RESEARCH COMMUNICATION

Gene targeting in the silkworm by use of a baculovirus

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The *Bombyx mori* fibroin light (L)-chain gene was cloned and the green fluorescent protein (GFP) gene inserted into exon 7. The chimeric *L-chain-GFP* gene was used to replace the *polyhedrin* gene of *Autographa californica* nucleopolyhedrovirus (AcNPV). This recombinant virus was used to target the *L-chain-GFP* gene to the *L-chain* region of the silkworm genome. Female moths were infected with the recombinant virus and then mated with normal male moths. Genomic DNA from their progenies was screened for the desired targeting event. This analysis showed that the chimeric gene had integrated into the *L-chain* gene on the genome by homologous recombination and was stably transmitted through generations. The chimeric gene was expressed in the posterior silk gland, and the gene product was spun into the cocoon layer.

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Lepidopteran insects and their cells are very useful as hosts for the production of heterologous proteins by baculovirus expression vectors (Luckow and Summers 1988; Miller 1988). However, gene expression is transient, because the infected cells ultimately die from virus infection and the *polyhedrin* promoter is expressed only during the very late phase of infection. For this reason, the *IE1* promoter from NPV (Jarvis et al. 1990; Jarvis 1993) or a densovirus promoter (Giraud et al. 1992) has been utilized for stable transformation of lepidopteran cells and constitutive expression of foreign genes. Attempts to develop similar transient expression systems in silkworms have failed, because plasmid DNAs injected into silkworm eggs were rapidly degraded (Nagaraju et al. 1996).

Although AcNPV can replicate in the silkworm, *Bombyx mori*, the AcNPV-infected larvae survive and grow without any symptoms of nuclear polyhedrosis (Mori et

al. 1995). Previously, we reported that AcNPV could be utilized as a vector for the transovarian transmission of foreign genes in the silkworm (Mori et al. 1995). The luciferase gene was introduced into the AcNPV genome, female fifth instar larvae were inoculated with the recombinant virus, and luciferase activity was detected in the larvae of subsequent generations, indicating the *luciferase* gene had been vertically transmitted. Densoviruses are also considered to be candidate transfer vectors for transgenesis of lepidopteran insect cells and stable expression of foreign genes (Giraud et al. 1992). On the other hand, transgenesis of *Drosophila melanogaster* is routinely accomplished by use of the *P*-element transposon and this has facilitated the analysis of developmental regulation of gene expression (Rubin and Spradling 1982). Recently, germ-line transformation of dipteran insects has been established for the purpose of genetically controlling the Mediterranean fruit fly (Handler et al. 1998; Jasinskiene et al. 1998), and mosquitoes have been genetically transformed with insect transposable elements *Hermes* and *Mariner* to control diseases such as yellow fever and malaria (Zwiebel et al. 1995; Coates et al. 1998).

In this report we examine the transgenesis by homologous recombination to carry out gene targeting and ferry transgene into the silkworm *B. mori*.

Results and Discussion

Because fibroin, which consists of heavy (H) and light (L) chains, is one major protein of silk and the most abundant protein produced by the silkworm, the *fibroin* promoter would be a useful promoter for the production of large quantities of heterologous proteins. We constructed a vector for the purpose of gene targeting in the silkworm, *B. mori*. As shown in Figure 1A, the 5-kb upstream (long arm) and 0.5-kb downstream (short arm) sequence of exon 7 of the *L-chain* gene (Kikuchi et al. 1992) were cloned, and then a green fluorescent protein (GFP) gene (Chalfie et al. 1994) was inserted between the long- and short-arm sequences. The chimeric *L-chain-GFP* gene was transferred into the *polyhedrin* region of AcNPV, and the recombinant virus was used as a targeting vector. Homologous recombination was expected to occur between the long- and short-arm sequences common to both the chimeric *L-chain* gene in the virus and the *L-chain* gene in the *B. mori* genome. The chimeric gene should then be expressed as an *L-chain-GFP* fusion protein under the control of the *L-chain* promoter. The ORF of the chimeric gene was terminated by the stop codon (TAG) of the *GFP* gene (Fig. 1B). One-day-old fifth instar female larvae (F₀) were injected with 50 μ l containing 5×10^5 plaque forming units (PFU) of the recombinant virus by percutaneous inoculation. Approximately 50 animals were inoculated in each experiment (Table 1). Although normal larval-pupal ecdysis was observed, pupal-adult metamorphosis was arrested. Metamorphosis, however, resumed after administration of ec-

