Virology 403 (2010) 145–154

Contents lists available at ScienceDirect

Virology

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

Characterization of the gene expression profile of human *bocavirus*

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ARTICLE INFO

Article history:
Received 16 February 2010
Returned to author for revision 9 April 2010
Accepted 15 April 2010
Available online 10 May 2010

Keywords:
Human bocavirus
Gene expression
Protein

ABSTRACT

We have generated a quantitative transcription profile of human bocavirus type 1 (HBoV1) by transfecting a nearly full-length clone in human lung epithelial A549 cells as well as in a replication competent system in 293 cells. The overall transcription profile of HBoV1 is similar to that of two other members of genus *Bocavirus*, minute virus of canines and bovine parvovirus 1. In particular, a spliced NS1-transcript that was not recognized previously expressed the large non-structural protein NS1 at approximately 100 kDa; and the NP1-encoding transcripts were expressed abundantly. In addition, the protein expression profile of human bocavirus type 2 (HBoV2) was examined in parallel by transfection of a nearly full-length clone in A549 cells, which is similar to that of HBoV1. Moreover, our results showed that, unlike human parvovirus B19 infection, expression of the HBoV1 proteins only does not induce cell cycle arrest and apoptosis of A549 cells.

Introduction

Human *Bocavirus* (HBoV) is one of the recently identified respiratory viruses, and tentatively is classified in the genus *Bocavirus* within the subfamily of *Parvovirinae* of the *Parvoviridae* family (Cotmore and Tattersall, 2005). Other members in the *Bocavirus* genus are bovine parvovirus type 1 (BPV1) (Chen et al., 1986), minute virus of canines (MVC) (Schwartz et al., 2002). Different species of HBoV have been identified in humans including the prototype HBoV in respiratory samples as well as HBoV2 and HBoV3 in feces (Kapoor et al., 2009; Arthur et al., 2009).

HBoV1 was initially identified from nasopharyngeal aspirates of patients with lower respiratory infections (Allander et al., 2005). The HBoV1 genome has been frequently detected worldwide, ranging from 2% to 19% in respiratory specimens from children under 2 years old with acute respiratory illnesses (Allander et al., 2005; Arden et al., 2006; Arnold et al., 2006; Bastien et al., 2006; Choi et al., 2006; Foulongne et al., 2006; Ma et al., 2006; Sloots et al., 2006; Weissbrich et al., 2006; Lin et al., 2007, 2008). HBoV1 is associated with acute expiratory wheezing and pneumonia (Allander et al., 2007; Kahn, 2008; Schildgen et al., 2008), and is commonly detected in association with other respiratory viruses (Kahn, 2008; Schildgen et al., 2008). However, HBoV1 acute infection with high viral loads in respiratory samples (>10,000 copies/ml) and increased IgG or IgM detection have been frequently associated with acute respiratory illnesses (Allander et al., 2005; Kantola et al., 2008), which indicates an apparent etiological link to respiratory illnesses. In addition to respiratory illnesses, HBoV1 is associated with gastroenteritic diseases (Lee et al., 2007; Arnold et al., 2006; Lau et al., 2007; Vicente et al., 2007; Albuquerque et al., 2007), a characteristic that shares with two closely related animal bocaviruses. The largest genome of HBoV1 that has been sequenced is 5299 nts, which lacks both termini; therefore, it is not infectious. To date, both termini of the HBoV genome have not been sequenced; therefore, an HBoV1 infectious DNA clone has not been described. Recently, new species of human *Bocavirus*, HBoV2 and HBoV3, were identified in human stool specimens. HBoV2 has a genomic organization identical to that of HBoV1, but the HBoV2 NS1, NP1, and VP1 proteins have only 78%, 67%, and 80% identity to those of HBoV1, respectively. Further studies are necessary, however, to identify potential associations of HBoV2 and HBoV3 with clinical symptoms or disease (Kapoor et al., 2009; Arthur et al., 2009).

