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Mos1 transposon-based transformation of fish cell lines using baculoviral vectors

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ABSTRACT

Drosophila Mos1 belongs to the mariner family of transposons, which are one of the most ubiquitous transposons among eukaryotes. We first determined nuclear transportation of the *Drosophila Mos1*-EGFP fusion protein in fish cell lines because it is required for a function of transposons. We next constructed recombinant baculoviral vectors harboring the *Drosophila Mos1* transposon or marker genes located between *Mos1* inverted repeats. The infectivity of the recombinant virus to fish cells was assessed by monitoring the expression of a fluorescent protein encoded in the viral genome. We detected transgene expression in CHSE-214, HINAE, and EPC cells, but not in GF or RTG-2 cells. In the co-infection assay of the *Mos1*-expressing virus and reporter gene-expressing virus, we successfully transformed CHSE-214 and HINAE cells. These results suggest that the combination of a baculovirus and *Mos1* transposable element may be a tool for transgenesis in fish cells.

1. Introduction

DNA transposons of the *mariner* family are particularly widespread in nature [1] and are active in a broad range of species, including vertebrates. The sequence of *mariner*-like transposon *Mos1* was shown to be composed of a single open reading frame encoding a transposase and flanked terminal inverted repeats (IRs) [2]. The cut-and-paste type transposase reportedly binds to IRs and catalyzes the excision and subsequent insertion of the element from one genomic locus into another [3].

Transposon-based transgenesis has been widely used for various species. Although homologous recombination is the major technique used for transgenesis, the application of this method has been restricted because of its low frequency of recombination in non-embryonic cells. The recombination technique of a cultured cell genome has been used in genetic engineering fields, especially in the production of clone animals via nuclear transplantation. Therefore, developing nuclear recombination techniques for various organisms is of great significance.

The baculoviral vector has been used as a carrier of transgenes for various organisms including insects and mammals, and introduced the transient expression of a foreign gene [4]. *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) has an especially wide spectrum of infection without viral proliferation [5]. Because it is assumed that AcMNPV does not require specific protein receptors for penetrating into cells, AcMNPV vectors may be available for many types of vertebrates including fish [5]. The production of transgenic fish is under the spotlight and genetically modified salmons are now becoming more commercially available. Thus, the development of transgenic techniques is increasingly important. In this study, we constructed AcMNPV carrying the *Mos1* transposon as a transgene. Using recombinant AcMNPV, we assessed the capability of transgenesis in several practical fish cell lines.

2. Materials and methods

2.1. Cells

Oncorhynchus tshawytscha (Chinook salmon) embryo-derived CHSE-214 cells, *Oncorhynchus mykiss* (Rainbow trout) gonad-derived RTG-2 cells, *Paralichthys olivaceus* (Bastard halibut) embryo-derived HINAE cells, and *Cyprinus carpio* (Common carp) epithelioma-derived EPC cells were propagated in eagle's minimum essential medium (Nissui) supplemented with 10% FBS. *Epinephelus malabaricus* (Malabar grouper) fin-derived GF cells

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were propagated in basal media eagle (Sigma) supplemented with 10% FBS. CHSE-214, RTG-2, HINAE, and GF cells were maintained at 20 °C and EPC cells were maintained at 33 °C. *Spodoptera frugiperda* Sf9 cells were propagated at 26 °C in TC-100 medium supplemented with 0.26% triptose phosphate broth (Difco) and 10% FBS.

2.2. Plasmid construction

The CMV promoter was amplified by PCR using the pAcGFP1-Hyg-C1 plasmid (Clontech) as a template, followed by cloning into the pGEM-T easy plasmid (Promega). The *Eco*RI-*Pst*I fragment containing the CMV promoter was inserted into pEGFP-1 (Clontech) to generate pCMVp-EGFP. The *Mos1*-EGFP fusion protein expression plasmid was constructed as below. *Mos1* coding sequence was amplified from pAd31 plasmid [6] by PCR with the primer set (5′-CTGCAGATGTCGAGTTTCGTGCCGAAT-3′ and 5′-CCGCGGTTCAA AGTATTTGCCGTCGCT-3′), and was then cloned into the pGEM-T easy plasmid. The *Pst*I-*Sac*II fragment containing the *Mos1* coding sequence and *Eco*RI-*Pst*I fragment containing the CMV promoter were inserted into the pEGFP plasmid to generate pCMVp-*Mos1*EGFP (Fig. 1A).

