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A rapid method for gene expression analysis of Borna disease virus in neurons and astrocytes using laser microdissection and real-time RT-PCR

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Abstract

Laser microdissection combined with real-time RT-PCR represents a powerful method to analyse the transcription efficiency of defined cell types. Therefore, a RNA-preserving immunolabelling method was established to identify neurons and astrocytes in persistently BDV-infected rat brain sections for subsequent laser microdissection and quantitation of viral gene products by real-time RT-PCR. Firstly, to ensure an accurate measurement of viral RNA after immunolabelling, different reference genes (glyceraldehyde-3-phosphate dehydrogenase [GAPDH], succinate-ubiquinone reductase [SDHA], hypoxanthine phosphoribosyl-transferase-1 [HPRT]) were tested. Only normalisation with GAPDH yielded a stable relative expression of viral RNA encoding the nucleoprotein (BDV-N), the matrixprotein and the glycoprotein (intron I and intron II). The two remaining reference genes biased the ratios of BDV-transcripts in the immunolabelled brain sections significantly. Secondly, 100 immunolabelled neurons and astrocytes were harvested using laser microdissection and amplification of all viral transcripts revealed 681 and 168 (BDV-N), 573 and 254 (intron I), 324 and 133 (intron II) and 161 and 36 (GAPDH) absolute copy numbers in neurons and astrocytes, respectively. Thus, laser microdissection combined with real-time RT-PCR provides an effective tool for the analysis of cell-specific viral transcription efficiency and allows elucidating virus–host-interactions and virus persistence mechanisms in the CNS.

Keywords: Borna disease virus; Laser microdissection; Immunofluorescence; Neuron; Astrocyte; Real-time RT-PCR

1. Introduction

Borna disease virus (BDV) is the causative agent of the so called Borna disease, a mostly fatal neurological disorder of the central nervous system (CNS) which is characterised by a progressive meningoencephalomyelitis mainly affecting horses and sheep (Richt et al., 1995, 1997, 2007; Rott and Becht, 1995; Staeheli et al., 2000). Typically, BDV-infection leads to virus persistence in the CNS but the underlying regulatory strategies to achieve viral persistence and survival are incompletely understood. As an enveloped, negative-sense and single stranded RNA virus (Cubitt et al., 1994; Briese et al., 1994), BDV has been classified as the prototype of the newly established virus family *Bornaviridae* (Pringle, 1996) within the order *Mononegavirales*. The genome is arranged in a typical order of the *Mononegavirales* (Briese et al., 1994; Cubitt et al., 1994) comprising three transcription units with six major open reading frames (ORF). In 3′ to 5′ direction, the genome encodes the nucleoprotein (BDV-N), the negative regulator protein X (Wehner et al., 1997; Perez et al., 2003; Schneider et al., 2003), the phosphoprotein (BDV-P), the matrixprotein (BDV-M), the glycoprotein (BDV-GP) and at the 5′-end the viral polymerase (BDV-L; Briese et al., 1994; Cubitt et al., 1994; de la Torre, 2002; Tomonaga et al., 2002). Interestingly, the expression of BDV-M, BDV-GP and BDV-L is co-transcriptionally regulated by alternative splicing of intron I or intron II (Briese et al., 1994; Cubitt et al., 1994; Schneider et al., 1994; de la Torre, 2002; Tomonaga et al., 2002). In experimentally infected Lewis rats, viral RNAs and proteins are frequently found in neurons and astrocytes (Gosztonyi and Ludwig, 1995; Herden et al., 2000). The role of virus transcription efficiency in different host cells for the generation of virus persistence in the brain has not been

