Identification of RNA instability elements in Borna disease virus

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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.

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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...
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 Xhol 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% CO2 and seeded in 24-well plates at a density of 1 × 104 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). RNA pellets were resuspended in 40 μl 1× saline sodium citrate (SSC) containing 0.1% SDS for 15 min each. Hybridization signal was detected using the BrightStar ® BioDetectTM Kit (Ambion). Membranes were exposed to film (biomax light film; Kodak, Rochester, NY, USA) and using Quantity One software (Bio Rad, Hercules, CA, 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 H2O 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 containing 10,000 units/ml penicillin G and 10 mg/ml streptomycin at 37 °C and 5% CO2, and seeded in 24-well plates at a density of 1 × 104 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, 500 ng Renilla luciferase plasmid DNA and 750 ng pBluescript II SK(+) (Stratagene, La Jolla, CA, USA) plasmid DNA to adjust for total DNA amount. Transfection was carried out in 700 μl Opti-MEM with 3 μl LipofectamineTM 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).

2.4. 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′-TCAGCCGAGCGGATCCTGAT-3′) and luc1421Rev (5′-CGCTTATATCAGGATGTT-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 MAXiScribe® SP6/T7 Kit (Ambion, Austin, TX, USA) containing 10 mM biotinylated UTP (Ambion). RNA from transfected cells was size-fractionated by electrophoresis in a 1% formaldehyde gel and transferred to 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 H2O, 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® BioDetectTM 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 1885 to 4152 resulting in a significant reduction of reporter signal compared to the empty vector (B6, 22%; BR2, 15%; Fig. 1B). 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® BioDetectTM Kit (Ambion). Membranes were exposed to film (biomax light film; Kodak, Rochester, NY, USA) and using Quantity One software (Bio Rad, Hercules, CA, USA).

3.2. 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 2318 and 2418. Comparison of the empty vector (Luc) and a construct comprising nt 2918–3871 revealed no dra-
Fig. 1. (A) BDV genome organization and schematic of deletion constructs of the third transcription unit that were cloned downstream of the firefly luciferase gene under the control of a CMV promoter. (B) Luciferase assays of oligodendrocytes transfected with pLucB2 (B2), pLucB3 (B3), pLucB4 (B4), pLucB5 (B5) and pLucB6 (B6) (left panel) and with pLucBR2 (BR2), pLucBR3 (BR3), pLucBR4 (BR4), pLucBR5 (BR5) and pLucBR6 (BR6) (right panel). The value of firefly luciferase expression with the empty vector pLuc (Luc) was set at 100% (7689 ± 34 light units). Values in arbitrary light units were standardized with Renilla luciferase as internal control.

3.3. Analysis of instability domain activity

The two regions containing sequences for which the most pronounced reduction in reporter activity was observed (nt 2818–2918 and nt 4022–4071) were either deleted from the original BDV construct B6 (Fig. 3A), or cloned into an unrelated West Nile virus (WNV) sequence background (Luc2000) (Fig. 3B). To eliminate potential for differences in stability due to variations in length of BDV sequence, nt 3986–4072 (containing nt 4022–4071) and nt 2818–2918 were cloned into the WNV sequence background at a distance from one another similar to that in BDV. These insertions resulted in a 34% reduction in reporter signal (pLuc2000+1+2; Fig. 3C). Similarly, deletion of nt 2818–2918 and nt 3986–4072 from the BDV pLucB6 construct resulted in an increase in reporter signal...
from 17% to 64% (construct dIE1+2; Fig. 3C). Individual deletion of either domain alone from the BDV construct pLucB6 restored the reporter signal to an approximately similar level (dIE1 and dIE2; Fig. 3C). This result confirmed a synergistic interaction of the two domains, nt 2818–2918 (IE1) and nt 3986–4072 (IE2).

3.4. Reduced reporter gene expression reflects decreased mRNA levels

The capacity of IE1 and IE2 to influence RNA transcript levels was further tested through quantitation of the luciferase-containing transcripts by real-time PCR. Cells transfected with pLucB6 showed only 26% of the luciferase-containing RNA compared to cells transfected with the empty pLuc construct, whereas deletion of IE1 and IE2 from the pLucB6 construct (dIE1+2) resulted in an increase of luciferase-containing RNA to 73% in comparison to the pLuc control (Table 1). Similar results were obtained with IE1 and IE2 cloned into the unrelated WNV background. Analysis of cells transfected with pLuc2000+1+2 revealed a 60% decrease in luciferase-containing mRNA expression compared to cells transfected with the pLuc2000 control (Table 1). The correlation between the total RNA copy number and observed luciferase activity confirms that reduction in the luciferase reporter signal indeed reflects reduced mRNA levels rather than RNA retention in the nucleus. Northern Blot analyses further confirmed this observation; hybridization signal of the luciferase RNA probe showed that band intensity was reduced to 27% in B6 transfected cells compared to cells that were transfected with pLuc control plasmid (Fig. 4). The deletion of the destabilizing domains IE1 and IE2 from the B6 construct resulted in an intensity of 57%.

