Virus Research 144 (2009) 27–34

Contents lists available at ScienceDirect

Virus Research

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

Identification of RNA instability elements in Borna disease virus

Ulrike Siemetzki[a,b], Mundrigi S. Ashok[a,c], Thomas Briese[a], W. Ian Lipkin[a,*]

[a] Center for Infection and Immunity, Mailman School of Public Health, Columbia University, New York, NY 10032, USA
[b] NYC DOHMH Public Health Laboratory - Virus Identification Unit, New York, NY 10016, USA
[c] Sartorius Stedim India Pvt Ltd., Bangalore 562123, India

ARTICLE INFO

Article history:
Received 17 February 2008
Received in revised form 24 March 2009
Accepted 26 March 2009
Available online 5 April 2009

Keywords:
Borna disease virus
RNA instability
Gene expression
Transcription

ABSTRACT

Genome organization and gene expression of Borna disease virus (BDV) are remarkable for the overlap of open reading frames, transcription units and transcription signals, readthrough of transcription termination signals, differential use of translation initiation codons, and exploitation of the cellular splicing machinery. Here we report an additional control of gene expression at the level of mRNA stability. Levels of BDV proteins in infected cells do not correspond to the transcriptional gradient typically observed in non-segmented negative-sense RNA viruses. The third transcription unit of BDV's negative-sense RNA genome encodes viral proteins M, G and L. Analysis of the third transcription unit identified RNA-destabilizing domains with the most pronounced activity located in regions spanning nucleotides 2818–2918 (instability domain-1) and 4022–4071 (instability domain-2). Given that one domain maps to intron-2 and is thereby eliminated upon splicing, this represents an intriguing mechanism for regulating transcript levels independent of a transcriptional gradient. The presence of instability domains in introns offers a mechanism to create the observed discontinuous gradient M > L > G, compatible with the non-cytopathic, persistent infection that is characteristic for BDV, and provides a rationale for the use of alternative splicing by this unusual virus.

1. Introduction

Borna disease virus (BDV), a nonsegmented, negative strand RNA virus, establishes persistent central nervous system (CNS) infection and causes behavioral disturbances in warm-blooded animals (Ludwig et al., 1988; Rott and Becht, 1995). Notable features of its molecular biology include replication and transcription in the nucleus (Briese et al., 1992; Carbone et al., 1991), overlap of open reading frames (ORFs) and transcription units (Briese et al., 1994; Cubitt et al., 1994a; Schneemann et al., 1994), RNA splicing and differential use of transcription termination sites and translation initiation codons (Cubitt et al., 1994b; Schneemann et al., 1994; Schneider et al., 1997a, 1994), and the requirement for phosphorylation by kinases with limited distribution within the CNS (Schwemmle et al., 1997).

The BDV genome is organized into three transcription units. The first transcription unit codes for the viral nucleoprotein (N, p38/40). The second unit contains in overlapping reading frames coding sequences for proteins X (p10) and P (phosphoprotein, p23). The matrix protein (M, p16), the type I membrane glycoprotein (G, p57, gp94) and the RNA-dependent RNA polymerase (L, p190) are encoded by the third transcription unit (Schneemann et al., 1994; Walker et al., 2000). The first two transcripts are found at similar levels in infected cells and tissues, whereas the third transcript is expressed at lower levels (Briese et al., 1994; Walker et al., 2000). Thus, the 5′ to 3′ transcriptional gradient observed in other non-segmented, negative strand RNA viruses (Abraham and Banerjee, 1976) is modified in BDV.

A potential mechanism to explain the marked reduction in levels of RNA transcripts originating from the third transcription unit may be the presence of negative regulatory elements. RNA instability elements play crucial roles in the regulation of eukaryotic gene expression (Tourriere et al., 2002), and have been demonstrated in several viral systems (Maldarelli et al., 1991; Nasioulas et al., 1994; Saiga et al., 1997; Schneider et al., 1997c; Schwartz et al., 1992; Sokolowski et al., 1999; Sokolowski and Schwartz, 2001). Although such sequences are frequently located in untranslated regions (UTRs) and comprise AU-rich elements (AREs), destabilizing domains are also found in coding sequences and may not involve AREs (Sokolowski et al., 1998).