The transfer plasmid carrying the *Mos1* ITR element and reporter genes was constructed as follows. The polyhedrin promoter in pFastBac1 (Invitrogen) was removed by *Bst*I and *Bam*HI digestion followed by blunting and self-ligation to generate pFastBac1-Δpolh. The 5′-partial sequence of *Mos1* was amplified from the pAd31 plasmid by PCR with the primer sets (first: 5′-ATA-GATGTCTCGCAAACGTAAATATTTATCGATTGTCATAAAACTTTGAC-3′ and 5′-GTTTTTGCGTTTGAGCATCGTCTTCATCC-3′, then second: 5′-GTGTACAAGTAGGGAATGTCGGTTCGAACATATAGATGTCTCGCAA-3′ and 5′-GTTTTTGCGTTTGAGCATCGTCTTCATCC-3′). The amplified fragments were cloned into the pGEM-T easy plasmid. The cloned fragment was excised and inserted into the pFastBac1-Δpolh plasmid using the *Eco*RI enzyme. The other part of *Mos1* was obtained from the pAd31 plasmid and combined with the 5′-partial sequence of *Mos1* by using *Cla*I and *Hin*dIII to generate pFastBac1-*Mos1*. The AcGFP1 expression unit and hygromycin-resistant gene were amplified from the pAcGFP1-Hyg-C1 plasmid by PCR with the primer set (5′-GTCGACGGAAGAGTCCTGAGGCGGAA-3′ and GTCGACCCCTTTCGCCATGGTTGTGG-3′). Each fragment was cloned into the pGEM-T easy plasmid and the multiple cloning site of the plasmid containing AcGFP1 was deleted by the digestion of *Bgl*II and *Bam*HI followed by blunting and self-ligation. The cloned fragments were then excised by restriction enzymes (AcGFP1 expression unit: *Sph*I, hygromycin-resistant gene: *Sal*I) and inserted into the pFastBac1-Mos1 plasmid to generate pFastBac1-GHmos (Fig. 2B).

The transfer plasmid for constructing the *Mos1*-expression virus was generated as follows. The human elongation factor 1 α promoter (EF1αp) was obtained from the pEF-BOS plasmid [7] using *Hin*dIII and *Eco*RI enzymes. The fragment containing EF1αp was cloned into the pBlueScript SK(−) vector (Stratagene), and was then redigested with *Sal*I and *Eco*RI. The fragment containing *Mos1* transposase was cut out from the pAd31 plasmid with *Eco*RI and *Hin*dIII. These two fragments were inserted into the *Sal*I-*Hin*dIII sites of the pFasBac1-Δpolh plasmid, generating pFastBac1-TPmos (Fig. 2B).

2.3. Viruses

Recombinant baculoviruses were prepared using the Bac-to-Bac baculovirus expression system (Invitrogen) according to the manufacturer's instructions. The helper plasmid pFastBac1-GHmos and pFastBac1-TPmos gave recombinant baculoviruses AcB-GHmos and AcB-TPmos, respectively. The generated viruses were grown in Sf9 cells, and viral titers were determined by plaque assay according to Maeda's method [8]. Fish cells (3×10^6 cells per well in T25 tissue culture flask) were incubated with recombinant viruses (1 pfu/cell) for 1 h in 1 ml of serum-free medium for viral infections, and then 3 ml of medium containing 3% FBS was added. After a 1-week culture, the medium was replaced with medium containing 10% FBS and 0.5 mg/ml hygromycin, and cells were then cultured for 1 week. Hygromycin-selected cells were further maintained in the medium containing 10% FBS.

2.4. Transfection of plasmid DNA and confocal fluoromicroscopy

One microgram of plasmid DNA was mixed with 2 μl of HilyMax reagent (Dojindo) and was then added to 1×10^6 fish cells in serum-free medium for transfections of fish cell lines. After a 3-h

