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Marine birnavirus (MABV)'s 5′ terminal region of segment A acts as internal ribosome entry site (IRES)

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Eukaryotic translation is initiated by either cap-dependent or cap-independent way, and the cap-independent translation can be initiated by the internal ribosomal entry site (IRES). In this study, to know whether the 5'UTR leader sequence of marine birnavirus (MABV) segment A and segment B can act as IRES, bicistronic vectors harboring a CMV promoter-driven red fluorescent gene (mCherry) and poliovirus IRES- or MABV's leader sequence-driven green fluorescent gene (eGFP) were constructed, then, transfected into a mammalian cell line (BHK-21 cells) and a fish cell line (CHSE-214 cells). The results showed that the poliovirus IRES worked well in BHK-21 cells, but did not work in CHSE-214 cells. In the evaluation of MABV's leader sequences, the reporter eGFP gene under the 5'UTR leader sequence of MABV's segment A was well-translated in CHSE-214 cells, indicating 5'UTR of MABV's segment A initiates translation in the cap-independent way and can be used as a fish-specific IRES system. However, the 5'UTR leader sequence of MABV's segment B did not initiate translation in CHSE-214 cells. As the precise mechanism of birnavirid IRES-mediated translation is not known, more elaborate investigations are needed to uncover why the leader sequence of segment B could not initiate translation in the present study. In addition, further studies on the host species range of MABV's segment A IRES and on the screening of other fish-specific IRESs are needed.

Key words: IRES, Marine birnavirus, Bicistronic vector, CHSE-214 cells

Introduction

Most of eukaryotic mRNAs are translated through the canonical 5′-cap-dependent mechanism. However, some eukaryotic mRNAs and some viral RNAs do not follow the canonical translation way (Weingarten et al., 2016). Many mammalian RNA viruses, such as encephalomyocarditis virus (EMCV) and poliovirus (PV), use a non-canonical mechanism for their RNA translation, and the untranslated region (UTR) of those viruses contain a specific RNA sequence, which is called as internal ribosome-entry sites (IRESs), by which the viral RNAs are translated in the 5′-cap-independent manner (Dorner et al., 1982; Jang et al., 1988; Firth and Brierley, 2012). Unlike the cap-dependent translation process where translators recognize cap structures existing at the 5'UTR of mRNA and move along the mRNA and start translating when meet the appropriate initiation codon, IRES acts as a ribosome-binding site and stimulates directly the ribosome subunit to start translation from the 5'UTR region (Leppek et al., 2018). Several kinds of IRESs that were isolated from mammalian viruses have been used to express proteins in a cap-independent way in mammalian cells (Belsham and Brangwyn,

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1990; Tsilouama-Kohara et al., 1992), however, a few information is available on the IRES of fish viruses (Rivas-Aravena et al., 2017).

Viruses in the family *Birnaviridae* are bi-segmented double-stranded RNA viruses and the genome of birnaviruses is composed of two segments, the segment A encoding polyprotein (VP2-VP4-VP3) and the segment B encoding RNA-dependent RNA polymerase (VP1) (Von Einem et al., 2004). The 5′-terminal ends of both segments of birnaviruses do not contain a cap structure (Dobos, 1993), indicating the translation of both segments are initiated by cap-independent way. In fact, the infectious bursal disease virus (IBDV), a birnavirus infecting young chickens, solves the 5′ uncap by VP1/VP3-dependent translation initiation (Ye et al., 2018). Furthermore, Rivas-Aravena et al. (2017) reported that the infectious pancreatic necrosis virus (IPNV), a salmonid birnavirus, possesses an IRES in the 5′ UTR of the segment A.

Marine birnavirus (MABV) is a member of the genus *Aquabirnavirus* of the family *Birnaviridae*, and is known to infect mainly marine fish (Hosono et al., 1994; Zhang and Suzuki, 2003). In the present study, to know whether the leader 5′ UTR sequence of MABV segment A and segment B can act as IRES, bicistronic expression vectors harboring a cytomegalovirus (CMV) promoter-driven mCherry cassette (red fluorescence) and the 5′ UTR sequence of MABV segment A- or segment B-flanked enhanced green fluorescent protein (eGFP) cassette were constructed and evaluated the translation activity of the 5′ UTRs of MABV by observing GFP fluorescence in transfected fish cells.

