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Transgenesis approaches for functional analysis of peptidergic cells in the silkworm *Bombyx mori*

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Abstract

The domestic silkworm, *Bombyx mori* represents an insect model of great scientific and economic importance. Besides the establishment of a stable germline transformation using the *PiggyBac* vector, technically feasible methods for *in vivo* gene delivery and transient gene expression were developed using viral based vectors, especially Sindbis viruses and baculoviruses. The recombinant baculovirus, *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV), commonly used for large-scale protein production in permissive cell lines or insects, has been used for foreign gene transfer into specific peptidergic cells of *B. mori in vivo*. Since targeted gene expression is essential for functional analysis of neuropeptide genes and their receptors, the baculovirus-mediated gene transfer can serve as a reliable approach in reverse genetic studies in the silkworm. We review various strategies employing the baculovirus vector system for transient expression of molecular markers and transcription factors in specific peptidergic cells to investigate their roles in *B. mori*. We also use this system for functional analysis of neuropeptide signaling in the ecdysis behavioral sequence. Our data indicate that the AcMNPV vector is suitable for efficient delivery of foreign genes and their expression directed into specific peptidergic neurons and endocrine cells of *B. mori* larvae and pupae. However, some modifications of the vector and steps for optimization are necessary to minimize negative effects of viral infection on the host development. The transient gene expression using the AcMNPV and other virus vectors are promising tools for analysis of molecular mechanisms underlying various neuroendocrine processes during development of *B. mori*.

Keywords

Bombyx mori; Transgenesis; Baculovirus; Sindbis virus; Neuropeptides; ETH

1. Introduction

The fruit fly *Drosophila melanogaster* has been frequently used for genetic studies targeted at molecular mechanisms controlling insect development. This species is an excellent model for elucidation of basic principles of animal development and physiology, due to the availability of elaborated molecular genetic techniques and relatively easy manipulation of genes based on P-element transposon (Rubin and Spradling, 1982). However, flies are highly derived and thus not ideal representatives of most invertebrates in many aspects of their life. Molecular genetic approaches on other relevant invertebrate species are thus necessary to complement the

findings in flies and to provide more comprehensive insights into generally conserved physiological and developmental processes.

The silkworm, *Bombyx mori* became a very important insect model in the fields of genetics, biochemistry and physiology. In the past few years, rapid progress has been made in applying molecular and genomic technologies to the silkworm. Availability of complete genome sequences (Mita et al., 2004; Xia et al., 2004), development of molecular linkage maps, EST databases (Mita et al., 2003; Cheng et al., 2004), DNA chips possessing ~6000 EST for gene expression analysis (Ote et al., 2004) and techniques for efficient gene transfer and RNAi technologies render this insect species an excellent non-drosophilid model system for solving a broad range of biological questions. Furthermore, large body size and obvious developmental markers in all post-embryonic stages makes the silkworm amenable to physiological and behavioral studies not feasible in the tiny fruit fly larvae, pupae or adults.

The gene transfer methods and other techniques of reverse genetics provide powerful tools for functional analysis of genes and their products, and for elucidation of molecular mechanisms underlying a wide variety of biological processes. The example of *D. melanogaster* shows how transgenesis can be extremely helpful in understanding roles of genes in the formation and function of a living organism. The successful transgenesis of an additional insect species, *B. mori*, opens new prospects in basic and applied research.