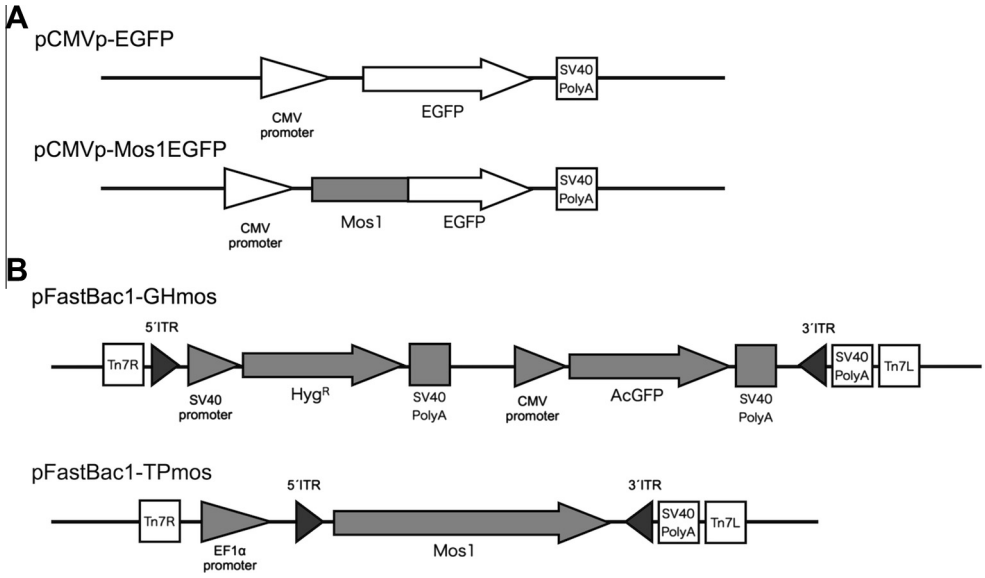

Fig. 1. Schematics of the constructed plasmids. (A) The structure of the EGFP-expression plasmid (pCMVp-EGFP, upper) and Mos1-EGFP fusion protein (pCMVp-Mos1EGFP, lower). (B) The structures of the donor plasmids for the construction of baculoviral vectors. pFastBac1-GHmos carries two reporter gene expression units (hygromicin-resistant gene and AcGFP1) with *Mos1* ITR sequences. pFastBac1-TPmos carries *Mos1* coding sequences under the control of the EF1α promoter. pFastBac1-GHmos and pFastBac1-TPmos were used to generate the recombinant baculoviruses AcB-GHmos and AcB-TPmos, respectively.

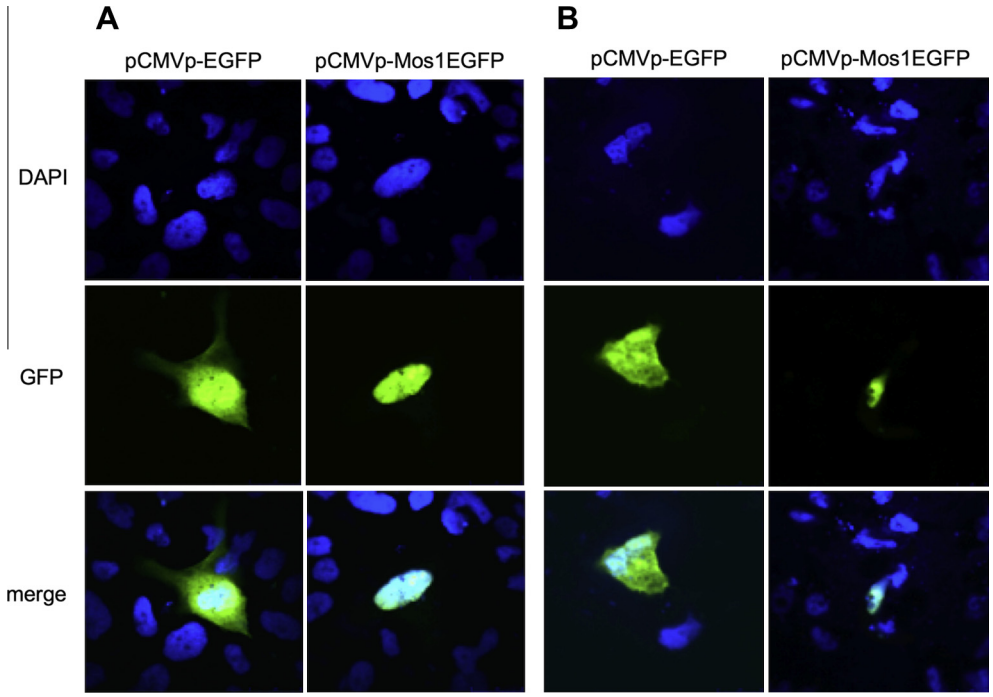

Fig. 2. Fluoromicroscopic images of CHSE-214 cells (A) and HINAE cells (B) transfected with the pCMVp-EGFP (left) or pCMVpMos1EGFP plasmid (right). Cells were fixed 48 h post-transfection and the distribution of GFP fluorescence was determined (upper: DAPI, middle: GFP, lower: merged images).

incubation period, the medium was removed and replaced with medium containing 10% FBS. Cells were fixed with 4% paraformaldehyde in PBS after 48 h and permeabilized with 0.5% TritonX-100 in PBS. Nuclei were counterstained with DAPI and fluorescent images were taken by a confocal laser scan microscope.

