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Evaluation of availability of various internal ribosome entry sites (IRESs) for bicistronic expression in different fish cell lines

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The internal ribosome entry site (IRES) elements from various RNA viruses are widely used to express proteins in bicistronic or multicistronic ways in mammalian cells. However, research on the application of IRESs in fish cells has been poorly conducted. In this study, to evaluate the availability of various viral IRESs in fish cells based on a bicistronic vector system, the translation activity of IRESs from viruses of invertebrates (aphid lethal paralysis virus ALPV; cricket paralysis virus, CrPV; and *Plautia stali* intestine virus, PSIV), of mammals (encephalomyocarditis virus, EMCV), and of fish (infectious pancreatic necrosis virus, IPNV; marine birnavirus, MABV; and snakehead retrovirus, SnRV) was analyzed in various fish cell lines originated from salmonid (chinook salmon embryonic cells, CHSE-214), cyprinid (Epithelioma papulosum cyprinid, EPC), and flatfish (Hirame natural embryonic cells, HINAE). Translation mediated by EMCV IRES was shown in all cell lines, but the activity was weak in CHSE-214 and HINAE cells while high activity was shown in EPC cells, suggesting that although EMCV IRES can be broadly used in various fish cells, the translational activity can be varied according to different cell lines. PSIV IRES showed moderate activity in EPC cells and low activity in CHSE-214 and HINAE cells. CrPV IRES showed weak activity in EPC and HINAE cells and no activity in CHSE-214 cells. In the case of ALPV IRES, green fluorescence was observed only in EPC cells but the activity was very weak. These results suggest that dicistroviral IRESs analyzed in this study would not be the best option for protein expression in various fish cells. The translation activity of SnRV IRES in fish cells was first verified in EPC cells, though the activity was very weak. The 5'UTR of MABV segment A showed high translation activities in all examined fish cell lines, suggesting that IRES of MABV can be used to develop multicistronic expression systems or cap-independent RNA-based translation systems in a wide range of fish cells.

Key words: Internal ribosome entry site (IRES), RNA viruses, Bicistronic expression system, Translational activity, Fish cell lines, MABV IRES

Introduction

The translation initiation of the vast majority of eukaryotic messenger RNAs occurs through a canonical, cap-dependent way involving the recruitment of 40S ribosomal subunit to the 5' cap of the mRNA, subsequent scanning, and generation of 80S complex at the AUG codon (Sonenberg and Hinnebusch, 2009). In this complex process, a large number of eukaryotic translation initiation factors (eIFs) such as eIF2, eIF3, eIF4, and initiator tRNA are involved (Komar and Hatzoglou, 2011). However, some eukaryotic mRNAs can initiate translation in a cap-independent way under stressed environments, physiological challenges, or apoptosis through the help of an internal ribosome

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entry site (IRES) (Spriggs *et al*., 2008; Godet *et al*., 2019). The IRES elements enable ribosomes to land the RNA and initiate translation through those of complicated secondary or tertiary structures assembled with stem loops and pseudoknots (Godet *et al*., 2019).

Due to the limited genome size, viruses have evolved various strategies to exploit the host gene expression system. One of the strategies is an IRES-mediated translation to promote viral protein synthesis (Walsh and Mohr, 2011). It has been known that diverse viruses with single-stranded RNA genomes have highly structured IRES in their non-coding region. Since the first discovery of IRES elements in the mRNA of *Picornaviridae* (Jang *et al*., 1988; Pelletier and Sonenberg, 1988), IRESs have been identified in other RNA viruses including *Dicistroviridae*, *Flaviviridae,* and *Iflaviridae* families, as well as in several retroviruses (Lu *et al*., 2006; de Breyne and Ohlmann, 2018; Jaafar and Kieft, 2019).