Materials and methods

Cells and virus

Chinook salmon embryo (CHSE-214) cells were grown in Leibovitz medium (L-15, Sigma) supplemented with 10% fetal bovine serum (FBS, Welgene) and 1% penicillin-streptomycin (Welgene) at 20℃. Baby hamster kidney (BHK-21) cells were grown in Dulbecco's modified Eagle's medium (DMEM, Welgene) supplement with 10% FBS and 1% penicillin-streptomycin at 37℃ with 5% CO_2.

MABV was propagated in a monolayer of CHSE-214 cells in the presence of 1% penicillin-streptomycin at 20℃. Cells displaying extensive cytopathic effect (CPE) were harvested and centrifuged at 4,000 g for 10 min at 4℃ and the supernatant was stored at -80℃.

Construction of bicistronic vectors

Bicistronic expression vectors were constructed based on pcDNA3.1(+). Human poliovirus IRES and eGFP sequence were amplified from pcDNA3-smURFP-IRES-eGFP (Addgene), and mCherry was amplified from pcDNA3.3-mCherry (Addgene) using primer sets polioIRES-F/poiloIRES-R, eGFP-F/eGFP-R, mCherry-F/mCherry-R, respectively (Table 1). To construct a vector harboring CMV promoter-driven mCherry and poliovirus IRES-driven eGFP (pCMV-mCherry-polioIRES-eGFP), each PCR amplified fragment using overlap cloner primer sets was treated with DpnI (Elpisbio, Korea) to eliminate non-amplified templates, then, the mixture was ligated using Overlap cloner Kit (Elpisbio, Korea).

To obtain the leader sequence of MABV segment A and segment B, total RNA was extracted from MABV-infected CHSE-214 cells using Hybrid R kit (Geneall) and synthesized cDNA using random hexamer (Promega). Using the above cDNA, each leader sequence of MABV leader A and B was amplified using primers sets leader A-F/leader A-R, leader B-F/leader B-R, respectively (Table 1). To construct pCMV-mCherry-MABVleaderA-eGFP, pCMV-mCherry-MABV leaderB-eGFP, each PCR amplified fragment was purified using a gel purification kit (Gel SV, Geneall) and subcloned into the above bicistronic expression vector using Overlap cloner Kit. The integrity of the DNA sequence of constructed plasmid was confirmed by sequencing.

Table 1. Primers used in this study

Primers		Sequence (5'-3')
For construction of pcDNA 3.1-mCherry-polio-eGFP		
Fragment #1	F	ATGGTGAGCAAGGGCGAGG
	R	CGCAATACCGGAGTACTCGAGTTACTTGTACAGCTCGTCC
Fragment #2	F	TACTCCGGTATTGCGGTACCC
	R	ATCTTGTTCAATCATCACGTGCTGATCAGATCCG
Fragment #3	F	ATGATTGAACAAGATGGATTGCACG
	R	TCAGAAGAACTCGTCAAGAAGGCGATAG
Fragment #4	F	GACGAGTTCTTCTGACACGTGCTACGAGATTTCGATTC
	R	GCCCTTGCTCACCATGGTGGCAAGCTTAAGTTTAAACGCTAG
For construction of pcDNA 3.1-mCherry- leader A/leader B-eGFP		
Fragment #1	F	TGCGGCGACCGAGTTGCTCTTGC
	R	TTACTTGTACAGCTCGTCCATGCC
Fragment #2	F	GCTAGCATGGTGAGCAAGGGCGAGGAGCTGTTCAC
	R	TCAGAAGAACTCGTCAAGAAGGCGATAG
Fragment #3	F	GACGAGTTCTTCTGACACGTGCTACGAGATTTCGATTC
	R	GCAACTCGGTCGCCGCATACACTA
Fragment #4A	F	GAGCTGTACAAGTAAGAAAGAGAGTTTCAACGTTAGTGGTAAC
	R	GCTCACCATGCTAGCGTAGATAGAATTGTAGGTTGTTTGTTAGAG
Fragment #4B	F	GAGCTGTACAAGTAAGGAAACAGTGGGTCAACGTTG
	R	GCTCACCATGCTAGCATCATGAGTTGTAGATGTAGAGGTTTTG
Cloning Primers		
Polio IRES	F	TACTCCGGTATTGCGGTACCC
	R	GATCCTATCCAATTCGCTTTATGATAAC
eGFP	F	ATGGTGAGCAAGGGCGAGG
	R	TTACTTGTACAGCTCGTCCATGCC
mCherry	F	ATGGTGAGCAAGGGCGAGG
	R	TTACTTGTACAGCTCGTCCATGCC
MABV leader A	F	GAAAGAGAGTTTCAACGTTAGTGGTAAC
	R	CCTCTAACAAACAACCTACAATTCTATCTAC
MABV leader B	F	GGAAACAGTGGGTCAACGTTGGTG
	R	CAAAACCTCTACATCTACAACTCATGAT

