Gene 575 (2016) 101–107

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Research paper

In vivo expression of human cytomegalovirus (HCMV) microRNAs during latency

Mesfin K. Meshesha [a], Zvi Bentwich [a], Semaria A. Solomon [a], Yonat Shemer Avni [a,b,*]

[a] The Shraga Segal Department of Microbiology, Immunology and Genetics, Faculty of Health Sciences, Ben Gurion University of the Negev, Beer Sheva 84105, Israel
[b] Laboratory for Clinical Virology, Soroka University Medical Center, Faculty of Health Sciences, Ben Gurion University of the Negev, Beer Sheva 84105, Israel

ARTICLE INFO

Article history:
Received 28 January 2015
Received in revised form 18 August 2015
Accepted 20 August 2015
Available online 21 August 2015

Keywords:
HCMV
MicroRNA
Latency
Monocytes
PBMC
Cytomegalovirus

ABSTRACT

Viral encoded microRNAs play key roles in regulating gene expression and the life cycle of human herpes viruses. Latency is one of the hallmarks of the human cytomegalovirus (HCMV or HHV5) life cycle, and its control may have immense practical applications. The present study aims to identify HCMV encoded microRNAs during the latency phase of the virus. We used a highly sensitive real time PCR (RTPCR) assay that involves a pre-amplification step before RTPCR. It can detect HCMV encoded microRNAs (miRNAs) during latency in purified monocytes and PBMCs from HCMV IgG positive donors and in latently infected monocytic THP-1 cell lines. During the latency phase, only eight HCMV encoded microRNAs were detected in PBMCs, monocytes and in the THP-1 cells. Five originated from the UL region of the virus genome and three from the US region. Reactivation of the virus from latency, in monocytes obtained from the same donor, using dexamethasone restored the expression of all known HCMV encoded miRNAs including those that were absent during latency. We observed a shift in the abundance of the two arms of mir-US29 between the productive and latency stages of the viral life cycle, suggesting that the star "passenger" form of this microRNA is preferentially expressed during latency. As a whole, our study demonstrates that HCMV expresses during the latency phase, both in vivo and in vitro, only a subset of its microRNAs, which may indicate that they play an important role in maintenance and reactivation of latency.

1. Introduction

Human cytomegalovirus (HCMV or HHV5) belongs to the beta subfamily of herpesviridae characterized by a linear dsDNA (Fields et al., 2001). HCMV is ubiquitous and a highly prevalent human pathogen, usually acquired at an early age. Primary HCMV infection in the immunocompetent host is usually asymptomatic and rarely causes illness. However, HCMV is a major public health challenge in immunocompromised patients such as those with HIV and in patients receiving immune-suppressant medications due to organ-transplantation. Furthermore, congenital HCMV is a leading cause of birth defects such as deafness, and neurodevelopmental aberrations (Beck et al., 2010; Cheeran et al., 2009; Schleiss, 2009; Crough and Khanna, 2009).

MicroRNAs are short non-coding regulatory RNAs. Many DNA viruses employ this stealth system to establish infection within the host environment by targeting host and their own target messenger RNAs, which has been previously reviewed in detail (Grundhoff and Sullivan, 2011). Over 250 virally encoded microRNAs have been documented in the miRBase, mostly from the herpesviruses (Griffiths-Jones, 2006). Like many other members of the herpesviruses, HCMV also encodes for microRNAs. The first 11 microRNA precursors from HCMV were discovered using cloning techniques and computational approaches (Pfeffer et al., 2005; Dunn et al., 2005; Grey et al., 2005). Using deep sequencing of short RNAs from HCMV infected Human Foreskin Fibroblast (HFF) cells, we and others discovered ten additional microRNAs of which six were from four novel precursors and four were from already known precursors (Meshesha et al., 2012; Stark et al., 2012). To date, 26 mature microRNAs of HCMV have been deposited in the miRBase and are scattered along both strands of its genome. Five pre-miRNAs originate from intergenic regions, another four are transcribed antisense to ORFs of annotated genes and mir-UL36 is encoded within an intron of the UL36 gene, (see reviews Hook et al. (2014); Dhuruvasan et al. (2011)).

