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Production and Characterization of the Recombinant Human μ-Opioid Receptor from Transgenic Silkworms

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The production of useful quantities of G protein-coupled receptors is a major problem not only for screening of various drug compounds but also in performing structural biology studies. To solve this problem, we investigated the possibility of using transgenic silkworms for the production of these receptors. Using the human μ-opioid receptor gene, we constructed three transgenic silkworm strains that produced μ-opioid receptors. The silkworms expressed significant amounts of the receptor in the fat body and silk gland. The product was evaluated using a saturation ligand-binding assay. The expressed receptor exhibited ligand affinity similar to that of an authentic sample, and the yield from the transgenic silkworm was comparable to that obtained using an Sf9-baculovirus expression system. As the mass rearing of transgenic silkworms has already been established, the silkworms can be adapted for production of large quantities of receptors.

Key words: G protein-coupled receptor, opioid receptor, *piggyBac* transposon elements, transgenic silkworm, UAS-Gal4 system.

Abbreviations: A3 promoter, silkworm cytoplasmic actin gene promoter; GPCRs, G protein-coupled receptors; UAS, upstream activating sequence for Gal4; IVR, inverse repeat sequence.

Mammalian cells respond to extracellular stimuli. For example, amines, amino acids, peptides, lipids, nucleotides, odorants and photons are recognized as stimulants. These stimuli are transmitted into the cells via G protein-coupled receptors (GPCRs), which are integral membrane proteins and represent the largest protein super-family in the human genome (*1*). The genome is estimated to encode nearly 850 members of this super-family, including several hundred 'orphan receptors', whose endogenous ligands have not yet been identified (*2*). Given that GPCRs play important roles in various physiological processes and cause many diseases, they are recognized as a major target for drug discovery. However, attempts to investigate the functions of GPCRs are often hampered by difficulties in obtaining the amounts of receptor required, as there are several problems with the existing heterologous expression systems.

Several high-yielding heterologous expression systems for GPCRs have been reported, and their potential for scale-up and applicability to drug screening and structural biological studies have been evaluated (*3*). Bacterial and yeast expression systems were thought to be useful because they have potential for large-scale cultivation. However, the number of recombinant GPCRs that have been expressed successfully and retained proper receptor function is rather limited in these systems (*3*). Expression using mammalian cells produces fully functional GPCRs, and several cell cultures are used for the expression of recombinant GPCRs and drug screening. Nevertheless, production of the receptors in mammalian cells is time-consuming and quite costly. In addition, it is difficult to cultivate mammalian cells on a large scale. The most common method used for obtaining recombinant GPCRs is the baculovirus expression system using insect cells, where it has been shown that numerous recombinant GPCR proteins possess the correct folding, post-translational modifications and ligand-binding activity (*3*). Thus, the baculovirus/insect cell-expression system is the most suitable for GPCR expression. In particular, Sf9 cells established from the moth *Spodoptera frugiperda* are the most popular insect cell line for the expression of GPCRs. Although cultivation on a large scale is possible, the equipment required and the cost of producing significant quantities of the receptors remain challenges. Development of a new production system is needed.

A recombinant protein production system using transgenic silkworms was developed (*4*). Here we used the *GAL4*/*UAS* system for the expression of the human μ-opioid receptor and characterized the product. We have shown that similar quantities of the functional receptor can be produced in the transgenic silkworm, compared to expression in insect cells. As mass rearing of silkworms has already been established, the method developed here will be useful for the mass production of GPCRs. Notably, this study is the first to report the production of recombinant membrane proteins using transgenic silkworms.

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MATERIALS AND METHODS

Animals—The silkworm strain w1-pnd was used to construct the transgenic silkworms. The diapausing strain w-1 was also used to maintain the transgenic strains. The GAL4 strains, 190-1 for FibL-GAL4 (*5*) and 192-1 for A3GAL (*6*), were used for driving the expression of the μ-opioid receptor gene regulated by the upstream activating sequence for Gal4 (UAS-μ). The silkworms were reared on an artificial diet (Nihon Nosanko) at 25°C. These strains are maintained at the Transgenic Silkworm Research Center, National Institute of Agrobiological Sciences in Tsukuba, Japan.