2. Transposon-based vectors

Transformation of *D. melanogaster* using the transposable P-element (Rubin and Spradling, 1982) raised hopes that this method will be available to other arthropods. However, P-element is strongly species-specific and non-functional outside of Drosophilidae (Handler et al., 1993). The subsequent search for new alternative transposable elements with more mobility properties was eventually successful. Using methodologies and techniques inspired by those in *Drosophila*, four transposable elements representing four different families of eukaryotic transposable elements have been identified for genetic transformation of non-drosophilid insects: *Minos* element isolated from *Drosophila hydei* (Franz and Savakis, 1991), the *Hermes* element from the house fly *Musca domestica* (Warren et al., 1994), the *Mos1* element from *Drosophila mauritiana* (Medhora et al., 1991) and the *PiggyBac* element, first detected in the baculovirus-infected cell culture of cabbage looper, *Trichoplusia ni* (Fraser et al., 1983). The lepidopteran-derived *PiggyBac* transposon appears to be the most promiscuous transposable element used for insect genetic transformation. The first successful transformation was accomplished in the medfly *Ceratitis capitata* (Handler et al., 1998) and notably, it was the first proof of transposon functionality in an insect order different from the original host. Shortly afterwards it was successfully used to transform a number of different insect species spanning three orders, including dipterans *Aedes aegypti* (Lobo et al., 1999), *D. melanogaster* (Handler and Harrell, 1999) and *M. domestica* (Peloquin et al., 2000), a coleopteran *Tribolium castaneum* (Berghammer et al., 1999) and lepidopterans *B. mori* (Tamura et al., 2000) and *Pectinophora gossypiella* (Peloquin et al., 2000). *PiggyBac*-based integration of exogenous DNA sequence into the recipient genome occurs through specific molecular mechanism for transposable element mobility and it is the same for all vector systems based on mobile genetic elements. All transposons contain terminal sequence motifs required for their identification and subsequent mobilization by a specific transposase encoded by the particular transposon itself. The transposon-mediated transformation requires separation of the transposase gene and the transposon backbone, rendering the transposon unable of autonomous mobility. The non-autonomous transposon bearing plasmid is injected into early pre-blastoderm insect embryos along with the helper plasmid providing transposase. The transposase supplied by the helper plasmid mediate excision of the transposon backbone from the second plasmid and its subsequent insertion into the host genome. Exogenous DNA

integrated into the genome of germ cells precursors is maintained and inherited in a lineage arising from particular germ cells.

PiggyBac is a vector of the first choice for genetic manipulation of non-drosophilid insects and it was the first efficient element for preparing stable transgenic lines of the silkworm *B. mori* (Tamura et al., 2000; Thomas et al., 2002). However, some limitations prevent establishment of silkworm transgenesis as a routine technique. These include a relatively long life cycle of *B. mori* and requirement of highly skilled microinjection technique for penetration of the hard egg shell. There are additional reasons why the germline transformation using transposon-based vectors systems is not feasible in most insect orders. Available transposons are often ineffective, most insect species have long or complicated life cycles, microinjection of eggs greatly affects survival of embryos, or screening and maintaining of transgenic lines is difficult. Therefore, alternative techniques are required for gene transfer and genetic manipulation of suitable insect models during their development.

One of the promising approaches to study gene functions includes the transient expression of foreign genes using virus vectors. In these cases of somatic transformation, the transgenes are not stably integrated into the host's genome and the successful gene transfer depends on the virus ability to infect target tissues of the permissive host. A simple injection of the virus construct into the host at any developmental stage provides excellent opportunities to introduce various genes or molecular markers into target cells or tissues and study functional outcomes of their expression. The virus systems are therefore relatively simple and effective tools for reporter assays, or physiological and behavioral analyses. Here, we summarize several gene transfer systems used for the silkworm transgenesis with emphasis on the baculoviruses as potential vectors for introducing genetic material into specific organs or cells.

3. The Sindbis virus

The alphavirus Sindbis is currently used as the highly efficient transducing agent in insect biology. Sindbis viruses (SINVs) are single stranded enveloped RNA viruses belonging to the family *Togaviridae* (Strauss and Strauss, 1994) that are naturally transmitted to a variety of hosts by mosquitoes and have no known pathological effects on the host insect. SINV has been engineered into a convenient system that can be used for transient gene expression in vertebrate and invertebrate organisms (Olson et al., 1998; Frolov et al., 1999; Pierro et al., 2003). With minimal cytotoxicity (Olson et al., 1998; Pierro et al., 2003), the virus can infect a wide range of insect species (Xiong et al., 1989; Strauss and Strauss, 1994) and it replicates in a variety of insect tissues, including the hemolymph, eyes, midgut, fat body, muscles, CNS and salivary glands (Olson et al., 2000, 2002; Pierro et al., 2003). The recombinant SINV has been successfully used for ectopic gene expression in various insects including mosquitoes (Olson et al., 1994; Higgs et al., 1995, 1996), a beetle *T. castaneum* (Lewis et al., 1999), a butterfly *Precis coenia* (Lewis et al., 1999), and the silkworm *B. mori* (Uhlřřová et al., 2003; Foy et al., 2004).

