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Review

Novel insights into the regulation of the viral polymerase complex of neurotropic Borna disease virus

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Abstract

Borna disease virus (BDV) genetic information is encoded in a highly condensed non-segmented RNA genome of negative polarity. Replication and transcription of the genome occurs in the nucleus, enabling the virus to employ the cellular splicing machinery to process primary transcripts and to regulate expression of viral gene products. BDV establishes a non-cytolytic, persistent infection that in animals is mainly restricted to neurons of the central nervous system. Based on these unique properties, BDV represents the prototype member of the virus family *Bornaviridae* in the order *Mononegavirales*. Analysis of molecular aspects of BDV replication has long been hampered by the lack of a reverse genetics system. Only recently, artificial BDV minigenomes permitted the reconstitution of the viral polymerase complex, allowing finally the recovery of BDV from cDNA. As in other families of the *Mononegavirales*, the active polymerase complex of BDV is composed of the polymerase (L), the nucleoprotein (N) and the phosphoprotein (P). In addition, the viral X protein was identified as potent negative regulator of polymerase activity. Protein interaction studies combined with minireplicon assays suggested that P is a central regulatory element of BDV replication that directs the assembly of the polymerase complex. Most intriguingly, BDV obtained from cDNA with variable genomic termini suggests a novel strategy for viral replication-control. BDV seems to restrict its propagation efficacy by defined 5′ terminal trimming of genomic and antigenomic RNA molecules. This review will summarize these novel findings and will discuss them in the context of BDV neurotropism and persistence.
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Keywords: Reverse genetics; Viral replication; Attenuation; Persistence; Genome trimming

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1. Introduction

Borna disease virus (BDV) is the causative agent of Borna disease (BD), a usually fatal meningoencephalitis, originally detected among horses in Germany. The classic hosts of BDV are horses, sheep and other farm animals and its endemic area is probably restricted to central Europe. However, serological evidence indicates an almost worldwide distribution of BDV and a broad host spectrum in warm-blooded vertebrates ranging from birds to non-human primates (Hornig et al., 2003; Staeheli et al., 2000). Natural transmission of BDV follows most likely the olfactory route since experimental intranasal inoculation is efficient and viral antigens are found in the olfactory bulb early in natural infection (Gosztonyi and Ludwig, 1984; Morales et al., 1988). Epidemiological data and the fact that high titers of infectious virus can be detected in the urine of persistently infected rats suggest that rodents might serve as natural reservoir and as vector for the transmission of BDV (Sauder and Staeheli, 2003; Sierra-Honigmann et al., 1993).

The broad host range of BDV and serological evidence (Billich et al., 2002) suggest that BDV or a BDV-like virus also might infect humans. Several reports have suggested a link between BDV infections and certain neuropsychiatric syndromes such as affective disorders, chronic fatigue syndrome, and schizophrenia (Bode et al., 1993; Rott et al., 1985; Schwemmle et al., 1999a). In neonate Lewis rats BDV can establish persistence in the central nervous system (CNS) that results in neuro-developmental and behavioral abnormalities in the absence of inflammation (Pletnikov et al., 2002). Infection of adult Lewis rats, in contrast, frequently induces an immune-mediated neurological disorder similar to BD (de la Torre, 2002a), which is characterized by partial ataxia of the hind legs, uncoordinated movement and massive weight loss of the animal. BDV infection of Lewis rats thus provides a valuable model system to study several important aspects of viral infection of the CNS. These include mechanisms for the induction of immunological tolerance and establishment of viral persistence, analysis of pathogen-induced neuro-developmental damage and their association with neuro-psychiatric disorders, and the induction of immune-mediated neuropathology. To gain insight into these complex processes, reverse genetics approaches are required that allow the detailed analysis of the molecular and cell biology of BDV infection.

1.1. Genome organization and regulation of gene expression

The negative-stranded RNA genome of BDV (Fig. 1) consists of approximately 8900 nucleotides and represents the smallest genome known amongst the *Mononegavirales*. Coding as well as regulatory sequences are highly conserved between different BDV strains (Kilbourne, 1991; Pleschka et al., 2001; Schneider et al., 1994a), suggesting not only high degree of genetic adaptation to non-cytolytic replication in the CNS, but also restricted genetic flexibility. Transcription of the genome is believed to occur sequentially starting at position +44 of the genomic 3′ end resulting in a transcription gradient, which however, is less pronounced than in most other *Mononegavirales*. The genome is organized in three transcription units (I–III) defined by three transcription initiation signals (S1–S3) and four transcription termination signals (T1–T4, see Fig. 1; Schneemann et al., 1994). BDV has a non-typical organization of the gene boundary regions. Instead of the characteristic T—intergenic region—S signal configuration of other *Mononegavirales*, BDV transcription units and transcriptive signals frequently overlap (Fig. 1; de la Torre, 2002b; Schneemann et al., 1995). Furthermore, transcripts derived from transcription unit III can undergo posttranscriptional modification by the cellular RNA splicing machinery, resulting in a complex pattern of viral RNA molecules in BDV-infected cells (Cubitt et al., 1994b; Schneider et al., 1994b; Tomonaga et al., 2000).

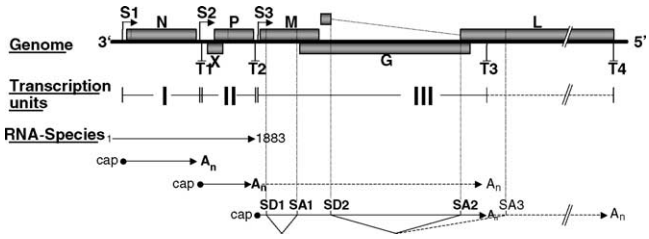

Fig. 1. Genomic organization and transcriptional map of BDV. Open reading frames are represented by grey boxes. The location of transcription initiation and transcription termination signals are indicated by S and T, respectively. Below the graph, the three transcription units are defined and primary transcripts as well as spliced RNA species are shown. The location of intron sequences are indicated by thin dashed lines and the splice donor sites (SD) and the splice acceptor sites (SA) are shown on the primary RNA transcripts of transcription units III. For details on the processing of the primary RNA transcripts see text.

The genes on the BDV genome are arranged in the typical order of the *Mononegavirales* and encode for at least six viral proteins. Beginning at the genomic 3′ end, transcription unit I (S1–T1) encodes the nucleoprotein (N). The S2- and T2-signals define the bi-cistronic transcription unit II, which contains overlapping open reading frames (ORF) for the X protein (X) and the phosphoprotein (P). Readthrough at T2 occurs with low frequency, resulting in a messenger RNA (mRNA) ranging from S2 to T3 that most likely serves as additional template for the translation of X and P proteins. All primary transcripts starting from S3 (transcription unit III) contain overlapping ORFs of the matrix (M) and the glycoprotein (G). Transcription from S3 usually terminates at T3, but in approximately 5% of all cases readthrough at T3 occurs and transcription terminates at T4 yielding a long primary transcript with the potential to code for the polymerase (L). All transcripts of unit III contain two intron sequences located within the M ORF (Intron 1) and the G ORF (Intron 2), respectively. Although both introns are efficiently removed from artificially expressed mRNA, splicing efficiency in BDV-infected cells is not complete, suggesting a

regulatory function of the viral polymerase complex in these splicing events (Jehle et al., 2000). Variable splicing of intron 1 and 2 thus enables BDV to regulate the expression of M, G and L (Fig. 1). M is translated from all mRNAs containing intron 1. All transcripts maintaining intron 2 encode G, but efficient translation of the G-ORF occurs only upon splicing of intron 1. Splicing of intron 2 from the primary transcript terminated at T4 creates a large ORF encoding L (p190), which is only translated efficiently if intron 1 is removed. Additional minor splicing products in BDV-infected cells indicate the existence of a third intron present only in transcripts terminated at T4, which is formed by the splice donor site of intron 2 (SD2) and an alternative splice acceptor site (SA3; Cubitt et al., 2001; Tomonaga et al., 2000). Splicing of intron 3 results in a shortened L ORF (p165) lacking the sequence coding for the N-terminal 300 amino acids of the L polymerase. Usage of SA3 in processing of viral transcripts in BDV-infected cells is downregulated by an exon-splicing suppressor sequence (Tomonaga et al., 2000). The relevance of intron 3 splicing in BDV replication has been questioned, since BDV strain No98 lacks the alternative splice acceptor site SA3 (Pleschka et al., 2001). In BDV-infected cells an additional subgenomic RNA species can be detected. Premature termination of genome replication at T2 results in an uncapped RNA molecule extending from nucleotides 1 to 1883 (Schneemann et al., 1994). The function of this uncapped sub-genomic RNA is unknown. The generally high but variable abundance of this molecule in infected cells suggests a possible role in the regulation of viral replication.

Finally, short non-coding regions of 52 and 43 nucleotides form the 5′- and the 3′-termini of the BDV genome, respectively (Briese et al., 1994). The non-coding regions contain the promoter elements that regulate the synthesis of (+) sense antigenomic RNA from (−) sense genomic RNA and vice versa. The terminal 20 nucleotides of the genome represent inverted terminal repeats with the potential to form a panhandle structure. As in other negative-strand RNA viruses, the termini are highly conserved and contain important elements for the initiation and regulation of viral replication (see below).

