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The full-length microRNA cluster in the intron of large latency transcript is associated with the virulence of pseudorabies virus

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ABSTRACT

Pseudorabies virus (PRV), the etiological pathogen of Aujeszky's disease, belongs to the *Alphaherpesvirus* subfamily. Large latency transcript (LLT), the most abundant PRV transcript, harbors a ~ 4.6 kb microRNA (miRNA) cluster-encoding intron. To investigate the function of the LLT miRNA cluster during the life cycle of PRV, we generated a miRNA cluster mutation virus (PRV-ΔmiR cluster) and revertant virus. Analysis of the growth kinetics of PRV-ΔmiR cluster-infected cells revealed significantly smaller plaques and lower titers than the wild-type and revertant viruses. The mutation virus exhibited increased IE180 and decreased EP0 expression. The clinical symptoms observed in mice infected with PRV-ΔmiR cluster revealed that the miRNA cluster is involved in the pathogenesis of PRV. Physical parameters, virus shedding assays, and the SN_{50} titers revealed that the miRNA cluster enhances PRV virulence in pigs. Collectively, our findings suggest that the full-length miRNA cluster is involved in PRV replication and virulence.

1. Introduction

Pseudorabies virus (PRV), belonging to the *Alphaherpesvirus* subfamily, is the etiological agent of Aujeszky's disease, which causes reproductive, neurological, and respiratory diseases in pigs. PRV is accepted as a model organism for the investigation of herpesvirus in neurophysiology and pathogenesis (Enquist, 2002; Klupp et al., 2004; Pomeranz et al., 2005). Although attenuated live or inactivated PRV vaccine campaigns are successfully conducted worldwide for disease eradication, outbreaks of PRV disease are still reported in swine populations (Hu et al., 2015; Yu et al., 2014). In its natural host, PRV establishes a lifelong latent infection in the trigeminal ganglia (TG) of the peripheral nervous system in infected pigs. Latent infected pigs then serve as the source of recurrent infection. However, the outcome of PRV infection in non-natural hosts is different, with cases of survival and latent infection being rarely observed and “mad itching” and rapid death being common (Mettenleiter, 2000; Pomeranz et al., 2005).

During latency, variable sizes of latency associated transcript (LAT) are transcribed in TG from the strand complementary to the EP0 and IE180 genes and overlapping the internal repeat sequence. One of the most abundant transcripts, large latency transcript (LLT), is the only transcriptionally active region of the PRV genome during latent infection (Cheung, 1991; Priola and Stevens, 1991). LLT is an 8.4 kb polyadenylated RNA, which is spliced to different sizes to yield a 4.6 kb stable intron (Cheung, 1991; Mahjoub et al., 2015). In herpes simplex virus 1 (HSV-1), the 8.3 kb LAT, which is homologous to PRV LLT, is associated with reactivation from latency in rabbits (Perng et al., 1994).

In our previous study, we reported the cluster of 11 miRNA genes of PRV in the infected porcine epithelial cell line (PK-15) (Wu et al., 2012). A similar miRNA cluster was identified in PRV using porcine immature dendritic cells (Anselmo et al., 2011). These studies confirmed that the miRNA cluster is completely included within the ~ 4.6 kb intron region of the LLT. Some researchers have proposed that these miRNAs play a pivotal role in the host–pathogen interaction network and in the viral self-regulatory sub-network. The detailed function of the LLT miRNA cluster requires investigation because it has been reported that the target genes include both viral and host genes (Wu et al., 2012).

Here, we generated a PRV mutant by replacing the full-length miRNA cluster in the LLT intron with an EGFP expression cassette. We report the involvement and importance of the LLT intron containing the miRNA cluster in viral replication and proliferation of PRV-Ea *in vitro*, and we also studied its function in virulence and pathogenicity in animals.

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Table 1
List of primers and oligonucleotides in this study.

Name	Sequence (5–3′)[a]	Location(nt)[b]
P1 (Intron upstream For)	GAAAGCTTCCCATAAAGCCAGTTGAAGACG	97222–97243
P2 (Intron upstream Rev)	TGGAATTCCCGGGCGAAGACAAACAAA	97754–97772
P3(Introndownstream For)	ATGAATTCCAGTGGGCGATGGTTGC	102141–102157
P4 (Intron downstream Rev)	AGCTCGAGAGATTTTTTGGGGAGATGGAG	102655–102676
P5 (LLT exon1–2 RT)	TAGAGGGTCTTGGGGATGTTGG	102369–102390
P6 (LLT exon1–2 For)	TTGTGCATGCACGGCAGCGT	97520–97539
P7 (LLT exon1–2 Rev)	ATGGTCCCGAGGGTCCCGGT	102272–102291
P8 (gD For)	TGCGCGCACCTGCTGTACTTTATC	121387–121410
P9 (gD Rev)	CCACCATGAAGTCGGTGAGGATGTT	121594–121617
P10 (β-actin For)	TGAAGGTAGTTTCGTGGATGC	944–964
P11 (β-actin Rev)	GACATCAAGGAGAAGCTGTGC	757–777
Anti-miR-LLT7 mimics	AUCCCCAUCAACCCCCCGG	–
Anti-NC mimics	CAGUACUUUUGUGUAGUACAA	–

[a] Underlined are introduced restriction enzyme sites.
[b] Positions correspond to GenBank accession number NC_006151.1 (P1-P9) and XM_003124280.4 (P10, P11).nt, nucleotide.