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dysteroid hormone (Mori et al. 1995). The average durations of the larval and pupal stages in the uninfected animals were 10 and 15 days, respectively, but the duration of the pupal stage of infected animals was a few days longer than in the uninfected animals. This phenomenon suggested that the ecdysteroid UDP-glucosyltransferase (*EGT*) gene of AcNPV was expressed, and the secreted EGT altered growth of the infected animals (Shikata et al. 1998). After infection with the recombinant baculovirus, no progeny virus was detected in hemo-

lymph at 5, 10, or 15 days postinoculation (p.i.), but 2.3×10^2 and 5.4×10^5 PFU/ml of progeny virus were detected in hemolymph at 20 and 25 days p.i., respectively. The progeny virus was propagated and the viral DNA was extracted. Restriction endonuclease analysis of the viral DNA indicated that the progeny virus was derived from the injected recombinant virus and not from the activation of a latent virus present in the silkworm (data not shown).

An outline of the screening method for gene targeting

Table 1. Frequency of gene targeting in the silkworm

Experiment	No. of inoculated F_0 larvae ^a	No. of F_0 larvae yielding GFP-positive embryos ^b (% positive)	No. of F_1 siblings containing <i>GFP</i> gene/no. tested (% positive) ^c	No. of F_2 larvae with targeted recombination ^{d,e}	
				male	female
1	51	3 (5.9)	2/125 (1.6) 4/141 (2.8) 4/119 (3.4)	0 1 1	0 0 0
2	47	1 (2.1)	0/149	N.D.	N.D.
3	46	1 (2.2)	10/138 (7.2)	2	1
4	53	2 (3.8)	1/110 (0.9) 4/108 (3.7)	0 0	0 1
5	55	1 (1.8)	4/156 (7.1)	1	0
Total	252	8 (3.2)	29/1046 (2.7)	5	2

^aFemale fifth instar larvae were inoculated with recombinant AcNPV and mated with normal moths.
^bGenomic DNA pools were prepared from 100 embryos from each inoculated F_0 larvae and tested for the presence of GFP by PCR with primers 5 and 6.
^cApproximately 100–150 siblings from each F_0 that produced PCR-positive embryos were reared, and individuals were tested for the presence of the *GFP* gene by PCR with primers 5 and 6.
^dGenomic DNA from F_2 larvae was analyzed by PCR using primers 7 and 8, which detect a novel DNA junction between GFP and chromosomal DNA.
^e(N.D.) Not determined.

is shown in Figure 2, and the frequency of gene targeting is summarized in Table 1. Virus-inoculated female moths were mated with a normal male moth (Fig. 2). The genomic DNA was extracted from 100 embryos and used as the template for PCR with primers 5 and 6 to screen for the presence of the *GFP* gene. Approximately 3% of the inoculated F_0 gave rise to F_1 embryos that were positive by PCR and dot blot hybridization (Table 1). The larvae from the remaining PCR-positive siblings were reared, whereas the PCR-negative siblings were discarded. About 100–150 F_1 individuals were collected, and hemocytes of each fifth instar larva were harvested from each individual separately. Genomic DNA was extracted from the hemocytes and screened by PCR analysis with primers 5 and 6. Overall, ~2.7% of all the F_1 individuals tested were positive for the *GFP* gene (Table 1). Male and female moths containing this gene were mated with normal moths, and their progenies (F_2) were reared. About 150 offspring were produced from each cross and hemocytes were harvested separately from each larvae in fifth instar. Genomic DNA from the hemocytes was assayed for gene targeting by a PCR screening method that specifically detects a novel DNA junction created by the targeting event. Only homologous recombination can juxtapose the two sequences that cre-

ate this junction, one from the *GFP* gene in the targeting vector, and the other within the downstream sequence beyond the end of the targeting vector (Fig. 1A). As shown in Figure 3A, the PCR reaction with genomic DNA (lane 3) and primers 7 and 8 amplified a 0.9-kb DNA fragment, showing that the targeting vector correctly recombined with the genomic DNA. The same results were obtained with the six other F_2 s. Nucleotide sequence analysis of the 0.9-kb PCR product from all F_2 animals indicated that one of homologous recombination occurred at the 3' end of the transgene in the targeting vector (Fig. 1B). After inoculation of 252 animals of the fifth instar larvae (F_0) with the recombinant AcNPV, the gene targeting event was ultimately detected in seven individuals that derived from different F_1 parents and, thus represent the distinct targeting events that occurred at a frequency of ~0.16% (Table 1).