1.2. Components of the viral polymerase complex

The active polymerase complex of non-segmented negative-stranded RNA viruses (NNS), termed ribonucleoprotein complex (RNP; Murphy and Lazzarini, 1974), consists of L, N and P associated with the viral RNA (Pattnaik et al., 1992). The N protein is the major component of the RNP and encapsidates the viral genome. BDV is unique amongst viruses of the *Mononegavirales*, since two isoforms of N (p39/p38) can be detected in infected cells that are synthesized by alternative usage of in-frame initiation codons. p38 lacks the N-terminal 13 amino acids (aa) of p39. These terminal aa harbor the only nuclear localization signal (NLS) identified in the N ORF (Kobayashi et al., 1998; Pyper and Gartner, 1997). p39, but not p38, supports replication and transcription of a BDV minigenome if expressed in the absence of the other isoform (Perez et al., 2003; Schneider et al., 2004b). Close to the N-terminus, N contains two independent P binding sites (Berg et al., 1998).

The P protein acts as co-transcriptional factor of the L polymerase, is involved in encapsidation of the viral RNA and further interacts with the small X protein (Schwemmle et al., 1998). Similar to N, two isoforms of BDV-P have been detected in infected cells, resulting from alternative usage of in-frame AUG-initiation codons (Kobayashi et al., 2000). The short isoform (P′) lacks the N-terminal 55 aa of P, and one of two independent NLS (Fig. 2; Schwemmle et al., 1999b; Shoya et al., 1998). Although P′ harbors all the sites required for the interaction with X, L, N and itself (Fig. 2) and is efficiently transported to the nucleus, it is unable to support reporter gene expression in a viral minireplicon system (Schneider et al., 2004a). Like other viral phosphoproteins, BDV-P becomes phosphorylated in vivo (Schwemmle et al., 1997). Since P is an integral component of the viral polymerase complex, P phosphorylation might be involved in the regulation of polymerase activity as has been demonstrated for human parainfluenza virus 3 (HPIV-3; Das et al., 1995) and vesicular stomatitis virus (VSV; Gao and Lenard, 1995b). However, the effects of BDV-P phosphorylation on replication and transcription of the BDV genome remain to be elucidated.

The X protein interacts with P (Schwemmle et al., 1998; Wolff et al., 2000). Direct interaction of X with N has been proposed (Malik et al., 1999), but was later shown to occur only indirectly (Wolff et al., 2000). Nuclear import of X is mediated by an unusual importin alpha motif (Wolff et al., 2002). Co-localization of X with P, N and L in nuclear dots suggests that X might associate with the viral polymerase complex. Indeed, a negative effect of X on reporter gene expression from a BDV minigenome has been demonstrated (see below).

The L protein harbors the RNA-dependent RNA polymerase activity and interacts with the P protein (Schneider

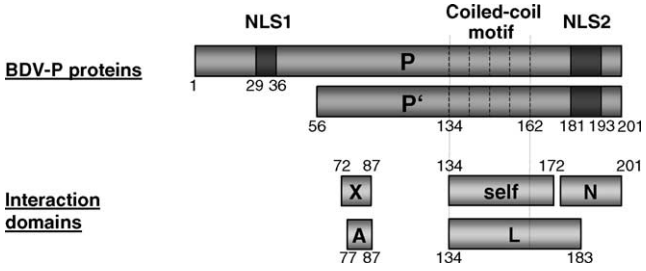

Fig. 2. Schematic representation of BDV-P protein. The long (P) and the short (P′) isoform of P are represented by grey bars. The two independent nuclear localization signals (NLS) are indicated by dark grey boxes and the heptad repeat motif responsible for multimerization of P is signified by five vertical dashed lines. The self, N, L and X boxes indicate the domains responsible for the interaction of P with the respective BDV protein. The A box indicates the domain responsible for interaction with amphoterine/HMG B1. Positions of amino acids defining the boundaries of the NLS, the heptad repeat motif and the various interaction domains are indicated.

et al., 2004a; Walker et al., 2000). Based on conserved in-frame initiation codons and alternative splicing of intron 3, the existence of two truncated isoforms (p180/p165) of BDV-L has been proposed (de la Torre, 2002b). However, no direct evidence for their presence in infected cells has been provided, nor was their functionality demonstrated in the BDV minireplicon assay. Interestingly, BDV polymerase activity is strongly inhibited by Ara-C (1-β-D-arabinofuranosylcytosine), a nucleoside analogue that specifically inhibits DNA polymerase (Bajramovic et al., 2002). Available data strongly suggest that Ara-C acts as competitive inhibitor of the viral polymerase (Volmer et al., 2005).

Several lines of evidence suggest that active viral polymerase complexes of *Mononegavirales* can incorporate cellular proteins. The transcriptase complex of VSV was shown to contain the cellular elongation factor α, the heat shock protein 60 and the capping enzyme guanylytransferase (Das et al., 1998; Qanungo et al., 2004). Viral components of the BDV polymerase complex were also shown to interact with cellular proteins. The N protein can bind to the Cdc2-cyclin B1 complex (Planz et al., 2003) and P interacts with the neurite outgrowth factor amphoterin/HMG-B1 (Kamitani et al., 2001). Direct incorporation of these cellular factors into the active BDV polymerase complex has not yet been demonstrated, but it remains possible that these or other cellular factors influence the replication of BDV.

1.3. Reverse genetics system

Due to the multiplication strategy of negative-strand RNA viruses, their naked genomic RNA by itself is not infectious. To reconstitute an active viral polymerase complex, the simultaneous expression of artificial mini- or full-length viral genomes together with viral protein components of the RNP is required. For a variety of viruses of the *Mononegavirales* such systems have been established in the past decade (Conzelmann, 2004). In these systems, expression of viral genome analogues is achieved by using either the RNA polymerase of bacteriophage T7 (T7pol), or the cellular RNA polymerase I (Pol I). Protein components of the viral polymerase complex are expressed from vectors controlled by either the T7pol- or a strong RNA polymerase II promoter. Successful reconstitution of the viral polymerase complex results in the expression of a reporter gene, usually the bacterial chloramphenicol transferase (CAT) gene, from an artificial minigenome, or in the recovery of replication-competent virus upon transfection of a cDNA encoding a full-length viral genome.

Two reverse genetics systems for BDV have been established using either T7pol or Pol I to express an artificial BDV minigenomic RNA, allowing for the first time the direct analysis of the BDV replication complex (Perez et al., 2003; Schneider et al., 2003). Despite the different strategies for the expression of viral RNA, both systems had the same requirements for polymerase activity. As in other *Mononegavirales*, active viral polymerase complex of BDV is composed of L, N and P proteins associated with the viral RNA. The presence of X strongly reduced expression of the CAT reporter gene and the activity of the BDV polymerase complex strongly depended on a defined N-to-P ratio. These insights into BDV replication were finally applied to recover recombinant BDV from plasmid DNA (Schneider et al., 2005). These novel reverse genetics systems have already facilitated the detailed analysis of the composition and regulation of the BDV polymerase complex and will further provide a powerful tool to study aspects of BDV multiplication. In the following chapters, the most important results will be presented in more detail.

2. BDV-P as regulator of the viral polymerase complex

Even before reverse genetics systems were available, several reports provided evidence that BDV-P has important regulatory functions in BDV replication. Analysis of viral proteins in acutely and persistently infected cells showed major alterations of the N and P protein contents during different phases of the BDV infection (Watanabe et al., 2000). A two-fold excess of N over P protein in acutely infected cells was altered to an up to eight-fold excess of P over N in persistently infected cells due to enhanced expression of P protein. Another study demonstrated that BDV-P mediates a viral restriction phenomenon called homologous interference that prevents the superinfection of BDV-infected cells with the same or a closely related strain of BDV (Geib et al., 2003). Moreover, stable expression of BDV-P rendered cells non-permissive to BDV infection, and transient expression of flag-tagged P strongly reduced viral protein expression in cells persistently infected with BDV (Geib et al., 2003). These data indicated that BDV replication and transcription is extremely sensitive to alterations in the stoichiometry of the various components of the polymerase complex. This information proved to be crucial for the establishment of a functional BDV minireplicon system.

