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Genomic features of the human bocaviruses

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Abstract

The human bocavirus (HBoV) was initially discovered in 2005 as the second pathogenic member of the parvovirus family, next to the human parvovirus B19. HBoV has since been shown to be extremely common worldwide and to cause a systemic infection in small children often resulting in respiratory disease. Three more, presumably enteric, human bocaviruses (HBoV2–4) have been identified in stool samples. Parvoviruses are assumed to replicate via their genomic terminal hairpin-like structures in a so-called ‘rolling-hairpin model’. These terminal sequences have recently been partially identified in head-to-tail HBoV-PCR amplicons from clinical samples, and are most likely hybrid relics of HBoV’s predecessors, namely bovine parvovirus 1 on the left-hand side and minute virus of canines on the right, shown for the first time in this article. Thereby, the replication model postulated for HBoV remains questionable as the occurrence of head-to-tail sequences is not a typical feature of the rolling-hairpin replication model. However, such episomes can also be persistent storage forms of the genome.

Keywords

genome structure; hairpin sequence; HBoV; human bocavirus; parvovirus; persistence; replication; transcription map

The human bocavirus (HBoV) was originally discovered in pooled nasopharyngeal aspirates using random PCR, high-throughput sequencing, and bioinformatics by Allander and coworkers in 2005 [1]. It was further found as a putative causative agent of respiratory disease in a series of pediatric patients, with the majority of the specimens containing no other respiratory viruses. Sequencing of the HBoV genome revealed two prototype isolates named HBoV ST1, consisting of 5217 nucleotides, and HBoV ST2, consisting of 5299 nucleotides. Based on phylogenetic analyses it was concluded that this novel virus is a parvovirus and would therefore represent the second human pathogen of this virus family, next to human parvovirus B19. It was also concluded that the viral genome was not fully

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deciphered, as the terminal hairpin-like structures, a typical feature of virtually all parvoviruses known so far, were absent in the prototype sequences of HBoV [1,2]. Later, three additional virus sequences, sequentially discovered in human stool samples, were phylogenetically characterized to be closely related with the human bocavirus and were thus named HBoV2, 3 and 4 [3–5]. For clarity, HBoV is therefore often denoted HBoV1.

Clinical impact & epidemiology

To a certain extent the clinical symptoms associated with the human bocaviruses depend on the particular HBoV species; HBoV1 infection is mainly associated with pediatric respiratory illness [6–8] but also gastrointestinal symptoms are often observed [9–11]. The prevalence of HBoV1 DNA in respiratory specimens has ranged from 2 to 19% and the most typical age for HBoV1 infection is 6–24 months [12–14]. In contrast, HBoV2–4 have been preferentially detected in stool samples and seems to be enteric [3–5,15–20]. HBoV2 has further been associated with gastrointestinal disease [4]. HBoV2–4 has so far not been detected in blood, whereas HBoV1 causes a systemic infection leading to a short-lived viremia and induction of specific antibodies [21–27]. To our knowledge, however, HBoV2–4 DNA has not been looked for in serum samples from patients with gastroenteritis, only from children with respiratory tract disease [27]. According to serologic studies, HBoV1 infection is extremely common, evidenced by adult seroprevalences approaching 100%. However, cross-reacting antibodies of the other human bocaviruses have been shown to strongly influence this figure [27]. Based on seroprevalences measured after competition with heterologous virus-like particles, the human bocavirus species most frequently infecting humans are, in descending order, HBoV1, HBoV2, HBoV3 and HBoV4 [27].

HBoV persistence

Two other human parvoviruses, B19 virus and adeno-associated virus (AAV), have been shown to commonly persist for decades in many different tissue types of constitutionally healthy people [28–33]. The mechanism for tissue persistence is debated. Viral DNA might be integrated into human chromosomes or stored as episomes, or it could even be encapsidated and be attached as full virions on the surface of follicular dendritic cells or be carried to tissues inside macrophages or other circulating cells.

HBoV1 DNA may reside in the upper airways for months after acute infection, either due to getting trapped in the nasopharynx, prolonged shedding or mucosal contamination from virus particles in the breathing air [34–37]. Similar tissue persistence as that of B19 virus or AAV, has, however, not been detected for HBoV, at least to the same extent [31,38–40]. Even though HBoV1 cause a systemic infection [21], persistence of HBoV should perhaps be looked for in respiratory or intestinal tracts.