In mammals, the IRES has been actively used to express several genes driven by the same promoter or to construct reporter systems by the insertion of an IRES cassette behind the 3' UTR of the gene of interest (Attal *et al*., 1999; Derrington *et al*., 2005). However, research on the application of IRESs in fish cells has been poorly conducted. So far, among IRES from mammalian viruses, only encephalomyocarditis virus (EMCV) IRES was identified to work in fish and fish cell lines. Fahrenkrug *et al.* (1999) confirmed that EMCV IRES was active in developing embryos of zebrafish, and Fukamachi *et al*. (2009) used the same IRES and confirmed its activity in medaka (*Oryzias latipes*). In chinook salmon embryo cells (CHSE-214 cells), EMCV IRES-mediated translation was also demonstrated (Rivas-Aravena *et al*., 2017). Liu *et al*. (2018) and Xu *et al*. (2019) used EMCV IRES for the co-expression of an antigen and a molecular adjuvant in DNA vaccines against Cyprinid herpesvirus-3 and *Vibrio anguillarum*, respectively. Recently, there have been several reports that genomic leader sequences from aquabirnaviruses are capable of cap-independent translation in a fish cell line. CHSE-214 cells infected with infectious pancreatic necrosis virus (IPNV) showed increased phosphorylation levels of eIF2α during the infection cycle (Gamil *et al*., 2015), and 5'UTR of segment A showed IRES activity (Rivas-Aravena *et al*., 2017). Kim and Kim (2021) also found that the 5'UTR of marine birnavirus (MABV)'s segment A acted as IRES in CHSE-214 cells.

In the present study, to develop efficient multicistronic vector systems in fish cells, we evaluated the availability of various viral IRESs in fish cells based on a bicistronic vector system. To accomplish this task, the translation activity of IRESs from viruses of invertebrates (ALPV, CrPV, and PSIV), of mammals (EMCV), and of fish (IPNV, MABV, and SnRV) was analyzed in various fish cell lines originated from salmonid, cyprinid, and flatfish.

Materials and Methods

Cells

Cell lines used in this study were chinook salmon embryo-214 (CHSE-214), epithelioma papulosum cyprinid (EPC), and hirame natural embryo (HINAE). CHSE-214 and HINAE cells were grown in Leibovitz-15 (L-15, Sigma) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin-streptomycin (Gibco) at 20°C. EPC cells were grown at 28°C using the same culture medium.

Construction of bicistronic vectors

To construct bicistronic vectors containing various IRES sequences, a previously constructed bicistronic vector containing MABV IRES between two reporter protein genes (Kim and Kim, 2021) was used. First, to manipulate the vector more easily, we inserted the sequence of NotI and Kpn2I into the 3' of mCherry gene and 5' of eGFP gene, respectively, by amplifying the vector using primer sets having overhang se-

quences, then the amplified vector was assembled with the EMCV IRES using Overlap Cloner DNA cloning kit (Elpisbio, Korea). The integrity of the resulting vector, the EMCV bicistronic vector, was verified by nucleotide sequencing (Macrogen, Korea) and used as a template for the construction of other bicistronic vectors used in this study.

To construct bicistronic vectors containing ALPV, CrPV, PSIV, IPNV, and SnRV, the EMCV bicistronic vector was digested with NotI and Kpn2I, then assembled with the PCR-amplified fragments from each of IRES sequences. The overlaps between the vector and insert were 15 bp, and the assembly was made using Gibson Assembly Cloning Kit (NEB). The information on IRES used in this study is shown in Table 1. The primer sets used in this study and the schematic presentation of bicistronic vectors are shown in Table 2 and Fig. 1, respectively.

Table 1. The information of IRES sequences used in this study

Origin	Location of IRES	Length (nt)	Reference
EMCV	5'UTR	572	pIRESneo3 vector (#631621, Addgene)
PSIV	IGR	291	AB006531
CrPV	IGR	208	AF218039
ALPV	IGR	184	AF536531
IPNV	5'UTR of segment A	119	AF343572
MABV	5'UTR of segment A	110	Kim and Kim (2021)
SnRV	Gag-pol	174	NC_001724