Vector Construction—To construct the vectors, the human μ-opioid receptor gene was amplified from a brain cDNA library (BIOMOL) using nested PCR with KOD polymerase (Toyobo). The primers used were: 5′-GC GCTTGGAACCCGAAAA-3′ (forward) and 5′-GTGAAG GTCGGAATGGCATG-3′ (reverse), for the first PCR. The second, nested, PCR was performed using the primers: 5′-TTTGCGGCCGCATGGACAGCAGCGC TGC C-3′ (forward) and 5′-TTTTCTAGATTAGGGCAACGGA GCAGTTTCT-3′ (reverse). The amplified fragment was ligated into the baculovirus transfer vector pFASTBacI (Invitrogen) at the NotI and XbaI sites. Synthetic DNA encoding for a histidine-tag was then inserted between the SalI and NotI sites. This construct was used for preparation of recombinant baculovirus using the Bac-to-Bac kit (Invitrogen) with Sf9 cells.

Preparation of the transfer vector for expression of the μ-opioid receptor gene in transgenic silkworms was carried out using the silkworm cytoplasmic actin gene promoter (A3 promoter) or the yeast UAS. PCR was performed to amplify a DNA fragment corresponding to the A3 promoter and histidine-tag using the primers: 5′-T TTGTCGACGCGCGTTACCATATATGGTG-3′ (forward) and 5′-AAAAGATCTTCTAGAAAAGCGGCCGCCATGAT GATGATGATGATGCATCTTGAATTAGTCTGCAAGAA AAGAA-3′ (reverse). Vector pPIGA3GFP (*4*) was used as the template. This PCR product was inserted between the *Sal*I and *Not*I sites instead of the histidine-tag-encoding sequence described above. Finally, the derived pFASTBac1 vector was digested with *Avr*II and blunt-ended, and then digested again with *BssH*II. The resulting fragment encoding the A3 promotor, histidine-tag, μ-opioid receptor cDNA and SV40 polyA signal sequence was purified. We subcloned this fragment into the *piggyBac* vector pBac[3xP3-DsRed], which carries a red fluorescent protein gene (DsRed) under the neuron-specific 3xP3 promoter as a marker for detecting the transgene using *Fse*I and *BssH*II (pBac[A3 μ/3xP3DsRed], Fig. 1).

Another transfer vector, pBac[UAS-μ/3xP3EGFP], which carries the UAS, histidine-tag, μ-opioid receptor cDNA and SV40 polyA signal sequence, was constructed by the same strategy, using primers: 5′-TTTGTCGACAA GCTTGCATGCCTGCAGG-3′ (forward) and 5′-AAAGCG GCCGCCATGATGATGATGATGATGCATCCAATTCCCT ATTCAGAGTTCTCTTC-3′ (reverse). The template used was pBacUAS-GFP (*5*), based on a *piggyBac* vector that carries an enhanced green fluorescent protein (EGFP) gene, pBac[3xP3-EGFPafm] (*7*). Plasmid construction was confirmed by DNA sequencing (DNA sequencer model 310, Applied Biosystems).