Due to double-stranded RNAs production during virus replication, SINV infection offers a tool to knock-down expression of specific genes in infected cells by RNA interference (RNAi). Successful RNAi gene silencing mediated by recombinant SINV has been reported in several mosquito species (Johnson et al., 1999; Adelman et al., 2001; Shiao et al., 2001; Attardo et al., 2003; Sanchez-Vargas et al., 2004) and in the silkworm *B. mori* (Uhlřřová et al., 2003). Suppression of the transcription factor Broad-Complex (BR-C) that is required for normal metamorphosis, reduced endogenous *BmBRC* mRNA levels in infected tissues and induced abnormalities corresponding to loss-of-function phenotypes previously observed in *BR-C Drosophila* mutants (DiBello et al., 1991; Uhlřřová et al., 2003).

However, Sindbis virus-mediated ectopic gene expression/RNAi silencing may be limited by restricted tissue tropism of viral infection. For instance, some *B. mori* organs such as gonads, Malpighian tubules and larval epidermis resist SINV infection (Uhlířová et al., 2003).

Drawbacks of SINV-based vectors also include instability of recombinant clones after multiple passages in cultured cells (Foy et al., 2004). Finally, the virus infects mammalian cells and thus SINV infection may be harmful to humans.

4. The baculoviruses

The baculoviruses are a large family of enveloped viruses with circular double-stranded DNA and genome size between 80 and 180 kbp. Baculovirus infectivity is restricted to arthropods, usually with a very limited species-specific tropism characteristic for individual members of *Baculoviridae*. Majority of baculoviruses are pathogenic to insects of the orders *Lepidoptera*, but also infect certain members of *Diptera* and *Hymenoptera* (Blissard et al., 2000). The family *Baculoviridae* is divided taxonomically into two genera: Granulovirus and Nucleopolyhedrovirus. Based on number of nucleocapsids per enveloped virion, the nucleopolyhedroviruses (NPVs) are further subdivided into two morphological groups: the single NPVs (SNPVs) and the multiple NPVs (MNPVs), which are more numerous (Vail et al., 1999; Volkman, 1997).

Autographa californica multiple nucleopolyhedrovirus (AcMNPV), originally isolated from the alfalfa looper *T. ni* (Vail et al., 1971), is the most extensively studied and best characterized NPV. Its genome has been completely sequenced (Ayres et al., 1994). It contains a circular double-stranded supercoiled DNA with a size of approximately 134 kbp packed in a rod-shaped capsid (Summers and Anderson, 1972). Like the other baculoviruses, AcMNPV has a unique, biphasic life cycle characterized by the production of two morphologically distinct forms at different times post-infection. Early after entry into host cell, the viral transcription and DNA replication occur in the nucleus of the cell and nucleocapsids are assembled. The budding of progeny particles through plasma membranes of infected cells result in the release of budded viruses (BV) and their systematic dissemination within all tissues of the host. In the late stages of infection, progeny virions become embedded within a proteinaceous matrix predominantly composed of a crystalline protein called polyhedrin (*polh*) which is produced by the powerful transcriptional activity of the *polh* promoter. Large quantities of the polyhedral inclusion bodies (also called polyhedra or occlusion bodies, OB) accumulate within the nucleus and are released into the body fluids after cell lysis. The ODV ultimately enter environment after disintegration of the dead host larva. In the occluded form, baculovirus particles are protected against exogenous factors and can persist in the environment scattered on the vegetation for a long time (Mazzone, 1985; Vlak and Rohrmann, 1985). Following ingestion of virion-containing polyhedra by susceptible insect species, polyhedron matrix is dissolved in alkaline environment of the midgut, embedded virus particles are released and infect epithelium cells to start a new life cycle. Polyhedral bodies thus allow horizontal spread of the virus among host individuals in the insect population (Fig. 1).

5. Baculoviruses as expression vectors

Certain baculoviruses have become important expression vectors for heterologous genes in insect cell lines and in the host larvae (Luckow and Summers, 1988; Fraser, 1992; King and Possee, 1992). However, the recombinant baculovirus construction is practically restricted to the AcMNPV and to some extent to the related *B. mori* single nucleopolyhedrovirus (BmSNPV) (Kost et al., 2005). Baculovirus-mediated gene expression has several advantages over other systems including the high expression level of foreign genes induced by strong promoters for the viral late genes, polyhedrin (*polh*) and *p10*. Due to dispensability of *polh* and *p10* for virus replication and propagation in insect cells, they can be replaced by foreign genes

and expressed in these loci. In addition, foreign proteins produced in eukaryotic milieu of insect cells often show posttranslational processing, modifications and/or multimeric assembly identical or similar to their natural homologs in the native organism (Luckow, 1991, 1993; O'Reilly et al., 1992a,b). Comparing to protein production in bacteria and yeast, the baculovirus system allows a large-scale production of authentic and biologically active eukaryotic proteins. The baculovirus capsid structure, as well as the large size and flexibility of the viral genome allow packing and expression of multiple smaller genes or very large single genes (French et al., 1990; Brown et al., 1991). Due to a narrow host range often restricted to specific insect species, baculoviruses are essentially nonpathogenic to other invertebrates, vertebrates and plants and are safe for the laboratory use.