A cell culture system of HBoV1 infection has been recently established (Dijkman et al., 2009); however, it is inefficient in that HBoV1 transcripts were only detected by reverse-transcription (RT)-PCR. Six transcripts of HBoV1 were identified from HBoV1-infected differentiated human airway epithelial cells (Dijkman et al., 2009). The abundance of these transcripts and their coding capabilities are not yet understood. Two non-structural proteins NS1 and NP1 were predicted, but the NS1 seems to lack the C-terminus compared to the full length NS1 of BPV1 and MVC (Qiu et al., 2007; Sun et al., 2009).

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doi:10.1016/j.virol.2010.04.014

The full length sequence of infectious MVC DNA (Genbank accession no.: FJ214110) shows 52.6% identity to HBoV1, while the NS1, NP1 and VP1 proteins of MVC are 38.5%, 39.9% and 43.7% identical to those of HBoV1, respectively (Sun et al., 2009). We have previously determined the transcription profiles of the BPV1 and MVC during infection (Qiu et al., 2007; Sun et al., 2009). In BPV1, the left ORF encodes the non-structural protein NS1, at approximately 100 kDa, and proteins at relatively small sizes are proposed as NS2 (Qiu et al., 2007; Lederman et al., 1987). The mid-ORF is thought to encode the BPV1 abundant non-structural protein NP1 at 28 kDa, and the right ORF contains the coding sequences for the overlapping capsid protein genes VP1 and VP2 (Qiu et al., 2007; Lederman et al., 1987). Both the NS1 and NP1 of MVC are important in DNA replication of MVC (Sun et al., 2009). The NP1 of HBoV1 and BPV1 can supplement the lack of function of the MVC NP1 in replication of an NP1 knock-out MVC infectious clone to some degree (Sun et al., 2009); however, the NP1 proteins of the bocaviruses share no similarity to any proteins of the other parvoviruses. Detection of HBoV proteins either during infection or in transfection has not been reported.

In this study, we generated a comprehensive transcription profile of HBoV1 by transfecting a replicative chimeric HBoV1 genome in 293 cells and a non-replicative genome in A549 cells. We studied the expression profiles of both the structural and non-structural proteins of HBoV in detail. Transcripts encoding the left ORF (NS1) are either spliced or unspliced at a small intron that lies in the middle of the genome. Thus, the spliced transcripts are able to encode a large nonstructural protein NS1 at approximately 100 kDa, which is comparable to the NS1 of MVC and BPV1; while the unspliced transcripts encode a relatively small nonstructural protein NS1-70 at approximately 70 kDa.

Results

Determine the relative abundance of HBoV1 transcripts by RNase protection assay (RPA)

A transcription map of the HBoV1 has been reported, which was obtained from HBoV1 RNA isolated from HBoV1-infected differentiated human airway epithelial cells (Dijkman et al., 2009). In that report, the HBoV1 transcripts were identified by reverse transcription (RT)-PCR; therefore, the relative abundance of the individual transcripts could not be determined. In other parvovirus systems, little difference has been seen between viral infection and plasmid transfection (Qiu et al., 2002, 2005, 2007; Sun et al., 2009). Therefore, we decided to examine the HBoV1 transcription profile in detail by transfection. To this end, we constructed an HBoV1 plasmid (pHBoV1) containing the full HBoV1 coding sequence (nt 1–5299) by amplifying a nearly full-length genome from an HBoV1-positive nasopharyngeal aspirate sample. The sequence of this clone was deposited in Genbank (access no.: GQ925675). We have found previously that the status of replication alters the transcription profile of human parvovirus B19 (B19V) (Guan et al., 2008). To observe the transcriptional profile in a replication-competent system, we inserted the HBoV1 genome into adeno-associated virus type 5 (AAV5) inverted terminal repeats (ITRs) so that it could be replicated while AAV5 Rep78 and necessary Ad5 genes were provided (Guan et al., 2008) in 293 cells (data not shown).