Construction of Transgenic Silkworms—Transgenic silkworm strains containing the μ-opioid receptor gene were constructed using the method of Tamura et al. (*4*, *8*). The plasmid DNA used for injection was purified using a QIAGEN Plasmid Midi Kit (Qiagen). The vector containing the μ-opioid receptor gene was mixed with a helper plasmid carrying the piggyBac transposase gene and injected into embryos of the w1-pnd strain at the preblastodermal stage. Hatched silkworms were raised until the adults mated within the same group or with host strain w1-pnd. The G1 embryos were screened 6–7 days after oviposition based on expression of DsRed or EGFP on their stemmata. The fluorescence of DsRed and EGFP was observed under a fluorescence microscope equipped with DsRed and GFP2 filter sets (Olympus). Transgenic silkworms identified from the screens were maintained by crossing with the diapausing w-1 strain. The strain with the μ-opioid receptor gene under the control of the actin A3 promoter was named 'A3' and was identified by red eyes. The other strain with UAS-μ, identified by green eyes, was mated with the transgenic strain having red eyes that carried the GAL4 gene under the control of the actin A3 gene promoter (A3-GAL4) or fibroin L-chain gene promoter (FibL-GAL4). The silkworms with UAS-μ and A3-GAL4 were named 'A3G4', and the insect cells with UAS-μ- and FibL-GAL4 were designated 'LG4'.

Expression in the Baculovirus-Sf9 System—The pFastBac1 derivative vector carrying the μ-opioid receptor gene (Fig. 1) was used to prepare recombinant baculovirus. The *Escherichia coli* DH10Bac, containing baculovirus Bacmid, was transformed using the pFastBac1 derivative and propagated on LB medium containing antibiotics (50 μg/ml kanamycin, 7 μg/ml gentamicin and 10 μg/ml tetracycline), 200 μg/ml Bluo-gal (Invitrogen) and 40 μg/ml isopropylthio-β-galactoside. The recombinant Bacmid was isolated from white colonies and was transfected into Sf9 cells to prepare recombinant baculoviruses using Cellfectin (Invitrogen). Harvested viruses were amplified and subsequently used to infect Sf9 cells, at a multiplicity of infection of approximately 10 at 28°C. At 48 h post-infection, Sf9 cells were harvested and used for the preparation of membrane protein.

Membrane Preparation—In addition to a posterior division of the silk gland and the fat body isolated from transgenic silkworms, the Sf9 cells expressing μ-opioid receptors were homogenized with a Potter-type glass homogenizer in a buffer containing 50 mM HEPES–KOH (pH 8.0), 10 mM $MgCl_2$, and a protease inhibitor cocktail (Sigma). The homogenate was centrifuged at 100,000 × *g* for 2 h (SCP70H ultracentrifuge and SW28 rotor; Hitachi), and then re-suspended in the same buffer. The protein concentration was determined using a protein assay kit (Bio-Rad).

Ligand-Binding Assay—Membrane protein samples (20 μg) containing the μ-opioid receptors were mixed with 100 μl of assay buffer [20 mM HEPES–KOH (pH 8.0), 1 mM EDTA, 160 mM NaCl, 0.5 mM dithiothreitol and various concentrations of [^{3}H]diprenorphine (PerkinElmer)]. To determine non-specific binding, 1 μM naloxone (Sigma) was added. Incubation was performed

