Cloning and Expression of *Bombyx mori* Silk Gland Elongation Factor 1γ in *Escherichia coli*


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Received September 3, 2001; Accepted October 29, 2001

Elongation factor 1 (EF-1) from the silk gland of *Bombyx mori* consists of α-, β-, γ-, and δ-subunits. EF-1α-GTP catalyzes the binding of aminoacyl-tRNA to ribosomes concomitant with the hydrolysis of GTP. EF-1γδ catalyzes the exchange of EF-1α-bound GDP for exogenous GTP and stimulates the EF-1α-dependent binding of aminoacyl-tRNA to ribosomes.

EF-1γ cDNA, which contains an open reading frame (ORF) encoding a polypeptide of 423 amino acid residues, was amplified and cloned by PCR from a silk gland cDNA library. The calculated molecular mass and predicted pl of the product were 48,388 Da and 5.84, respectively. The silk gland EF-1γ shares 67.3% amino acid identity with *Artemia salina* EF-1γ. The N-terminal domain (amino acid residues 1–211) of silk gland EF-1γ is 29.3% identical to maize glutathione S-transferase. We demonstrated that silk gland EF-1γ bound to glutathione Sepharose, suggesting that the N-terminal domain of EF-1γ may have the capacity to bind to glutathione.

Key words: elongation factor 1γ; glutathione S-transferase (GST); glutathione; *Bombyx mori*

Elongation factor 1 (EF-1) catalyzes the binding of aminoacyl-tRNA to the ribosome concomitant with the hydrolysis of GTP. EF-1 consists of four different subunits with molecular masses of 51 kDa (α), 49 kDa (γ), 33 kDa (δ), and 26 kDa (β) in vertebrates and insects, which correspond to EF-1α, EF-1γ, EF-1β, and EF-1β′ in plants, respectively. EF-1α is responsible for the binding of aminoacyl-tRNA to the ribosome with concomitant hydrolysis of GTP. The EF-1βγδ complex catalyzes the exchange of EF-1α-bound GDP for exogenous GTP. Both EF-1β and EF-1δ possess guanine nucleotide exchange activity. EF-1γ acts in concert with EF-1β to facilitate the exchange of EF-1α-bound GDP for GTP.

We have cloned the cDNAs encoding silk gland EF-1α, EF-1β which corresponding to EF-1β′ in the old nomenclature, and EF-1δ. EF-1α, EF-1β, and EF-1δ consist of 463 amino acids, 222 amino acids, and 262 amino acids, respectively. The C-terminal halves of silk gland EF-1β (amino acid residues 91–222) and silk gland EF-1δ (amino acid residues 131–262) share 72.7% amino acid identity. The carboxy-terminal region, amino acid residues 189–222, of silk gland EF-1β bound to silk gland EF-1α in vitro.

The EF-1αβγδ complex forms a complex with valyl-tRNA synthetase from mammalian cells. Mammalian valyl-tRNA synthetase contains an additional N-terminal extension compared to the corresponding yeast enzyme of about 200 amino acid residues which bears strong sequence similarity to the N-terminal moiety of EF-1γ. The N-terminal extension of valyl-tRNA synthetase is required for the binding to EF-1δ to form the complex.

Immunocytochemical and subcellular fraction studies on human fibroblasts have demonstrated that EF-1βγδ is localized in the endoplasmic reticulum (ER) region. The presence of EF-1βγδ in the ER is in agreement with the observation that EF-1γ binds specifically to membranes and tubulin. EF-1γ may

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*Abbreviations*: EF-1, elongation factor 1; ORF, open reading frame; GST, glutathione S-transferase; ER, endoplasmic reticulum; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; IPTG, isopropyl-β-D-thiogalactopyranoside; SDS-PAGE, SDS-polyacrylamide gel electrophoresis
act as an anchor to bind the EF-1βγδ complex to the ER.\textsuperscript{13}