2.1. The N-to-P stoichiometry regulates BDV polymerase activity

The first experiments to establish the BDV minireplicon system were designed to determine the optimal stoichiometry of viral protein components for the formation of functional RNPs (Schneider et al., 2003). Constant amounts of plasmids encoding N and L were co-transfected with variable amounts of P plasmid resulting in 1:1, 10:1 and 100:1 ratios of N-to-P plasmid ratios. Expression of the CAT reporter gene was efficient upon transfection of a 10:1 ratio of N and P plasmids, strongly reduced at a 100:1 ratio and completely abolished if N and P plasmids were used at a ratio of 1:1. Further titration experiments demonstrated that the N-to-P and not the L-to-P ratio was critical for polymerase activity and that an approximately 20-fold molar excess of N- over

P- encoding plasmids is optimal for efficient CAT expression from the BDV minigenome. Using flag-tagged versions of BDV-N and -P, it was shown that the level of expressed proteins corresponds well with the amount of transfected plasmids. The importance of the N–P stoichiometry for BDV polymerase activity was further underscored by data showing that additional expression of the p38 isoform of BDV-N restored polymerase activity in cells expressing unfavorably high amounts of P protein (Schneider et al., 2004b). Since p38 is unable to directly support transcriptional activity of the polymerase complex, this finding suggests that additional expression of p38 positively affected the assembly of the RNP and/or the processivity of the viral polymerase complex by shifting the N-to-P ratio, rather than by simply increasing the number of transcriptionally active RNPs. To the best of our knowledge, no other virus of the *Mononegavirales* requires such a high N-to-P ratio for optimal replication and is completely inhibited by equimolar concentrations of N and P. The stringent control of the BDV replication through variable amounts of P might be a prerequisite for BDV persistence in the CNS.

Compared to other *Mononegavirales*, BDV propagation is notoriously slow in tissue culture and in experimentally infected animals. It has been speculated that slow propagation of BDV is due to an inefficient polymerase complex that is unable to provide sufficient amounts of viral proteins and genomes for faster spreading of the virus. Data obtained from the minireplicon system do not support this view. At optimal N-to-P ratios, CAT expression from the BDV minireplicon system was comparable to that from the minireplicon of the fast-spreading measles virus (own unpublished data). In this context it is important to note that even in the acute phase of BDV infection, the N-to-P ratio is roughly 2:1, which appears to be suboptimal for viral replication (Watanabe et al., 2000). This suggests that BDV may generally restrict its propagation efficacy by maintaining a suboptimal high level of P protein. Together with the observation that the N-to-P ratio in BDV-infected cells undergoes major alterations in the course of infection, this suggests that BDV may employ variable expression of P protein to achieve attenuated viral replication. The molecular mechanism(s) involved in the regulation of BDV-P protein levels in infected cells are still not well characterized, but might involve the interaction of P with the viral X protein (see also chapter 3).

2.2. The role of BDV-P in assembly and transcriptional activity of the viral polymerase complex

The function of P proteins in assembly and activity of viral polymerase complexes has been extensively studied for several viruses. These studies identified not only structural and functional similarities, but also important differences between the P proteins of different viruses of the *Mononegavirales*. For a better understanding of the structure–function relationship of BDV-P, I will first try to give a short summary on the current view on P function in other NNS viruses.

The P proteins of the *Mononegavirales* are multifunctional proteins with the ability to form a variety of hetero- and homo-oligomeric protein complexes. Most P proteins are between 400 and 600 aa in size and have a similar organization of protein interaction domains. The architecture of the C-terminal part of P is conserved and contains three distinct domains that mediate self-oligomerization, interaction with L and interaction with the RNA-associated N protein. The self-oligomerization domain contains a heptad-repeat motif, which suggests the formation of a coiled-coil structure between several P monomers (Choudhary et al., 2002; Curran et al., 1995a; Schwemmle et al., 1998). P multimerization is essential, since deletion or mutation of the heptad-repeat motif abrogated polymerase activity in viral minireplicon systems (De et al., 2000; Gao and Lenard, 1995a, 1995b). In all *Mononegavirales* examined so far, the predominant form of P in infected cells was biochemically shown to be a trimer (Curran et al., 1995a; De et al., 2000; Gao and Lenard, 1995b). However, the crystal structure of the self-oligomerization domain of the Sendai virus (SeV) P protein indicated a tetrameric coiled-coil (Tarbouriech et al., 2000a, 2000b). The discrepancy between the biochemical and the structural analysis might reflect the influence of other domains on P multimerization and suggests that several biologically active multimers of P might exist. The L binding domain of P is located between the self-oligomerization domain and the C-terminal nucleocapsid-binding domain (NBD). Two fundamentally different interactions between P and N can occur. One interaction mediates the association of the P–L complex to the nucleocapsid and is essential for assembly of the viral polymerase complex. The other interaction mediates the binding of P to free N molecules (N^0) that presumably prevents aggregation of N and helps to form the encapsidation complex (N^0–P), which is essential for specific and efficient packaging of viral RNA. Since unproductive association of the rare L protein with the abundant N^0 molecule would sequester viral polymerase activity, viruses must have evolved mechanisms that prevent P from simultaneously interacting with L and N^0. In the P proteins of a variety of *Mononegavirales*, additional domains close to the N-terminus have been identified which specifically interact with N^0 (Curran et al., 1995b). The existence of a second interaction domain suggests a possible mechanism to ensure exclusive interaction of the P–L complex with the nucleocapsid. P could undergo structural alterations upon binding to L that prevents the N-terminal domain from interacting with N^0. In addition, N might alter its conformation upon association with the viral RNA exposing a previously inaccessible binding site for the P–L complex and simultaneously hiding the site responsible for the P–N^0 interaction. The mechanisms which regulate the assembly of viral polymerase complexes are still not understood in detail and might further involve interaction of the polymerase complex with promoter sequences or secondary structures of the nucleocapsid such as panhandle formation. It might well be that viruses of the *Mononegavirales* follow different strategies to achieve correct and efficient formation

of active polymerase complexes. In any case, available data suggest that P is a highly versatile protein with a high degree of structural flexibility to achieve its many functions. In this context it is of interest to note that the P proteins of *Mononegavirales* have a high content of intrinsically disordered regions (Karlin et al., 2003, 2002). This indicates that these proteins can adopt different structural conformations depending on their interaction partners. Intrinsically disordered regions were also identified at the C-terminus of the N protein (termed Ntail) of most *Mononegavirales* (Bourhis et al., 2004; Karlin et al., 2003), which contain the sites of P attachment to the nucleocapsid. The crystal structure of the NBD of measles virus (MV) P was determined (Johansson et al., 2003). The NBD is a stable 3-helix bundle that forms a central hydrophobic cleft. This structure suggests a model for the association of the P–L complex to the nucleocapsid during assembly and transcription of the RNA. The interaction of MV–P with the nucleocapsid was shown to induce the structural transition of Ntail, resulting in the formation of a short amphipathic helix that can accommodate its hydrophobic surface in the hydrophobic cleft of the NBD (Kingston et al., 2004b; Longhi et al., 2003). The resulting hydrophobic interaction is rather weak with a dissociation constant of 35 μM at 37 °C (Kingston et al., 2004a), which might allow the successive association and dissociation of NBD and Ntail domains during RNA synthesis. This process would nicely fit the "cart wheeling" model for the processivity of viral polymerase complex proposed for SeV (Curran, 1998). The model suggests that the P multimers provide the "legs" on which the polymerase "walks" along the nucleocapsid during RNA synthesis by simultaneous making and breaking of P–N contacts. The movement of the polymerase may be guided by the consecutive interaction of L with downstream P multimers associated to the nucleocapsid. Such a model could readily explain the sensitivity of viral polymerase complexes to altered N-to-P ratios. Limiting as well as excessive amounts of P proteins bound to the nucleocapsid would directly affect processivity of the polymerase. If the cart wheeling model is correct, viral P proteins not only serve structural functions in assembly of the viral polymerase complex by providing a scaffold on which the complex is assembled, but also directly regulate viral replication.

How does BDV-P fit into this complex picture? The finding that BDV-L, N and P are sufficient to reconstitute active BDV polymerase complexes demonstrates that BDV-P provides all the auxiliary functions required for viral RNA synthesis (Perez et al., 2003; Schneider et al., 2003). BDV-P consists of 201 aa and thus is two- to three times smaller than P proteins of most other *Mononegavirales*. This compact structure is reflected in the organization of important interaction domains (Fig. 2). As in other viruses the self-oligomerization domain contains a heptad-repeat motif that is essential for activity of the viral polymerase complex and the predominant form of BDV-P in infected cells is likely to be a trimer (Schneider et al., 2004a). The self-oligomerization and the N-binding domains have been mapped to the C-terminal portion of the protein (Schwemmle et al., 1998). Using a mammalian-two-hybrid system we recently mapped the L-binding domain between the self-oligomerization and the N-binding domain (Schneider et al., 2004a), demonstrating that BDV-P has the same overall architecture as other viral P proteins (Fig. 2). However, in contrast to other P proteins, the tight clustering of interaction domains in the C-terminal 70 aa of BDV-P results in a partial overlap of the central L interaction domain with the self-oligomerization domain and possibly with the N-binding site (Fig. 2). This has important functional implications for the assembly of the BDV polymerase complex. We were able to show that L can interact with P multimers, indicating that despite an overlap of the interaction domains, L–P and P–P interactions do not interfere with each other. In contrast, the interaction of L and P was efficiently blocked in the presence of free N protein. This represents the first direct demonstration that a particular P molecule of a member of the *Mononegavirales* cannot simultaneously interact with L and N^0. Since BDV-P seems to use the same domain for the interaction with N^0 and the nucleocapsid, this finding strongly supports the view that structural alterations either in the C-terminal NBD of BDV-P and/or in the N protein must occur during assembly of the BDV polymerase complex. Indeed, a program that predicts intrinsically disordered protein regions (PONDR) with high confidence (>99.6%) indicates a high structural flexibility of BDV-P (Fig. 3, top). In contrast to other *Mononegavirales* (Karlin et al., 2003), the C-terminal part of BDV-P is predicted to be highly unstructured, supporting the view that the NBD of BDV-P might adopt different conformations during P–N^0 and P–L-nucleocapsid interaction. PONDR further predicts an intrinsically disordered region at the C-terminus of BDV-N (residues 319–358, Fig. 3, bottom) similarly to the situation in other *Mononegaviruses*, where an intrinsically disordered C-terminal region provides the nucleocapsid-binding site for P (Bourhis et al., 2004; Longhi et al., 2003). Interestingly this region has not yet been implicated in the interaction of BDV-N and P. Two domains were identified that mediate the interaction of N with P, which are both localized in the N-terminal half of the N protein (Berg et al., 1998). In analogy to other *Mononegavirales*, it is tempting to speculate that the N-terminal interaction domains serve the interaction of N^0 with P and the C-terminal unstructured region mediates the assembly of the viral polymerase complex. Such differential interaction of P with N^0 and the nucleocapsid would be in accordance with the cart-wheeling model and could explain the high sensitivity of BDV replication to high amounts of BDV-P.