3. Results

Transposases require nuclear transportation to rearrange genomic sequences. Therefore, we constructed the expression vector of the *Mos1*-EGFP fusion protein to visualize the distribution of *Mos1* in fish cell lines (Fig. 1A). Because the nuclear localization signals of *Drosophila Mos1* were previously shown to be positioned at 131–133 a.a. and 142–146 a.a. [9], and the arginine of position 132 appears contribute to DNA digestive activity [10], the fusion protein was designed to keep these elements. The pCMVp-EGFP or pCMVp-Mos1EGFP plasmid was transfected to CHSE-214 and HINAE cells and EGFP fluorescence was then determined. EGFP driven by the CMV promoter was detected throughout the cell, whereas the *Mos1*-EGFP gave merged images with DAPI in both cells (Fig. 2). These results indicated that the *Mos1* region in the fusion protein possessed the nuclear localization signal function in fish cells.

We next constructed recombinant baculoviruses that possessed marker genes sandwiched with *Mos1* IR sequences (AcB-GHmos) or *Mos1* transposase (AcB-TPmos) (Fig. 1B). The marker genes in AcB-GHmos was transposed in the presence of *Drosophila Mos1* transposase. AcB-TPmos encoded the *Drosophila Mos1* gene under the control of EF1α promoter. To maintain the AcB-TPmos viral genomic structure, *Mos1* expression in insect cells was restricted by removing the polyhedrin promoter from the vector.

The infectivity of the recombinant baculoviral vector in fish cell lines was determined using AcB-GHmos. AcB-GHmos carried the AcGFP1 gene under the control of the CMV promoter, presenting the transient expression of AcGFP1 from the viral genome in infected cells. Fluorescent microscopy 7 days post-infection showed AcGFP1 signals in CHSE-214, HINAE, and EPC cells (Fig. 3), with the highest frequency of transduction in CHSE-214 cells (Table 1). Although EPC cells produced AcGFP1 signals, some EPC cells showed detachment after a viral infection, and this became more significant when the amount of the virus was increased (data not shown). On the other hand, AcGFP1-positive cells were not detected in GF or RTG-2 cells. Thus, the baculoviral vectors successfully infiltrates into CHSE-214 and HINAE cells.

We next attempted to transform CHSE-214 and HINAE cells with *Drosophila Mos1* transposase. *Mos1* transposase was expressed from the recombinant baculoviral vector AcB-TPmos and transferred the marker genes (hygromycin-resistant gene and AcGFP1 gene) from the AcB-GHmos vector. The co-infection of AcB-TPmos and AcB-GHmos vectors showed AcGFP fluorescent expression in CHSE-214 and HINAE cells 7 days post-infection (Fig. 4A and 4B). The hygromycin selection of CHSE-214 cells for 4 weeks after infection resulted in the formation of colonies that expressed AcGFP1. AcGFP1 expression was prolonged even after further passages (at least 13 weeks post-infection) (Fig. 4C). This result indicated that the marker genes were integrated into the cell genome by *Mos1* transposase.

4. Discussion

The AcMNPV vector showed infectivity to CHSE-214, HINAE, and EPC cells with the expression of AcGFP1 under the control of the CMV promoter. Unfortunately, GF and RTG-2 cells inoculated with AcMNPV vectors did not show expression of the marker gene. This result was consistent with a previous report that demonstrated the insusceptibility of RTG-2 cells to AcMNPV [5]. Because we used a viral concentration up to 10 pfu/cell in this study, it is possible that cells showed susceptibility to higher concentrations of baculoviral vectors. For example, EPC cells demonstrated dose-dependent transduction efficiency of the baculoviral vector and cells showed only 2% infectivity on m.o.i. of 100 [5]. Another possible problem is the efficiency of

