (*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 bEF1α gene, which has about 20 fold more intensive promoter activity than BmA3 promoter. Moreover, the bEF1α promoter was normally regulated in Bm5, Sf9, and S2 cells. Therefore, we suggest that bEF1α 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 1α, 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 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., 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 (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 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 germine 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-
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 1α 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±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 bEF1α 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 (bEF1α) 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 EcoR I, Xho I and EcoR I/Xho 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 bEF1α mRNA, 5’-RACE PCR was carried out with poly (A”) RNA using CapFishing™ 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 bEF1α fragment. These primers were used in 5’-RACE in conjunction with the anchor primer (5’-RACE primer: 5’-AGTGAAGGCGCCAGTATGAAAGGT- GATCGG-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 test the promoter activity of the constructs. The Dual Vector Kit (Promega, USA) were used to generate luciferase plasmids in view of the reporter gene constructed from firefly luciferase (pGL3-Basic Vector) and renilla luciferase using pRL-SV40 vector. The constructs were transfected into a cell line derived from B. mori (BmA3 promoter activity uses a plasmid pGL3-Basic Vector containing the firefly luciferase gene) using liposome (Lipofectamine Plus, Invitrogen, USA) and luciferase activity was measured using a Dual Luciferase Reporter Assay System (Promega, USA).

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 bEF1α gene.

<table>
<thead>
<tr>
<th>Name</th>
<th>Position</th>
<th>Sequences</th>
<th>Forward/ reverse</th>
<th>Construct name</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1</td>
<td>-905/-885</td>
<td>5’-CTCGAGCTGTCACCTCTCAAAATTTGG-3’</td>
<td>F</td>
<td>pGL3-bEF1α-D1</td>
</tr>
<tr>
<td>D2</td>
<td>-702/-682</td>
<td>5’-CTCGAGAGATTTTCTCTAATTGG-3’</td>
<td>F</td>
<td>pGL3-bEF1α-D2</td>
</tr>
<tr>
<td>D3</td>
<td>-504/-484</td>
<td>5’-CTCGAGTCGATCTACGAAATGCTAATGCTA-3’</td>
<td>F</td>
<td>pGL3-bEF1α-D3</td>
</tr>
<tr>
<td>D4</td>
<td>-244/-222</td>
<td>5’-CTCGAGATT TTGGTAAGATTGTGA-3’</td>
<td>F</td>
<td>pGL3-bEF1α-D4</td>
</tr>
<tr>
<td>D5</td>
<td>-98/-78</td>
<td>5’-CTCGAGCAAGACACATTTGAGGGTTTTT-3’</td>
<td>F</td>
<td>pGL3-bEF1α-D5</td>
</tr>
<tr>
<td>R1</td>
<td>+1/+38</td>
<td>5’-AGATCTCACGGATTACCAACTGCGGTG-3’</td>
<td>R</td>
<td></td>
</tr>
</tbody>
</table>
USA) was used to measure promoter activity. For this assay, 5 serial deleted fragments of the 5'-flanking region of b bEF1α 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-bEF1α-D1 (-905/+38), pGL3-bEF1α-D2 (-702/+38), pGL3-bEF1α-D3 (-504/+38), pGL3-bEF1α-D4 (-244/+38), and pGL3-bEF1α-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 × 10⁵ 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 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) 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 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 elongation factor 1α of B. mori.

Structure analysis of full-length bEF1α 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 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 bEF1α (B. mori elongation factor 1α) because it has high homology (99%) with elongation factor 1α of B. mori. EF-1α promotes the GTP dependent binding of aminoacyl-tRNA to A site of ribosome (Hershey, 1991). The amino acid sequence of EF-1α 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 GHVDHGKT (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 bEF1α at amino acid residues 14-22, 91-94, and 153-156, respectively (Fig. 2).

Characterization of bEF1α mRNA expression
To confirm whether bEF1α mRNA was ubiquitously expressed or not in B. mori, RT-PCR was performed. There were a little difference in expression level of the b bEF1α mRNA according to each tissue and development.

![Fig. 1. Expression pattern according to tissue and development stage of bEF1α 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, epicdermis; 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.](image-url)
nal stage of 5th instar larvae, but the bEF1α mRNA was ubiquitously expressed in all tissues and developmental stages tested (Fig. 1). As these results, we suppose that bEF1α cDNA has potential of development as a valuable promoter for ubiquitous and powerful expression of a heterologous gene.

**Structural analysis of bEF1α genomic DNA**

A positive phage clone was isolated from the screening of a B. mori genomic DNA library with bEF1α cDNA probes labeled with $[^{32}P]$dATP. It was including the complete genomic sequence containing 906 bp of the 5'-flanking region (GenBank accession number: JQ638952).

**Fig. 2.** The genomic nucleotide and deduced aa sequences of B. mori elongation factor 1α (bEF1α) 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.
A Strong Transcription Activity of the *Bombyx mori* Elongation Factor 1α Promoter

**A**

![Graph](image)

**B**

![Graph](image)

**C**

![Graph](image)

Fig. 3. Analysis of bEF1α deletion constructs by dual luciferase reporter gene activity. (A) Various lengths of 5’-DNA sequences of bEF1α 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-bEF1α-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 bEF1α 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), bEF1α 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 bEF1α 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 bEF1α gene.

**Activity of bEF1α promoter**

To investigate the activity of bEF1α promoter, a 944 bp bEF1α 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-bEF1α-D1 (-905/+38), pGL3-bEF1α-D2 (-702/+38), pGL3-bEF1α-D3 (-504/+38), pGL3-bEF1α-D3 (-244/+38), and pGL3-bEF1α-D5 (-98/+38) were co-transfected with control plasmid into Bm5 cells. The promoter activity of each
activity was measured, respectively. The bEF1 promoter was inserted into Bm5, Sf9 and S2 insect cells, and then their promoter activity was determined. Among these 5 deletion constructs, the second construct, pGL3-bEF1α-D2 had the highest effect on transcriptional activity, with approximately 116% activity compared to pGL3-bEF1α-D1. The transcriptional activities of other deletion constructs from pGL3-bEF1α-D3 to pGL3-bEF1α-D5 were gradually decreased in a size-dependent manner. The pGL3-bEF1α-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-bEF1α-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-bEF1α-D2 was 20.1 fold higher than that of BmA3 promoter (Fig. 3B).

To confirm whether the bEF1α promoter also is normally regulated at other insect cell lines, the pGL3-bEF1α-D2 and pGL3-BmA3 plasmids were transfected into Bm5, Sf9 and S2 insect cells, and then their promoter activity was measured, respectively. The bEF1α 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 the bEF1α 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


Transgenic silkworms produce recombinant human type III
Tomita M, Hino R, Ogawa S, lizuka M, Adachi T, Shimizu K,
Sotoshiro H, Yoshizato K (2007) A germline transgenic silk-
worm that secretes recombinant proteins in the sericin layer
CecC, a cecropin gene expressed during metamorphosis in
Germ line transformation of the silkworm, Bombyx mori,
using the transposable element Minos. Mol. Genet. Genom-
ics 277, 213-220.
inducible transgenic expression in the silkmoth Bombyx
Wurm FM (2003) Human therapeutic proteins from silk-
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 mamma-
lian glycoproteins produced by baculovirus-madiated gene
Zhao A, Zhao T, Zhang Y, Xia Q, Lu C, Zhou Z, Xiang Z, Nak-
agaki 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.