3. BDV-X as regulator of viral polymerase activity

BDV-X acts as strong negative regulator of polymerase activity in the minireplicon system (Perez et al., 2003; Schneider et al., 2003). Using X mutant proteins that lost the ability to interact with BDV-P, we were able to demonstrate that

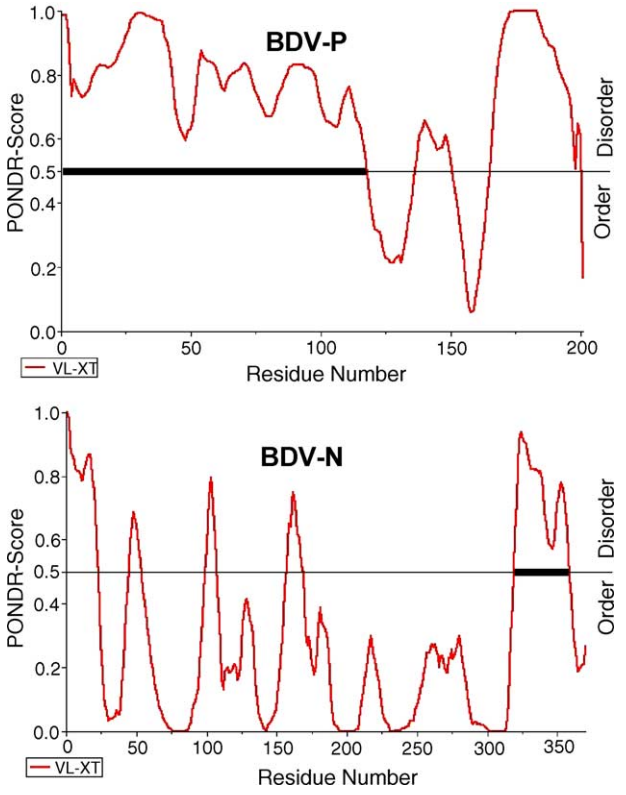

Fig. 3. PONDR prediction of intrinsically disordered regions. Sequences were submitted to the PONDR server (http://www.pondr.com/) using the default integrated predictor VL-XT (Li et al., 1999; Romero et al., 2001). Access to PONDR was provided by Molecular Kinetics (Pullman, WA, USA) under license from the WSU Research Foundation. PONDR is copyright © 1999 by the WSU Research Foundation, all rights reserved. Predictions are shown for BDV-P (top) and BDV-N (bottom). The thick middle lines indicate disordered regions of 40 aa or larger for which a very good confidence of prediction (>99.6%) exists.

the inhibitory activity of X is mediated through its interaction with P (Poenisch et al., 2004). Previous studies had suggested that X might exercise its inhibitory activity by sequestration of BDV-P to the cytoplasm, thereby limiting the number of P molecules available for nuclear replication (Kobayashi et al., 2003). However, clear cytoplasmic retention of P was only observed when a five-fold excess of X over P encoding plasmids were co-transfected. Since titration experiments in the minireplicon system later showed that already very low concentrations of X (at an X-to-P ratio of 1:5) strongly inhibit viral RNA synthesis (Schneider et al., 2003), other mechanisms might be involved in the regulatory function of X. The recent observation that X preferentially interacts with a monomeric form of BDV-P (Schneider et al., 2004a) suggests that the X–P interaction might limit the number of P molecules available for multimerization or, alternatively, could interfere with the correct multimerization of P. Both events would result in a reduced number of transcriptionally active P trimers. Furthermore, it was shown that X does bind to P–L as well as to P–N^0 complexes (Schneider et al., 2004a). This suggests that X is able to act directly on the RNP to reduce RNA synthesis either by interference with polymerase activity or by inhibition of encapsidation of viral RNA.

An important question that needs to be solved to understand the function of X in BDV replication is how the expression of X and P is regulated in BDV-infected cells. X and P are encoded by overlapping ORFs in transcription unit II (Fig. 1). The X initiation codon is localized 48 nucleotides downstream of the 5′ cap of the mRNA and 49 nucleotides upstream of the P initiation codon. Furthermore, the X initiation codon is embedded in a better Kozak context than the P initiation codon. Thus, standard ribosomal scanning for initiation codons would result in a predominant translation of the X ORF. Since even equimolar concentrations of X and P completely block viral RNA synthesis in the minireplicon system (Schneider et al., 2003), it seems likely that BDV employs unknown regulatory mechanisms to reduce expression of X or to enhance expression of P. Downregulation of X protein levels could be achieved by enhanced degradation of X compared to P. Our observation that transient expression of X protein from plasmid DNA results in long-lasting expression of high levels of X protein argues against enhanced X protein degradation. However, the half-lives of X and P proteins in BDV-infected cells remain to be determined. Alternative mechanisms might include ribosomal shunting or internal ribosomal entry which both result in preferential usage of internal initiation codons.

Finally, it should be mentioned that the regulatory function of X in BDV-infected cells might differ from its function in the minireplicon system. The minireplicon system is optimized for maximal activity of the viral polymerase complex. Under these conditions it is well conceivable that the addition of a factor that interferes with optimal P activity results in decreased RNA synthesis and lower reporter gene expression. As described before, in BDV-infected cells, especially in the persistent phase of infection, an excess of P protein compared to N protein is present that most likely restricts BDV replication (Watanabe et al., 2000). In such an environment, X–P interaction might no longer result in inhibition of the polymerase complex. On the contrary, X might neutralize excessive amounts of P protein at the site of replication, thus preventing BDV replication from ending in a deadlock from which it can no longer escape. If true this would suggest that X is involved in the reactivation of RNA synthesis during persistence.

4. Regulation of BDV replication through terminal modification of the viral genome

Genome and antigenome synthesis of negative-strand RNA viruses are initiated at promoter sequences of about 20 nucleotides that are located at the very 5′ and 3′ ends of the viral genome and antigenome, respectively. These promoters represent inverted terminal repeats (ITR) with a high degree of sequence complementarity, which have the potential to form a panhandle structure with matching 5′ and 3′

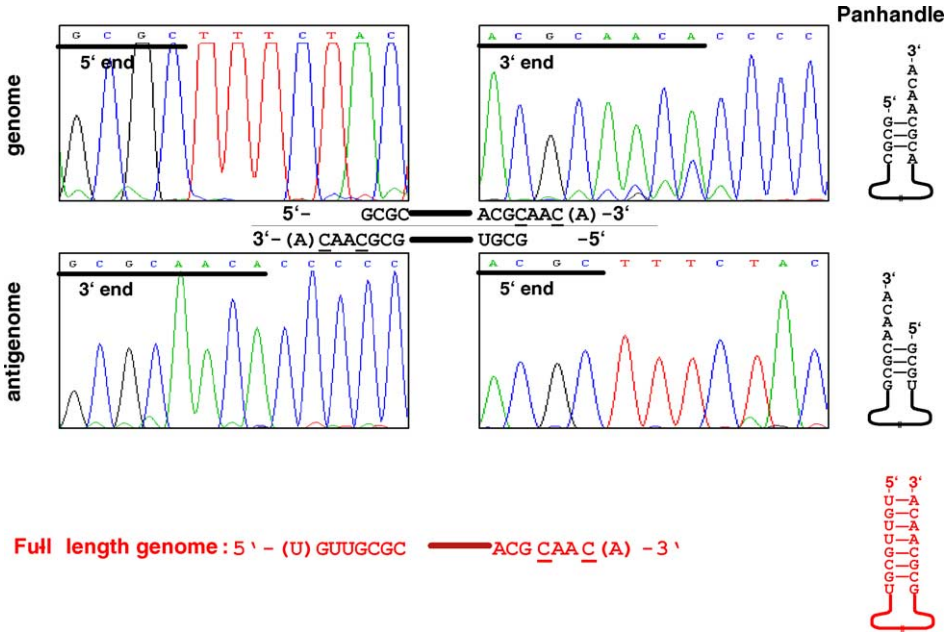

Fig. 4. Terminal genomic and antigenomic sequences of BDV strain He/80 determined by 5′ and 3′ RACE experiments. Electropherograms show the bulk sequence of amplified PCR-fragments from near the 3′ termini (including part of the C tail) and from near the 5′ termini (including part of the synthetic RNA oligo). The deduced panhandle structures and the alignment of genome and antigenome as well as the complete BDV genome sequence and the panhandle with complete 5′ and 3′ ITR are shown schematically. Terminal nucleotides, which are most likely added by the BDV polymerase during the termination process, are presented in brackets. The C nucleotides located in position 2 and position 5 from the 3′ end of the genome and the antigenome are underlined.

