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Original article

Development of a novel Borna disease virus reverse genetics system using RNA polymerase II promoter and SV40 nuclear import signal

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Abstract

Borna disease virus (BDV) is a noncytolytic, neurotropic RNA virus that replicates and transcribes in the nucleus of infected cells. Therefore, efficient synthesis of BDV RNA in the nucleus is critical for the development of a reverse genetics system for this virus. Here, we report the development of such a system using the RNA polymerase II (Pol II) promoter. The BDV minigenome cDNA was flanked by hammerhead ribozyme and hepatitis delta ribozyme sequences and inserted downstream of the Pol II promoter. To improve the efficacy of minigenome expression, we estimated the effects of several signal sequences within the minigenome constructs. We found that insertion of the SV40 nuclear import sequence into the Pol II constructs significantly enhances the replication of the minigenome even in cells lacking the SV40 large T antigen. This novel system is theoretically applicable to any mammalian cell line and would be valuable for analyzing host- or cell-type-dependent differences in BDV replication and production. We could demonstrate here the cell-type-dependent inhibitory effect of the viral protein X on BDV polymerase activity. This system may be useful for various research fields not only of BDV but also of other negative-sense RNA viruses.

Keywords: Borna disease virus; Negative-strand RNA virus; Reverse genetics; RNA polymerase II promoter; SV40 nuclear import signal

1. Introduction

Borna disease virus (BDV) is an enveloped virus with a nonsegmented, negative-strand RNA genome that causes highly neurotropic and noncytopathic infections [1,2]. This virus is unique among known animal mononegaviruses in that it has a nuclear site for the replication and transcription of its genome. At least six different proteins are encoded in the BDV genome. Based on their positions in the viral genome, these proteins are considered the counterparts of nucleoprotein (N), phosphoprotein (P), matrix protein (M), surface glycoprotein (G) and RNA-dependent RNA polymerase (L), respectively, found in other mononegaviruses. A small open-reading frame (ORF), which overlaps the P ORF, encodes another major protein (X) of BDV [3,4]. Recent reports have demonstrated that X may be a negative regulator of BDV polymerase activity [5,6].

As with other RNA viruses, BDV needs to form a ribonucleoprotein (RNP) complex, which consists of the genomic RNA encapsidated by N, in order to replicate in mammalian cells. This complex is the minimum unit of infectivity, and the synthesis of individual mRNAs or full-length antigenomic RNAs is initiated with viral polymerase unit, containing L and P. Thus, coexpression of the N, P and L with a synthetic

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negative-strand RNA genome will require reconstituting the biologically active BDV RNP. Recently, a reverse genetics system for BDV was established to analyze the viral gene activities in replication and transcription and to artificially produce this virus [5–7]. As a result, the rescue of functional nucleocapsid complexes from cloned cDNAs has been reported for all of the mononegavirus families [8,9]. In most systems, including that for BDV, the synthesis of negative-sense viral RNAs from cDNA clones is conducted using the RNA polymerase I (Pol I) or T7 RNA polymerase (T7) promoter [8–11]. Both the Pol I and T7 promoter systems were highly efficient for the generation of negative-sense viral RNAs, but appear to contain restrictions in the selection of cell types used for the transfection of cloned viral cDNAs. In the T7 system, infection with a recombinant vaccinia virus, vTF7-3 [12], or use of a constitutively expressing cell line is inevitably required to supply T7 RNA polymerase. Besides, the species-specific recognition of the Pol I promoter by a transcription factor narrows the range of cell types that can be used for the production of viral RNAs [13]. In addition, since BDV replicates and transcribes in the nucleus, the efficient production or nuclear import of the minigenome RNA into the nucleus could be critical for the replication of the synthesized BDV RNA [1,2]. Because T7 RNA polymerase functions in the cytoplasm, the BDV minigenome expressed by the T7 promoter must enter the nucleus after the production in the cytoplasm to initiate replication. Furthermore, although Pol I accounts for as much as 80% of all the transcriptional activity in the nucleus [14,15], this system may not be adequate to express a long, single RNA molecule, such as a mononegavirus genome.