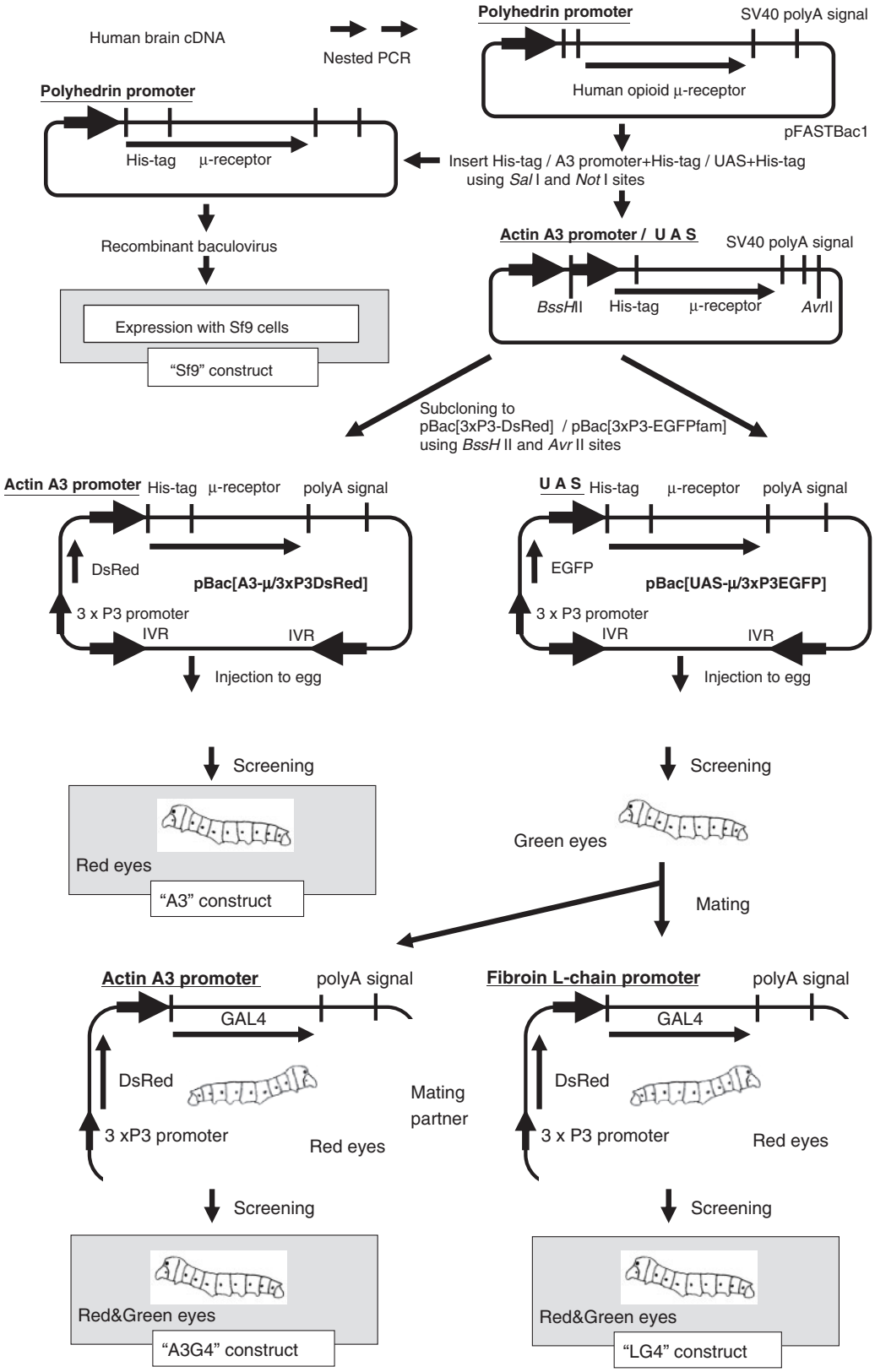

Fig 1. **Overview of the vector and strain constructions.** UAS, upstream activating sequence for GAL4; IVR, inverted terminal repeat sequence of the transposon *piggyBac*.

at 30°C for 90 min in 96-well plates. The reaction mixtures were filtered with a GF/B glass fiber filter (Whatman) and washed three times with cold 20 mM potassium phosphate buffer (pH 7.0). The [^{3}H]diprenorphine trapped by the filter was measured in a liquid scintillation counter (LS6500, Beckman Coulter).

Western Blots—Membrane protein samples prepared from the transgenic silkworms (30 μg) were dissolved in 2.5% SDS on ice for 5 min and separated using 12.5% SDS–PAGE. After electrophoresis, the proteins were transferred to a nylon membrane and incubated with primary antibody (1:10,000 dilution of anti-histidine-tag; Santa Cruz Biotechnology), followed by the secondary antibody (1:15,000 dilution of horseradish peroxidase-labelled anti-rabbit IgG; GE Healthcare). Visualization was performed using an ECL Advance kit (GE Healthcare).