Since, the baculovirus-insect cell expression system was introduced (Smith et al., 1983; Pennock et al., 1984), it has become widely utilized for routine recombinant protein production in diagnostics, therapeutics or structural and functional studies (Kost et al., 2005). This system have also been used for the baculovirus surface display strategies based on expression of foreign peptides and proteins on the surface of virus particles, which could serve as immunogens for stimulation of mammalian immune system (Lindley et al., 2000). The discovery that baculoviruses can enter some mammalian cell lines (Hofmann et al., 1995; Boyce and Bucher, 1996) further broadened their application and appropriately modified recombinant baculoviruses are now considered as attractive vectors for gene delivery into mammalian cells (Kost and Condreay, 2002). Environmental applications include utilization of several species-specific baculoviruses for biological control of various insect pests (Inceoglu et al., 2001).

6. The recombinant baculoviruses

Foreign genes were originally inserted into the baculovirus genome by highly inefficient homologous recombination (Smith et al., 1983). The target gene was first inserted into a transfer vector (plasmid) containing suitable virus promoter flanked by the baculovirus DNA sequence derived from the non-essential locus, such as *polh* gene. Insect cells cotransfected with the transfer plasmid and baculovirus genomic DNA produced a mixture of parental and recombinant viruses with frequency of about 0.1–1%. The visual identification of recombinants was very difficult, since it was based on altered morphology of occlusion-negative baculovirus plaques compared to strong background of wild-type parental plaques (Summers and Smith, 1987). Improvement of the recombinant efficiency came with linearization of the parent AcMNPV baculovirus genome at the unique (Bsu36I) restriction site located in the *polh* locus that increased the percentage of obtained recombinant viruses to approximately 30% (Kitts et al., 1990). To obtain an even higher proportion of recombinants (80% or more), multiple Bsu36I sites were introduced into the baculovirus DNA (Kitts and Possee, 1993). Bsu36I digestion resulted in removing of an essential viral gene (ORF 1629) downstream of the *polh* gene. The homologous recombination with the transfer plasmid carrying the ORF 1629 and a gene of interest restored this essential locus in majority of the viable progeny. However, this approach still takes more than a month to purify plaques, amplify the virus, and confirm the desired recombinants.

Efforts to eliminate the plaque assay led to development of the *in vitro* bacterial transposition method (Luckow et al., 1993; Anderson et al., 1996), which became commercially available as the Bac-to-Bac[®] system (Invitrogen, Carlsbad, CA, USA). This technique employs the bacterial transposon Tn7 for site-specific transposition of foreign genes from the donor plasmid into the AcMNPV baculovirus DNA in *Escherichia coli*. The gist of the method is construction of the recombinant plasmid containing target gene(s) flanked with inverted terminal repetitions of Tn7 elements (Tn7L and Tn7R). The recombinant donor plasmid is subsequently transformed into *E. coli* cells containing the bacmid (baculovirus shuttle vector) with the Tn7 target site (mini-*att*Tn7). Site-specific transposition of the mini-Tn7 element from the donor

plasmid to the mini-*att*Tn7 target site on the bacmid DNA is mediated by transposition proteins provided by helper plasmid and results in integration of the target gene into the viral genomic DNA. The *E. coli* colonies containing recombinant bacmids are selected and easily identified by antibiotic resistance and by color, since the transposition results in disruption of the *lacZ* gene. The recombinant baculovirus DNA (bacmid) is then used to transfect insect cells and produce the recombinant virus suitable for various applications (Fig. 2). This method eliminates multiple rounds of purification and amplification of the virus and reduces time for selection of recombinant viruses. Since the inserted gene of interest is under transcriptional control of the strong viral *polh* promoter, it is expressed in high levels instead of the polyhedrin protein (Kost et al., 2005; Motohashi et al., 2005). The budded form of the AcMNPV lacking the polyhedrin bodies cannot infect permissive larvae by oral ingestion, so it is safe to use recombinant baculoviruses in the laboratory. Although the parallel bacmid system using BmSNPV has been developed (Motohashi et al., 2005), it is not commercially available.