3.5. Differential regulation of RNA stability through alternative splicing of instability domains

Given that IE1 is located in intron-2, we hypothesized that alternative splicing might contribute to the control of BDV gene
Fig. 3. (A) Map of the BDV third transcription unit with nt regions 2818–2918 and 3986–4072 indicated. (B) Both or individual regions were deleted from plucB6 and constructs cloned downstream of the firefly luciferase gene, resulting in pluc-dIE1 (BDV 1888–4154 with deletion of nt 2818–2918), pluc-dIE2 (BDV 1888–4154 with deletion of nt 3986–4072) and pluc-dIE1+2 (BDV 1888–4154 with deletion of nt 2818–2918 and 3986–4072). A non-specific sequence derived from West Nile virus was cloned downstream of the luciferase gene (pLuc2000) and the BDV regions nt 2818–2918 and nt 3986–4072 were introduced at a similar distance from one another as found in BDV resulting in pLuc2000+1+2. (C) The left panel shows Luciferase assay of oligodendrocytes transfected with pLuc, pLucB6, pLuc-dIE1, pLuc-dIE2, pLuc-dIE1+2 and pLuc2000. The right panel shows the Luciferase assay of cells transfected with pLuc2000 and pLuc2000+1+2. In both panels the expression of the unmodified luciferase clone (6785 ± 52 light units) or the luciferase clone with the non-specific sequence (6337 ± 41) was set to 100%, respectively. Values in arbitrary light units were standardized with Renilla luciferase as internal control.

Table 1

<table>
<thead>
<tr>
<th>Plasmid</th>
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<th>% b</th>
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</tr>
<tr>
<td>pLucB6</td>
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<td>26.1</td>
</tr>
<tr>
<td>pLuc-dIE1+2</td>
<td>1733.2</td>
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<tr>
<td>pLuc2000</td>
<td>9037.3</td>
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<tr>
<td>pLuc2000+1+2</td>
<td>3620.4</td>
<td>40.1</td>
</tr>
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</table>

| b Quantitated by real-time PCR and normalized to cellular porphobilinogen mRNA.
| b Relative level in reference to empty vector plasmid pLuc (100%).

expression through effects on the interaction between IE1 and IE2. We therefore analyzed the relative stability of alternative splice products generated from the 2.8 kb primary transcript of the BDV third transcription unit (1.4, 1.5, and 2.6 kb).

The primary 2.8 kb transcript and its 2.6, 1.5, 1.4 kb splice products were cloned downstream of the luciferase reporter gene to generate constructs pLuc2.8, pLuc2.6, pLuc1.5, and pLuc1.4, respectively (Fig. 5A). Consistent with the results for plucB6/plucBR2 in the previous experiments (Fig. 1B), the BDV 2.8 kb construct pLuc2.8 resulted in a reduction of reporter signal to 15% of the control values (Fig. 5B). The pLuc2.6 construct, which contains both IE1 and IE2 but lacks intron-1 (nt 1932–2025), showed a comparable reduction in reporter signal. In contrast, the pLuc1.5 construct, which lacks IE1
due to splicing and removal of intron-2, showed only a reduction in reporter signal to 60% of the control, which was again consistent with the results in the previous experiment where constructs comprised deletion of IE1 or IE2 from the BDV sequence (Fig. 3C). A higher reduction to 41% of the control was observed with the pLuc1.4 construct, which lacks both introns (Fig. 5B). This suggests that an additional region in intron-1, included in nt 1888–2263 (see B2 in Fig. 1), may participate in modulating RNA expression.

4. Discussion

mRNA turnover plays an important role in the control of eukaryotic gene expression. The process of transcript degradation typically includes a poly(A)-shortening endonucleolytic cleavage that is controlled by trans-acting factors binding to cis-acting elements within the 3′-UTR of mRNAs (Brewer, 1999; Couttet et al., 1997; Korner et al., 1998). However, in the case of mammalian c-fos, c-myc, and β-tublin, mRNAs encode instability determinants in their coding regions (Bernstein et al., 1992; Gay et al., 1987; Herrick and Ross, 1994; Schiavi et al., 1994; Shyu et al., 1991; Yen et al., 1988). RNA instability elements comprising AU-rich elements (AREs) (Wilson and Brewer, 1999) and other instability determinants have also been described in coding regions of Human immunodeficiency virus type 1 (HIV-1) (Lee and Rossi, 2004; Maldarelli et al., 1991), Human papillomavirus type 16 (HPV-16) (Sokolowski et al., 1998), and Human T-cell leukemia virus type 1 (HTLV-1) (Saiga et al., 1997). In HIV-1, mRNA instability elements are found in Gag/Pol and Env transcripts (Cochrane et al., 1991; Nasioulas et al., 1994; Schneider et al., 1997c; Schwartz et al., 1992), and interactions reported between the AU-rich instability element in the 5′-portion of gag and nuclear factors PSF and p54nrb (Zolotukhin et al., 2003). In HPV-16, instability elements are reported not only in the 3′-UTR (Sokolowski et al., 1999; Sokolowski and Schwartz, 2001; Wiklund et al., 2002), but also in the coding region where cis-acting AREs are located primarily in the 5′-half of the L1 gene (Tan et al., 1995); inhibitory elements lacking AUUUA or UUUUU motifs but displaying a 60% A + U content are located in the 5′-end of the L2 gene (Sokolowski and Schwartz, 2001; Sokolowski et al., 1998). HTLV-1 contains an RNA instability element within the pol region (Saiga et al., 1997), but although the HTLV-1 pol sequence contains a typical AU-motif, mutation of this motif does not affect RNA stability; thus, other, to be defined determinants must be implicated.

We have identified at least two RNA instability domains in the coding region of the third transcription unit of BDV. One of the domains is located within nt 2818–2918; the other within nt 4021–4072. These regions do not contain AUUUA or UUUUU motifs and show A + U contents of only 58% (IE1) and 48% (IE2). The activity of the domains is modified by context and modulated through synergistic interaction between the domains. Placement of IE1 and IE2 into a WNV sequence background (Luc2000/Luc2000+1+2)
resulted in a reduction in reporter signal by 34% rather than the 83% observed in the BDV sequence background. Likewise, deletion of nt 3871 located the main determinant. Genes Dev. 6 (4), 642–654.