To confirm further that the targeting event occurred by homologous recombination, Southern blot analysis was performed. Genomic DNA was digested with *AccI* and *ScaI*, and Southern blot hybridization was performed with the *GFP* gene or a portion of the *L-chain* gene as a probe (Fig. 3B). Two bands with the expected sizes were detected when the *GFP* probe was hybridized to the genomes from the seven animals from which the 0.9-kb PCR product had been amplified (Fig. 3C). With the *L-chain* gene as a probe, a single band was detected in the genome from control larvae and two additional fragments were detected in the seven larvae that scored positive for the 0.9-kb PCR product (Fig. 3C). This result showed that the chimeric gene had been introduced into the *L-chain* gene of the transgenic silkworms by a gene-targeting event and that one copy was present per haploid genome of each individual.

When the silk gland was dissected and exposed to long wavelength UV light, emission of green fluorescence was observed from that of the *GFP* gene-targeted silkworm larvae and no green fluorescence was observed from control larvae (Fig. 3D). The green fluorescence was also observed from the larval intersegmental membrane, because the intersegmental membrane forms a flexible joint and is translucent. Two subunits of fibroin (H and L chains) are synthesized in the posterior silk gland, and the organ is located in the posterior abdomen. Therefore, we concluded that the chimeric *L-chain-GFP* gene was probably expressed in the posterior silk gland. SDS-PAGE analysis revealed a new protein with a molecular mass of 52 kD and decreased amounts of L chain (25 kD) in the silk protein of an F_2 animal as compared with a normal animal (Fig. 3E). The molecular mass of the new protein correlated to with that of the chimeric protein consisting of L chain (25 kD) and GFP (27 kD). Furthermore, the new protein band reacted with an antibody against GFP (Fig. 3E), indicating that the silk protein prepared from cocoons of targeted F_2 animals was composed of the chimeric *L-chain-GFP* gene. Cocoons produced by *GFP* gene-targeted silkworms did not appear any different from normal cocoons and green fluorescence was not observed under the irradiation of long wavelength UV light. It is necessary to study the physi-

Figure 2. Scheme of the screening method for analysis of gene targeting.

Figure 3. Screening for gene targeting. (A) Screening by PCR analysis. Primer 7 (5'-ACTACAAGACACGT-GCTG-3') in the *GFP* gene and primer 8 (5'-AGCATGACAACAG-TACCG-3') in the intron of downstream of exon 7 were synthesized to screen silkworms for homologous recombination events by PCR. As the normal *L-chain* gene contained no binding sites for primer 7 and the targeting vector contained no binding sites for primer 8, only the homologous recombinant allele could contain both priming sites. The targeting event was demonstrated by amplification of the 0.9-kb PCR product. (Lane 1) Negative control containing water instead of template DNA; (lane 2,3) genomic DNA from hemocytes of a control larva and a tested larva, respectively. The size (kb) of the DNA markers is shown at left. (B) Gene map of the periphery of exons 6 and 7. The *AccI* and *ScaI* sites are indicated in the *L-chain* gene (top) and the chimeric gene (bottom). The lengths of restriction enzyme fragments and each probe are also shown. (C) Southern blot analysis of silkworm genomic DNAs. Genomic DNA was digested with *AccI* and *ScaI* and analyzed by Southern blot hybridization with probes for the *GFP* gene (left) or the *L-chain* (exon 7) gene (right). (Lane 1) Genomic DNA from a control larva; (lanes 2–8) genomic DNA from larvae in which the 0.9-kb PCR product was amplified. (D) Green fluorescence under the irradiation of long wavelength UV light. Silk glands were dissected (left) and exposed to long wavelength ultraviolet light (right). Silk glands from the control animal and the *GFP* gene-targeted animal are shown as control and GFP, respectively. (E) SDS-PAGE (left) and Western blot analysis (right) of silk protein. (Lane 1) Normal cocoon layer; (lane 2) cocoon layer from the *GFP* gene-targeted animal. Antibody used in the Western blot analysis was specific for GFP. The sizes (kD) of protein markers is shown at left.

ochemical properties of the cocoons and the raw silk. These findings indicated that the chimeric protein was synthesized under the control of the *L-chain* promoter in the posterior silk gland and secreted into the cocoon layer.

When male and female F_2 animals were mated with normal animals and segregation of the *GFP* gene in the F_3 generation was analyzed by dot blot hybridization, about one-half of the individuals contained this gene (Table 2). When the *GFP* gene-targeted F_3 animals were crossed with normal animals and the *GFP* gene-targeted animals, about one-half and three-fourths of the individuals scored positive for the *GFP* gene, respectively (Table 2). These results indicate that the chimeric gene was entirely localized to a single chromosome in the F_2 generation of the silkworm and that a germ-line integration event had occurred at the F_1 stage. Although the genomic DNA from individuals of each generation was used as a template for detection of AcNPV DNA, the viral DNA was detected in only the F_0 and F_1 generations.

