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Elementary research into the transformation BmN cells mediated by the *piggyBac* transposon vector

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ABSTRACT

To generate stable transformants of *Bombyx mori* silkworm BmN cells continuously expressing a target gene from a *piggyBac*-derived vector, BmN cells were transfected with a *piggyBac* vector containing a neomycin-resistance gene, green fluorescent protein gene, and human insulin-like growth factor I gene (hIGF-I) and a helper plasmid containing the *piggyBac* transposase sequence under the control of the *B. mori* actin 3 (A3) promoter. With the antibiotic G418, we selected stably transformed BmN cells expressing hIGF-I from the *piggyBac*-derived vector containing a neomycin-resistance gene driven by the *ie*-1 promoter from the *B. mori* nucleopolyhedrovirus. However, no stably transformed BmN cells transformed with the *piggyBac* element vector containing an SV40-promoter-driven neomycin-resistance gene were isolated. Determined with an enzyme-linked immunosorbent assay, the expression level of hIGF1 was about 7.8 ng in 5×10^5 cells in which the h*IGF*-I gene was driven by the sericin-1 promoter, and 147.5 ng in 5×10^5 cells in which the h*IGF*-I was under control of *B. mori* fibroin heavy chain gene (*fib*-H) promoter with its downstream signal peptide sequence. Analysis of the chromosomal insertion site by inverse PCR showed that the exogenous DNA was inserted into the cell genome randomly or at a TTAA target sequence, characteristic of *piggyBac* element transposition. These results are particularly important because *piggyBac* has been suggested for use in the transgenesis of silkworm cells.

1. Introduction

The TTAA-specific transposon *piggyBac* is rapidly becoming a highly useful transposon in genetically engineering a wide variety of species, particularly insects. *piggyBac* is a class II transposable element, originally discovered as an IFP2 element from the lepidopteran insect cell line, TN-368, which insertionally inactivates certain baculovirus genes, producing mutants with a distinctive plaque phenotype known as "few polyhedra" (Fraser et al., 1985). The *piggyBac* element is a short inverted terminal repeat (ITR) transposable element, 2.5 kb long, with 13-bp ITR sequences and a single 2.1 kb open reading frame (Cary et al., 1989). Functionally, *piggyBac* encodes a transposase with a precise cut-and-paste mechanism and a unique preference for TTAA sites (Fraser et al., 1995, 1996; Elick et al., 1996). Therefore, it is considered to be the type element of the TTAA-specific transposon family (Fraser, 2000). The mobility and transposition functions of *piggyBac* were established and exploited to develop an important system for insect germline transformation (Handler et al., 1998). Recently, the *piggyBac* system has been used to transform many other types of organisms, ranging from the protest *Lasmodium falciparum* (Balu et al., 2005) to the mouse *Mus musculus* (Ding et al., 2005), and to transfer genes into mammalian cells, including human cells (Wilson et al., 2007). Thus, *piggyBac* is widely and increasingly applied to study gene function, gene therapy, transposon-based mutagenesis of cell lines, and the continuous production of recombinant proteins.

The silkworm is not only a very useful animal model for research, but also an economically important insect. *piggyBac* has been used in research to identify silkworm gene function (Inouea et al., 2005), in a transgenic silkworm bioreactor (Cao et al., 2006; Ogawa et al., 2007), and to confer enhanced resistance to nucleopolyhedrovirus on the silkworm (Kanginakudru et al., 2007). Insect cell lines have a wide range of applications in the production of recombinant proteins, the identification of gene functions, studies of baculoviruses, and the analysis of apoptosis in insect cells (Wickham et al., 1992). Although several insect cell lines have been used for stable transformation, only a few have been reported in *Bombyx mori* cells.

hIGF-I is a growth factor with clinical significance in medicine, essential for cell proliferation and used therapeutically in treating various diseases including diabetes mellitus (Xie et al., 2008), improving diabetic wound healing (Hirsch et al., 2008). Since insulin and hIGF-1 share some overlapping roles, hIGF-I may become a substitute therapeutic agent in subjects with certain defects in their insulin receptor signaling (Panahi et al., 2004). Patel et al. (2009) overexpressed the h*IGF*-I gene in normal human fibrob-