Classification of HBoV

The four human bocavirus species, HBoV1–4, are proposed members of the family *Parvoviridae*, subfamily *Parvovirinae*, genus *Bocavirus*. The preliminary taxonomic classification was based on phylogenetic analyses that revealed the highest similarity of HBoV to two animal parvoviruses that were the founders of the genus *Bocavirus*, the bovine parvovirus 1 (BPV1) and the minute virus of canines (MVC), leading to the hypothesis that HBoV has an ancient zoonotic origin [1]. This hypothesis is supported by the fact that among the additional newly discovered porcine, gorilla, chimpanzee and sea lion bocaviruses, the nonhuman primate bocaviruses in particular display a high similarity to HBoV [41–47]. HBoV1 is classified as an autonomous virus based on the fact that it may occur without accompanying co-pathogens and that it can replicate in primary human cell culture in the absence of helper viruses [48]. It was assumed that HBoV, similar to other

parvoviruses, packages linear ssDNA into the progeny virus particles. Indeed, by NASBA, an isothermal amplification method able to detect and differentiate single-stranded templates, HBoV was shown to preferentially package the negative ssDNA into capsids [49].

The classification of HBoVs as parvoviruses is, in addition to phylogeny, also based on their genome organization: a large left open reading frame (ORF) encodes the nonstructural protein, NS1, that during the replication cycle of parvoviruses acts as a multifunctional protein, being essential in DNA replication, cell cycle arrest, and gene transactivation [50–55]. The middle ORF is a unique feature of the *Bocavirus* genus and encodes an additional nonstructural protein, a nuclear phosphoprotein NP1, which in MVC has been shown to be an essential part of the replication machinery [56]. The large right-hand ORF encodes two proteins, the structural components VP1 and VP2.

In addition, 3D structure analyses of purified virus-like VP2 particles by 7.9-Å resolution cryo-electron microscopy followed by image reconstruction, revealed many features in common with parvoviruses, such as a protrusion at the threefold axis, a depression at the twofold axis, and a channel at the fivefold axis surrounded by a canyon. It also revealed a smooth HBoV1 capsid topology most similar to that of the human parvovirus B19 [57].

Genetic divergence between the human bocaviruses

Notably, based on phylogenetic analyses of the amino acid sequences deduced from the *VP1/2* gene, HBoV1 is more divergent from HBoV2–4 (~20%) than these are from each other (~10%); it was thus concluded that HBoV1 may have evolved from a primary enteric pathogen to a respiratory pathogen [5]. The genetic intraspecies diversity of the enteric viruses is much greater than that seen for HBoV1. Furthermore, the enteric viruses seem to have recombinant origins, including a high level of HBoV2 intraspecies recombination [5]. More detailed genomic analyses of HBoV species revealed a very high likelihood that HBoV3 is a progeny form of a recombination event between HBoV1 and 4 [18].

HBoV genome & its replication

Genomic analyses of human HBoV strains are difficult, mainly owing to technical limitations in virus culture, which has been reported only once [48], and lack of full-length infectious clones. However, two recent studies have revealed insights showing that the HBoV transcriptome strongly resembles its animal relatives [48,58].

In general, parvovirus genomes consist of a linear ssDNA that encodes the viral proteins; the coding sequence is flanked by terminal imperfect palindromes or inverted repeats that form hairpin-like structures and are responsible and essential for viral genome replication [59–62]. The current replication model for parvoviruses claims that the parvoviruses replicate their genomes via their terminal hairpin-like structures in a so-called ‘rolling-hairpin’ model, which is a derivative of the classic ‘rolling-circle’ replication mechanism [59–62]. The annealed 3′ hairpin end functions as a primer for the cellular polymerase in the elongation of a complementary strand to the rest of the viral genome, making it double-stranded – a form also needed for transcription. This initial duplex monomeric replicative form, with a single covalently closed left-terminal sequence, is then ligated with itself to close the right end as well, after which NS1 is needed to open the covalently closed duplex at the replication origin, adjacent to the right hairpin. The resulting 3′ terminal gap in the parental strand is then filled in using the transferred sequence as a template. NS1 remains covalently bound to the free 5′ terminus, along with an 18–26-nucleotide-long tether, and is important in the unfolding and displacement of the hairpin [63]. This hairpin transfer thus replaces the original hairpin sequence with its inverted complement at each round of replication, forming

two alternating hairpin forms, called ‘flip’ and ‘flop’. This rolling-hairpin mechanism results in multimeric DNA concatemers, where the coding sequence has been copied twice as often as the termini, in a head-to-head or tail-to-tail manner, which means that the replication intermediates form large mirrored sequences.