termini (Fig. 4). Such panhandle structures were found to be important regulatory elements of replication for members of the *Orthomyxoviridae* (Brownlee and Sharps, 2002; Cheong et al., 1999; Flick and Hobom, 1999; Hsu et al., 1987) and *Bunyaviridae* (Barr and Wertz, 2004; Kohl et al., 2004; Ronnholm and Pettersson, 1987) families. In case of the *Mononegavirales*, it is unclear whether a panhandle structure is indeed formed during viral replication (Wertz et al., 1994), or whether the terminal complementarity of the genome simply reflects similar sequence requirements of the genomic and antigenomic promoters (Tapparel and Roux, 1996).

Sequence analysis of the BDV genome indicated that the ITRs of BDV are unusual amongst the *Mononegavirales*, since they appeared to be non-complete (Briese et al., 1994; Cubitt et al., 1994a; Pleschka et al., 2001). The 5′ terminus of the viral genome lacks four terminal nucleotides required for perfect complementarity with the 3′ terminus. Since the terminal nucleotides of the genomes are of utmost importance for the replication of negative-strand RNA viruses, the unusual structure of the published BDV ITRs had to be confirmed before a full-length rescue system was established. In contrast to previous attempts where analysis was restricted to the BDV genome, this time the genomic and the antigenomic terminal sequences were determined by 5′ and 3′ RACE experiments. The outcome of this analysis was most surprising. As shown in Fig. 4, both the BDV genome and the BDV antigenome carried a defined 5′ terminal deletion resulting in 3′ overhangs of four nucleotides at both sides of aligned genomic and antigenomic RNA molecules or in potential panhandle structures (Schneider et al., 2005). Maintenance of genetic information requires perfect complementarity between the genome and the antigenome. The unusual terminal structure of BDV thus implies that the majority of viral genomes and antigenomes represent 5′ terminal truncated subgenomic RNA species and furthermore suggests the existence of only a very small population of full-length RNA molecules. To test this hypothesis two full-length cDNA clones were created differing only in four nucleotides. One encoded a genome with a non-complete 5′ ITR (rBDVnc), as previously published, and the other encoded the theoretical full-length BDV genome with complete 5′ and 3′ ITRs (rBDVc). Using a newly established BDV rescue system both recombinant viruses were recovered from cDNA (Schneider et al., 2005). Although successful recovery of rBDVnc suggested that a complete 5′ ITR is not essential for BDV replication, spreading efficiency and production of infectious particles of rBDVnc were strongly impaired. In contrast, growth kinetics and infectivity of rBDVc were indistinguishable from those of parental BDV, demonstrating that the full-length molecule with matching terminal ends represents the complete BDV genome. Analysis of the terminal sequences of rBDVc showed that the majority of RNA genomes and antigenomes were again trimmed at their 5′ ends (Schneider et al., 2005), resulting in the same terminal sequences as shown for parental BDV in Fig. 4. Since the rBDVc genome originated from a cDNA encoding the full-length molecule, it seems that the subgenomic RNA molecules are produced by programmed terminal trimming during BDV replication (see model presented in Fig. 5A).

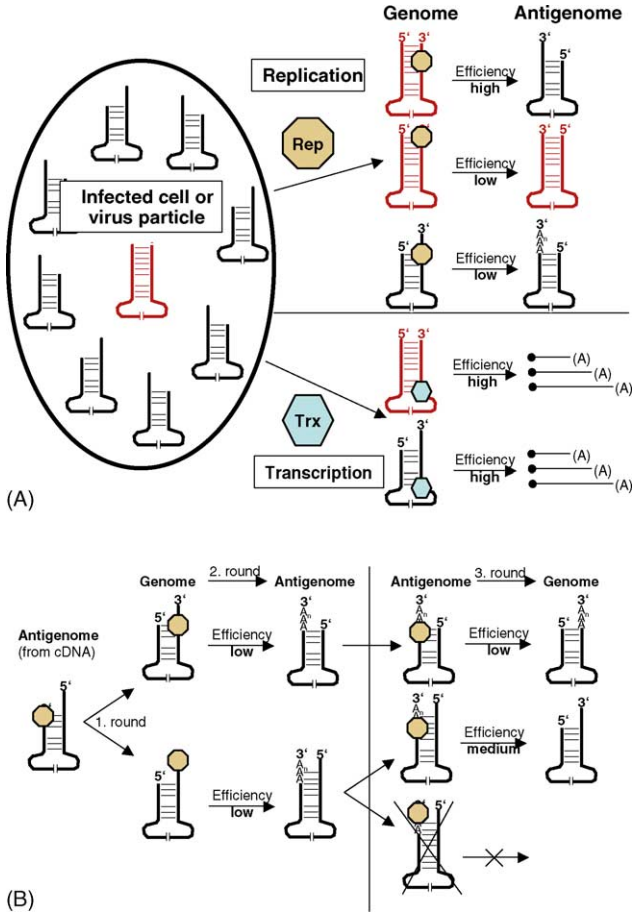

Fig. 5. Regulation of BDV replication through 5′ terminal trimming of genomic and antigenomic RNA molecules. (A) Model for the replication and transcription of parental BDV and rBDVc. Schematic drawing depicting proposed preferential replication of full-length BDV genomes and preferential production of trimmed subgenomic RNA molecules. The initiation sites of the replicase complex (Rep, yellow octagon) and the transcriptase complex (Trx, blue hexagon) are indicated. Our model predicts that genomes with 5′ terminal truncations are inefficiently replicated, but efficiently transcribed. (B) Model for the replication of rBDVnc. Schematic drawing showing three rounds of replication starting with the antigenome derived from cDNA. Our model proposes that full-length genomes and antigenomes are not being produced explaining the reduced replication capacity of rBDVnc. The addition of up to five non-template-encoded A nucleotides at the 3′ ends of genomes and antigenomes derived from the replication of subgenomic RNA molecules are indicated by a 3′ terminal stretch of three A nucleotides in the panhandle presentation. Note that the existence of panhandles in BDV replication has not been formally proven yet.

The terminal sequences of rBDVnc were almost identical to those of rBDVc and parental BDV, except that the antigenome of rBDVnc lacked a correct 3′ terminus (as expected from the sequence of the full-length cDNA clone). Unexpectedly, the four 3′ terminal nucleotides were not simply missing in the antigenomic RNA molecule, but rather were replaced by a stretch of up to five non-template-encoded A nucleotides (Schneider et al., 2005). This indicates that the BDV polymerase, like other RNA polymerases, preferentially terminates RNA synthesis by addition of an A nucleotide. Thus, the 3′ terminal A nucleotides of the BDV genome and antigenome (Fig. 4) are most likely not encoded by the viral genome but rather are added by the BDV polymerase during the termination process. The fact that rBDVnc was unable to produce a correct antigenomic 3′ end supports the view that rare full-length RNA molecules serve as template for antigenome and probably also genome synthesis (Fig. 5A). If alternative mechanisms other than replication of a full-length template strand were responsible for the production of the 3′ overhangs, rBDVnc should have been able to produce a correct 3′ terminus of the antigenome, because the truncated genomic 5′ termini of rBDVnc, rBDVc and parental BDV were identical. The frequency of these full-length RNA molecules in infected cells as well as in virus particles seems too low for detection by analysis of PCR fragments. In fact, when more than 150 individual plasmid clones derived from PCR fragments representing the genomic 5′ end of rBDVc were analyzed, no sequence corresponding to a complete 5′ ITR was found. The majority of viral RNA molecules nevertheless seem to have complete 3′ termini, which can only occur by replication of the full-length template molecule. This indicates that initiation of replication occurs preferentially at full-length RNA genomes and antigenomes, suggesting that efficient initiation of BDV replication requires complementarity of the 5′ and 3′ termini (Fig. 5A). Since it is difficult to apprehend how the 5′ end should influence the efficiency of replication initiation at the 3′ end of the same RNA molecule other than by direct interaction of the two ITRs, available data strongly support the view that the ITRs of BDV form some sort of panhandle structure to initiate replication.