P-element transgenesis of *D. melanogaster* or of other insects with transposons is dependent on the random integration of a foreign gene (O'Brochta and Atkinson 1996). Recombinant baculovirus-mediated transgenesis of the silkworm allowed specific alterations in a target

sequence. Targeted disruption of an endogenous gene would permit analysis of gene function through the pro-

Table 2. Segregation patterns of the *GFP* gene in the F_3 and F_4 generation

Mating	No. of F_3 or F_4 animals tested ^a	No. of GFP-positive larvae (% tested)
F_3		
Control (♀) × F_2 (♂) from exp. 1) ^b	92	43 (46.7)
Control (♀) × F_2 (♂) from exp. 5) ^b	95	49 (51.6)
F_2 (♀ from exp. 3) × control (♂) ^b	96	46 (47.9)
F_2 (♀ from exp. 4) × control (♂) ^b	90	44 (48.9)
F_4		
F_3 (♀) × control (♂) ^c	91	42 (46.2)
Control (♀) × F_3 (♂) ^c	98	50 (51.0)
F_3 (♀) × F_3 (♂) ^c	96	70 (72.9)

^aGenomic DNA from hemocytes of each 5th instar larva (F_3) was extracted, and the *GFP* gene was analyzed by PCR amplification.

^b F_2 male and female animals were derived from each experiment (Table 1).

^c*GFP* gene-targeted F_3 male and female animals were obtained by the cross of F_2 generation.

duction of gene knockouts in the silkworm. In addition, homologous recombination of a foreign gene downstream from a powerful promoter, such as the *fibroin* promoter, would allow large-scale and constitutive production of a useful protein in the silkworm (Marshall 1998). Thus, the genetic manipulations described in this study will be important in clarifying the regulation of gene expression and introducing new traits into beneficial insects.

Materials and methods

Construction of targeting vector and PCR amplification

PCR primers were synthesized for the 5.0-kb long-arm and the 0.5-kb short-arm DNA fragments according to the published sequence data for the *L-chain* gene (Kikuchi et al. 1992). The genomic DNA of fifth instar larvae was extracted from hemocytes by use of a DNA extraction kit (Stratagene). The primers synthesized for PCR amplification of long-arm DNA fragment were: primer 1 as a forward primer (5'-AATTAGCTCTAGATGAGCTCCCGGCGTACC-3') and primer 2 as a reverse primer (5'-CAACTAAGGGATCCGCGTCATTACCGTTGC-3'). In the reverse primer, the AAGCCGGTTCGCG sequence included in the exon 7 of *L-chain* gene was converted into AAGGGATCCGCG, resulting in a creation of a *Bam*HI site. PCR amplification was carried out with the LA PCR kit (TaKaRa) with 0.4 mM of each primer and 200 ng of genomic DNA as template. After heating the reaction mixture (50 μ l) in MicroAmp reaction tubes (PE Applied Biosystems) at 94°C for 1 min in a Gene AmpPCR System 2400 (PE Applied Biosystems), amplification was carried out for 30 cycles of denaturation (20 sec at 98°C) and annealing extension (15 min at 68°C). A final 10-min step at 72°C was performed at the completion of these cycles. The long arm was excised with *Xba*I and *Bam*HI, and the fragment was inserted into baculovirus transfer vector pVL1392 (PharMingen) at the *Xba*I and *Bam*HI sites to obtain pVLFL-5K. Primer 3 (5'-TACCCACTGTCCAATCCACCG-3') and primer 4 (5'-CCGGCTTAGTTGCTAATGCTC-3') were synthesized for amplification of the short-arm DNA fragment. Primer 5 (5'-GGAGAAGAACTTTTCAC-TGGAG-3') and primer 6 (5'-ATCCATGCCATGTGTAATCCC-3') were used to screen for the *GFP* gene. Primer 7 (5'-ACTACAAGACACGT-GCTG-3') in the *GFP* gene and primer 8 (5'-AGCATGACAACAG-TACCG-3') in the intron downstream of exon 7 were synthesized for screening the targeting event. These PCR amplifications were carried out in a reaction mixture (50 μ l) containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 200 mM dNTP, 2 mM MgCl₂, 0.4 mM of each primer, 2.5 units of *rTaq* DNA polymerase (TOYOBO), and 200 ng of the genomic DNA as template. MicroAmp reaction tubes were used with AmpliWax PCR Gems (PE Applied Biosystems) for an auto-hot start. After heating the reaction mixture at 80°C for 5 min and cooling to 5°C for 30 sec in a Gene AmpPCR System 2400 (PE Applied Biosystems), amplification was carried out for 35 cycles of denaturation (1 min at 94°C), annealing (1 min at 55°C) and extension (2 min at 72°C). A final 7-min step at 72°C was performed at the completion of these cycles. PCR products were cloned into pCR2.1 vector (Invitrogen). The short arm was excised with *Eco*RI and inserted into the pGFPcDNA vector (Clontech) at the dephosphorylated *Eco*RI site to construct pGFP-0.5K. *Bam*HI and *Stu*I fragments were separated from pGFP-0.5K and ligated with an *Bam*HI adaptor at the *Stu*I site. The resulting 1.2-kb *Bam*HI fragment composed of the *GFP* gene and the short-arm DNA fragment was inserted into pVLFL-5K at the dephosphorylated *Bam*HI site, and the recombinant baculovirus transfer vector, pAcFLGFP, was constructed. *Spodoptera flugiperda* IPLB-SF21AE cells were transfected with 5 μ g of the recombinant transfer vector and 0.5 μ g of linearized AcNPV DNA. Baculogold Baculovirus DNA (PharMingen). A recombinant AcNPV (targeting vector) rescued by the transfer vector was isolated and plaque purified.