Underlined nucleotides indicate overlapped sequence.

Transfection and fluorescence observation

BHK-21 cells and CHSE-214 cells were cultured into 6 well plates 24 h prior to transfection, then, cells were transfected with 2 μg of pCMV-mCherry-polio IRES-eGFP, pCMV-mCherry-MABVleaderA-eGFP, or pCMV-mCherry-MABVleaderB-eGFP using FuGENE HD (Promega) according to the manufacturer's protocol. Cells were washed at 12 h post-transfection and medium was replaced. Expressed red fluorescence (mCherry) and eGFP were observed using a fluorescent microscope.

Results

Bicistronic vectors harboring a CMV promoter-driven red fluorescent gene (mCherry) and poliovirus

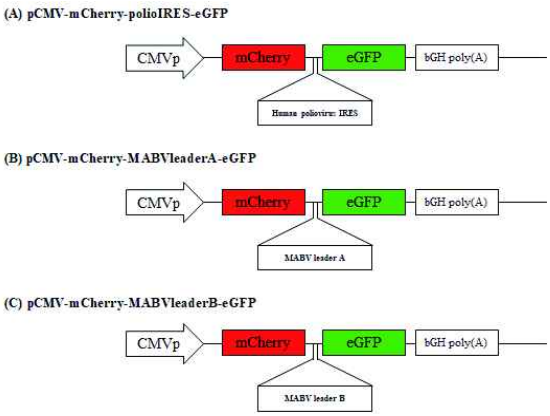

Fig. 1. Schematic representation of the bicistronic vectors that harboring CMV promoter-driven mCherry and (A) poliovirus IRES-driven, (B) MABV segment A leader-driven, or (C) MABV segment B leader-driven eGFP.

IRES- or MABV's leader sequence-driven green fluorescent gene (eGFP) were successfully constructed (Fig. 1) and were used to test the cap-independent translation ability in fish cells (Fig. 1).

To know whether the human poliovirus's IRES can work in fish cells, the vector pcDNA3.1-mCherry-polioIRES-eGFP was constructed (Fig. 1A) and transfected into a mammalian cell line, BHK-21 cells, and a fish cell line, CHSE-214 cells. Under the fluorescent microscope, BHK-21 cells showed both red (mCherry) and green (eGFP) fluorescence, while CHSE-214 cells showed only red florescence (Fig. 2).

