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Genome trimming by Borna disease viruses: Viral replication control or escape from cellular surveillance?

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Abstract. Persistence of RNA viruses is frequently associated with non-uniform terminal nucleotide deletions at both ends of the viral genome, which are believed to restrict viral replication and transcription during persistent infection. Borna disease virus (BDV), a negative strand RNA virus with no recognizable acute phase, quickly establishes persistence. We recently demonstrated that the vast majority of BDV genomes and antigenomes possess uniformly trimmed 5′ termini, even if the virus is recovered from complementary DNA encoding a hypothetical full-length viral genome. Here we discuss different mechanisms which might lead to the selective 5′-terminal trimming of the BDV genome and subsequent retrieval of the lost genetic information. We further discuss possible benefits of genome trimming in the light of recent findings that terminal RNA structures are recognized by intracellular sensors which trigger innate immunity. We hypothesize that 5′-terminal genome trimming might represent a smart strategy of BDV to evade the antiviral host response.

Keywords. RNA virus, viral persistence, genome termini, terminal deletion, Borna disease virus, innate immune system, 3′ overhangs, 5′ triphosphate, RIG-I.

Genome trimming by Borna disease virus

The terminal nucleotides of RNA virus genomes contain promoter elements that direct the association with the viral polymerase and determine the initiation sites for genome and antigenome synthesis. In the case of negative strand RNA viruses the nucleotides at both ends of the genome usually represent perfect inverted terminal repeats (ITRs) with matching 5′ and 3′ ends of up to 20 nucleotides. The ITRs possess the potential to form a stem-loop structure by direct interaction of the complementary sequences, which results in non-covalent circularization of the viral RNA. This structure has been designated a panhandle. For members of the *Orthomyxoviridae* [1–4] and *Bunyaviridae* [5–7] panhandle formation has been demonstrated experimentally and was shown to be required for efficient viral genome replication. For members of the order *Mononegavirales* it is still under debate whether panhandles are indeed formed during replication [8, 9].

Several unique features characterize the biology of Borna disease virus (BDV) [10]. It is the only member of the order *Mononegavirales* which replicates and transcribes its genome in the nucleus of infected cells [11]. Its propagation in cultured cells and infected animals is strictly non-cytolytic and results in obligatory persistence of the virus [12]. Although BDV efficiently infects cultured cells derived from various

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species and tissues, replication *in vivo* is restricted mainly to neurons of the central nervous system (CNS) [13]. Studies from several laboratories indicate that the ITRs of the BDV genome are exceptional amongst members of the *Mononegavirales* as they appear to possess heterogeneous termini and lack perfectly matching 5′ and 3′ ends [10, 14–16]. Alignment of the ITR in a panhandle configuration results in an overlap of 4 nucleotides at the 3′ end of the genome (Fig. 1). The non-complete ITRs are functional and support reporter gene expression when tested in artificial minireplicon systems [17, 18]. Analysis of the terminal sequences of the BDV antigenome show that the situation is even more peculiar than previously anticipated. Like genomic ITRs, antigenomic ITRs appear to be non-complete, so that alignment of the terminal sequences in the panhandle configuration generates a four-nucleotide overlap of both 3′ ends [19]. Thus, the vast majority of genomic and antigenomic RNA molecules present in BDV-infected cells are not perfect mirror images of each other (Fig. 1). This constellation is not compatible with conventional wisdom regarding sequence requirements for maintenance of genetic information. To explain this unexpected finding, we previously postulated that full-length genomic RNA with complete ITRs must exist (Fig. 1), but that it occurs at very low frequency only [19]. We tested this hypothesis by generating recombinant BDV from complementary DNA (cDNA) encoding viral genomes with complete (rBDVc) ITRs. We found that rBDVc replicated very efficiently, like non-recombinant wild-type BDV [19]. Interestingly, the genomes and antigenomes of rBDVc possessed uniformly trimmed 5′ termini, strongly indicating that BDV employs an as yet undefined mechanism to maintain trimmed 5′ termini throughout its replication cycle. We also generated a virus (rBDVnc) with a non-complete, pre-trimmed 5′ terminus. The virus was unable to fully reconstitute the missing genetic information [19]. The propagation of rBDVnc was strongly attenuated, but the virus was able to establish a persistent infection in Vero cells [19], suggesting that replication of trimmed genomes and antigenomes was possible but inefficient. To account for these findings, we proposed a model which suggests that efficient initiation of genome and antigenome synthesis requires the presence of the complete ITR in the hypothetical full-length BDV genome (Fig. 1). We further postulated that initiation occurs with highest frequency at an internal C nucleotide located four nucleotides upstream of the 3′ end [19, 20]. According to this model, genome trimming serves to restrict BDV propagation while maintaining efficient transcription of viral genes. It is in accordance with the observation that despite the presence of high amounts of viral transcripts and proteins, only a very few infectious particles can be isolated from the brain of persistently infected animals.