We used mRNAs isolated from two sources for transcript mapping: those from A549 cells transfected with pHBoV1, and those from 293 cells transfected with p5TRHBoV1/pHIVAV5Rep78/phelper. RT-PCR and 5′/3′ RACE were performed as previously described (Qiu et al., 2002, 2005, 2007; Sun et al., 2009), and gel purified PCR fragments were further sequenced (data not shown). The RNA landmarks determined by transfection of p5TRHBoV1 in 293 cells, which were the same as those determined by transfection of pHBoV1 in A549 cells, are depicted and diagramed to scale in Fig. 1A. These landmarks basically confirmed the transcription units as identified in the previously reported virus infection system (Dijkman et al., 2009), with the exception of an extra splice site (A1-1) in the first intron. This A1-1 splice site is present in BPV1 RNA during BPV1 infection (Qiu et al., 2007), but not in MVC RNA from MVC infection (Sun et al., 2009).

To determine the relative abundance of each HBoV1 mRNA, we used seven anti-sense HBoV1 probes to protect individual HBoV1 mRNA. A schematic diagram of the seven anti-sense HBoV1 probes with their putative protected bands and nucleotide numbers (nt) is shown in Fig. 1A.

Probe PD1

Probe PD1, spanning the putative promoter (P3) and the first donor site (D1), protected bands of 133 and 55 nts. These bands mapped the RNA initiation site at nt 187 and the first splice donor site (D1) at nt 242. Similar to that of BPV1 and MVC, the first exon of HBoV is short, containing only 55 nts (Fig. 1B, lane 2). Approximately 75% of HBoV1 RNAs were spliced at the D1 donor site. Multiple bands centralized at nt 55 were considered as spliced RNA (Qiu et al., 2002).

Probe PA1-1

Probe PA1-1, spanning the first acceptor site of the first intron (A1-1), protected bands of 182 and 120 nts (Fig. 1B, lane 3). The 120-nt band mapped the first 3′ splice acceptor site at nt 920, which is similar to the location of the acceptor site in BPV1 RNA. In addition, as seen in BPV1, only a small portion of RNAs, less than 10% of spliced RNA at the D1 donor site, were spliced at the A1-1 acceptor site (Qiu et al., 2007).

Probe PA1-2 and PA1-2/D2

Probe PA1-2, spanning the second acceptor of the first intron (A1-2), protected bands of 162 and 117 nts (Fig. 1C, lane 2). Probe PA1-2/D2, spanning the second acceptor of the first intron (A1-2) and the donor site of the small intron (D2), protected bands of 225, 122 and 168 nts (Fig. 1C, lane 3). These bands protected from both probes confirmed the location of the A1-2 acceptor and the D2 donor at nt 2043 and nt 2165, respectively. Similar to BPV1 and MVC, the majority of HBoV1 RNAs protected by probe PA1-2/D2 were spliced at both sites, resulting in the abundant band present at 122 nts (approximately 90%). Only approximately 5% of RNAs in this region were unspliced at the A1-2 acceptor site, which resulted in the band at 168 nts (spliced at D2) and the band at 225 nts (unspliced at D2). Interestingly, this probe did not detect RNAs that were spliced at A1-2 and remained unspliced at the D2 site (which would have generated a band at 181 nts).

Probe A2/D3

Probe PA2/D3, which spans the A2 acceptor and the D3 donor sites, protected bands at 241, 123, 192 and 167 nts. These bands confirmed the usage of the A2 site at nt 2235 and the D3 site at nt 2358 (Fig. 1D, lane 2). Approximately half of the RNAs that spliced the small intron (D2–A2) were not further spliced at the third intron (D3–A3), which resulted in the 192-nt band; another half were spliced at D3, which resulted in the 123-nt band. These spliced mRNAs at 123 nts (spliced at both A2 and D3, Fig. 1D, lane 2) represent capsid protein-encoding mRNAs, which account for one third of the RNAs protected by probe A2/D3. Only a minor portion of RNAs, at a size of 167 nts, were unspliced at the A2 site and spliced at the D3 site (Fig. 1D, lane 2), which was not seen in RNAs from transfected A549 cells (data not shown); therefore, we did not include this transcript in the final transcription profile.

Probe PA3

Probe PA3, which spans the A3 acceptor site, protected two bands at 159 and 107 nts. Thus the A3 acceptor site was mapped to nt 2995