In previous work we noted that only low levels of BDV G or L protein were obtained with eukaryotic expression plasmids (Walker et al., 2000). These results, together with the observation that low levels of mRNAs derived from the third transcription unit and their

* Corresponding author at: Center for Infection and Immunity, Mailman School of Public Health of Columbia University, 722 West 168th Street, Room 1801, New York, NY 10032, USA. Tel.: +1 212 342 9033; fax: +1 212 342 9044.
E-mail address: wil2001@columbia.edu (W.I. Lipkin).

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doi:10.1016/j.virusres.2009.03.016

cognate proteins are present during BDV infection *in vitro* and *in vivo*, led us to speculate that BDV might regulate gene expression through RNA-destabilizing sequences not related to AREs.

2. Methods

2.1. Plasmid constructs

All vectors used in this study were generated from pcDNA3 (Invitrogen, Carlsbad, CA, USA). The firefly luciferase gene was inserted downstream of the CMV promoter between restriction sites BamHI and EcoRV. The downstream NotI and XhoI sites were used to accommodate constructs representing the BDV third transcription unit, or non-specific DNA obtained from *West Nile virus* (nt 8870–11027; Genbank accession number AF196835), respectively. As an internal control for transfection efficiency we used the pRL-TK *Renilla* luciferase vector (Promega, Madison, WI, USA). The vector contains *Renilla* luciferase cDNA under the control of the herpes simplex virus thymidine kinase promoter to provide low to moderate levels of *Renilla* luciferase expression in co-transfected mammalian cells.

2.2. Cell transfection and Luciferase assay

Oligodendrocytes (OL) were cultured in Dulbecco's modified Eagles Medium (DMEM) with 10% fetal calf serum, 5 mM L-glutamine, 10,000 units/ml penicillin G and 10 mg/ml streptomycin at 37 °C and 5% CO_2 and seeded in 24-well plates at a density of 1×10^5 cells/well for transfection. Transient transfection of the cells was performed using 1 μg total DNA consisting of 200 ng of the respective plasmid DNA construct, 50 ng *Renilla* luciferase plasmid DNA and 750 ng pBluescript II SK(+) (Stratagene, La Jolla, CA, USA) carrier DNA to adjust for total DNA amount. Transfection was carried out in 700 μl Opti-MEM with 3 μl Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA). Cells were incubated for 12 h before the medium was replaced. After another 8 h the cells were harvested for luciferase or RNA analyses. Luciferase activity in cell extracts was determined using the Dual Luciferase Reporter Assay System (Promega, Madison, WI, USA).

2.3. RNA extraction, reverse transcription (RT) and real-time polymerase chain reaction (PCR)

Total RNA was extracted using TRI Reagent (Molecular Research Center, Cincinnati, OH, USA). RNA pellets were resuspended in 40 μl H_2O and treated with DNase I (Ambion, Austin, TX, USA) at 37 °C for 2 h. Reverse transcription was carried out using 200 ng total RNA with random hexamers in a total volume of 22 μl using TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed in an ABI PRISM 7700 Sequence Detector (Applied Biosystems) using 5 μl cDNA in a reaction volume of 25 μl containing 300 nM of each primer, 200 nM of probe and 1× real-time PCR mix (TaqMan Universal PCR Master Mix; Applied Biosystems). Activation of UNG (2 min at 50 °C) and AmpliTaq (10 min at 95 °C) was followed by 45 cycles of PCR (15 s at 95 °C and 1 min at 60 °C). Each cDNA sample was analyzed in duplicate. Primers used for detection of luciferase transcripts were Luc-1582F (5′-TCG AAG TAT TCC GCG TAC GTG) and Luc-1655R (5′-GCC CTG GTT CCT GGA ACA A) and the probe Luc-1604T (5′-FAM-TGT TCA CCT CGA TAT GTG CAT CTG TAA AAG CA-TAMRA). For the amplification of porphobilinogen sequence as an internal standard, primers were PD10-76F (5′-ATT CGG GGA AAC CTC AAC ACC) and PD11-229R (5′-GGC CCA CAG CAT ACA TGC AT) and the probe was PDT-174T (5′-TET-AGG ATC TGC CCA ACC CGG TTG TGC-TAMRA).