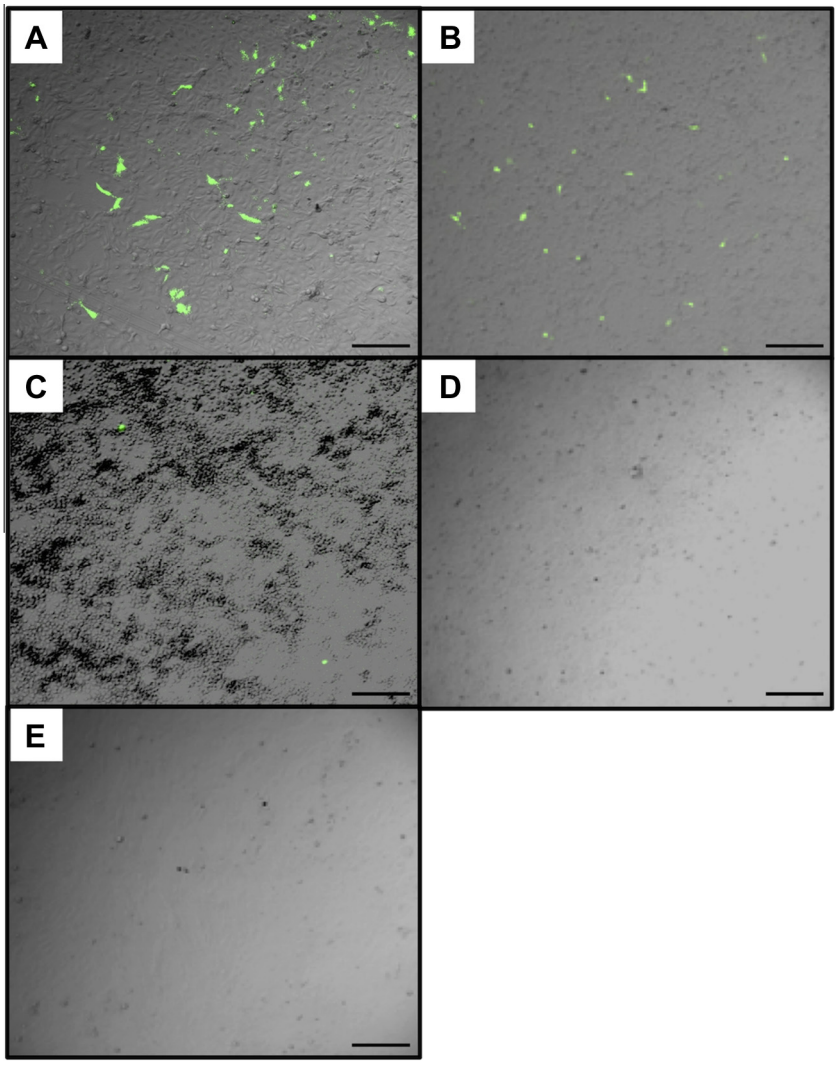

Fig. 3. Fluoromicroscopic images of fish cell lines. The recombinant baculoviral vector AcB-GHmos was infected into CHSE-214 (A), HINAE (B), EPC (C), GF (D), or RTG-2 (E) cells and cultured for 7 days. GFP fluorescent images were merged with phase contrast light microscopic images. The scale bars in the each panel indicate 200 μm.

Table 1
The transduction efficiency of the baculoviral vector (AcB-GHmos) to fish cell lines.

Cells	Total number of cells	AcGFP1-positive cells	Frequency
CHSE-214	7.3×10^4	515	7.1×10^{-3}
HINAE	4.4×10^4	117	2.7×10^{-3}
EPC	2.6×10^4	33	1.3×10^{-3}

The number of GFP-positive cells was determined using fluoromicroscopy 7 days post-infection, as shown in Fig. 3. Frequency was calculated by dividing the number of AcGFP1-positive cells by the total number of cells determined using a hemocytometer.

gene expression. The expression of foreign genes is known to be restricted in mammalian cells in a histone deacetylation-mediated manner [11], [12], and [13]. A previous study also showed that LacZ expression from a baculoviral vector was significantly increased by a histone deacetylase inhibitor in fish EPC cells [5]. Thus, the susceptibility of fish cells lines to baculoviral vectors varies among each cell line and appears to depend on the efficiency of viral infection and transgene expression.