7. Baculovirus-mediated gene transfer in *B. mori*

As mentioned above, AcMNPV is the most commonly used baculovirus for targeted expression of foreign genes. In practical applications, cells or larvae of AcMNPV parental organisms *Spodoptera frugiperda* and *T. ni* are often used as hosts for the recombinant virus. The silkworm *B. mori* is a native host for BmSNPV and was previously recognized as non-permissive to AcMNPV infection (Rahman and Gopinathan, 2004; Shikata et al., 1998), although the viruses share over 90% genome identity (Gomi et al., 1999). The first successful gene introduction in *B. mori* was accomplished by the recombinant BmSNPV-mediated *in vivo* expression of chorion genes controlled by their own regulatory elements. Infection of the silkworm pupae by the recombinant virus caused transient and tissue specific expression of chorion (Iatrou and Meidinger, 1990).

The recombinant BmSNPV containing several promoters for prothoracitropic hormone (PTTH) and bombyxin were used for targeted expression of the enhanced green fluorescent protein (*egfp*) in the brain of silkworm larvae. Strong EGFP expression was detected in the brain neurosecretory cells producing these neuropeptides (Moto et al., 2003). These data indicate that recombinant baculoviruses can serve as an efficient transduction system permitting cell-specific expression of transferred genetic material in *B. mori*. Surprisingly, the recombinant AcMNPVs carrying the same neuropeptide-EGFP constructs were ineffective in transient gene expression of *B. mori* (Moto et al., 2003).

However, more recent studies revealed 17 different silkworm strains permissive to AcMNPV infection (Guo et al., 2005a,b). The genetic basis underlying differences in a susceptibility of various silkworm strains to this virus is still unknown. Genetic cross experiments indicated the presence of a single dominant gene (or a set of genetically linked genes) that may be responsible for the resistance of non-permissive silkworms to the AcMNPV infection. However, these antiviral genes have not been identified and molecular mechanisms suppressing the viral infection remain enigmatic (Guo et al., 2005b).

The additional polyvoltinne N4 strain was found permissive to AcMNPV infection and used for specific expression of *egfp* gene under three neuropeptide promoters for PTTH, bombyxin and diapause hormone-pheromone biosynthesis activating hormone (Shiomi et al., 2003). Following infection of larvae and pupae with recombinant AcMNPV bearing *egfp* reporter and neuropeptide promoters, the green fluorescence was detected in a small number of neurons in the CNS naturally producing these particular neuropeptides (Figs. 3 and 4A) (Shiomi et al., 2003). The baculovirus expression system was also used to determine role of the myocyte enhancer factor 2 in transcription of the PTTH gene (Shiomi et al., 2005). This technology is potentially useful for examination of cell-specific gene expression patterns when the

neuropeptide or mRNA levels are relatively low. These data indicate that AcMNPV-mediated gene transfer system can be used for various *in vivo* studies including identification of *cis*-regulating elements, detection and visualization of changes in gene expression during development, and functional analysis of genes and their products.

Disadvantages of these *in vivo* applications include pathogenic effects of the AcMNPV on its permissive host which may affect functions of introduced gene(s) in later stages of infection. Severe developmental defects are caused by the baculovirus ecdysteroid UDP-glucosyltransferase (EGT) which inactivates ecdysteroids in the host hemolymph, alters larval development and suppresses metamorphosis depending on sensitivity of different *B. mori* strains (O'Reilly, 1995). Larvae or pupae of sensitive strains usually die 1–2 weeks after AcMNPV injection. These alterations of larval development were prevented by disruption of the non-essential early gene encoding ETG (Shikata et al., 1998; Guo et al., 2005a). Another alternative is to use semi-permissive strains (e.g. Kosetsu), which in many cases develop normally and show obvious expression of the reporter EGFP gene in target cells (Daubnerová and Žitňan, unpublished).