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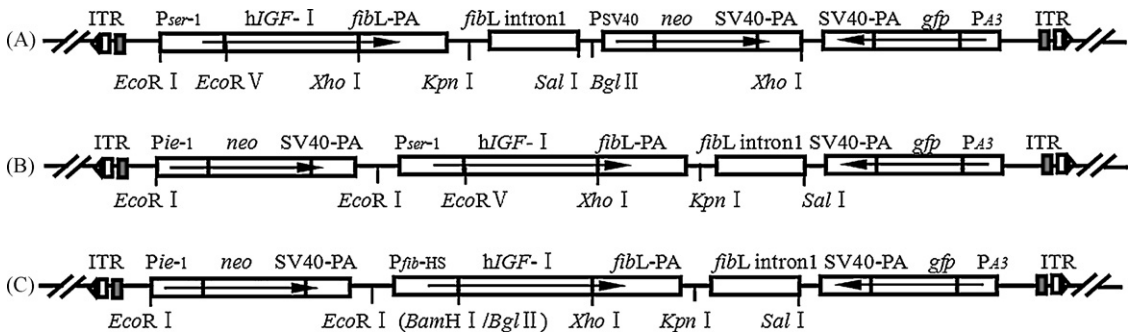

Fig. 1. Construction of recombination transgenic vectors. The construction of pigA3GFP-ser-hIGF-SV40-neo, pigA3GFP-ser-hIGF-ie-neo and pigA3GFP-fibHS-hIGF-ie-neo is shown in A, B, C, respectively. The plasmid pigA3GFP-ser-hIGF-SV40-neo contains the h*IGF1* gene under the control of the *B. mori Ser*-1 promoter, the partial intron 1 sequence of the *B. mori* fibroin light chain gene, the *neo* gene driven by the SV40 promoter, the *B. mori A3* cytoplasmic actin gene promoter fused to the GFP-encoding sequence, and the SV40 3′ untranslated sequences. The plasmid pigA3GFP-ser-hIGF-ie-neo contains the h*IGF*-I gene under the control of the *B. mori ser*-1 promoter and the *neo* gene driven by the immediate early-stage gene promoter of the *B. mori* nucleopolyhedrovirus, the partial intron 1 sequence of the *B. mori* fibroin light chain gene, and the *gfp* expression cassette under the control of the *B. mori A3* cytoplasmic actin gene promoter. The plasmid pigA3GFP-fibHS-hIGF-ie-neo contains FibHS-hIGF expression cassette, the *neo* gene driven by the immediate early-stage gene promoter of the *B. mori* nucleopolyhedrovirus, the partial intron 1 sequence of the *B. mori* fibroin light chain gene, and the *gfp* expression cassette under the control of the *B. mori A3* cytoplasmic actin gene promoter. ITR, inverted terminal repeats of *piggyBac*; $P_{ser\text{-}1}$, *B. mori* sericin 1 promoter; P_{SV40}, SV40 promoter; $P_{ie\text{-}1}$, immediate early-stage gene promoter of *B. mori* nucleopolyhedrovirus; P_{A3}, *B. mori A3* cytoplasmic actin gene promoter; $P_{fib\text{-}HS}$, fib-H promoter with its downstream signal peptide sequence hIGF-I, human insulin-like growth factor 1 gene; neo, neomycin-resistance gene; GFP, green fluorescent gene; intron, partial intron 1 sequence of the *B. mori* fibroin light chain gene; fibL-PA, polyA signal sequence of the *B. mori* fibroin light chain gene; SV40-PA, SV40 3′ untranslated sequence.

lasts, leading to cells that expressed 4 ng of hIGF-1 per 10^6 cells per 24 h with biological activity.

Although several insect cell lines have been used for stable transformation, only a few have been reported in *B. mori* cells. To establish a gene expression system for the stable expression of h*IGF*-I in transformation BmN cells mediated by *piggyBac*-derived vectors, we constructed the transgenic vector with expression elements including a neomycin-resistance gene (*neo*), the green fluorescent protein gene (*gfp*), and h*IGF*-I, transfected together with a helper plasmid (transposase donor) into BmN cells. Then, we screened for G418-resistant cells expressing h*IGF*-I. Our results show that in the stably transformed line of BmN cells, the plasmids had inserted precisely at the TTAA nucleotide motif and also in a random manner.