The amino acid sequence of the N-terminal domain of EF-1γ from \textit{Trypanosoma cruzi} shows significant homology to class θ glutathione S-transferases (GST)\textsuperscript{12} and the N-terminal extension of valyl-tRNA synthetase. Other investigators have reported similar observations on EF-1γ from humans, \textit{Artemia salina}, \textit{T. cruzi}, a fungus, and a yeast.\textsuperscript{13} We have observed that rice EF-1γ has significant homology to GST and significant GST activity.\textsuperscript{14} The GST-like domain of EF-1γ appears to participate in the detoxification of lipophilic compounds in \textit{T. cruzi}, which overexpresses EF-1γ, is strongly resistant to clomipramine, a lipophilic compound.\textsuperscript{15}

In order to characterize the GST-like domain of EF-1γ, we obtained a EF-1γ cDNA clone by PCR amplification from a silk gland cDNA library, and expressed it in \textit{E. coli} as a His-tagged protein. The N-terminal domain (amino acid residues 1–211) of silk gland EF-1γ was 29.3\% identical to maize GST. We found that His-tagged EF-1γ bound to glutathione Sepharose.

### Materials and Methods

\textbf{Isolation of mRNA from silk gland and construction of silk gland cDNA library.} Total RNA was prepared from the posterior silk gland of \textit{Bombyx mori} at the 3rd day of the 5th instar by the acid-phenol extraction method.\textsuperscript{16} Poly-(A)+ RNA was purified from the total RNA using Oligo-dT30 cellulose (Stratagene). Subsequently, a silk gland cDNA library was constructed in λgt 11 using the cDNA Cloning System (Amersham Pharmacia Biotech) according to the manufacturer’s instructions.

\textit{EF-1γ} cDNA amplification by PCR using degenerate primers. The EF-1γ-specific primers used were designed based on several stretches of amino acid sequence conserved among \textit{Drosophila melanogaster}, \textit{Artemia}, rabbit, \textit{Xenopus}, and humans corresponding to the amino acid sequences 17-KALIAAQY-23: K17-Y23 sense primer (5′-AAR GCN TTG ATH GCN GCN CAR TA-3′), 71-NAIAYYV-77: N71-V77 sense primer (5′-AAY GCN ATH GCN TAY TAY GT-3′), 376-DWQIDYE-382: D376-E382 antisense primer (5′-TAR TAR TCN ACY TGC CAR TC-3′), 384-YDKKLDP-391: Y384-P391 antisense primer (5′-GGR TCC AGY TTY TTC CAR TCR TA-3′). The first PCR was done using cDNA prepared from the silk gland cDNA library as a template, and K17-Y23 sense primer and Y384-P391 antisense primer. The PCR reaction mixture was composed of 50 pmol of each primer, 5 ng of cDNA as a template, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl$_2$, 0.2 mM dNTPs, and 1.0 unit of Taq DNA polymerase in a final volume of 25 μl. The amplification conditions were as follows: 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The cycle was repeated 25 times, preceded by 3 min at 95°C, and followed by 5 min at 72°C.

The nested PCR was done using the DNA amplified by the first PCR as a template, and N71-V77 sense primer and D376-E382 antisense primer, under the same conditions as for the first PCR. The DNA fragment amplified by the nested PCR was subcloned into pT7Blue with a Perfectly Blunt Cloning kit (Novagen) according to the manufacturer’s instructions.

\textbf{Screening of \textit{EF-1γ} cDNA.} About 5 × 10$^4$ recombinant phages were plated on a lawn of \textit{E. coli} Y1090 strain and the phages were screened by plaque hybridization using the nested-PCR-amplified \textit{EF-1γ} cDNA as a probe. The nested-PCR-amplified \textit{EF-1γ} cDNA was labelled with Alkphos Direct (Amersham Pharmacia Biotech). Hybridization was done at 55°C overnight according to the manufacturer’s instructions. Hybridization signals were detected using X-ray films. Clones that hybridized with the probe in the second and third screenings were isolated. Positive λDNAs were isolated using a QIAGEN Lambda Midi kit (Qiagen). The phage DNAs were cleaved with the restriction enzyme \textit{Bam} HI and ligated to pUC19 at the \textit{Bam} HI site.