We have been using the convenient Bac-to-Bac[®] baculovirus expression system (Invitrogen) for gene introduction and transient genetic manipulation of permissive silkworm strains to elucidate questions regarding neuropeptide signaling necessary for normal development and behaviors. Particularly, we investigate signaling mechanisms between endocrine Inka cells and different peptidergic neurons in the CNS of *B. mori*. Inka cells produce ecdysis triggering hormones (ETH) which act on their receptors in the CNS to orchestrate the ecdysis behavioral sequence (Žitňan et al., 2007). ETH receptors (ETHR) have been identified and characterized in *D. melanogaster* and *Manduca sexta* (Iversen et al., 2002; Park et al., 2003; Kim et al., 2006a). Further studies showed that two subtypes of ETH receptors (ETHR-A and ETHR-B) are differentially expressed in specific peptidergic neurons of the CNS. Upon ETH action, these receptor neurons subsequently release multiple neuropeptides which control different phases of the ecdysis sequence (Kim et al., 2006a,b). The Bac-to-Bac[®] system provides a convenient and promising tool to investigate mechanisms of expression, release and function of different cells and bioactive molecules in the complex cascade regulating the insect ecdysis sequence. The permissive N4 strain and semi-permissive Kosetsu strain, or their hybrids are ideal for various experiments focused on neuroendocrine events during the ecdysis sequence. So far, we successfully achieved transient EGFP expression in Inka cells using the promoter for ETH (Fig. 4B), while the upstream regulatory sequences for various neuropeptide genes were used for EGFP expression in specific CNS neurons (Daubnerová, Roller, Žitňan, unpublished). These data indicate that the Bac-to-Bac[®] system is suitable for targeted expression of various molecular markers, receptors and regulatory factors to study the ETH-driven peptide signaling cascade in *B. mori*. Examples include cell-specific gene silencing by RNAi, cell ablation using apoptosis-promoting genes (e.g., *reaper*, *grim*, *hid*), expression of calcium indicator GCaMP for monitoring activity of individual cells, and expression of receptors or transcription factors for activation or inhibition of neurons by appropriate ligands. The *B. mori* is therefore an attractive model organism for analysis of complex neuroendocrine interactions at cellular and molecular levels.

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Fig. 1.

Bi-phasic replication cycle of the AcMNPV. Occlusion derived virions (ODV) released from polyhedra in the alkaline environment of the insect midgut fuse with midgut epithelial cells to trigger primary infection. The viral transcription and replication is initiated upon entering the nucleus. Budding nucleocapsids obtain their final envelope from the host cell membrane enriched in viral glycoproteins. Budded viruses (BV) subsequently start new cycle of infection by entering next susceptible cells through an endocytotic process and spread infection throughout the larva. During the later stages of infection, progeny virus particles become embedded within the matrix of occlusion body protein (polyhedrin). Large numbers of polyhedra or occlusion bodies (OB) accumulate in the host during infection and enter

environment upon cell lysis and larval body disintegration to further spread virus infection to surrounding hosts.

Fig. 2.

Generation of the recombinant baculovirus using the Bac-to-Bac[®] expression system. The recombinant donor vector with a gene of interest under control of the *polh* promoter is transformed into DH10Bac[™] competent *E. coli* cells containing a parent bacmid as well as helper plasmid providing transposition enzymes. Integration of the expression cassette in the baculovirus DNA is achieved by site-specific transposition between the Tn7 elements (Tn7L and Tn7R) of the pFastBac[™] donor vector and the mini-*att*Tn7 target site in the bacmid. Since the mini-*att*Tn7 attachment site is inserted in the reading frame of *lacZa* gene, successful transposition disrupts the expression of LacZa peptide allowing comfortable detection of bacterial colonies containing recombinant bacmid. The extracted recombinant baculovirus DNA is then transfected into cultured insect cells to produce the recombinant baculovirus. Generated recombinant virus particles are subsequently amplified by repeated rounds of infection and after determination of virus titer by plaque assay, the recombinant virus can be used for different purposes. The drawing is modified from the Guide to baculovirus expression vector systems (BEVS) and insect cell culture techniques (Invitrogen Instruction Manual).

Fig. 3.

Schematic drawing of the pFastBac plasmid containing a neuropeptide promoter and *egfp*. The upstream region of a neuropeptide gene is fused with *egfp* to drive its expression. The promoter-EGFP expression cassette is inserted into a polylinker of the pFastBac-HTB vector (Invitrogen) and used for preparation of the recombinant bacmid DNA.

Fig. 4. Targeted EGFP expression induced by PTTH and ETH promoters using the baculovirus expression system. (A) EGFP expression in two pairs of neurosecretory cells in the brain producing PTTH. (B) EGFP expression in the endocrine Inka cell producing ETH. Scale bars; 50 μ m in (A) and 200 μ m in (B).