

**Short Communication A reverse genetics system for Borna disease virus**

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Received 26 June 2003

Accepted 29 July 2003

Ahead-of-print 6 August 2003

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**Borna disease virus (BDV) is an enveloped virus. Its non-segmented, negative-stranded RNA genome has the coding capability for six main polypeptides and has an organization characteristic of members of the order *Mononegavirales*. However, based on its unique genetics and biological features, BDV is considered to be the prototypic member of a new virus family, *Bornaviridae*. Here, the establishment of a reverse genetics system for BDV is described. Intracellular synthesis of a BDV RNA analogue or minigenome (MG) from a plasmid was driven by RNA polymerase I. Co-transfection with plasmids expressing the BDV polymerase (L), nucleoprotein (N) and phosphoprotein (P) under the control of RNA polymerase II allowed for BDV MG replication and expression. This process depended on a delicate N : P ratio, whereas the L : P ratio was less critical. Two isoforms of N, Np40 and Np38, are present in BDV-infected cells but only Np40 was strictly required for virus polymerase activity. BDV p10 polypeptide encoded by the P gene exhibited a strong inhibitory effect on BDV MG expression.**

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Borna disease virus (BDV) causes CNS disease that is frequently manifested by behavioural abnormalities (Ikuta *et al.*, 2002; Pletnikov *et al.*, 2002; Rott & Becht, 1995). Evidence indicates that the natural host range of BDV, as well as its prevalence and geographical distribution, are very broad (Hatalski *et al.*, 1997; Ikuta *et al.*, 2002; Richt *et al.*, 1997; Richt & Rott, 2001; Rott & Becht, 1995; Staeheli *et al.*, 2000). Moreover, serological data and molecular epidemiological studies indicate that BDV can infect humans and might be associated with certain neuropsychiatric disorders (Billich *et al.*, 2002; Carbone, 2001; Planz *et al.*, 2002; Richt *et al.*, 1997; Richt & Rott, 2001; Rott & Becht, 1995; Staeheli *et al.*, 2000). Both virus and host factors contribute to a variable period of incubation, as well as significant heterogeneity, in the symptoms and pathology associated with BDV infection (Gonzalez-Dunia *et al.*, 1997; Hatalski *et al.*, 1997; Ikuta *et al.*, 2002; Richt *et al.*, 1997; Rott & Becht, 1995; Staeheli *et al.*, 2000).

BDV is an enveloped virus with a non-segmented, negative-stranded RNA genome. Its genome is about 8.9 kb long, the smallest among known negative-stranded RNA viruses, and has an organization similar

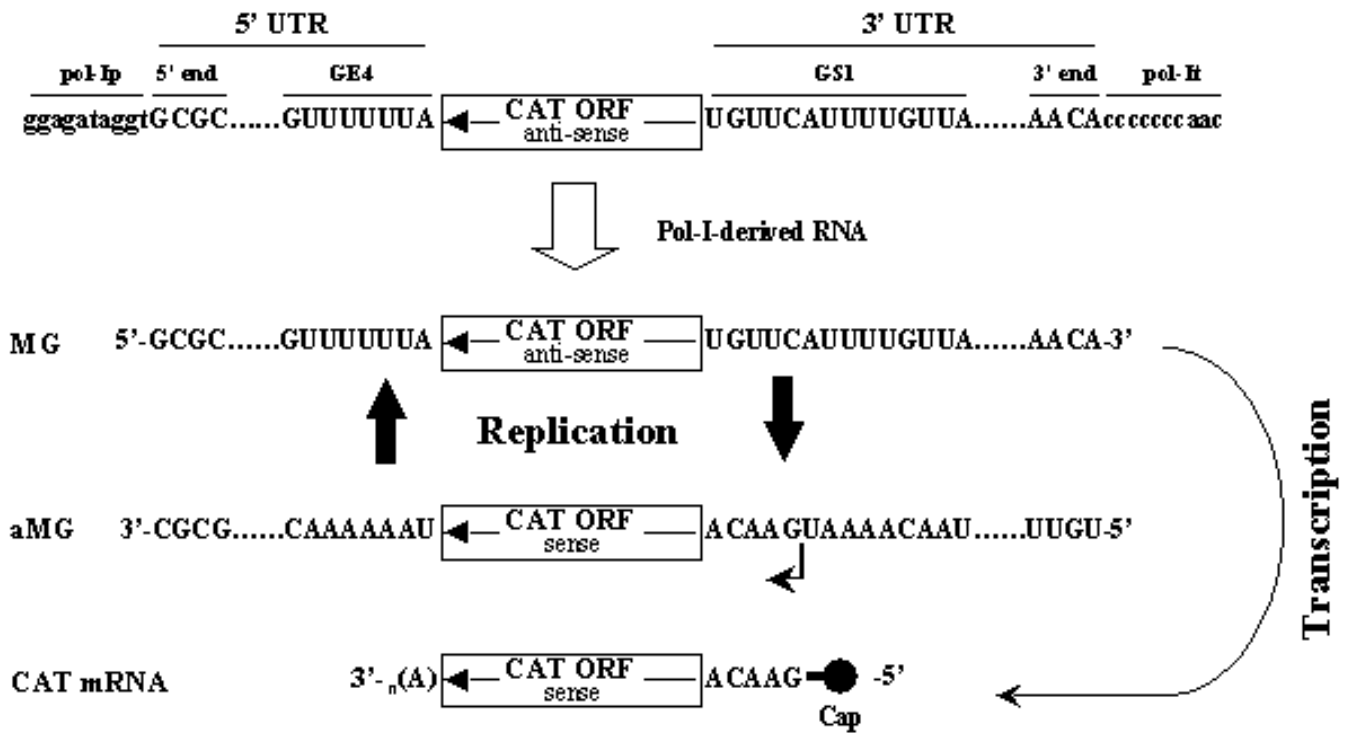
to that of other mononegaviruses (de la Torre, 1994; Schneemann *et al.*, 1995). Six major ORFs are found in the BDV genome sequence (de la Torre, 1994; Schneemann *et al.*, 1995). Based on their positions in the viral genome (3'-N-p10/P-M-G-L-5'), together with their biochemical and sequence features, these polypeptides are the counterparts of the nucleoprotein (N), phosphoprotein (P), transcriptional activator, matrix (M) protein, surface glycoprotein (G) and polymerase (L), respectively, found in other negative-stranded RNA viruses (Tordo *et al.*, 1992). BDV has the property, unique among known animal negative-stranded RNA viruses, of a nuclear site for the replication and transcription of its genome (Briese *et al.*, 1992; Cubitt & de la Torre, 1994). In addition, BDV uses a remarkable diversity of strategies, including RNA splicing, to regulate its genome expression (Cubitt *et al.*, 2001; de la Torre, 1994; Schneemann *et al.*, 1995; Tomonaga *et al.*, 2000). Based on its distinct genetic and biological features among known negative-stranded RNA viruses, BDV is considered to be the prototypic member of a new virus family, *Bornaviridae*, within the order *Mononegavirales*.