Table 2. Primers used in this study

Primers		Sequences (5'-3')
For the construction of a bicistronic vector harboring EMCV IRES		
Vector	F	TCCGGAATGGTGAGCAAGGGCGAGGAGCTGTTCAC
	R	GCGGCCGCTTACTTGTACAGCTCGTCCATGCCGCCGGTGGAGTG
EMCV	F	GAGCTGTACAAGTAAGCGGCCGCCCCCTCTTCCCTCCCCCCC
	R	GCCCTTGCTCACCATTCCGGATTATCATCGTGTTTTTCAAGGAAACCAC
For the construction of bicistronic vectors haboring ALPV, CrPV, PSIV, IPNV and SnRV IRES		
PSIV	F	GAGCTGTACAAGTAAGCGGCCGCCGACACGCGGCCTTCCAAGCAG
	R	GCCCTTGCTCACCATTCCGGACTTTGATTGGGCAGTTGTATCTCTGC
CrPV	F	GAGCTGTACAAGTAAGCGGCCGCAGTACCCTTCACCAAAGCAAAAATG
	R	GCCCTTGCTCACCATTCCGGAGGTATCTTGAAATGTAGCAGGTAAATTTCTTAG
ALPV	F	GAGCTGTACAAGTAAGCGGCCGCAATTACTAATTTGATCTTTAGGTTATAATGTTAGGAC
	R	GCCCTTGCTCACCATTCCGGAAGGTGAGTTTCTGACCTATCTGACC
IPNV	F	GAGCTGTACAAGTAAGCGGCCGCGGAAAGAGAGTTTCAACGTTAGTGG
	R	GCCCTTGCTCACCATTCCGGAGATTCATGTAGATAAGAATTGTTGGTTGTTTG
SnRV	F	GAGCTGTACAAGTAAGCGGCCGCGGCTACGCCTTAGCCCAACAATAC
	R	GCCCTTGCTCACCATTCCGGATGGTGGTCCTGCTCTGAGAAC

Bold font: restriction enzyme; underline: overlapped sequences for assembly

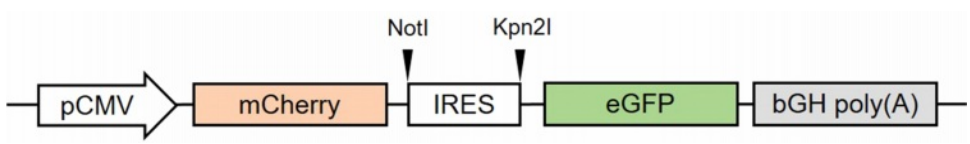

Fig. 1. Schematic presentation of bicistronic vector used in this study. The vector contains IRES sequence between two reporter protein genes, mCherry and enhanced green fluorescent protein (eGFP) gene, and the backbone vector contained cytomegalovirus (CMV) immediate early promoter and bovine growth hormone (bGH) poly A signal.

Transfection and fluorescence observation

Cells were seeded to about 80% confluency in a 35 mm dish on the previous day of transfection. Each of the bicistronic vectors containing ALPV, CrPV, PSIV, EMCV, IPNV, MABV and SnRV IRES (3000 ng) was transfected using Fugene HD transfection reagent (Promega) in accordance with the manufacturer's instructions. The cells were incubated at each cell's optimal incubation temperature. When the fluorescence of mCherry appeared strongly, cells were photographed to evaluate translational activity.

Results and Discussion

IRES elements were first reported in poliovirus (PV) and EMCV RNAs which are members of the family *Picornaviridae* (Jang *et al*., 1988; Pelletier and Sonenberg, 1988). Picornaviruses are non-enveloped, positive single-stranded RNA viruses, of which the viral genome has an internal ribosome entry site (IRES) element at 5'UTR and intergenic region EMCV IRES is widely used in experimental and pharmaceutical applications because of its high activity in various cell lines and accurate translation ability (Borman *et al*., 1995; Bochkov and Palmenberg, 2006; Martínez-Salas et al., 2015). In the present study, translation mediated by EMCV IRES was shown in all cell lines (Fig. 2A, Table 3), in particular, high activity was observed in EPC cells. However, the translational activities in other cell lines were weak, suggesting that although EMCV IRES can be broadly used in various fish cells, translational activity can vary according to the types of fish cell lines.