\textbf{Rapid amplification of cDNA ends (RACE).} The 5′-terminal region of \textit{EF-1γ} cDNA was amplified by \textbf{5′-RACE} using cDNA from the silk gland as a template and using primer sets consisting of \textit{EF-1γ}-specific primer (5′-GGC AAG TGT GAT TCT CTC GGA-3′) and λgt11 primer (5′-GGT GGC GAC GAC TCC TGG AGC CCG-3′) or of \textit{EF-1γ}-specific primer and λgt11 Rev primer (5′-TGG ACA CCA GAC CAA CTG GTA ATG-3′). The reaction mixture was the same as that for the first PCR reaction mixture. The amplification conditions were as follows: 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. The cycle was repeated 25 times, preceded by 3 min at 95°C, and followed by 5 min at 72°C. The DNA fragment amplified by \textbf{5′-RACE} was subcloned into pT7Blue and sequenced as described below.

\textbf{Nucleotide sequence analysis.} Nucleotide sequencing using the dideoxy chain termination method was done using a Thermo Sequenase kit with double-stranded templates. The oligonucleotide primers used for cycle sequencing were Cy5-M13 universal, Cy5-M13 reverse, Cy5-T7, and Cy5-T7 terminator primers. After the cycle sequencing reaction, samples were electrophoresed at 1,500 V and signals were detected with an ALFexpress DNA sequencer (Amersham Pharmacia Biotech).
Expression of His-tagged EF-1γ in E. coli. For expression in bacteria, an E. coli expression vector, pET-15b, was used. To obtain a cDNA fragment containing the coding region of EF-1γ, PCR was done using the silk gland cDNA as a template with 5'-primer 5'-GGA ATT CCA TAT GCC GGC CGG GGT ACT TTA-3' (underlining indicates the Nde I recognition site) and 3'-primer 5'-CCG CTC GAG TCA CTT GAA TAT CTT GCC CTG-3' (underlining indicates the Xho I recognition site). The reaction mixture was composed of 50 pmol of each primer, 5 ng of cDNA as a template, 1.25 units of ExTaq DNA polymerase, and 5 μl of 10×-buffer for ExTaq (Takara) in a final volume of 50 μl. The amplification conditions were as follows: 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. The cycle was repeated 30 times, preceded by 3 min at 94°C, and followed by 5 min at 72°C. To confirm that the DNA amplified by PCR was cDNA encoding silk gland EF-1γ, the DNA was subcloned into pT7Blue and sequenced using the ALFexpress DNA sequencing system.

After confirmation of the DNA sequencing, the DNA fragment encoding EF-1γ was excised by digestion with Nde I and Xho I, and ligated into the expression vector pET-15b that had previously been digested with the same enzymes. The resulting construct, pET-EF1γ, was sequenced using the ALFexpress DNA sequencing system to confirm the integrity of the junctions, and was used to transform E. coli BL21(DE3)pLysS.

Purification of His-tagged EF-1γ. The transformed bacteria were grown to an A600 of 0.8 (400 ml) and induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 18 h at 25°C. The bacteria were then harvested by centrifugation at 5,000 rpm for 10 min at 4°C and resuspended in 10 ml of ice-cold buffer A [20 mM Tris-HCl, pH 8.0, 0.5 M NaCl, and 20% glycerol]. The cells were lysed by sonication for 10 min in buffer A containing 2% Triton X-100 and centrifuged at 10,000 × g for 20 min at 4°C. The supernatant was put on a HisTrap column according to thesupplier’s instructions (Amersham Pharmacia Biotech).

Binding of His-tagged EF-1γ to glutathione Sepharose. Nine micrograms of purified His-tagged EF-1γ and 20 μl of a 50% slurry of glutathione Sepharose were incubated in buffer B [50 mM Tris-HCl pH 8.0, 75 mM KCl, 10 mM MgCl2] at 4°C for 1 h. The Sepharose beads were then washed three times with 500 μl of buffer B containing 0.1% Tween 20. The proteins bound to the Sepharose beads were eluted with 50 μl of 10 mM glutathione in buffer B. The eluent was analyzed by SDS-PAGE followed by staining with Coomassie Brilliant Blue.

Results and Discussion

Cloning and nucleotide sequence of silk gland EF-1γ

The first PCR was done by using the silk gland cDNA library (5 ng) as a template, K17-Y23 sense primer, and Y384-P391 antisense primer. Analysis of the amplified DNA by 1.4% agarose gel electrophoresis indicated that the 1.1-kbp DNA was apparently a single band.