As with other negative-stranded RNA viruses, the template of the BDV polymerase is exclusively a nucleocapsid consisting of the genomic RNA encapsidated tightly by the virus N protein. The nucleocapsid associated with the viral polypeptides of the polymerase complex forms a ribonucleoprotein that is active in transcription and replication and which is also the minimum unit of infectivity (Conzelmann, 1998; Garcia-Sastre & Palese, 1993; Tordo *et al.*, 1992). In contrast to positive-stranded RNA viruses, de-proteinized genome RNAs of negative-stranded RNA viruses cannot function as mRNAs and are not infectious. Thus, generation of biologically active synthetic virus from cDNA requires *trans*-complementation by all viral proteins involved in virus replication and transcription. These considerations have hindered severely the application of recombinant DNA technology to the genetic analysis of negative-stranded viruses, including BDV. However, during the last decade, significant progress has been made in this area and for many negative-stranded RNA viruses, short model genomes could be encapsidated and expressed, either by infectious helper viruses or by plasmid-encoded proteins (Conzelmann, 1998; Garcia-Sastre & Palese, 1993; Neumann *et al.*, 2002). This latter approach is based on the co-expression of both genome analogues with pre-determined termini and viral proteins from transfected plasmids. This approach has revolutionized analysis of *cis*-acting sequences and *trans*-acting proteins required for virus replication, transcription, maturation and budding. Moreover, it has allowed the generation and rescue of infectious viruses entirely from cloned cDNAs for members of several different families of negative-stranded RNA viruses (Conzelmann, 1998; Garcia-Sastre & Palese, 1993; Neumann *et al.*, 2002). The aim of this work was to establish the initial steps in the development of similar procedures to investigate the molecular biology of BDV.

We have established an RNA pol I/pol II system for the intracellular reconstitution of BDV replication and transcription. For this purpose, the sequence of a BDV minigenome (MG) was cloned in the

polymerase I (pol I) expression vector pRF42 (Flick & Pettersson, 2001). In the resulting pol I-MG construct (Fig. 1), RNA pol I initiates transcription after the murine pol I promoter (pol-Ip) at the first 5' end position (G) of the BDV genome. Pol I transcribes the BDV 5' UTR, including the transcription/termination/polyadenylation signal (GE4) (genome polarity) for the BDV L protein (Schneemann *et al.*, 1994), the CAT reporter gene in anti-sense polarity and the BDV 3' UTR, encompassing the transcription initiation signal (GS1) (genome polarity) for the BDV N protein (Schneemann *et al.*, 1994). Thereafter, it terminates when reaching the murine pol I terminator (pol-It) located downstream of the 3' UTR sequences. This obviates the need for a *cis*-acting ribozyme to generate the correct 3' end. The 5'- and 3'-termini in our BDV MG correspond to those determined for BDV strain He80 (Cubitt *et al.*, 1994; Pleschka *et al.*, 2001). We selected murine pol-Ip because it has been shown to work well in BHK-21 cells (Flick & Pettersson, 2001; Pinschewer *et al.*, 2003), a cell line that is easy to transfect and supports BDV multiplication. Upon encapsidation of the primary pol I transcript, BDV polymerase reconstituted intracellularly is expected to use it as a template for synthesis of full-length anti-MG (aMG) RNA (replicate) and subgenomic CAT mRNA (transcript) (Fig. 1), which can be assessed conveniently by determining levels of CAT activity in cell lysates.