Quantitative RT–PCR—Total RNA fractions from the fat body and the posterior division of the silk gland of the transgenic silkworms were isolated using an RNeasy kit (Qiagen). RT–PCR was performed using the QuantiTect SYBR Green RT–PCR kit (Qiagen) with primers 5′-T TTGCGGCCGCATGGACAGCAGCGCTGCC-3′ and 5′-TT TTCTAGATTA GGGCAACGGAGCAGTTTCT-3′. The temperature program consisted of 50°C for 30 min for reverse transcription, followed by 45 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 1 min, for PCR. All reactions were monitored and quantified using iCycler iQ (Bio-Rad). The PCR product was confirmed by 1.5% agarose gel electrophoresis. The amounts of μ-opioid mRNA in different strains and tissues were normalized relative to the amount of actin mRNA that was amplified using primers 5′-GGATCGCTATGA CAAACTTAAGAG GA-3′ and 5′-ATTGTGAACTAGG ACCTTACGGAATC-3′.

RESULTS AND DISCUSSION

To compare the expression of the μ-opioid receptor gene in three types of transgenic silkworms (A3, LG4 and A3G4), the amount of mRNA in the silk gland and fat body was determined using quantitative RT–PCR (Fig. 2). The silkworm A3 promoter is a very strong, non-tissue-specific promoter. The fibroin L-chain promoter has been reported to be very strong (*5*), but it only drives expression in the posterior division of the silk gland. Therefore, it is reasonable that large amounts of mRNA were accumulated in the fat body of the larvae in the A3 and A3G4 transgenic silkworm strains. It is also reasonable that large amounts of mRNA appeared in the silk gland, but little appeared in fat bodies in the LG4 strain. For the A3G4 strain, higher expression was observed in the fat body than in the silk gland. When the expression of the two strains A3 and A3G4 was compared, the latter strain tended to express larger amounts of transcript, suggesting that use of the *GAL4/UAS* system in the silkworm tends to result in higher promoter activity.

The ligand-binding assay for [^{3}H]diprenorphine (Fig. 3) and Western blot analysis (Fig. 4) on are combinant μ-opioid receptor in transgenic silkworms revealed that μ-opioid receptors were expressed in the fat body or silk gland of these transgenic strains. The fat body of the A3G4 strain had an almost equal amount of μ-opioid receptors as the Sf9 cells. Although quantitative RT–PCR results showed that the LG4 construct led to greater mRNA expression than did the A3 construct in the silk gland, almost equal amounts of the receptors were found between them.

We surmise that the receptor-folding and translocation steps in the cell membrane, rather than transcription and translation, may be rate limiting for μ-opioid receptor expression. The optimization of translocation steps, for example, employment of a suitable N-terminal leader sequence, may be useful in achieving higher yields for our transgenic system.

Figure 3 also shows that the obtained K_d values were similar to reported values, using the recombinant receptor from Sf9 cells (Table 1) (*9*) and that the expressed μ-opioid receptors are active structures. To investigate the functionality of the human μ-opioid receptor on silkworm cells, we tried to observe agonist effects using a [^{35}S]GTPγS-binding assay (*10*) in a preliminary study. When we compared the radioactivity of [^{35}S]GTPγS bound on membranes prepared from silk glands and fat bodies with and without 10 μM leucine encephalin, we did not find any significant differences (data not shown). These results indicated that the μ-opioid receptors expressed on silkworm cells did not couple with endogenous G proteins. In a previous report, mammalian muscarinic receptors expressed using Sf9 cells did not couple with endogenous G proteins (*11*). From these results, we assume that mammalian GPCRs do not couple with insect G proteins. To observe GPCR–Gα protein coupling using a transgenic silkworm system, we are now preparing new transgenic constructs that express human GPCR–Gα fusion proteins (*10*).