The mechanism responsible for the generation of 5′ terminally trimmed RNA molecules has not yet been fully elucidated. Specificity of the truncation argues against RNA degradation, although it is conceivable that genome trimming involves a replication-associated endonuclease activity that specifically removes four nucleotides from the 5′ end of the nascent transcripts. However, this mechanism could not fully explain the attenuated phenotype of rBDVnc and furthermore would involve the first description of a nuclease activity associated with the polymerase complex of a virus of the *Mononegavirales*. We favor the alternative possibility presented in the model in Fig. 5A. The model suggests that the BDV replication complex usually initiates RNA synthesis at the internal C residue in position 5 from the 3′ end of the template strand (Fig. 4) generating subgenomic RNA molecules with restricted replication competence. Only rarely, the BDV polymerase initiates at the C residue in position 2 from the 3′ end (Fig. 4) of the template strand resulting in the production of full-length RNA molecules. The frequency of initiation might depend on the different sequence context in which the two C nucleotides are embedded. In contrast to rBDVc, rBDVnc is lacking the C residue in position 2 of the antigenomic RNA and therefore is unable to produce full-length RNA molecules as indicated in the model presented in Fig. 5B. The fact that rBDVnc is viable in the absence of full-length RNA

molecules suggests that replication of trimmed genomic and antigenomic RNA molecules can occur (Fig. 5B), although with strongly reduced efficacy resulting in the observed attenuation of rBDVnc propagation and infectivity (Schneider et al., 2005). This view is further supported by a sequence polymorphism found in both strands at the position of the penultimate C nucleotide by 3′ RACE experiments (Fig. 4; Schneider et al., 2005). All electropherograms indicated a dominant C nucleotide and a less frequent A nucleotide in this position. The C/A polymorphism was found in all three viruses examined (Schneider et al., 2005). This finding suggests that most RNA molecules encoded a C residue in position 2 and thus originated from replication of full-length templates. A minority of the RNA molecules seems to encode an A instead of a C residue in this position, which is best explained by replication of subgenomic RNA molecules in which RNA synthesis is frequently terminated by addition of non-template encoded 3′ terminal A nucleotides as demonstrated for the antigenome synthesis of rBDVnc (Schneider et al., 2005). This situation is predicted by the models presented in Fig. 5A and B and provides direct evidence for the existence of full-length and subgenomic RNA molecules with different replication competence.

How might BDV benefit from restricting the number of fully replication competent genomes and antigenomes? Despite the pronounced differences in infectivity and spreading efficiency of rBDVnc and rBDVc, nearly identical levels of viral RNA and proteins were found in persistently infected cells (Schneider et al., 2005). This surprising result suggested that absence of complementary ITRs does not so much affect transcription of the BDV genome (Fig. 5A), but heavily delays replication (Fig. 5B) and thus the production of templates for the transcription of mRNA. Late in infection, rBDVnc seems to overcome the restriction of delayed genome replication. However, early in infection, the inability of rBDVnc to produce sufficient amounts of primary transcripts due to inefficient genome amplification might result in an increased number of abortive infections. Such a scenario may readily explain the low number of infectious particles detected and the reduced spreading capacity of rBDVnc (Schneider et al., 2005). Although this provides only indirect evidence, it strongly suggests that restriction of the number of fully replication competent genomes in rBDVc and parental BDV has a similar function. The low percentage of viral genomes able to efficiently transmit infectivity to neighboring cells might control virus propagation. Attenuation of viral propagation might be a prerequisite for non-cytolytic replication and persistence of BDV.

Efficient transcription of mRNA from truncated BDV genomes suggests that replication and transcription are differentially regulated. This supports a two-entry site model for the initiation of BDV transcription and replication (Fig. 5A) as has been shown for VSV (Whelan and Wertz, 2002). It is thus well conceivable that similar to VSV (Qanungo et al., 2004), BDV replicase and transcriptase complexes may represent distinct entities with different subunit composition.

5. Perspectives

The ability to recover recombinant BDV from cDNA represents a technical break-through in several aspects. It is the first rescue system for a non-segmented RNA virus with nuclear replication and the first rescue system for an RNA virus with predominant persistent replication. Insights gained from analysis of the BDV replication cycle might not only improve our understanding of the replication of *Mononegavirales*, but also provide essential information for the establishment of reverse genetics systems for severe human pathogens with predominant persistent replication such as Hantaanvirus.

Nuclear replication and persistent propagation of BDV in the CNS of infected animals suggested the existence of exclusive and stringent mechanisms for the regulation of BDV replication. Analysis of BDV polymerase complex using novel reverse genetics approaches has highlighted the unique position of the *Bornaviridae* amongst the *Mononegavirales*. In vivo reconstitution assays demonstrated an extraordinary sensitivity of the BDV polymerase complex to altered P protein expression and identified the small viral X protein as negative regulator. To elucidate the molecular basis for the regulatory functions of P and X, further studies involving recombinant viruses with different X-to-P stoichiometry will be essential. The function of the viral N protein also deserves further attention. Based on the recently published crystal structure of BDV-N (Rudolph et al., 2003) it might be possible to insert structure-guided mutations into the N protein, which specifically target specific functions of N. Analysis of the effects of these mutations will improve our knowledge of the formation of functional RNP structures in negative-strand RNA viruses. The ability to generate predetermined mutations within the protein coding regions of the BDV genome further offers a powerful tool to analyze other important aspects of the viral replication cycle, including assembly, budding and entry of BDV.

Recombinant BDV created by the reverse genetics approach provided strong evidence that genome replication is not only regulated at the level of protein–protein interaction, but also by direct modification of the genetic information. Although similar phenomenon have been described for Seoul virus (Meyer and Schmaljohn, 2000), a member of the Hantavirus genus, the specific truncation of exactly four nucleotides from the 5′ termini of viral RNA molecules is unprecedented among the negative-strand RNA viruses. A detailed analysis of underlying mechanisms involving determination of sequence requirements for promoter function might enable the production of a recombinant BDV that is no longer able to modify its genome. Such a virus might offer novel insights into the establishment of viral persistence in the CNS.

Recombinant BDV containing an additional transcription unit might represent a valuable vector system for the long-term expression of foreign genes in the CNS of infected animals. Viruses expressing certain neurotrophines might help to elucidate the mechanism(s) involved in BDV interference

with neuronal plasticity (Gonzalez-Dunia et al., 2005). Such viruses might further provide novel experimental models to study the effect of cellular factors on various aspects of brain development and CNS architecture as well as for the experimental therapy of neurodegenerative disease.

Although BDV has not yet been fully recognized as human pathogen, the unique genome structure, the unusual regulation of transcription and replication and the highly neurotropic propagation of BDV resulting in CNS persistence justify the intensive analysis of the replication cycle of this intriguing virus. Insights into the molecular and cellular biology of BDV replication obtained by reverse genetics approaches will not only improve our understanding of the replication of negative-strand RNA viruses in general, but also offer novel insights into virus-induced CNS pathology and associated diseases.

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References

Bajramovic, J.J., Syan, S., Brahic, M., de la Torre, J.C., Gonzalez-Dunia, D., 2002. 1-beta-D-arabinofuranosylcytosine inhibits Borna disease virus replication and spread. J. Virol. 76, 6268–6276.

Barr, J.N., Wertz, G.W., 2004. Bunyamwera bunyavirus RNA synthesis requires cooperation of 3′- and 5′-terminal sequences. J. Virol. 78, 1129–1138.

Berg, M., Ehrenborg, C., Blomberg, J., Pipkorn, R., Berg, A.L., 1998. Two domains of the Borna disease virus p40 protein are required for interaction with the p23 protein. J. Gen. Virol. 79, 2957–2963.

Billich, C., Sauder, C., Frank, R., Herzog, S., Bechter, K., Takahashi, K., Peters, H., Staeheli, P., Schwemmle, M., 2002. High-avidity human serum antibodies recognizing linear epitopes of Borna disease virus proteins. Biol. Psychiatry 51, 979–987.

Bode, L., Ferszt, R., Czech, G., 1993. Borna disease virus infection and affective disorders in man. Arch. Virol. Suppl. 7, 159–167.

Bourhis, J.M., Johansson, K., Receveur-Brechot, V., Oldfield, C.J., Dunker, K.A., Canard, B., Longhi, S., 2004. The C-terminal domain of measles virus nucleoprotein belongs to the class of intrinsically disordered proteins that fold upon binding to their physiological partner. Virus Res. 99, 157–167.

Briese, T., Schneemann, A., Lewis, A.J., Park, Y.S., Kim, S., Ludwig, H., Lipkin, W.I., 1994. Genomic organization of Borna disease virus. Proc. Natl. Acad. Sci. U.S.A. 91, 4362–4366.

Brownlee, G.G., Sharps, J.L., 2002. The RNA polymerase of influenza A virus is stabilized by interaction with its viral RNA promoter. J. Virol. 76, 7103–7113.

Cheong, H.K., Cheong, C., Lee, Y.S., Seong, B.L., Choi, B.S., 1999. Structure of influenza virus panhandle RNA studied by NMR spectroscopy and molecular modeling. Nucleic Acids Res. 27, 1392–1397.