In the present study, to generate an efficient system of BDV reverse genetics, we cloned a cDNA encoding the reporter protein, chloramphenicol acetyltransferase (CAT), in the negative-sense orientation between the 5′ (trailer) and 3′ (leader) untranslated region (UTR) sequences of the BDV genome. The BDV minigenome cDNA was flanked by hammerhead ribozyme (HamRz) and hepatitis delta ribozyme (HdRz) sequences and inserted downstream of the RNA polymerase II (Pol II) promoter, such as cytomegalovirus (CMV) immediate-early promoter, and a hybrid promoter composed of the CMV immediate-early enhancer fused to the chicken β-actin promoter (CAG) [16]. This novel reverse genetics system of BDV does not require T7 RNA polymerase and, in principle, is applicable to all mammalian cell lines. To improve the efficiency of minigenome expression in the nucleus, we estimated the effects of several signal sequences, including HamRz, the simian virus 40 (SV40) origin/promoter sequence and polyadenylation (poly-A) signal, within the minigenome constructs. We suggested that the SV40 origin sequence, which encodes the plasmid nuclear import signal, increases the nuclear level of plasmid DNAs, leading to the efficient replication of the BDV minigenome even in the SV40 large T antigen (LT)-negative cell lines. This method could be useful not only for the development of production of recombinant BDV in mammalian cells, but also for a comparison of the replication properties of BDV in various cell lines from different species. Using this novel system, we demonstrated that the viral protein X exhibits a cell-type-specific inhibitory effect on BDV polymerase activity.

2. Materials and methods

2.1. Virus and cell lines

The OL cell line derived from a human oligodendroglioma [17] was cultured in Dulbecco's modified Eagle's medium (DMEM)-high glucose (4.5%) supplemented with 10% fetal bovine serum (FBS), 100 U of penicillin G per ml, 100 μg of streptomycin per ml, and 4 mM glutamine. The HEK293T (293T; human embryonic kidney), Vero (monkey kidney) and N2a (mouse neuroblastoma) cells were cultured in DMEM-low glucose (1.0%) supplemented with 10% FBS. The BHK (baby hamster kidney) cells were grown in minimum essential medium supplemented with 10% FBS.

2.2. Plasmid construction

To generate CMV-driven BDV minigenome vector, pCMV-HR (Fig. 1A), chemically synthesized oligonucleotides encoding HamRz [18,19] and BDV 5′ UTR sequences were annealed and ligated into the *Kpn*I and *Xho*I sites of the vector pcDNA3 (Invitrogen, San Diego, CA). The resulting plasmid was digested with *Eco*47III and *Xba*I sites, and the BDV 3′ UTR fused with HdRz, which was amplified from a plasmid phuPol I-MG [5], was inserted into the plasmid together with a cDNA clone encoding the CAT gene to yield the plasmid pc-HR. Finally, pCMV-HR was generated by the insertion of a *Bgl*II and *Xba*I fragment of pc-HR into the *Bam*HI and *Xba*I sites of pBluescript SKII(−) (Stratagene, La Jolla, CA). The CAG-driven BDV minigenome vector pCAG-HR (Fig. 1A) was generated by subcloning the CAG promoter into the pBluescript SKII(−) plasmid (pBS-CAG). The region spanning the HamRz and HdRz sequences of pCMV-HR was amplified by PCR and inserted into the blunt-ended *Sal*I and *Eco*RI sites of pBS-CAG. pCMV-HR-BGH, which contains a poly-A signal derived from bovine growth hormone (BGH), was constructed by inserting the BGH poly-A signal into the *Xba*I site of pCMV-HR. The pCMV-HR and pCAG-HR plasmids containing the SV40 origin/promoter, pCMV-HRSV and pCAG-HRSV were constructed as follows. Three different regions within the SV40 origin/promoter, SV1, SV2 and SV3 (Fig. 3A), were amplified from pEGFP-N1 by PCR (Clontech Laboratory, Inc., Palo Alto, CA) and inserted into the *Not*I site of the Pol II-driven minigenome vectors. Plasmids phuPol I-MG were kindly provided by Dr. J.C. de la Torre (The Scripps Research Institute, La Jolla, CA) and described elsewhere [5]. The plasmid pCXN2-P was constructed by inserting gel-extracted fragments from pcD-P into the *Eco*RI and *Xho*I sites of pCXN2. Detailed information about the primers and PCR procedures used to generate these plasmids is available from the authors. Nucleotide sequences of the recombinant plasmids were confirmed by DNA sequencing.