Dicistroviridae is a family of infecting invertebrates and is composed of at least 15 species (Bonning and Miller, 2010). In their genome, the translation initiation of both non-structural and structural genes is mediated by IRES elements located at 5'UTR and IGR region, respectively (Hertz and Thompson, 2011). To date, there has been no report on the translation activity of dicistroviral IRESs in fish and fish cell lines. In this study, we first explored the translation activity of IGR IRESs of ALPV, CrPV, and PSIV in various fish cell lines. The IRES of PSIV showed moderate activity in EPC cells and low activity in CHSE-214 and HINAE cells (Fig. 2B, Table 3). Although the IRES of CrPV is known to work in a wide range of cell lines and animals (Johnson

Table 3. Translation efficiency of IRESs in various fish cell lines

	CHSE-214	EPC	HINAE
EMCV	O 12/31 (38.7%)	O 102/102 (100%)	O 17/62 (27.4%)
PSIV	O 8/22 (36.3%)	O 38/57 (66.6%)	O 18/64 (26.8%)
CrPV	X	O 15/142 (10.5%)	O 3/78 (3.8%)
ALPV	X	O 11/70 (15.7%)	X
IPNV	O 5/49 (10.2%)	X	O 22/57 (38.5%)
MABV	O 33/35 (94.2%)	O 59/59 (100%)	O 60/60 (100%)
SnRV	X	O 5/43 (11.6%)	X

Translation efficiency is indicated by the percent of cell numbers showing eGFP/mCherry.