Choudhary, S.K., Malur, A.G., Huo, Y., De, B.P., Banerjee, A.K., 2002. Characterization of the oligomerization domain of the phosphoprotein of human parainfluenza virus type 3. Virology 302, 373–382.

Conzelmann, K.K., 2004. Reverse genetics of *mononegavirales*. Curr. Top. Microbiol. Immunol. 283, 1–41.

Cubitt, B., Ly, C., de la Torre, J.C., 2001. Identification and characterization of a new intron in Borna disease virus. J. Gen. Virol. 82, 641–646.

Cubitt, B., Oldstone, C., de la Torre, J.C., 1994a. Sequence and genome organization of Borna disease virus. J. Virol. 68, 1382–1396.

Cubitt, B., Oldstone, C., Valcarcel, J., Carlos de la Torre, J., 1994b. RNA splicing contributes to the generation of mature mRNAs of Borna disease virus, a non-segmented negative strand RNA virus. Virus Res. 34, 69–79.

Curran, J., 1998. A role for the Sendai virus P protein trimer in RNA synthesis. J. Virol. 72, 4274–4280.

Curran, J., Boeck, R., Lin-Marq, N., Lupas, A., Kolakofsky, D., 1995a. Paramyxovirus phosphoproteins form homotrimers as determined by an epitope dilution assay, via predicted coiled coils. Virology 214, 139–149.

Curran, J., Marq, J.B., Kolakofsky, D., 1995b. An N-terminal domain of the Sendai paramyxovirus P protein acts as a chaperone for the NP protein during the nascent chain assembly step of genome replication. J. Virol. 69, 849–855.

Das, T., Gupta, A.K., Sims, P.W., Gelfand, C.A., Jentoft, J.E., Banerjee, A.K., 1995. Role of cellular casein kinase II in the function of the phosphoprotein (P) subunit of RNA polymerase of vesicular stomatitis virus. J. Biol. Chem. 270, 24100–24107.

Das, T., Mathur, M., Gupta, A.K., Janssen, G.M., Banerjee, A.K., 1998. RNA polymerase of vesicular stomatitis virus specifically associates with translation elongation factor-1 alphabetagamma for its activity. Proc. Natl. Acad. Sci. U.S.A. 95, 1449–1454.

De, B.P., Hoffman, M.A., Choudhary, S., Huntley, C.C., Banerjee, A.K., 2000. Role of NH(2)- and COOH-terminal domains of the P protein of human parainfluenza virus type 3 in transcription and replication. J. Virol. 74, 5886–5895.

de la Torre, J.C., 2002a. Bornavirus and the brain. J. Infect. Dis. 186 (Suppl 2), S241–S247.

de la Torre, J.C., 2002b. Molecular biology of Borna disease virus and persistence. Front. Biosci. 7, d569–d579.

Flick, R., Hobom, G., 1999. Interaction of influenza virus polymerase with viral RNA in the 'corkscrew' conformation. J. Gen. Virol. 80, 2565–2572.

Gao, Y., Lenard, J., 1995a. Cooperative binding of multimeric phosphoprotein (P) of vesicular stomatitis virus to polymerase (L) and template: pathways of assembly. J. Virol. 69, 7718–7723.

Gao, Y., Lenard, J., 1995b. Multimerization and transcriptional activation of the phosphoprotein (P) of vesicular stomatitis virus by casein kinase-II. EMBO J. 14, 1240–1247.

Geib, T., Sauder, C., Venturelli, S., Hassler, C., Staeheli, P., Schwemmle, M., 2003. Selective virus resistance conferred by expression of Borna disease virus nucleocapsid components. J. Virol. 77, 4283–4290.

Gonzalez-Dunia, D., Volmer, R., Mayer, D., Schwemmle, M., 2005. Borna disease virus interference with neuronal plasticity. Virus Res. (this issue).

Gosztonyi, G., Ludwig, H., 1984. Borna disease of horses An immunohistological and virological study of naturally infected animals. Acta Neuropathol. (Berl) 64, 213–221.

Hornig, M., Briese, T., Lipkin, W.I., 2003. Borna disease virus. J. Neurovirol. 9, 259–273.

Hsu, M.T., Parvin, J.D., Gupta, S., Krystal, M., Palese, P., 1987. Genomic RNAs of influenza viruses are held in a circular conformation in virions and in infected cells by a terminal panhandle. Proc. Natl. Acad. Sci. U.S.A. 84, 8140–8144.

Jehle, C., Lipkin, W.I., Staeheli, P., Marion, R.M., Schwemmle, M., 2000. Authentic Borna disease virus transcripts are spliced less efficiently than cDNA-derived viral RNAs. J. Gen. Virol. 81, 1947–1954.

Johansson, K., Bourhis, J.M., Campanacci, V., Cambillau, C., Canard, B., Longhi, S., 2003. Crystal structure of the measles virus phos-

phoprotein domain responsible for the induced folding of the C-terminal domain of the nucleoprotein. J. Biol. Chem. 278, 44567–44573.

Kamitani, W., Shoya, Y., Kobayashi, T., Watanabe, M., Lee, B.J., Zhang, G., Tomonaga, K., Ikuta, K., 2001. Borna disease virus phosphoprotein binds a neurite outgrowth factor, amphoterin/HMG-1. J. Virol. 75, 8742–8751.

Karlin, D., Ferron, F., Canard, B., Longhi, S., 2003. Structural disorder and modular organization in Paramyxovirinae N and P. J. Gen. Virol. 84, 3239–3252.

Karlin, D., Longhi, S., Receveur, V., Canard, B., 2002. The N-terminal domain of the phosphoprotein of Morbilliviruses belongs to the natively unfolded class of proteins. Virology 296, 251–262.

Kilbourne, E.D., 1991. New viruses and new disease: mutation, evolution and ecology. Curr. Opin. Immunol. 3, 518–524.

Kingston, R.L., Baase, W.A., Gay, L.S., 2004a. Characterization of nucleocapsid binding by the measles virus and mumps virus phosphoproteins. J. Virol. 78, 8630–8640.

Kingston, R.L., Hamel, D.J., Gay, L.S., Dahlquist, F.W., Matthews, B.W., 2004b. Structural basis for the attachment of a paramyxoviral polymerase to its template. Proc. Natl. Acad. Sci. U.S.A. 101, 8301–8306.

Kobayashi, T., Shoya, Y., Koda, T., Takashima, I., Lai, P.K., Ikuta, K., Kakinuma, M., Kishi, M., 1998. Nuclear targeting activity associated with the amino terminal region of the Borna disease virus nucleoprotein. Virology 243, 188–197.

Kobayashi, T., Watanabe, M., Kamitani, W., Tomonaga, K., Ikuta, K., 2000. Translation initiation of a bicistronic mRNA of Borna disease virus: a 16-kDa phosphoprotein is initiated at an internal start codon. Virology 277, 296–305.

Kobayashi, T., Zhang, G., Lee, B.J., Baba, S., Yamashita, M., Kamitani, W., Yanai, H., Tomonaga, K., Ikuta, K., 2003. Modulation of Borna disease virus phosphoprotein nuclear localization by the viral protein X encoded in the overlapping open reading frame. J. Virol. 77, 8099–8107.

Kohl, A., Dunn, E.F., Lowen, A.C., Elliott, R.M., 2004. Complementarity, sequence and structural elements within the 3′ and 5′ non-coding regions of the Bunyamwera orthobunyavirus S segment determine promoter strength. J. Gen. Virol. 85, 3269–3278.

Li, X., Romero, P., Rani, M., Dunker, A.K., Obradovic, Z., 1999. Predicting protein disorder for N-C- and internal regions. Genome Inform. Ser. Workshop Genome Inform. 10, 30–40.

Longhi, S., Receveur-Brechot, V., Karlin, D., Johansson, K., Darbon, H., Bhella, D., Yeo, R., Finet, S., Canard, B., 2003. The C-terminal domain of the measles virus nucleoprotein is intrinsically disordered and folds upon binding to the C-terminal moiety of the phosphoprotein. J. Biol. Chem. 278, 18638–18648.

Malik, T.H., Kobayashi, T., Ghosh, M., Kishi, M., Lai, P.K., 1999. Nuclear localization of the protein from the open reading frame x1 of the Borna disease virus was through interactions with the viral nucleoprotein. Virology 258, 65–72.

Meyer, B.J., Schmaljohn, C., 2000. Accumulation of terminally deleted RNAs may play a role in Seoul virus persistence. J. Virol. 74, 1321–1331.

Morales, J.A., Herzog, S., Kompter, C., Frese, K., Rott, R., 1988. Axonal transport of Borna disease virus along olfactory pathways in spontaneously and experimentally infected rats. Med. Microbiol. Immunol. (Berl) 177, 51–68.

Murphy, M.F., Lazzarini, R.A., 1974. Synthesis of viral mRNA and polyadenylate by a ribonucleoprotein complex from extracts of VSV-infected cells. Cell 3, 77–84.

Pattnaik, A.K., Ball, L.A., LeGrone, A.W., Wertz, G.W., 1992. Infectious defective interfering particles of VSV from transcripts of a cDNA clone. Cell 69, 1011–1020.