Although the level of GPCR expression in the transgenic silkworm is nearly the same as that observed in Sf9 cells, the most attractive advantages of using the silkworm system are cost and the suitability for

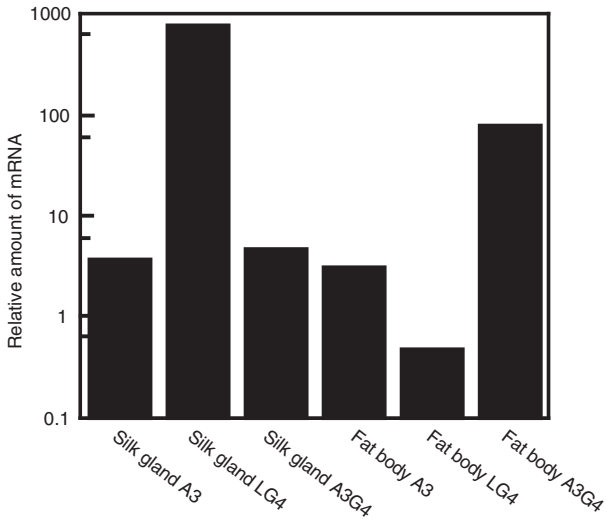

Fig 2. **Relative amounts of μ-opioid receptor mRNA in the silk gland and fat body of transgenic silkworms A3, LG4 and A3G4.** The level of mRNA was determined by quantitative RT–PCR, and the data obtained were normalized relative to endogenous actin mRNA.

large-scale production. One silkworm larva has 300–500 mg of fat body and 0.5–1 g of silk gland (wet tissue weight). We estimate that one larva is equivalent to ~20–30 ml of Sf9 culture and expresses 150–250 ng of μ-opioid receptor. The transgenic silkworms constructed here will be applied to large-scale receptor production and receptor preparation. These advances will develop a new field in insect technology.

In addition to these positive features, there are still a few areas that require improvement regarding μ-opioid receptor purification for structural and biological studies. One is the method of preparing membrane fractions. Membranes are usually prepared following a stepwise centrifugation protocol in which the nuclei and organelles are removed first by low-speed centrifugation. However, in our experiments, we observed approximately 40–60% of the total [^{3}H]diprenorphine-binding activity in the precipitate when we collected the plasma membrane in the supernatant after centrifugation at $3{,}000 \times g$ for 10 min. As silkworm tissues seemed to be tougher than cultured cells, the homogenization we used might have been insufficient to completely disrupt the silk gland and fat body. To recover all of the [^{3}H]diprenorphine-binding activity, we skipped the low-speed centrifugation and directly precipitated the cell membranes using ultracentrifugation. The resulting membrane fractions contained