Abbreviations: cDNA, DNA complementary to RNA; dsDNA, double stranded DNA; EBV, Epstein–Barr virus; GB, major envelop glycoprotein gene; HCMV, human cytomegalovirus; HFF, human foreskin fibroblast cells; HIV, human immunodeficiency virus; IE, immediate early; Ig, immunoglobulin; LUNA, latency unique nuclear antigen; MIEP, major immediate early promotor; miRNAs, microRNAs; MOI, multiplicity of infection; mRNA, messenger RNA; PBMC, peripheral mononuclear cells; PBS, phosphate buffer saline; PCR, polymerase chain reaction; PMA, phorbol 12-myristate 13-acetate; pre-miRNA, microRNA precursor; qPCR, quantitative polymerase chain reaction; qRTPCR, quantitative real time polymerase chain reaction; RISC, RNA-Induced Silencing Complex; RT, reverse transcriptase; RTPCR, real time polymerase chain reaction; UL, unique long; UL81-82as, unique long 81-82 antisense; US, unique short; vIL-10, CMV interleukin.

* Corresponding author at: Department of Virology and Molecular Genetics, Faculty of Health Sciences, Ben Gurion University of the Negev, Beer Sheva 84105, Israel.
E-mail address: yonat@bgu.ac.il (Y.S. Avni).

http://dx.doi.org/10.1016/j.gene.2015.08.040
0378-1119/

All of the HCMV microRNAs discovered so far have been detected in infected fibroblast cells during productive infections. This has been mostly due to lack of appropriate cell-lines or in vivo animal models for studying HCMV latency. The exact location of HCMV latency has not yet been determined but cells of the myeloid lineage such as CD14+ monocytes (Hargett and Shenk, 2010; Taylor-Wiedeman et al., 1991), CD34+ hematopoietic stem cells (Mendelson et al., 1996; Weekes et al., 2013) and immature dendritic cells (Senechal et al., 2004) have all been shown to carry viral genomes without virus replication, as is the case during latency. However, since virus harboring monocytes are quite rare (0.004% to 0.02%) (Slobedman and Mocarski, 1999) an in vivo study of HCMV latency is still a difficult task. Recently, THP-1 monocyte-like cell lines have been described that support HCMV latency and share a number of similar characteristics with infected monocytes, such as methylation patterns on the Major Immediate Early Promoter (MIEP) of latent HCMV (Saffert and Kalejta, 2006). In addition, infected THP-1 cells harbor viral DNA in the absence of both virus replication and genes associated with productive infection such as the Immediate Early (IE1 and IE2), Early genes and Late genes such as *UL55* (Smuda et al., 1997). A number of HCMV latency-associated transcripts have also been described in these cells, which together with the characteristics listed above, can serve as hallmarks of HCMV latency.

Understanding HCMV miRNA expression during the virus's latency phase offers great potential for therapy, as it may prevent reactivation of the virus from latency. Moreover, in vivo expression of microRNAs during latency has not been reported so far. In this study we present evidence that HCMV expresses a subset of its microRNAs during its latent phase in freshly isolated PBMC and monocytes of latently infected individuals, and demonstrate that the in vitro model of HCMV infected THP-1 monocytic cell lines lends support to studies of microRNAs during latency.

2. Materials and methods

2.1. Cells and viruses

Low passage clinical isolate of HCMV was propagated on MRC-5 and human foreskin fibroblast (HFF) cells and aliquots were kept in −70 °C. Viral titers were determined using CMV IFA kit (Light Diagnostics, USA). HFF cells were cultured in BIO-AMF complete media (Biological Industries, BeitHaemek, Israel). MRC-5 cells were grown in BIO-AMF complete media (Biological Industries, Beit-Haemek, Israel) supplemented with 10% fetal bovine serum (Gemini), 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.292 mg/ml glutamine (Biological Industries, BeitHaemek, Israel).