2.4. Northern Blot analysis

To generate a probe for Northern Blot analysis a 370 nucleotide (nt) fragment of the luciferase gene was amplified with the primers luc1051fwd (5′-TCTGACGCAGGCAGTTCTATG-3′) and luc1421rev (5′-GCGTTATTTATCGGAGTTG-3′). The amplicon was cloned into TOPO TA (Invitrogen, Carlsbad, CA, USA) and the plasmid linearized by restriction digest at the 5′-end of the luciferase fragment. The linearized plasmid was then transcribed *in vitro* with the MAXIscript® SP6/T7 Kit (Ambion, Austin, TX, USA) including 10 mM biotinylated UTP (Ambion).

RNA from transfected cells was size-fractionated by electrophoresis in a 1% formaldehyde gel and transferred to a positively charged nylon membrane (Ambion, BrightStar®-Plus Positively Charged Nylon Membrane; Ambion, NorthernMax® One-Hour Transfer Buffer). The RNA was cross-linked and prehybridized for 2 h at 65 °C (5.85 ml H_2O, 500 μl 100× Denhardt's solution, 250 μl 20% SDS, 100 μl salmon sperm DNA (10 mg/ml), 100 μl yeast t-RNA (10 mg/ml), 200 μl 0.5 M EDTA, and 3.0 ml 20× SSC). Thereafter, 100 ng of the *in vitro* transcribed RNA probe was added and hybridized overnight at 65 °C. Subsequent wash steps included 2 washes with 2× SSC and 0.1% SDS for 5 min each and 2 washes with 0.1× SSC and 0.1% SDS for 15 min each. Hybridization signal was detected using the BrightStar® BioDetect™ Kit (Ambion). Membranes were exposed to film (biomax light film; Kodak, Rochester, NY, USA) and using Quantity One software (Bio Rad, Hercules, CA, USA).

3. Results

3.1. The third transcription unit of BDV contains sequences that modulate RNA expression

BDV uses alternative splicing to generate a series of transcripts from its third transcription unit. The presence or absence of destabilizing sequences in these transcripts may contribute to the regulation of their relative abundance. To investigate the presence of sequences that potentially destabilize transcripts of the third transcription unit, we initially analyzed a construct representing the primary 2.8 kb transcript. A series of 5′ to 3′, and 3′ to 5′ deletion constructs were created between nt positions 1885 and 4154 of BDV strain V (Genbank accession number NC_001607) and cloned downstream of the firefly luciferase gene in pcDNA Luc (Fig. 1A). Individual constructs were transfected into oligodendrocytes (OL cells) along with a *Renilla* luciferase expressing plasmid to control for transfection efficiency.

Constructs spanning the entire region from nt 1888 to 4154 and 1885 to 4152 resulted in a significant reduction of reporter signal when compared to the empty vector (B6, 22%; BR2, 15%; Fig. 1B). Analysis of the 5′ and 3′ deletion construct series indicated several critical domains: effects on reporter signal was observed for nt 3871–4154, and nt 2263–2918 (3′ deletion series); and for nt 2913–3650 and nt 3650–3855 (5′ deletion series). These findings suggested that the function of some of these domains is modulated by the presence or absence of neighboring sequences.

3.2. Mapping of destabilizing regions

The region spanning nt 2263–2918 that resulted in a reduction of reporter signal was further mapped using an additional set of deletion constructs (BR1.1 through BR1.7; Fig. 2A). Comparison of constructs BR1.1 and BR1.2 implicated a region between nt 2318 and 2418. Comparison of constructs BR1.6 and BR 1.7 implicated a region between nt 2818 and 2918. Comparison of the empty vector (Luc) and a construct comprising nt 2918–3871 revealed no dra-