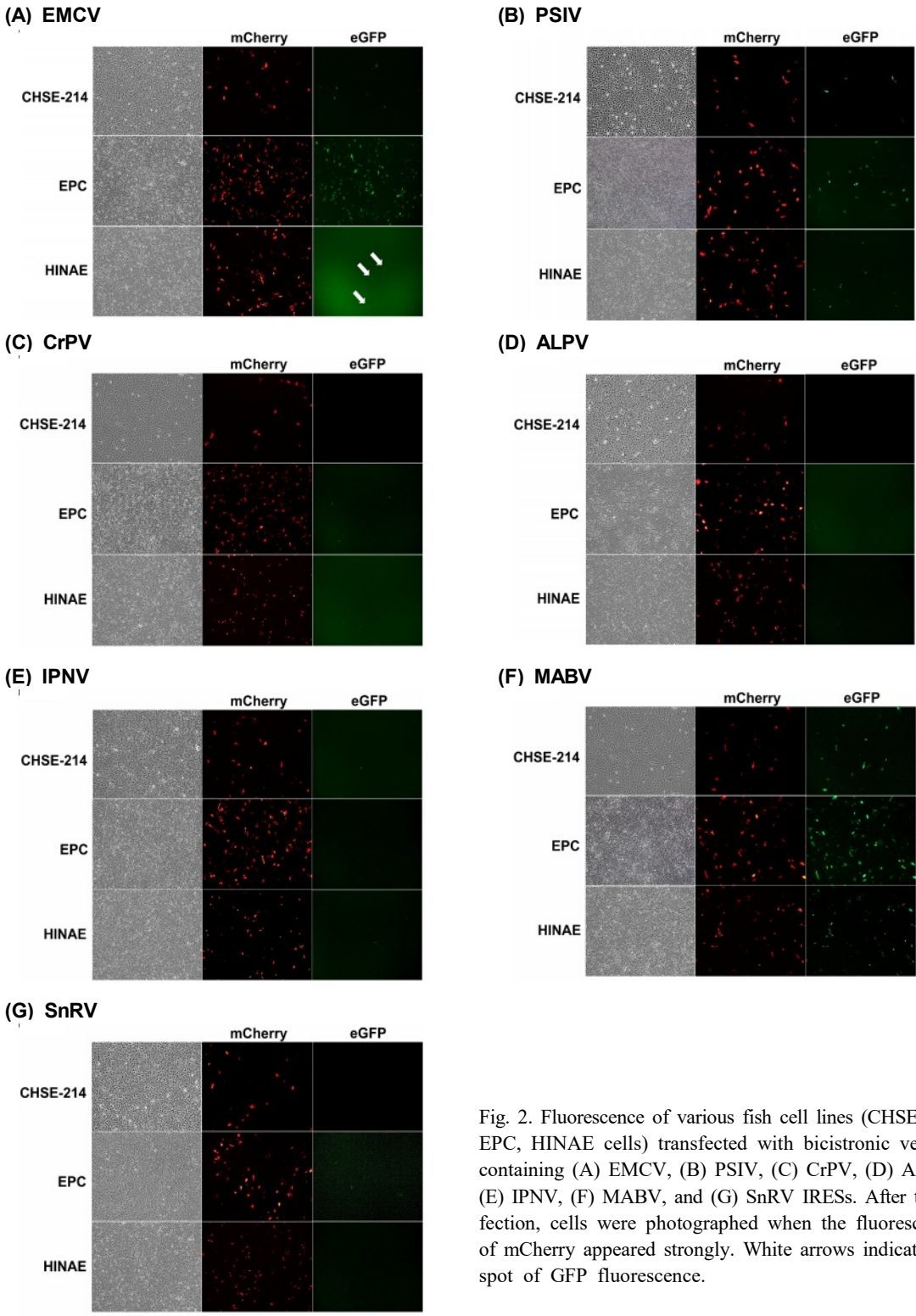

Fig. 2. Fluorescence of various fish cell lines (CHSE-214, EPC, HINAE cells) transfected with bicistronic vectors containing (A) EMCV, (B) PSIV, (C) CrPV, (D) ALPV, (E) IPNV, (F) MABV, and (G) SnRV IRESs. After transfection, cells were photographed when the fluorescence of mCherry appeared strongly. White arrows indicate the spot of GFP fluorescence.

et al., 2017), in this study, weak activity was observed in EPC and HINAE cells and there was no activity in CHSE-214 cells (Fig. 2C, Table 3). In the case of ALPV IRES, green fluorescence was observed only in EPC cells, but the activity was very weak (Fig. 2D, Table 3). These results suggest that dicistroviral IRESs analyzed in this study would not be the best option for protein expression in various fish cells.

Recently, there have been several reports that leader sequences of aquabirnaviruses are capable of cap-independent translation in fish cells. Birnaviruses are non-enveloped, bi-linear segmented double-stranded RNA viruses (Delmas *et al*., 2019). Both segments do not have a cap structure at 5'UTR (Dobos, 1993), indicating that they are translated by a cap-independent mechanism. In this study, 5'UTR IRES of IPNV segment A showed very weak translational activity in CHSE-214 and HINAE cells, and no activity in EPC cells (Fig. 2E, Table 3). Previously, Rivas-Aravena *et al*. (2017) confirmed different efficiencies and structural changes of IPNV IRES according to temperature. Therefore, it is possible that no activity in EPC cells shown in this study was influenced by the high temperature (28°C) effect. Meanwhile, MABV IRES was highly active in all cell lines (Fig. 2F, Table 3), suggesting that MABV IRES would be highly useful in protein expression in various fish cells.

Since Hart *et al*. (1996) suggested a cap-independent IRES mechanism in the expression of SnRV leader peptide (LP) protein, there has been no study to verify the translation activity of SnRV IRES. In this study, we first identified the translation activity of SnRV IRES in EPC cells, though the activity was very low (Fig. 2G, Table 3).

In conclusion, we analyzed the translational efficiency of viral IRESs in various fish cell lines. As IRES activity can be strictly dependent not only on host factors but also on environmental conditions (Licursi *et al*., 2015; Lozano and Martínez-Salas, 2015), different translation activities of IRESs shown in this study are thought to be influenced by cell-type specificity or temperature sensitivity of each viral IRES. In this study, we found that the 5'UTR of MABV segment A had high translation activity in all examined fish cell lines that were derived from salmonid, cyprinid, and flatfish, suggesting IRESs of MABV can be used to develop multicistronic expression systems or cap-independent RNA-based translation systems in a wide range of fish cells.

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