The 1.1-kbp DNA was excised from the gel and used as a template for nested PCR. The nested PCR was done by using the primer set of N71-V77 sense primer and D376-E382 antisense primer, and 2 ng of the 1.1-kbp DNA as a template under the same conditions as for the first PCR. Analysis of the amplified DNA by 1.4% agarose gel electrophoresis showed a single band with the expected molecular mass of 0.9 kbp. The 0.9-kbp DNA was excised from a 1.4% agarose gel, subcloned into pT7Blue, and sequenced by the dideoxy chain termination method using a Thermo sequenase kit. The deduced amino acid sequence encoded by the cDNA showed significant homology with EF-1γ from Artemia salina and Drosophila melanogaster.

We obtained 8 positive clones from 5 × 104 independent plaques of a silk gland cDNA library by in situ plaque hybridization using the 0.9-kbp cDNA as a probe. None of these positive clones included the complete reading frame of EF-1γ. The largest insert DNA consisted of 1,558 nucleotides, from nucleotide number 46 to 1603.

To obtain the 5'-terminal end of the EF-1γ cDNA, the 5'-RACE method was done using ExTaq DNA polymerase for the DNA amplifications. After gel-purification, the resulting fragments were subcloned into the EcoRV site of plasmid pT7Blue and sequenced as described above. Sequence analysis of the inserts from 7 individual colonies indicated that these inserts had the same sequence as EF-1γ cDNA in the region from position 1 to position 480. The nucleotide sequence of EF-1γ cDNA revealed by the 5'-RACE method is underlined in Fig. 1.

The nucleotide sequence of the EF-1γ cDNA and the deduced amino acid sequence are shown in Fig. 1. The EF-1γ cDNA consists of 1,603 nucleotides, with an open reading frame (ORF) encoding a polypeptide of 423 amino acid residues with a calculated molecular mass of 48,388 Da, which agrees with the molecular mass measured by SDS-PAGE. The predicted pI of 5.84 was higher than the predicted pIs of both EF-1β and EF-1δ. A cytoplasmic polyadenylation element, UUUUUUAU, and a polyadenylation signal, AAUAAA, were found in the 3'-UTR.

Amino acid sequence comparison between silk gland EF-1γ and EF-1γ from other species

The deduced amino acid sequence of silk gland EF-
showed a high degree of similarity to those of other EF-1\_\text{g} reported previously (Fig. 2). It shared 67.3\%, 64.3\%, 57.7\%, 57.1\%, 57.1\%, 33.9\%, 33.0\% and 28.4\% amino acid identity with EF-1\_\text{g} of those species. Alignment of the amino acid sequences showed some highly conserved regions in all of the EF-1\_\text{g}.

Thr230 of \textit{X. laevis} EF-1\_\text{g} is phosphorylated by \textit{cdc2} kinase during meiotic cell division in \textit{Xenopus} oocytes.\textsuperscript{17} Candidate sites for analogous phosphorylation (T\_W\_S P\_x) have been identified in EF-1\_\text{g} from \textit{H. sapiens}, rabbit, and \textit{S. cerevisiae}, but not in EF-1\_\text{g} from silk gland, \textit{A. salina}, \textit{D. melanogaster}, \textit{O. sativa}, or \textit{T. cruzi}. The N-terminal halves of \textit{Artemia} EF-1\_\text{g} (amino acid residues 1–240) and \textit{Artemia} EF-1\_\text{b} (amino acid residues 1–103) are attached to each other.\textsuperscript{18} The interaction sites of EF-1\_\text{g} are expected to be located in the conserved regions of the N-terminal domain of EF-1\_\text{g}.

\textbf{GST-like domain of silk gland EF-1\_\text{g}}

The amino acid sequence of the N-terminal domain (1–211) of silk gland EF-1\_\text{g} was 29.3\% identical to that of maize GST. The crystal structure...