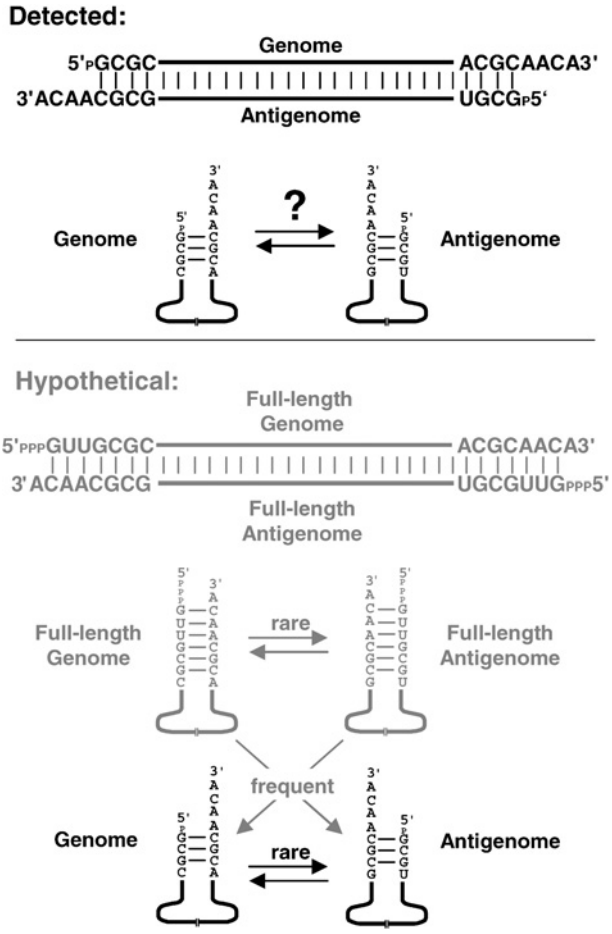

Figure 1. Alignment of 5' trimmed BDV genome and antigenome as complementary strands and in the panhandle configuration to show the terminal 3' overhangs and to indicate the previously postulated model for maintaining genetic information [19]. The detected forms of the BDV genome and antigenome are shown in black. The hypothetical full-length genome and antigenome are shown in gray.

Alternative models for genome trimming by BDV

Although the proposed mechanism can explain the replication behavior and terminal structures of rBDVc and rBDVnc fairly satisfactorily, several observations are difficult to reconcile with this model. First, none of the various attempts to determine the terminal sequences of the BDV genome provided direct physical evidence for the putative full-length BDV genome. In our own study, we analyzed more than 150 individual polymerase chain reaction (PCR) fragments amplified from the 5′ terminus of the

rBDVc genome, but failed to detect a single untrimmed molecule [19]. Studies by others who used different technical approaches generated similar results [10, 14, 15]. Although it is conceivable that the frequency of the hypothetical full-length molecule is simply too low to be detected by 5′ RACE (5′ amplification of cDNA ends) technology, it may be that BDV employs alternative mechanisms to generate 5′-trimmed genome ends. Second, it is difficult to explain with this model why the 5′ termini of the BDV genome and antigenome seem to carry monophosphates rather than triphosphates as expected if the ends of these RNA molecules are generated by a standard transcription initiation process [15, 19]. Thus, the 5′ termini of the BDV genomes and antigenomes seem to be processed by pyrophosphatases or endonucleases. The lack of obvious nuclease or phosphatase domains in any of the BDV proteins suggests that the critical enzymatic activity is of cellular origin. Since BDV genome trimming generates nucleotide overlaps on the 3′ ends, it is tempting to speculate that nuclear members of the highly conserved double-stranded RNA-specific endoribonuclease III (RNase III) family are involved in the trimming process. In this scenario, trimming would have to occur during interaction of the ITRs, which is in accordance with the models presented below.