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elucidated so far. To date, this can be easily investigated by laser microdissection, which represents a smart and established research tool allowing the proper isolation of a homogeneous cell preparation from a complex tissue such as brain (Prosniak et al., 2003; Burbach et al., 2004). In combination with real-time RT-PCR, it is possible to analyse exactly viral RNA profiles in defined cell types *in vivo* (Schütze and Lahr, 1998). Recently, we were successful in isolating BDV-specific RNA from hematoxylin and eosin (H&E) stained rat hippocampus using laser microdissection (Porombka et al., 2006). However, common histochemical staining procedures are usually inadequate to differentiate especially astrocytes from the surrounding neuroparenchyma. Astrocytes and neurons can be definitely identified using immunolabelling protocols for specific marker proteins such as neuronal nuclei protein (NeuN) and glial fibrillary acidic protein (GFAP). Both have already been successfully applied for laser microdissection and subsequent RNA quantitation (Prosniak et al., 2003; Burbach et al., 2004). Usually, isolation of single cell provides only small amounts of RNA which limits the number of genes to be investigated. Therefore, only one reference gene is used for the accurate normalisation of relative RNA copy numbers in single cells to avoid unnecessary consumption of the valuable RNA. In the present report, we searched for (i) one reference gene for the reliable normalisation of BDV-specific transcripts in immunolabelled neurons and astrocytes and we were interested (ii) whether immunolabelled neurons and astrocytes yielded still enough RNA for cell specific viral RNA expression analysis using laser microdissection and pressure catapulting (LMPC) in combination with real-time RT-PCR. Therefore, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), succinate-ubiquinone reductase (SDHA) and hypoxanthine phosphoribosyl-transferase-1 (HPRT) were tested as potential reference genes ensuring a steady ratio of BDV-RNA copy numbers in immunolabelled brain sections compared with unlabelled brain tissues. Real-time RT-PCR analysis was performed using primer and double dye oligoprobe combinations corresponding to BDV-N-specific transcripts (BDV-N +ssRNA) and parts of the BDV-M ORF (designated as intron I +ssRNA) and BDV-GP ORF (designated as intron II +ssRNA). The reliable quantitation of BDV-RNA might serve as an important framework for the successful study of viral transcription efficiency in defined brain cell types. This, in turn, might improve essentially the knowledge of virus-host-interactions on a cellular level which possibly underlie and facilitate viral persistence strategies.

2. Materials and methods

2.1. Virus infection of rats

Thirty days old Lewis rats were infected intracerebrally in the left frontal cortex with 10^4 $ID_{50}\,ml^{-1}$ of infectious brain tissue (BDV strain 5/25/92) as described (Herden et al., 2005). Rat brains were collected in the chronic phase of the disease at 60 and 75 days post-infection (dpi; $n = 3$). Brains were immediately embedded in Tissue Tek® Compound, frozen and stored at −80 °C until use.

2.2. Preparing PEN-membrane slides

Cryo sections of BDV-infected rat brains were prepared as described (Porombka et al., 2006) to investigate the potential reliability of GAPDH, SDHA and HPRT as reference genes for the correct normalisation of BDV-transcript values in immunolabelled brain sections (brain samples deriving from rats killed at 60 dpi) and to determine the amount of BDV-N-, intron I- and intron II-RNA in neurons and astrocytes labelled by immunofluorescence (brain samples deriving from rats killed at 75 dpi). PEN-membrane slides (P.A.L.M. Microlaser Technologies AG, Bernried, Germany) were irradiated with UV light for 30 min, subsequently covered with 50 μl poly-L-lysin 0.1% (w/v) (Sigma–Aldrich Chemie GmbH, Munich, Germany) and air-dried for 30 min. Under RNase free conditions, serial transversal hippocampal sections (bregma −2.12 to −4.80; Paxinos and Watson, 1998) with a thickness of 6 μm were cut at −20 °C. The sections were mounted on PEN-membrane slides and fixed for 5 min in 70% icecold ethanol (Karl Roth GmbH + Co. KG, Karlsruhe, Germany) prepared with DEPC-treated water. All sections were air-dried for 10 min and stored separately at −80 °C until use.

2.3. Preparing brain sections for the evaluation of reference genes

2.3.1. Native brain tissue

To determine the amount of viral RNA and reference genes in unlabelled brain tissue, sections were thawed for 10 min at room temperature under a laminar flow. Subsequently, one brain section of each animal ($n = 3$; 60 dpi) was collected by using a sterile scalpel. Each brain section was transferred into a 1.5 ml collection tube containing 350 μl lysis buffer (Qiagen, Hilden, Germany). Samples were vortexed for 30 s and stored at −80 °C until RNA isolation.

2.3.2. Immunofluorescence labelling of neurons

To define the amount of BDV-specific RNA and cellular reference genes in the immunolabelled brain tissue, brain sections ($n = 3$; 60 dpi) were thawed for 10 min at room temperature under a laminar flow. Sections were blocked for 3 min with 1% bovine serum albumin in PBS pH 7.4 containing 3% Triton X-100. Brain sections were incubated with a monoclonal anti-neuronal nuclei (NeuN) antibody solution (Chemikon, Hampshire, UK, 1:50 in PBS), containing RNaseOut™ recombinant ribonuclease inhibitor (Invitrogen™, Karlsruhe, Germany) in a final concentration of 1 unit/μl for 5 min at room temperature. To remove the liquid, the antibody solution was decanted laterally and sections were covered three times with 600 μl PBS for 15 s. After incubation with a Cy3-conjugated goat anti-mouse secondary antibody (Dianova, Hamburg, Germany, 1:50 in PBS, pH 7.4) for 5 min in the dark at room temperature, sections were rinsed carefully three times with 600 μl PBS for 15 s. Sections were air-dried in the dark for 10 min and NeuN-labelled brain tissue was collected as described in Section 2.3.1. All liquids were prepared using DEPC-treated water.