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Rana grylio virus as a vector for foreign gene expression in fish cells

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ABSTRACT

In the present study, *Rana grylio* virus (RGV, an iridovirus) thymidine kinase (TK) gene and viral envelope protein 53R gene were chosen as targets for foreign gene insertion. ΔTK-RGV and Δ53R-RGV, two recombinant RGV, expressing enhanced green fluorescence protein (EGFP) were constructed and analyzed in *Epithelioma papulosum cyprinid* (EPC) cells. The EGFP gene which fused to the virus major capsid protein (MCP) promoter p50 was inserted into TK and 53R gene loci of RGV, respectively. Cells infected with these two recombinant viruses not only displayed plaques, but also emitted strong green fluorescence under fluorescence microscope, providing a simple method for selection and purification of recombinant viruses. ΔTK-RGV was purified by seven successive rounds of plaque isolation and could be stably propagated in EPC cells. All of the plaques produced by the purified recombinant virus emitted green fluorescence. However, Δ53R-RGV was hard to be purified even through twenty rounds of plaque isolation. The purified recombinant virus ΔTK-RGV was verified by PCR analysis and Western blotting. These results showed EGFP was expressed in ΔTK-RGV infected cells. Furthermore, one-step growth curves and electron microscopy revealed that infection with recombinant ΔTK-RGV and wild-type RGV are similar. Therefore, RGV was demonstrated could be as a viral vector for foreign gene expression in fish cells.

1. Introduction

Iridoviruses are large, icosahedral, enveloped DNA viruses that replicate in the nucleus and cytoplasm of infected cells (Williams et al., 2005). These viruses can infect a variety of invertebrates and poikilothermic vertebrates such as crustaceans, fish, reptiles and amphibians (Henderson et al., 2001; Williams, 1996). As an aquatic virus, iridovirus was increasingly taken into account because it caused heavy economic losses in farmed food and ornamental fish, as well as posed great threat to wildlife conservation. The researches about iridovirus focused on complete sequence determination (Eaton et al., 2007; Huang et al., 2009; Jancovich et al., 2010; Shi et al., 2010; Wong et al., 2011; Zhang et al., 2004), transcriptome and proteome analysis (Chen et al., 2006, 2008; Dang et al., 2007; Ince et al., 2010; Luo et al., 2009; Majji et al., 2009; Song et al., 2006) and individual gene identification (Ao and Chen, 2006; Lin et al., 2008; Wan et al., 2010; Wang et al., 2008; Zhao et al., 2007, 2008b), such as RGV thymidine kinase (TK) gene and envelope protein 53R. TK gene, an early gene of RGV, exists in many large DNA viruses and encodes a cytoplasmic protein (Zhao et al., 2009). 53R gene, a core gene of the family *Iridoviridae*, encodes an envelope protein of RGV (Eaton et al., 2007; Zhao et al., 2008a). Moreover, 26 core genes and some sequences with promoter activity were also identified in iridovirus (Eaton et al., 2007; Pallister et al., 2005; Willis et al., 1984).

There are a variety of viral vectors (e.g. recombinant virus) used in molecular biology. Recombinant virus could be used for studying individual gene function, constructing delivery vehicle for biological control of some species or diseases. To date, recombinant viruses have been well-studied in some large DNA viruses, such as African swine fever virus (ASFV) (García et al., 1995; Oliveros et al., 1999; Rodríguez et al., 1992, 2009; Epifano et al., 2006), vaccinia virus (Satheshkumar et al., 2009; Sood et al., 2008; Szajner et al., 2001; Resch et al., 2005), human cytomegalovirus (Marchini et al., 2001; Xu et al., 2004) and so on. However, most of the above researches are based on mammalian DNA virus, and knowledge about recombinant aquatic DNA virus (such as iridovirus) is very limited (Pallister et al., 2007).