2.2. Monocytes

Twenty ml of whole blood was collected in EDTA tubes from three volunteers (age of S1, S2 and S3, was 36, 34 and 62 years old, respectively). Donors' sera were tested for CMV-IgG and CMV-IgM antibodies status (DiaSorin, Italy), and for CMV-DNA (RealStar CMV PCR Kit, Altona, Germany). Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-Hypaque (Nycomed Pharma, Oslo, Norway) density gradient centrifugation. Monocytes were isolated from human PBMCs using anti-CD14 magnetic bead separation (Miltenyi Biotec GmbH) according to the manufacturer's instructions, or alternatively by adherence. Briefly: PBMC were incubated for 1 h in PBS at 37 °C, 5% CO_2 in a 6 well plate. Non-adherent cells were then aspirated and adherent cells were further incubated in RPMI for 3 h followed by excess washing with PBS to remove residual lymphocytes. Purified monocytes and THP-1 cells were subjected to RNA extraction or cultured in the presence of RPMI 1640 medium (Invitrogen) supplemented with 10% heat inactivated fetal bovine serum (Gemini), 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.292 mg/ml glutamine (Beit-Haemek, Israel). To reactivate the virus from the monocytes of CMV-antibodies-positive donors, cells were first treated with 100 ng/ml phorbol 12-myristate 13-acetate (PMA). After 24 h, cells were inspected under microscope for adherence and differentiation. Differentiated monocytes were then cultured for 10 days in the presence of 600 nM dexamethasone.

THP-1 cells were infected at a MOI (multiplicity of infection) of 3 with a low-passage clinical isolate of HCMV, while HFF and MRC-5 cells were infected at an MOI of 2. HFF and MRC-5 cells were inoculated for 2 h at 37 °C on a rocker. After the incubation, the inoculum was removed, the cells were washed three times with sterile PBS and fresh medium was added. For the latency experiment, THP-1 cells and virus stock was centrifuge at 3000 r.p.m. for 90 min at 37 °C. After centrifugation, THP-1 cells were washed three times with sterile PBS, resuspended in fresh media and cultured in 25 cm^2 flask. To induce differentiation of THP-1 cells and monocytes, PMA was added into the culture media at a concentration of 100 ng/ml and the cells were transferred to 6 well-plates. In THP-1 cells differentiation was induced 10 days post infection.

2.3. Viral latency

To establish viral latency in vitro, THP-1 cells were infected in suspension as described above and infected cells were cultured for 10 days. Media was refreshed every 3 days. To confirm maintenance of latency, first, the presence of viral DNA in the cells but not in the culture supernatant was assayed by real-time quantitative PCR (qRT-PCR). Secondly, expression of latency-associated viral transcripts was measured by RT-PCR for: UL138, vIIL-10, and UL81-82as (LUNA). Thirdly, disappearance of immediate early and late viral transcripts (UL122 and UL55) and their reappearance following reactivation was determined.

2.4. Nucleic acid extraction and amplification

DNA was isolated from both culture supernatant (200 μl) and cells (5×10^4 cells in 200 μl of PBS) using a mini blood kit (QIAGEN, Hilden, Germany) and following the manufacturers protocol. RNA extraction was performed using TRI reagent (Sigma, St Luis, MO, USA) according to the manufacturer's instruction. Aliquots of total RNA were used directly for quantitation of gene expression by RT-PCR or subjected to small RNA library construction for microRNA profiling.

qRT-PCR was used to determine the efficiency of viral genome delivery to the infected THP-1 cells. Viral genomes were quantified with forward and reverse primers and a probe against the viral glycoprotein-B gene (Table 1). Unknown sample values were determined on the basis of a standard curve generated from a 10-fold serial dilution of known copy numbers of HCMV stocks. qRT-PCR reactions contained 5 μl of 200 μl extracted DNA, 5 μM primers, 2.5 μM probe, 12.5 μl LC480 master mix (Roche Diagnostics), and nuclease-free

Table 1
Forward and reverse primers and probes used for qRTPCR.

Genes	Primers and probe sequence
UL122	5′-GACCCTGATAATCCTGACCAGT-3′
	5′-CAACATAGTCTGCAGGAACG-3′
	5′-FAM-TGGCCTTGGTCACGGGTGTCTC-BHQ-1-3′
UL55	5′-TGGGCGAGGACAACGAA-3′
	5′-TGAGGCTGGGAAGCTGACAT-3′
	5′-FAM-TGGGCAACCACCGCACTGAGG-BHQ-1-3′
LUNA	5′-ATGACCTCTCCTCCACACC-3′
	5′-GACGCTATATTTAGGGCTTCC-3′
UL138	5′-ACGGGTTTCAACAGATCGAC-3′
	5′-TGCGCATGTTTTTGAGCTAC-3′
Vl-IL10	5′-TGTTGAGGCGGTATCTGGAGA-3′
	5′-CCGTCTTGAGTCCGGGATAG-3′
β-actin	5′-CATTGCCGACGGATGCA-3′
	5′-GCCGATCCACACGGAGTACT-3′