2. Materials and methods

2.1. Cells and virus

PK-15 and MDBK cell lines were obtained from the China Center for Type Culture Collection (Wuhan), and were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Gibco, USA) in a 5% CO_2 environment at 37 °C. PRV-Ea, a wild-type PRV strain, was propagated in PK-15 cells (Wang et al., 2017).

2.2. Construction of the mutant virus (PRV-ΔmiR cluster) and the revertant virus (PRV-ΔmiR cluster-R)

To generate a miRNA cluster-mutant virus, a transfer vector was constructed. Upstream (567 bp) and downstream (552 bp) homologous arms of the LLT intron were cloned into the eukaryotic expression vector pcDNA3.1(+) with primer pairs P1/P2 and P3/P4, respectively (Table 1). In the transfer vector, the miR cluster containing 11 miRNAs was replaced by an EGFP expression cassette (BD Biosciences Clontech), including CMV promoter, EGFP open reading frame and SV40 poly(A). PK-15 cells were cotransfected with PRV-Ea genomic DNA (Yao et al., 2015) and the LLT miRNA cluster mutation transfer vector using Lipofectamine®2000 (Invitrogen, USA). Recombinant virus was plaque purified (Hu et al., 2015).

To construct the revertant virus, PK-15 cells were cotransfected with PRV-ΔmiR cluster genomic DNA and a plasmid containing the full-length sequence of the PRV LLT intron. In the revertant virus, the EGFP expression cassette was replaced with the full-length LLT intron. PRV-ΔmiR cluster and PRV-ΔmiR cluster-R were confirmed by polymerase chain reaction (PCR) with primer pairs P1/P4 (Table 1) and DNA sequencing.

2.3. Plaque morphology

Analysis of plaque morphology was performed as described previously (Hu et al., 2015). Briefly, MDBK cell monolayers were inoculated with 100 PFU of virus, overlaid with 1.6% carboxymethyl cellulose, fixed with 10% neutral formalin at 48 h post infection (hpi), and then stained with 0.35% crystal violet solution. Plaques were imaged under a microscope at a 40× magnification. Plaque diameters were measured using the software Adobe Acrobat 7.0 Professional and plaque areas were calculated using Microsoft Office Excel 2007.

2.4. One-step growth kinetics

PK-15 cells were infected with virus at a multiplicity of infection (MOI) of 5, and harvested at 0, 3, 6, 9, 12, 18, 24, and 30 hpi. Viral titers were determined on MDBK cells as described previously (Liu et al., 2008).

2.5. Western blot analysis

To generate rabbit anti-PRV IE180 polyclonal antibody, New Zealand white rabbits (1.5–2.0 kg weight) were obtained from Hubei Research Center of Laboratory Animals and PRV IE180 polypeptide (sequence: AGDGGAPPQRQPRRC) was synthesized. Rabbits were immunized three times with IE180 polypeptide, and anti-sera were validated by western blot for specificity.

PK-15 cells were infected with virus at a MOI of 5, and harvested at different time points with RIPA buffer (Sigma-Aldrich, USA). Per sample, 20 μg of protein was loaded onto SDS-PAGE gels (10% acrylamide) and transferred to a Hybond-P membrane (GE, USA), which was subsequently blocked with 5% bovine serum albumin diluted in Tris-buffered saline, supplemented with 0.05% Tween (TBS-T). The blocking reagent was removed, and membranes were rinsed three times in TBS-T for at least 15 min each time. Primary antibodies were incubated at 4 °C overnight. Rabbit anti-PRV IE180 peptide polyclonal antibody was used at a dilution of 1:500. Mouse anti-PRV EP0 polyclonal antibody from Dr. Chun-mei Ju was used at a dilution of 1:500. Mouse anti-GAPDH monoclonal antibody (CWBIO, China) was diluted at 1:5000. Primary antibody was removed, and incubated with secondary antibodies conjugated with horseradish peroxidase (HRP) for 3 h at room temperature. Bands were detected by chemiluminescence using the ECL Prime kit (GE) and visualized using a ChemiDoc MP imager (Bio-Rad, USA) according to the manufacturer's instructions.

2.6. RNA extraction and northern blot analysis

Prv-miR-LLT1 was detected by northern blot analysis (Wu et al., 2012). Briefly, 20 μg of extracted total RNA mixture for prv-miR-LLT1 was electrophoresed in 15% polyacrylamide gel containing 8 M urea. The separated RNA was transferred completely to Hybond-N + nylon membrane (GE, USA), and cross-linked with UV light. DNA probes with sequences complementary to prv-miR-LLT1 (5′-GCGACGGACCCAGGG GTGAGA-3′) and U6 (5′-AAAATATGGAACGCTTCACGAA-3′) were perfectly labeled with [γ-^{32}P] ATP (Wu et al., 2012). The cyclone storage phosphor system was used to detect the northern blot signal.

2.7. RT-qPCR analyses

An RT-qPCR analysis was performed as described previously (Mahjoub et al., 2015) with some modifications. PK-15 cells were infected with virus at a MOI of 5, and total RNA was harvested at 12 hpi by Trizol (Invitrogen, USA). Per sample, 1 μg of total RNA was reverse