Rana grylio virus (RGV) is a pathogenic agent that has resulted in high mortality in cultured pig frog (*Rana grylio*) (Zhang et al., 2001). Previous reports have revealed that RGV is a member of family *Iridoviridae* and is closely related to frog virus 3 (FV3), the type species of the genus *Ranavirus* (Zhang et al., 1999, 2001, 2006). Cellular changes and some other viral proteins involved in RGV replication and assembly were investigated and analyzed (Huang et al., 2006, 2007; He et al., 2010; Ke et al., 2009; Kim et al., 2010; Sun et al., 2006). The large genome size and many open reading frames implied that RGV could be genetically manipulated by

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Table 1
Primers for plasmids construction and PCR analysis (enzyme cleavage site is underlined).

Primers	Primers sequences (5′-3′)
P1	CTCGGTACCAACCTCTGAGAAAG (*KpnI*)
P2	TGATACAAGCTTCAGTTACAGAAGACAT (*HindIII*)
P3	AGAAAGCTTTTCCTTTTTATTATAAGATAC (*HindIII*)
P4	CTCAAGCTTAACCTCTGAGAAAGC (*HindIII*)
P5	GATGAATTCACCAGTTACAGAAGACAT (*EcoRI*)
P6	ATCGAATTCATGGTGAGCAAGGG (*EcoRI*)
P7	TTGCTCGAGCCACAACTAGAATGC (*XhoI*)
P8	GAAGGTACCCATCGCAAACGC (*KpnI*)
P9	CCCAAGCTTCGGATTTCTTTG (*HindIII*)
P10	GTCGGCGCCAAAATCTTTCTG (*NarI*)
P11	AGAGTCGACCTCCCCAAAGACTCTC (*SalI*)
P12	GAGAAGCTTACCAGGACCTGTACT (*HindIII*)
P13	GTAGTCGACCAGGTTTAGGCCAG (*SalI*)
P14	TTTGGTACCTCTCCGAAAAAGTG (*KpnI*)
P15	TTCGAATTCAGCATTCCAAGAAC (*EcoRI*)
P16	CTCGTCGACA ACCTCTGAGAAAGC (*SalI*)
P17	TTGGGTACCCCACAACTAGAATGC (*KpnI*)
P18	CTCAAGCTTGGAACTTTCTATGACA (*HindIII*)
P19	TAGGAATTCATAGACAACACAAGACG (*EcoRI*)

homologous recombination. In this study, two recombinant *Rana grylio* viruses (ΔTK-RGV and Δ53R-RGV) expressing EGFP gene were constructed through homologous recombination in EPC cells, the features of ΔTK-RGV were further characterized by different methods.

2. Materials and methods

2.1. Virus and cell line

Rana grylio virus (RGV) was used in the study. *Epithelioma papulosum cyprinid* (EPC) cells were maintained in TC199 medium supplemented with 10% fetal bovine serum (FBS) at 25 °C. RGV propagation and viral titer determination were performed as described previously (Zhang et al., 1999, 2006)

2.2. Promoter activity detection

The promoter activity was determined by luciferase assay. A 221 bp DNA fragment named p50 containing the nucleotide sequence from −205 to +16 relative to the translation initiation codon of RGV MCP gene, was obtained by PCR from RGV DNA using primers P1/P2 (All the primers used in the study were listed in Table 1). Another 205 bp DNA fragment named p50′ containing the nucleotide sequence from −205 to −1 relative to the translation initiation codon of RGV MCP gene, was obtained by PCR using primers P1/P3. The two DNA fragments were digested with *KpnI* and *HindIII*, and then inserted into *KpnI*–*HindIII*-treated plasmid pGL3-basic (Promega, U.S.A.) to generate plasmids pGL3-p50 and pGL3-p50′, respectively.

To assay the promoter activity of p50 and p50′, EPC cells grown in 24-well plates were transfected with plasmids pGL3-basic, pGL3-p50 and pGL3-p50′ using Lipofectamine 2000 (Invitrogen, U.S.A.) according to the manufacturer's protocol, respectively. Plasmid phRL-TK (Promega, U.S.A.), which was used as an internal control, was co-transfected with these plasmids at the same time. After 24 h transfection, cells were infected with RGV at multiplicity of infection (MOI) of 1, and then harvested at various intervals (10, 20, 30 h) after infection. The cells were lysed with lysis buffer (Promega, U.S.A.) and luciferase activity was measured using a dual-luciferase reporter assay kit (Promega, U.S.A.) with a Junior LB9509 Luminometer (Berthold, Germany). Relative luciferase activity was normalized to the amount of *Renilla* luciferase internal control. The results showed p50 has stronger promoter activity than p50′, and thus p50 was used. The nucleotide sequence of p50 was submitted to Genbank (Genbank ID: JN018069).