Until the beginning of this year the terminal sequences were not yet deciphered for the human bocaviruses; two recent studies have, however, narrowed this gap in our knowledge [64,65]: Lüsebrink and coworkers have identified an unknown sequence of HBoV1 in different patient samples and in the so-far only cell-culture isolate, Bonn 1 [48]. The novel sequence was in a head-to-tail orientation (Figure 1) of previously described terminal genomic (but not hairpin) sequences covalently linked by a sequence that share high similarity to parts of the terminal hairpins of the prototypic bocaviruses BPV1 and MVC [64]. The right-hand ‘tail’ of the identified HBoV1 head-to-tail junction [64] contains a sequence, (5'-GCGCCTTAGTTATATATAACAT-3'), identical with the right-end hairpin (REH) of MVC [56]; while the left-end ‘head’ shows two sequences (3'-CGCGCGTA-5' and 3'-GATTAG-5') identical with the left-end hairpin (LEH) of BPV1 [56,66] (Figure 2). We hypothesize that the HBoV1 terminal repeats are most likely hybrid relics of HBoV's predecessors, of BPV1 at the left-hand side, and MVC at the right terminal sequence, shown for the first time in this review. Since the LEH of HBoV1 in the sequenced episome contains identical sequences with a ‘bubble’ on the stem and with parts of the ‘rabbit ear’ in the BPV1 LEH (Figure 2), we predict that the HBoV1 LEH likely resembles the BPV1 LEH.

Those head-to-tail sequences of Lüsebrink *et al.* [64] were unexpected as replicative forms, as they did not fit into the replication model of the rolling hairpin but gave rise to the hypothesis that the human bocaviruses may use an alternative route of replication, namely the classical rolling-circle model (Figure 1). The observations by Lüsebrink *et al.* were confirmed by a recent study by Kapoor and coworkers, who also detected head-to-tail sequences in intestinal biopsies of patients infected with HBoV3 [65]. However, the head-to-tail junction obtained from the HBoV3-infected tissues revealed a sequence that was different from that of HBoV1 (Figure 2C) [65], which only contained two sections of identical sequences in the REH. If a classical rolling-circle replication were to take place, the majority of progeny genomes would be newly synthesized from the episome in a single orientation. However, while it has been shown that HBoV1 mainly packages the minus strand, in about 10% of observed isolates the positive strand is encapsidated [49]. This portion of plus-stranded genomes could perhaps be produced from 10% of the episomes replicating the other strand by rolling-circle replication running ‘counterclockwise’ to that of the majority of episomes, or perhaps from an additional less-effective rolling-hairpin replication, depending on the structure of the terminal sequences. As a matter of speculation, in the case of a true rolling-circle replication, the restriction site for the separation of progeny concatemers could be located in the linker sequence. However, rolling-circle replication is not a proven model for parvoviruses, and should be further elucidated. The new terminal ‘linker’ sequence [64] was missing in the first sequencing analysis of HBoV1 [1], was not as long in the HBoV3 sequence [65], and has been shown to be variable in AAV [67]. To conform to the classical view of parvovirus hairpins and replication models, further hitherto-unknown sequences that could form ‘rabbit ear’ hairpins may also be present in the HBoV LEH, but may have been left out during formation or amplification of the episome. Parvovirus hairpins are notoriously difficult to amplify and to clone. Apparently, then, the structures of the HBoV1 LEH and REH still require further investigation.

In a study of a novel porcine bocavirus-like virus, PPV4, of an unassigned genus, a similar head-to-tail orientation was detected [42]. Likewise, the DNA of wild-type AAV2, a parvovirus of the *Dependovirus* genus, has been detected as head-to-tail junctions both as

episomes in human tissues [67], and as integrated proviruses in cell cultures [68], with extensive rearrangements and deletions in the terminal repeat sequences. We believe that both the HBoV1 and HBoV3 episomes are either forms of replication intermediates or storage forms of persistent viral genomes in tissues.