\textbf{Cloning and Expression of EF-1\_\text{g}}

The predicted amino acid sequence is shown below the nucleotide sequence. Amino acids are numbered beginning with the N-terminal residue. The nucleotide sequence identified by the 5\_\text{RACE} method is underlined. The arrow under the nucleotide sequence indicates the EF-1\_\text{g}-specific primer position used for 5\_\text{RACE}. The putative cytoplasmic polyadenylation element and polyadenylation signal are boxed. The asterisk indicates the translational termination codon. The sequence has been submitted to DDBJ with accession no. AB046361.
Fig. 2–1. Amino Acid Sequence Alignment of the Cloned Bombyx mori Silk Gland EF-1γ and Those from Other Species.

The amino acids conserved among more than five species are shown in gray boxes. Gaps introduced for good alignment are indicated by dashes. "Consensus" shows amino acid residues that are conserved in more than five (lowercase letters) or all of the nine aligned sequences (capital letters).

Expression and purification of His-tagged EF-1γ

To express EF-1γ in E. coli, cleavage sites for restriction enzymes Nde I and Xho I were introduced at the ends of the ORF by PCR. An Nde I-Xho I fragment encoding EF-1γ was inserted into the E. coli expression vector pET-15b. The resulting vector, pET-EF1γ, was used to transform E. coli BL21(DE3) pLysS, and expression of EF-1γ was induced by IPTG.

After cultivation, the cells were disrupted by sonication. Total proteins in the extract of the induced E. coli were analyzed by SDS-PAGE. A protein of 48 kDa was detected after induction by IPTG (Fig. 4), and reacted with specific anti-EF1γ antiserum (data not shown), indicating that the 48-kDa protein was His-tagged EF-1γ. As the 48-kDa band of His-tagged EF-1γ was recovered in both the soluble and insoluble fractions separated by centrifugation (data not shown), the His-tagged EF-1γ was...
Cloning and Expression of EF-1γ

purified from the soluble fraction using a HisTrap kit according to the supplier’s instructions. The yield of His-tagged EF-1γ was about 25 mg from the soluble fraction of the extract from 400 ml of induced culture.

**Binding of EF-1γ to glutathione Sepharose**

The binding of His-tagged EF-1γ to a glutathione matrix was examined. Nine micrograms of purified His-tagged EF-1γ was mixed with 20 μl of glutathione Sepharose (50% slurry), and the mixture was incubated at 4°C for 1 h. After the Sepharose beads were washed three times with 500 μl of a buffer containing 0.1% Tween 20, the proteins bound to the Sepharose beads were eluted with 50 μl of 10 mM glutathione. The eluent was analyzed by SDS-PAGE, which showed that EF-1γ specifically bound to glutathione Sepharose (Fig. 4).

As the amino acid residues of various GSTs which correspond to silk gland 51–56 and 69–74 are well conserved and responsible for the binding of glutathione, these sequences in silk gland and rice EF-1γ are thought to be responsible for the binding of EF-1γ to the glutathione Sepharose. Although the physiological meaning of the glutathione-binding activity of EF-1γ is unknown, it is tempting to speculate that glutathione regulates translation at the elongation step. As it is well known that reduced glutathione stimulates translation and oxidized glutathione inhibits translation,21,22) EF-1γ may play a role as a sensor to redox state in cytosol.

As we have observed that rice EF-1γ has low but
significant GST activity, the comparisons between plant (rice) and animal (silk gland) EF-1γ will facilitate the analysis of the function of glutathione in the elongation step.

The fact that overexpression of EF-1γ in Trypanosoma cruzi causes resistance to the tricyclic antidepressant drug clomipramine,5) suggest also that the GST-like domain of EF-1γ is involved in detoxification in vivo of some exogenous or endogenous toxins.

The fact that the amino acid sequence of EF-1γ showed significant similarity with those of GST and valyl-tRNA synthetase (Fig. 3), suggests that these three proteins have evolved from a common ancestor. However, no GST and glutathione-binding activities have been detected in valyl-tRNA synthetase. It is interesting that the sister proteins valyl-tRNA synthetase and EF-1γ participate in the formation of a multi-subunit complex containing EF-1 and valyl-tRNA synthetase.8) The GST-like domain of EF-1γ is thought to be involved in the binding with EF-1δ.9)

Acknowledgment

We thank Prof. Hiroshi Matsuzawa, Aomori University, for helpful discussions.

References