2.3. p50 promoting EGFP gene expression

In another way, we testified whether p50 could promote EGFP expression by RGV inducing. p50 was generated by PCR using the primers P4/P5, digested with *HindIII* and *EcoRI*, and then inserted into plasmid pCDNA3.1(TaKaRa, Japan) to generate plasmid pCDNA3.1-p50. A 0.95 kb DNA fragment containing the EGFP gene and polyadenylation signals was obtained by PCR from the pEGFP-N3 plasmid using primers P6/P7. The PCR product was cut with *EcoRI* and *XhoI*, and then cloned into digested pCDNA3.1-p50 to generate plasmid pCDNA3.1-p50-EGFP. To ensure that the EGFP gene is downstream of p50, this insert was analyzed by PCR amplification and DNA sequencing (data not shown). The constructed plasmid was further transfected into cells, EGFP gene expression was tested in the presence or absence of RGV. EPC cells grown on coverslips in 6-well plates were transfected with plasmid pCDNA3.1-p50-EGFP and then infected or mock infected with RGV at MOI of 1. At 24 h post-infection, cells were rinsed with PBS and fixed with 4% paraformaldehyde for 15 min. The cells were then rinsed with PBS and permeabilized with 0.2% Triton X-100 for 15 min. Then, staining of viral and cellular DNA with Hoechst 33342 (Sigma, U.S.A.). Finally, the cells were rinsed with PBS, mounted with 50% glycerol and visualized under fluorescence microscope (Leica, Germany).

2.4. Recombinant plasmids construction

RGV TK gene and 53R gene (nucleotide positions 92147–92734, 58770–60338 in RGV genome, respectively) were chosen as target genes for recombinant plasmids construction. To insert the foreign gene into TK site, two DNA fragments (TKL and TKR, TKL nucleotide positions from −492 to +226, TKR nucleotide positions +370 to +1103 relative to the translation initiation codon of TK gene) containing flanking and partial coding sequence of TK gene were used as homologous arms for recombination. To insert the foreign gene into 53R site, two DNA fragments (53R-L and 53R-R, 53R-L nucleotide positions from −678 to +285 and 53R-R positions from +1282 to +2201 relative to the translation initiation codon of 53R gene) containing flanking and partial coding sequence of 53R gene were used as homologous arms. EGFP fused to the virus promoter p50 (p50-EGFP) from plasmid pCDNA3.1-p50-EGFP was used as a marker gene for recombinant virus selection and purification.

TKL was generated by PCR using primers P8/P9, digested with *KpnI* and *HindIII*, and then inserted into *KpnI*–*HindIII*-digested pGL3-basic to generate pGL3-TKL. TKR was obtained by PCR using the primers P10/P11, digested with *NarI* and *SalI*, and cloned into *NarI*-*SalI*-treated plasmid pGL3-TKL to generate plasmid pGL3-TK. p50-EGFP was generated by PCR using primers P4/P7, digested with *HindIII* and *XhoI*, and then inserted into *HindIII*–*XhoI*-digested plasmid pGL3-TK to generate pGL3-p50-EGFP-TK. 53R-L and 53R-R were obtained by PCR using primers P12/P13 and P14/P15, respectively. p50-EGFP was generated by PCR using primers P16/P17. The construction of recombinant plasmid pUC19-p50-EGFP-53R was the same as pGL3-p50-EGFP-TK. The schematic diagram of two plasmids construction was shown in Fig. 1.

Both the plasmids were confirmed by restriction enzyme digestion and DNA sequencing, and then were used for recombinant virus construction.