HBoV transcriptome

So far, there is, however, only limited information on the transcriptome of human bocaviruses. Two studies investigated the transcription of viral DNAs, making use of primary cell culture and a transfection approach into permanent cell culture [48,58]. The first study, carried out by Dijkman and coworkers, identified five transcripts including spliced variants, most likely coding for the proteins NS1, NP1, UP1/NP1, VP1/VP2 and UP2/VP1/VP2 [48]. This study also revealed the existence of a putative additional protein encoded by the ORF_x region. However, all these HBoV1 transcripts were determined only from analysis of HBoV mRNAs using reverse transcription PCR. Therefore, the abundance and significance of each species of mRNA transcript remained unknown [48]. The predicted NS1 protein, namely NS1-70 in Figure 3, from the map obtained in the study by Dijkman and coworkers [48], is only 639 amino acids in length, which is much shorter than the MVC [56] and BPV NS1 [69], and does not share homology with them at the C-terminus. A larger HBoV1 NS1 that is 781 amino acids in length ('NS1' in Figure 3) was predicted to be encoded by HBoV1 R1 mRNA transcripts that are spliced from the D2 donor and A2 acceptor sites (Figure 3). The expression of NS1 was demonstrated by Chen and coworkers by transfection of 293 cells with a replication-competent HBoV1 genome [58]. The HBoV1 NS1 shares two conserved C-terminal motifs with the MVC and BPV NS1, 740WGERLGLI747 and 757PIVLXCFE764, which are likely transactivation domains [70]. The transfected replication-competent HBoV1 plasmid expresses both the large NS1 and the small NS-70 [58]. In addition, the D2-A2 intron as well as the NS1 C-terminal domain that overlaps with the NP1 ORF, are well-conserved among HBoV genotypes 1–4 [5,41]. Therefore, we believe that the large NS1 should be expressed during HBoV1 infection. A summarized transcription map of HBoV1 produced from the results of virus infection and plasmid transfection is presented in Figure 3. Furthermore, transfection of a near-full length HBoV2 clone produced the same profile of protein expression with detection of NS1, NS1-70, NP1, VP1 and VP2 at sizes similar to those of HBoV1 [58], as well as the same transcription profile [Qiu *et al.*, Unpublished Data]. These findings suggest that HBoV1 and HBoV2 are very similar genetically, and that HBoV3 and HBoV4 likely share a similar gene expression profile to that of HBoV1 and HBoV2. The UP1 protein, which contains the entire NS1 C-terminus (pale bars in Figure 3), was not detected by transfection [58]. Notably, the AUG start codon for the putative UP1 is conserved among HBoV genotypes 1–4. Further examination of HBoV1 protein expression during virus infection is warranted.

Conclusion

Our knowledge of the HBoV genome and its replication is to a large extent limited by the lack of permissive cell lines and infectious clones, and by the restricted availability of high titers of the virus itself. Based on the detection and sequencing of covalently closed circular head-to-tail genomic structures, the terminal sequences of HBoV have been partially identified. We suggest the HBoV1 termini to be hybrid relics of HBoV's predecessors, BPV on the left, and MVC on the right termini. The head-to-tail orientation in these episomes has prompted speculation of an alternative replication model for HBoV, since the classic rolling-hairpin replication model of parvoviruses does not include such structures. However, these episomes could also be storage structures for persistent infection.

Future perspective

Novel parvoviral species and genotypes will undoubtedly be identified in both animals and humans, and new disease associations will be documented. Even new human bocaviruses will perhaps be detected. Genomic analyses of human bocaviruses are difficult, mainly due to technical limitations in virus culture and the lack of full-length infectious clones. However, recent studies have revealed insights into the previously unknown terminal sequences and into the HBoV transcriptome that strongly resembles its animal relatives. These studies are only the starting point for more research on the biology of the highly interesting bocaviruses; the most urgently-needed tools are infectious clones and simpler culturing methods. With their development, the functions of NP1 and the putative UP1 and ORF_x proteins in the life cycle of HBoV infection, will be elucidated. Parvoviruses have been shown to replicate by a model called rolling-hairpin replication. However, this has been challenged in the case of HBoV, an issue that needs to be settled in the near future. More details of replication and protein function will emerge with future research. Moreover, episomal structures have been identified that could be forms of genome persistence in tissues. The frequency and the molecular and cellular mechanisms of HBoV persistence, as well as the possible pathogenic potential of persistence, are further interesting topics for future research.

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Executive summary

Taxonomy & clinical picture

- Human bocaviruses 1–4 (HBoV1–4) are, together with the prototypic bovine parvovirus and the minute virus of canines, proposed members of the *Bocavirus* genus in the *Parvoviridae* family. Parvoviruses are small nonenveloped ssDNA viruses.
- HBoV1 is the most common HBoV species and causes a systemic infection in small children, often resulting in respiratory disease.
- Three other HBoVs, HBoV2–4, have been identified in stool samples and are presumably enteric.

Genetic divergence of HBoV1–4

- HBoV1 is more divergent from HBoV2–4 (~20%) than these are from each other (~10%).
- The genetic intraspecies diversity of HBoV2–4 is much greater than that seen for HBoV1, and they seem to have recombinant origins.