Dot blot hybridization

Following PCR amplification, 10 μ l of the PCR sample was immobilized on a nylon membrane (Amersham) with a vacuum manifold. *GFP* cDNA was excised from the pGFPcDNA vector and used as a probe for hybridization. Detection of PCR products was carried out with the ECL direct nucleic acid labeling and detection system (Amersham).

Analysis of nucleotide sequence

The nucleotide sequence of the intron-exon boundary of the *L-chain* gene was determined by the dideoxytermination method carried out with a 373A DNA Sequencing system (PE Applied Biosystems).

Southern blot analysis and Western blot analysis

The *GFP* gene was excised from the pGFPcDNA vector (CLONTECH) with *Bam*HI and *Eco*RI and used as a probe. A probe for the *L-chain* gene was prepared by PCR amplification. The primers were designed to amplify a part of the *L-chain* gene (Fig. 3B). Primer 9 (5'-TTTTTAATTATC-CACAGCTC-3') and primer 10 (5'-GTCTGTTTTATATAGGTAG-3') were synthesized. The PCR amplification was carried out in a reaction mixture (50 μ l) containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 200 mM dNTP, 2 mM MgCl₂, 0.4 mM of each primer, 2.5 units of *rTaq* DNA polymerase (Toyobo), and 200 ng of the genomic DNA as template. MicroAmp reaction tubes were used with AmpliWax PCR Gems (PE Applied Biosystems) for an auto-hot start. After heating the reaction mixture at 80°C for 5 min and cooling to 5°C for 30 sec in a Gene AmpPCR System 2400 (PE Applied Biosystems), amplification was carried out for 35 cycles of denaturation (1 min at 94°C), annealing (1 min at 55°C) and extension (2 min at 72°C). A final 7-min step at 72°C was performed at the completion of these cycles. Southern blot analysis was performed on silkworm genomes after digestion with *Acc*I and *Sac*I (Fig. 1A). Electrophoresed genomic DNA was transferred to Hybond-N membranes (Amersham), and hybridization was performed with a commercially available digoxigenin DNA labeling and detection kit (Boehringer Mannheim) according to the supplier's instructions. Western blot analysis for chimeric protein expression was performed on silk proteins from the cocoon layer. To dissolve the silk protein, 150 ml of 70% LiSCN was added to 5 mg of the cocoon layer, and 40 ml of 62.5 mM Tris-HCl buffer (pH 6.8) containing 2% SDS and 5% 2-mercaptoethanol was added to 10 ml of the dissolved silk protein. The silk protein (20 μ l) was then subjected to 12.5% SDS-PAGE, and the proteins were transferred to nitrocellulose. The nitrocellulose blot was placed in protein-blocking solution [20 mM Tris-HCl, 500 mM NaCl at pH 7.5 (TBS) containing 3% gelatin] for 1 hr, and the blots were treated overnight with rabbit anti-GFP antibody (CLONTECH). The blots were then treated with goat anti-rabbit IgG (H+L) horseradish peroxidase conjugate (Bio-Rad) at a 1:3000 dilution for 1 hr, and Konica Immunostaining HRP-1000 was used for signal detection.

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