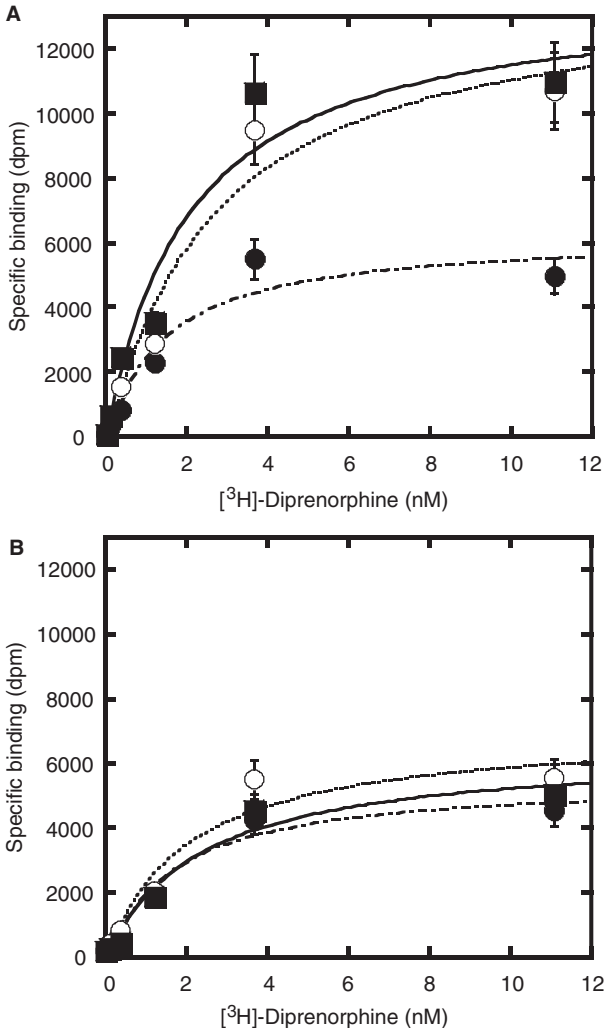

Fig 3. **Representative results for the saturation ligand-binding assay of μ-opioid receptors in the fat body and the posterior silk gland, using [^{3}H]diprenorphine.** (A) A3 fat body (closed circles), A3G4 fat body (closed squares) and Sf9 (open circles); (B) A3 posterior silk gland (closed circles), A3G4 posterior silk gland (closed squares) and LG4 posterior silk gland (open circles). Non-specific binding, measured in the presence of 1 μM naloxone, was subtracted. Each point represents the average of three determinations ($n = 3$). The result is representative of three independent experiments.

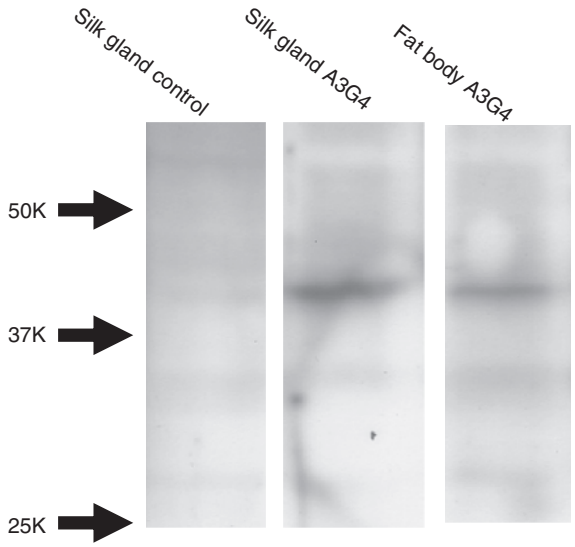

Fig 4. **Western blot analysis of extracts from the silk gland and fat body of A3G4 transgenic silkworms.** Membrane protein samples (30 μg) were separated using 12.5% SDS–PAGE. The proteins were transferred to a nylon membrane and incubated with primary antibody (1:10,000 dilution of anti-histidine-tag; Santa Cruz Biotechnology), followed by secondary antibody (1:15,000 dilution of horseradish peroxidase-labelled anti-rabbit IgG; GE Healthcare). Visualization was performed using an ECL Advance kit (GE Healthcare).

Table 1. **B_{max} and K_d values for the recombinant μ-opioid receptor determined by the ligand-binding assay (Fig. 3).**

	Silk gland			Fat body			
	A3	LG4	A3G4	A3	LG4	A3G4	Sf9
B_{max} (pmole/mg)	0.17 ± 0.045	0.20 ± 0.078	0.19 ± 0.09	0.19 ± 0.090	ND	0.41 ± 0.20	0.40 ± 0.20
$K_{d\ (nM)}$	1.7 ± 0.59	2.0 ± 1.2	1.9 ± 1.3	1.4 ± 0.95	ND	2.1 ± 1.4	2.0 ± 1.3

ND, not detected.

unsuitable components for receptor purification, although these components did not inhibit our receptor activity measurements. The other area of concern is the genetic purification of the transgenic strains in order to obtain greater expression. In this article, we used genetically mixed transgenic silkworms with different genetic characteristics such as different recombination positions on the chromosomes and different copy numbers. Therefore, we are now investigating the isolation and cloning of high-expression lines. Moreover, comparing the expression yields from different GPCRs as well as different constructions and lines will allow the identification of new features for a transgenic silkworm system. The characterization of high-expression clones is our next goal, and we will publish data on this in the near future.

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CONFLICT OF INTEREST

None declared.

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