2. Materials and methods

2.1. Construction of plasmid pigA3GFP-ser-hIGF-SV40-neo

To construct the *piggyBac*-derived plasmid pigA3GFP-ser-hIGF-SV40-neo, which contains both the *B. mori* sericin-1 (ser-1)-promoter-driven h*IGF*-I gene and the SV40-promoter-driven *neo* gene, the partial intron 1 (fib-L-intron 1) sequence of the fibroin light chain gene was amplified by PCR using specific primers Fib-L-5 (5′-CGGATATCTATGGGCTCCAGTAACC-3′, underlining indicates the EcoRV site) and Fib-L-6 (5′-GCGTCGACGGTCAGGTTAGATTAACGGG-3′, underlining indicates the SalI site) from the silk-gland genomic DNA of the silkworm Haoyue strain. The purified product was digested with SalI and EcoRV, and ligated into the vector pBluescript II SK (+) (Invitrogen Corporation) to construct the plasmid pSK-fib-L-intron 1. The sequence of fib-L-intron 1 was deposited in GenBank (accession no. DQ679478). The plasmid pSK-fib-L-intron 1 was digested with SalI and EcoRV and then ligated into pigA3GFP (Tamura et al., 2000) with the *B. mori A3*-promoter-driven *gfp* gene digested with SalI and SmaI to generate the recombinant plasmid pigA3GFP-fib-L-intron. The fragment containing the SV40-promoter-driven *neo* gene was amplified from the plasmid pcDNA3.1 (Invitrogen Corporation) with primers neo-1 (5′-GCAGATCTGTGGAATGTGTGTCAGTTAGGG-3′, underlining indicates the BglII site) and neo-2 (5′-AGCTCGAGCTAGAGGTCGACGG-3′, underlining indicates the XhoI site). The PCR product was digested with BglII and XhoI, then ligated into the plasmid pigA3GFP-fib-L-intron 1 to generate the plasmid pigA3GFP-intron 1-SV40-neo. To construct the plasmid pSK-ser-hIGF1-polyA, the partial sequence of the h*IGF-1* gene, which encodes the bioactive peptide of human insulin-like growth factor I (Rinderknecht and Humbel, 1978), was amplified from the plasmid pET28a-hIGF-I (Xu et al., 2006) with primers IGF-I-1 (5′-TGGATATCATGGGACCGGAGACGCTCTGC-3′, underlining indicates the EcoRV site) and hIGF-I-2 (5′-ATCTCGAGAAGCTTAAGCTGACTTGGCAGGCTTG-3′, underlining indicates the XhoI and HindIII sites), then digested with XhoI and EcoRV and ligated into pSK-ser-DsRed-polyA (Zhu et al., 2007), from which the DsRed fluorescent protein gene fragment had been excised with the same enzymes. The plasmid pSK-ser-hIGF1-polyA was cut with KpnI and EcoRI, and the isolated h*IGF*-I gene driven by the *B. mori* sericin-1 promoter was cloned into the KpnI/EcoRI sites of the plasmid pigA3GFP-intron 1-SV40-neo to generate the plasmid pigA3GFP-ser-hIGF-SV40-neo (Fig. 1A).

To identify the plasmid pigA3GFP-hIGF-SV40-neo, the primer pairs Ser-1/Ser-2, hIGF-1-1/hIGF-1-2, TPFib-L-3 (5′-GGCTCGAGCAAATTGTGTTTGCGTTAGG-3′)/TPFib-L-4 (5′-GCGGTACCCACTGTCCAATCCACCGTC-3′), Fib-L-5/Fib-L-6, DEGFP-1 (5′-TGGAATTCATGGTGAGCAAGGGCGAGG-3′)/DEGFP-2 (5′-TTGGATCCTTACTTGTACAGCTCGTCCATG-3′), and neo-1/neo-2 were used to detect the *ser*-1 promoter, the h*IGF*-I gene, the polyA signal sequence of the *B. mori* fibroin light chain gene, the partial intron 1 sequence of the fibroin light chain gene, the *gfp* gene, and the SV40 promoter fused to the neomycin-resistance gene, respectively.

2.2. Construction of plasmid pigA3GFP-ser-hIGF-ie-neo

To construct the *piggyBac*-derived plasmid pigA3GFP-ser-hIGF-ie-neo, which contains both the h*IGF*-I gene under the control of the *B. mori* ser-1 promoter and the *neo* gene driven by the immediate early-stage gene (*ie*-1) promoter of the *B. mori* nucleopolyhedrovirus, the h*IGF*-I expression cassette excised from the plasmid pSK-ser-hIGF1-polyA was ligated to plasmid pigA3GFP-fib-L-intron 1, which was digested with KpnI and EcoRI, to generate plasmid pigA3GFP-ser-hIGF-fib-L-intron 1. The *ie*-1-promoter-driven *neo* gene was isolated from the plasmid pIE-Neo (Zhou et al., 2007) by digestion with EcoRI, and cloned into the EcoRI site of the plasmid pigA3GFP-ser-hIGF-fib-L-intron 1 to form pigA3GFP-ser-hIGF-ie-neo (Fig. 1B).

To identify the plasmid pigA3GFP-ser-hIGF-ie-neo, the primer pairs Ser-1/Ser-2, hIGF-1-1/hIGF-1-2, TPFib-L-3 (5′-GGCTCGAGC-