The baculoviral DNA that entered mammalian cell nuclei was present as an episome and degraded within several days [14]. PCR with viral-genome specific primers could not detect AcMNPV in the hygromycin-resistant GFP-positive CHSE-214 cells established in this study (data not shown). Therefore, the baculoviral vector expression system in mammalian cells was used as a transient gene expression system without the risk of viral replication [15] and [16]. This approach can also be applied to other vertebrate cells including fish [5]. In addition to this, a combination of a baculoviral vector and the transposon *sleeping beauty* enabled stable transgene expression in human HEK293 cells [17]. Because DNA transposons of the *mariner* family are particularly widespread in nature and are active in a broad range of species [1], we chose *Drosophila Mos1* as a candidate of a wide spectrum transposon that is expected to function in fish cells [18]. As expected, the *Drosophila Mos1*-EGFP fusion protein expressed from the baculoviral vector was functionally localized to fish cell nuclei, and the functions of marker genes on the baculoviral vector sandwiched between two IRs were stably conferred to fish cells by the combination of AcMNPV and *Mos1*. Considering the wide spectrum of AcMNPV infection and conservation of *mariner* transposons, the baculovirus-*Mos1* system may be function in other eukaryote cells, although some improvements are required.

Phylogenetic studies of *mariner* transposons have provided compelling evidence for the occurrence of horizontal transfer across species during evolution [19], [20], and [21]. Despite the extensive amount of evidence on the horizontal transfer of *mariner*

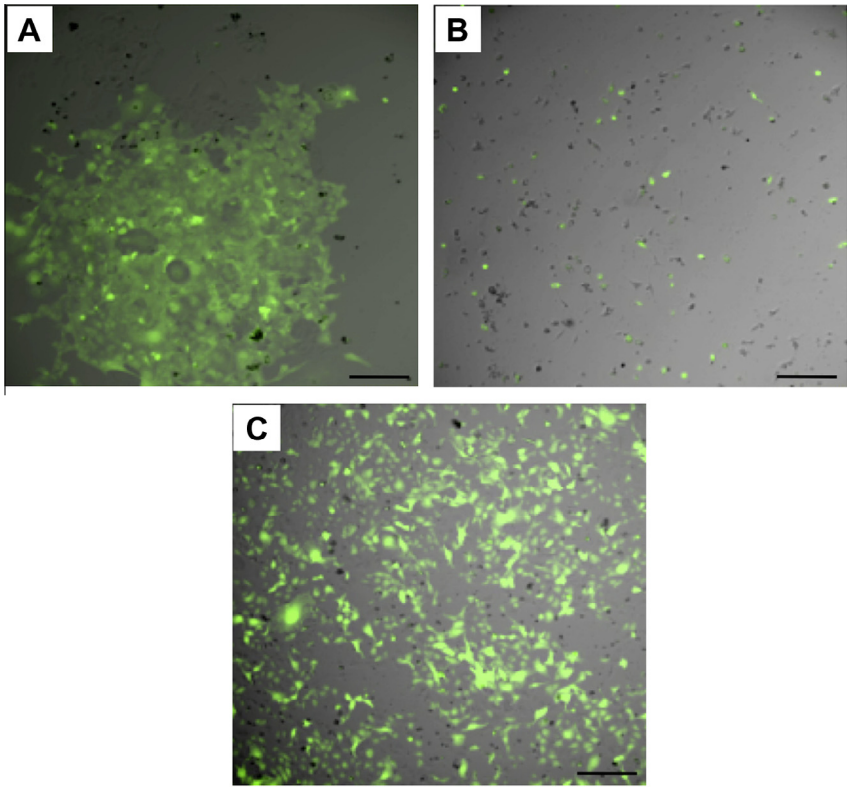

Fig. 4. Fluoromicroscopic images of fish cell lines co-infected with the recombinant baculoviruses AcB-GHmos and AcB-TPmos 4 weeks post-infection. CHSE-214 (A) or HINAE (B) cells were cultured in the presence of hygromycin for 4 weeks post-infection. CHSE-214 cells formed a colony with AcGFP1 expression and were further cultured for 9 weeks (13 weeks from the viral infection) with passages. The scale bars in the each panel indicate 200 μm.

transposons, the vectors that carry these elements have not yet been identified. Because class II transposons move in a DNA form, a DNA virus could be one of the candidates of a vector for *mariner* transposons in nature. The results of this study demonstrated that, at least in a laboratory condition, the baculovirus possessed the ability to transfer mariner transposons into non-host cells through the typical infection process.

In conclusion, the baculovirus-*Mos1* combination system worked successfully in some fish cell lines and allowed stable transgene expression in these cells. Although the experiments shown here were carried out on cultured cell lines, this method may be applicable on an individual level because of the broad utility of the baculovirus and *mariner*.

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