To better account for the observations described above, we would like to propose two alternative models for how BDV is generating its trimmed 5′ ends (Fig. 2). These models include scenarios in which internal sequences are used as templates to retrieve the genetic information lost by 5′ trimming of the genome. In the first model (Fig. 2a) the nascent strand disengages after the polymerase has reached the 5′ end of the template and anneals to the partially complementary sequence of its own 5′ ITR. Intrastrand and possibly also interstrand switches of nascent RNA molecules and viral RNA-dependent RNA polymerases occur during transcription of the coronavirus genome [21]. Evidence has been provided that interstrand transfer of RNA primer occurs during initiation of genome synthesis in influenza virus [22]. In the case of BDV, the new template would then direct the synthesis of the missing 3′-terminal nucleotides before termination occurs. There is evidence that the 3′-terminal A residues on both RNA strands are not template-encoded but rather added by the viral polymerase during the termination process [19]. In this model, genome trimming by a nuclease would occur during or after termination of RNA synthesis. In a second model, the nascent strand disengages from the template after the polymerase has reached the 5′ end of the template and anneals to the 5′ G of an internal 5′-GUUG-3′ motif near the 3′ end of the template, as shown in Fig. 2b. If the polymerase continued the polymerization process, the CAAC motif of the 3′ end would be retrieved. The recognition of a distinct 5′-GUUG-3′ motif and termination of RNA synthesis might be directed by structural constraints and/or cis-acting regulatory sequences. As in the model above, we would assume that termination would trigger the non-templated incorporation of an A residue and the endonucleolytic cleavage of the 5′ end. This scenario would require that the 5′ and 3′ ends of the template strand form an ITR and remain in close proximity during synthesis of the nascent RNA molecule.

The proposed new models can explain how 3′ overhangs are retrieved in the absence of a hypothetical full-length genome. It should be noted, however, that the new models cannot explain the observation that rBDVnc was unable to restore the missing genetic information [19], arguing in favor of the originally proposed model.

Potential beneficial role of genome trimming during persistent infection

Genome trimming by BDV and other RNA viruses has generally been associated with attenuation of viral replication and transcription during viral persistence [20, 23–25]. Recent work indicates additional functions for terminal deletions of viral genomes during viral persistence, namely preventing the induction of an innate immune response. Induction of the innate immune system depends on the recognition of viral components, such as double-stranded RNA (dsRNA) by host pattern-recognition receptors [26, 27]. The retinoic acid-induced gene I (RIG-I), an RNA helicase, has been identified as a pattern-recognition receptor responsible for the detection of most negative-strand RNA viruses [28]. Studies from different laboratories recently showed that RIG-I recognizes the 5′ triphosphate groups of viral RNA molecules rather than dsRNA, as previously suggested [29–31]. This finding suggests that BDV and other persisting RNA viruses might delete nucleotides from the 5′ termini of their genomic RNA molecules to eliminate terminal 5′ triphosphate groups.

Another highly suggestive observation concerning the function of terminal deletions during persistent infection of RNA viruses comes from the analysis of the interferon (IFN)-inducing potential of small interfering RNAs (siRNAs) [32]. siRNAs and cellular microRNAs (miRNAs) are generated by the cleavage of larger precursor molecules by the cellular RNase III Dicer, which results in 2-nucleotide-long 3′ overhangs on both strands. Detailed analysis of chemically