Genome structure

- The HBoV genome is linear ssDNA, over 5 kb in length.
- The termini of most parvoviruses are imperfect palindromes or inverted repeats forming terminal hairpins with U-, T- or ‘rabbit ear’-like structures. Some parvoviruses have identical termini while others have nonidentical ones. Bocaviruses are in the latter category.
- The terminal sequences of HBoV have not yet been deciphered in full. However, in head-to-tail episomal structures, new sequences were detected: the right-hand terminus of HBoV1 had some identical short sequences with that of the minute virus of canines and the left-hand terminus with that of prototypic bovine parvovirus, but no actual hairpins could yet be modeled with the existing HBoV sequence.
- HBoV3 episomes with similar head-to-tail structures but with slightly shorter junction sequences have been detected in intestinal tissue.

Replication models

- Parvoviruses use cellular polymerases for their replication and transcription, and therefore require rapidly dividing cells in S phase. The *in vivo* host cells for HBoV1–4 are unknown. HBoV1 has so far been cultured only in primary airway epithelial cells differentiated into pseudo-stratified human airway epithelium.
- Parvoviruses generally replicate by rolling-hairpin replication, a model that is able to explain the occurring hairpin transfers. Replication results in multimeric DNA concatemers, where the coding sequence has been copied twice as often as the termini, in a head-to-head or tail-to-tail manner.
- The recently revealed HBoV1 and HBoV3 head-to-tail junction sequences do not fit into this rolling-hairpin model. It was thus suggested that HBoV would replicate differently from the other known parvoviruses, namely along the rolling-circle model, or the episomes would be storage forms of persistent infection.

Transcription map

- HBoV makes use of all three reading frames in one direction.
- Two putative nonstructural proteins, NS1 and NS1-70, are encoded by the left side and two partly overlapping structural proteins, VP1 and VP2, on the right side of the genome. In the middle is a shorter open reading frames for a nuclear phosphoprotein, NP1, that is unique for bocaviruses. In addition, two further small open reading frames have been suggested based on reverse transcription PCR, but have not been confirmed by protein expression.
- All the HBoV mRNA transcripts are processed from one single precursor mRNA, which is transcribed from the promoter at map unit 3, through alternative splicing as well as alternative polyadenylation. These features are similar to those of parvovirus B19 and Aleutian mink disease virus.

Future perspective

- Infectious clones and permissive cell lines will show whether HBoV replication is unique among parvoviruses and whether the head-to-tail episomes are replicative intermediate forms or storage structures in persistent infection of our tissues.

Figure 1. Proposed alternative model of human bocavirus DNA replication

Based on the sequence analyses of the head-to-tail PCRs, it was suggested that HBoV replicates in a classical rolling-circle [64] rather than rolling-hairpin model, as occurs in other parvoviruses [62]. Progeny DNA of negative polarity is replicated from the positive-strand genome that circularizes by a so-far unknown mechanism. The model is supported by the nucleotide sequence of the head-to-tail PCR products and is in line with the fact that HBoV progeny genomes are of negative-strand polarity, which can be explained by the stoichiometry of newly synthesized negative-strand DNA [49].

HBoV: Human bocavirus.

Figure 2. Structures of human bocavirus palindromic terminal repeats

(A) The structures of the MVC REH and BPV1 LEH are depicted with HBoV-identical sequences shown in boxes. The MVC and BPV1 sequences refer to GenBank accession no. FJ214110 and DQ335247, respectively. (B) The newly identified sequence of the HBoV head-to-tail junction is shown between the vertical arrows [64]. Identical sequences on either the MVC REH or BPV LEH are boxed. The black HBoV1 sequence refers to accession no. DQ000496 in GenBank [1]. Sequences identical to the HBoV3 episome are underlined. (C) The HBoV3 head-to-tail junction is shown between the vertical arrows [65]. Sequences identical to the HBoV1 head-to-tail junction are underlined and nucleotides differing from the HBoV1 sequence are shown in gray.

BPV: Bovine parvovirus; HBoV: Human bocavirus; LEH: Left-end hairpin; MVC: Minute virus of canines; REH: Right-end hairpin.

Figure 3. Genetic map of human bocavirus 1
The genetic map of HBoV1 is shown, with transcription and RNA processing units presented in nucleotide numbers referring to the GenBank accession no. DQ000496 (without including nucleotides of the LEH and REH, depicted in gray). Eight major species of HBoV mRNA transcripts are shown with their relative abundance and sizes (minus a poly-A tail of ~150 nt) [47,57]. ORFs are depicted in different shades and patterns, representing each reading frame. Both predicted proteins from the coding capability of each species of the HBoV mRNA transcript and proteins detected from transfection are shown on the right-hand side. Expression of the ORFx and UP1 has not been determined.
LEH: Left-end hairpin; ORF: Open reading frame; REH: Right-end hairpin.