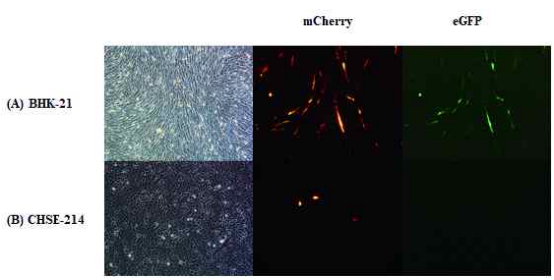

Fig. 2. Evaluation of the working of the human poliovirus IRES in BHK-21 cells and CHSE-214 cells. Cells were transfected with pCMV-mCherry-polioIRES-eGFP, then, red and green fluorescence was observed under a fluorescent microscope.

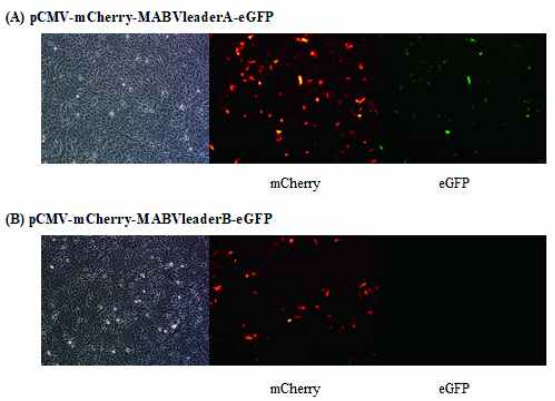

Fig. 3. Evaluation of the cap-independent translation of MABV leader sequences in CHSE-214 cells. Cells were transfected with (A) pCMV-mCherry-MABVleaderA-eGFP and (B) pCMV-mCherry-MABVleaderB-eGFP, then, red and green fluorescence was observed under a fluorescent microscope.

To examine whether the MABV's segment A and B's leader sequences can play as an IRES, CHSE-214 cells were transfected with pCMV-mCherry-MABVl eaderA-eGFP or pCMV-mCherry-MABVleaderB-eGFP vector. In CHSE-214 cells transfected with pCMV-mCherry-MABVleaderA-eGFP, both red and green fluorescence were observed, while in cells transfected with pCMV-mCherry-MABVleaderB-eGFP, only red fluorescence was observed (Fig. 3).

Discussion

Although the cap-independent IRES-mediated translation is an uncanonical mechanism in eukaryotes, many mammalian viruses and cellular mRNAs exploit IRES for their translation (Baird et al., 2006), suggesting the important role of IRES in the regulation of translation in eukaryotes. In viruses belonging to Birnaviridae, the cap-independent translation has been reported chicken IBRD (Ye et al., 2018) and a piscine IPNV (Rivas-Aravena et al., 2017). In the present study, we have demonstrated that the translation of MABV also initiated by the cap-independent way. In the present results, a reporter gene under the 5'UTR

leader sequence of MABV's segment A was translated in CHSE-214 cells, indicating 5'UTR of MABV's segment A initiates translation in the cap-independent way.

As both segment A and segment B of birnavirid viruses do not have a cap structure at the end of 5'UTR, not only the segment A but also the segment B of MABV has to be translated in the cap-independent way. However, in this study, the 5'UTR leader sequence of MABV's segment B did not initiate translation in CHSE-214 cells. To be functional, IRES should interact with several cofactors in host cells, and different IRESs differ in the requirement of those factors (Martinez-Salas et al., 2018). As the precise mechanism of birnavirid IRES-mediated translation is not known, more elaborate investigations are needed to uncover why the leader sequence of segment B could not initiate translation in the present study.

As IRES can be used for the co-expression of multiple genes by one promoter system, it is advantageous to construct multicistronic vectors in a compact form. However, IRES ability is usually tissue and species specific (Schumacher et al., 1999; Plank et al., 2013). In the present study, poliovirus IRES worked well in a mammalian cell line (BHK-21 cells), but did not work in a fish cell line (CHSE-214 cells). Therefore, to use the IRES system for fish, fish-specific IRESs should be developed. As the present MABV's segment A leader sequence showed the characteristics of IRES in CHSE-214 cells, it is applicable to the development of compact polycistronic vectors for fish. Further studies on the host species range of MABV's segment A IRES and on the screening of other IRESs for fish use are needed.

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