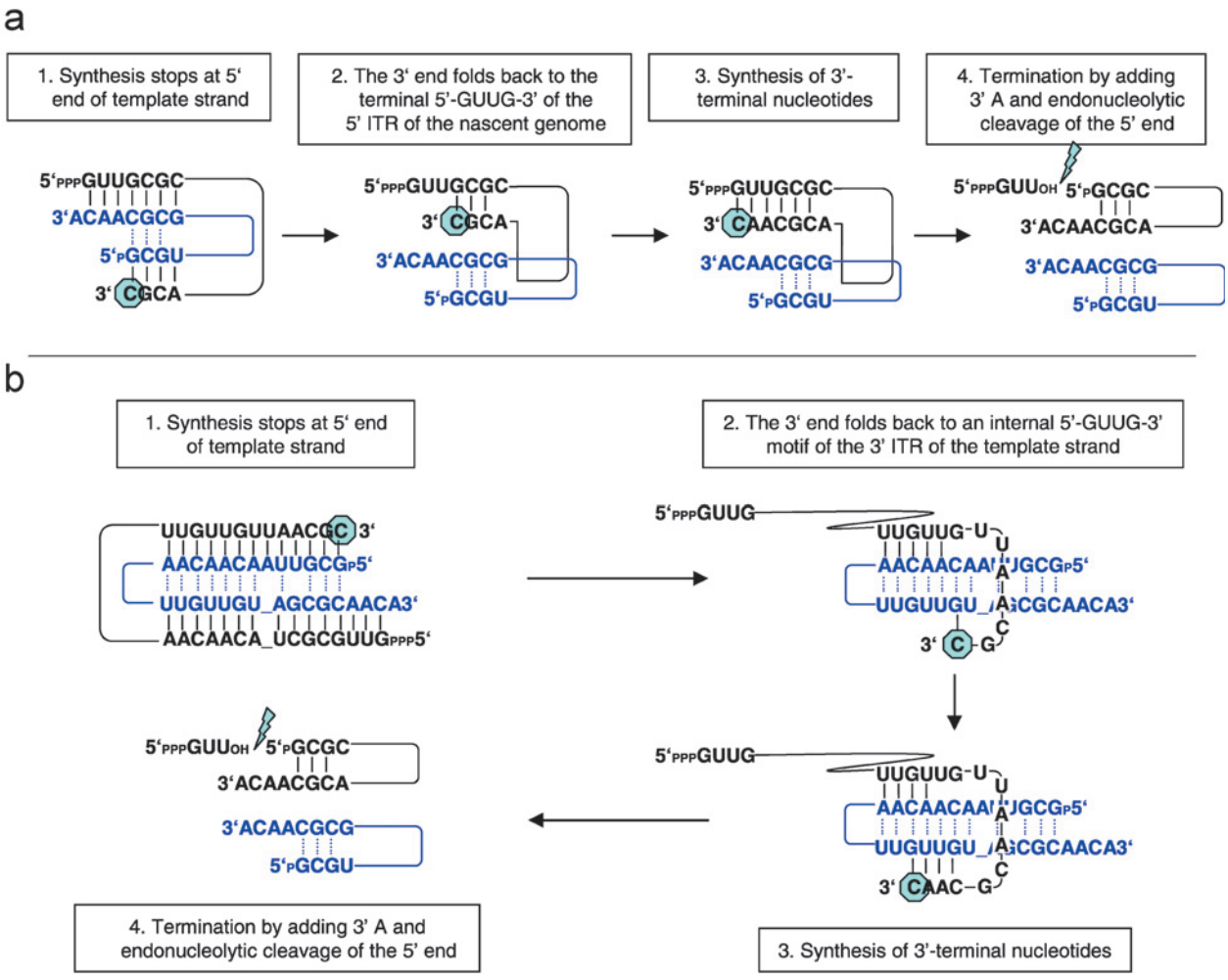

Figure 2. Schematic representation of two models for generating 3′ overlapping nucleotides in the absence of the hypothetical full-length RNA molecules. The template for synthesis of the overlapping nucleotides might be provided by the 5′ terminal 5'GUUG3' motif of the newly synthesized BDV genome (a) or by an internal 5′GUUG3′ close to the 3' end of the template strand (b). The template strand (antigenome) is shown in blue color and the newly synthesized genome is shown in black. The blue octagon indicates the position of the viral replicase complex. The blue flash symbol marks the site where endonucleolytic cleavage might occur.

synthesized siRNA molecules with different terminal structures showed that molecules composed of RNA strands with perfectly matching 5′ and 3′ ends strongly induced IFN production, whereas molecules with 3′ overhangs did not. siRNAs with 5′ overhangs induced intermediate IFN levels [32]. It was further shown that terminal 3′ overhangs prevent efficient unwinding of double-stranded siRNA molecules, which is required to induce efficient signaling of RIG-I and other cytoplasmic RNA helicases that recognize viral dsRNA, such as the melanoma differentiation-associated gene 5.

Direct experimental evidence for downmodulation of the innate immune response by viral genome trimming has not been provided yet. However, it is appealing to think that BDV genome trimming generates termini with a 5′ monophosphate and overlapping nucleotides at the 3′ end, which are almost optimally suited to prevent recognition by RIG-I. The terminal heterogeneities found in genomes of other RNA viruses [23–25, 33, 34] similarly result in frequent elimination of 5′ triphosphate groups and the generation of overlapping nucleotides. Nuclear replication of BDV is not in conflict with the postulated function of BDV genome trimming in the evasion of RIG-I-mediated host cell responses. It has been demonstrated that RIG-I (which is mainly localized in the cytoplasm) is essential for interferon synthesis in response to infection with influenza virus [28], which also replicates in the nucleus. Furthermore, evidence has been provided that RIG-I recognizes viral ribonucleoprotein (RNP) complexes [35]. BDV assembly occurs outside the nucleus [36], and thus it is likely that BDV RNPs are accessible to cytoplasmic sensors such as RIG-I. Although formal proof is still needed, these observations suggest that

terminal genome deletions might support RNA virus persistence by preventing recognition of viral RNA by the cellular innate immune system in persistently infected cells.

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