Perez, M., Sanchez, A., Cubitt, B., Rosario, D., de la Torre, J.C., 2003. A reverse genetics system for Borna disease virus. J. Gen. Virol. 84, 3099–3104.

Planz, O., Pleschka, S., Oesterle, K., Berberich-Siebelt, F., Ehrhardt, C., Stitz, L., Ludwig, S., 2003. Borna disease virus nucleoprotein interacts with the CDC2-cyclin B1 complex. J. Virol. 77, 11186–11192.

Pleschka, S., Staeheli, P., Kolodziejek, J., Richt, J.A., Nowotny, N., Schwemmle, M., 2001. Conservation of coding potential and terminal sequences in four different isolates of Borna disease virus. J. Gen. Virol. 82, 2681–2690.

Pletnikov, M.V., Moran, T.H., Carbone, K.M., 2002. Borna disease virus infection of the neonatal rat: developmental brain injury model of autism spectrum disorders. Front. Biosci. 7, d593–d607.

Poenisch, M., Unterstab, G., Wolff, T., Staeheli, P., Schneider, U., 2004. The X protein of Borna disease virus regulates viral polymerase activity through interaction with the P protein. J. Gen. Virol. 85, 1895–1898.

Pyper, J.M., Gartner, A.E., 1997. Molecular basis for the differential subcellular localization of the 38- and 39-kilodalton structural proteins of Borna disease virus. J. Virol. 71, 5133–5139.

Qanungo, K.R., Shaji, D., Mathur, M., Banerjee, A.K., 2004. Two RNA polymerase complexes from vesicular stomatitis virus-infected cells that carry out transcription and replication of genome RNA. Proc. Natl. Acad. Sci. U.S.A. 101, 5952–5957.

Romero, P., Obradovic, Z., Li, X., Garner, E.C., Brown, C.J., Dunker, A.K., 2001. Sequence complexity of disordered proteins. Proteins 42, 38–48.

Ronnholm, R., Pettersson, R.F., 1987. Complete nucleotide sequence of the M RNA segment of Uukuniemi virus encoding the membrane glycoproteins G1 and G2. Virology 160, 191–202.

Rott, R., Herzog, S., Fleischer, B., Winokur, A., Amsterdam, J., Dyson, W., Koprowski, H., 1985. Detection of serum antibodies to Borna disease virus in patients with psychiatric disorders. Science 228, 755–756.

Rudolph, M.G., Kraus, I., Dickmanns, A., Eickmann, M., Garten, W., Ficner, R., 2003. Crystal structure of the borna disease virus nucleoprotein. Structure (Camb) 11, 1219–1226.

Sauder, C., Staeheli, P., 2003. Rat model of borna disease virus transmission: epidemiological implications. J. Virol. 77, 12886–12890.

Schneemann, A., Schneider, P.A., Kim, S., Lipkin, W.I., 1994. Identification of signal sequences that control transcription of borna disease virus, a nonsegmented, negative-strand RNA virus. J. Virol. 68, 6514–6522.

Schneemann, A., Schneider, P.A., Lipkin, W.I., 1995. The atypical strategies used for gene expression of Borna disease virus, a nonsegmented, negative-strand RNA virus. Uirusu 45, 165–174.

Schneider, P.A., Briese, T., Zimmermann, W., Ludwig, H., Lipkin, W.I., 1994a. Sequence conservation in field and experimental isolates of Borna disease virus. J. Virol. 68, 63–68.

Schneider, P.A., Schneemann, A., Lipkin, W.I., 1994b. RNA splicing in Borna disease virus, a nonsegmented, negative-strand RNA virus. J. Virol. 68, 5007–5012.

Schneider, U., Blechschmidt, K., Schwemmle, M., Staeheli, P., 2004a. Overlap of interaction domains indicates a central role of the P protein in assembly and regulation of the Borna disease virus polymerase complex. J. Biol. Chem. 279, 55290–55296.

Schneider, U., Naegele, M., Staeheli, P., 2004b. Regulation of the Borna disease virus polymerase complex by the viral nucleoprotein p38 isoform. Brief report. Arch. Virol. 149, 1409–1414.

Schneider, U., Naegele, M., Staeheli, P., Schwemmle, M., 2003. Active Borna disease virus polymerase complex requires a distinct nucleoprotein-to-phosphoprotein ratio but no viral X protein. J. Virol. 77, 11781–11789.

Schneider, U., Schwemmle, M., Staeheli, P., 2005. Genome trimming: a unique strategy for replication control employed by Borna disease virus. Proc. Natl. Acad. Sci. U.S.A. 102, 3441–3446.

Schwemmle, M., De, B., Shi, L., Banerjee, A., Lipkin, W.I., 1997. Borna disease virus P-protein is phosphorylated by protein kinase Cepsilon and casein kinase II. J. Biol. Chem. 272, 21818–21823.

Schwemmle, M., Jehle, C., Formella, S., Staeheli, P., 1999a. Sequence similarities between human bornavirus isolates and laboratory strains question human origin. Lancet 354, 1973–1974.

Schwemmle, M., Jehle, C., Shoemaker, T., Lipkin, W.I., 1999b. Characterization of the major nuclear localization signal of the Borna disease virus phosphoprotein. J. Gen. Virol. 80, 97–100.

Schwemmle, M., Salvatore, M., Shi, L., Richt, J., Lee, C.H., Lipkin, W.I., 1998. Interactions of the borna disease virus P, N, and X proteins and their functional implications. J. Biol. Chem. 273, 9007–9012.

Shoya, Y., Kobayashi, T., Koda, T., Ikuta, K., Kakinuma, M., Kishi, M., 1998. Two proline-rich nuclear localization signals in the amino- and carboxyl-terminal regions of the Borna disease virus phosphoprotein. J. Virol. 72, 9755–9762.

Sierra-Honigmann, A.M., Rubin, S.A., Estafanous, M.G., Yolken, R.H., Carbone, K.M., 1993. Borna disease virus in peripheral blood mononuclear and bone marrow cells of neonatally and chronically infected rats. J. Neuroimmunol. 45, 31–36.

Staeheli, P., Sauder, C., Hausmann, J., Ehrensperger, F., Schwemmle, M., 2000. Epidemiology of Borna disease virus. J. Gen. Virol. 81, 2123–2135.

Tapparel, C., Roux, L., 1996. The efficiency of Sendai virus genome replication: the importance of the RNA primary sequence independent of terminal complementarity. Virology 225, 163–171.

Tarbouriech, N., Curran, J., Ebel, C., Ruigrok, R.W., Burmeister, W.P., 2000a. On the domain structure and the polymerization state of the sendai virus P protein. Virology 266, 99–109.

Tarbouriech, N., Curran, J., Ruigrok, R.W., Burmeister, W.P., 2000b. Tetrameric coiled coil domain of Sendai virus phosphoprotein. Nature Struct. Biol. 7, 777–781.

Tomonaga, K., Kobayashi, T., Lee, B.J., Watanabe, M., Kamitani, W., Ikuta, K., 2000. Identification of alternative splicing and negative splicing activity of a nonsegmented negative-strand RNA virus Borna disease virus. Proc. Natl. Acad. Sci. U.S.A. 97, 12788–12793.

Volmer, R., Bajramovic, J.J., Schneider, U., Ufano, S., Pochet, S., Gonzalez-Dunia, D., 2005. Mechanism of the antiviral action of 1-(-D-arabinofuranosylcytosine (Ara-C) on Borna disease virus. J. Virol. 79, 4514–4518.

Walker, M.P., Jordan, I., Briese, T., Fischer, N., Lipkin, W.I., 2000. Expression and characterization of the Borna disease virus polymerase. J. Virol. 74, 4425–4428.

Watanabe, M., Zhong, Q., Kobayashi, T., Kamitani, W., Tomonaga, K., Ikuta, K., 2000. Molecular ratio between borna disease viral-p40 and -p24 proteins in infected cells determined by quantitative antigen capture ELISA. Microbiol. Immunol. 44, 765–772.

Wertz, G.W., Whelan, S., LeGrone, A., Ball, L.A., 1994. Extent of terminal complementarity modulates the balance between transcription and replication of vesicular stomatitis virus RNA. Proc. Natl. Acad. Sci. U.S.A. 91, 8587–8591.

Whelan, S.P., Wertz, G.W., 2002. Transcription and replication initiate at separate sites on the vesicular stomatitis virus genome. Proc. Natl. Acad. Sci. U.S.A. 99, 9178–9183.

Wolff, T., Pfleger, R., Wehner, T., Reinhardt, J., Richt, J.A., 2000. A short leucine-rich sequence in the Borna disease virus p10 protein mediates association with the viral phospho- and nucleoproteins. J. Gen. Virol. 81, 939–947.

Wolff, T., Unterstab, G., Heins, G., Richt, J.A., Kann, M., 2002. Characterization of an unusual importin alpha binding motif in the borna disease virus p10 protein that directs nuclear import. J. Biol. Chem. 277, 12151–12157.