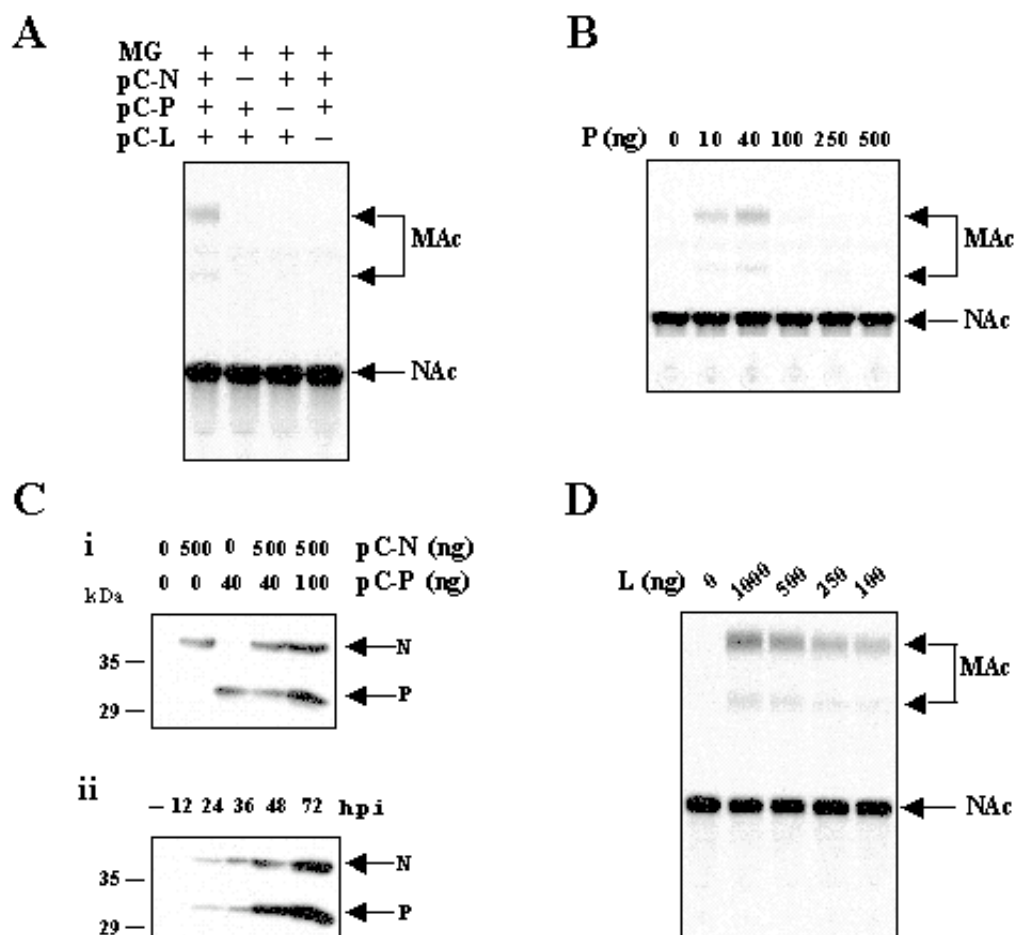
First, we examined the minimal virus *trans*-acting factors required for BDV polymerase activity. Cells were co-transfected with the plasmid expressing the BDV pol I-MG construct and different combinations of pC plasmids expressing BDV N, P and L genes under the control of a modified chicken  $\beta$ -actin promoter (Niwa *et al.*, 1991). As with many other negative-stranded RNA viruses (Conzelmann, 1998; Garcia-Sastre & Palese, 1993; Neumann *et al.*, 2002), we found that BDV L, N and P constituted the minimal virus *trans*-acting factors required for MG expression (Fig. 2A). Expression of CAT correlated with the detection of the predicted subgenomic CAT mRNA and aMG RNA species (Fig. 3C). Hybridization with a CAT sense riboprobe detected BDV MG RNA only in the presence of L. This suggests that pol-Ip-driven MG RNA is unstable, or is produced at very low levels, and, therefore, only the fraction of MG RNA encapsidated and amplified correctly via replication by the virus polymerase can be detected by Northern blotting. The anti-sense CAT riboprobe detected a broad RNA band of about 0.8 kb (Fig. 3C). This band corresponded to the CAT mRNA and aMG RNA species, which, due to their similar sizes, were unresolved under the Northern blot conditions used.



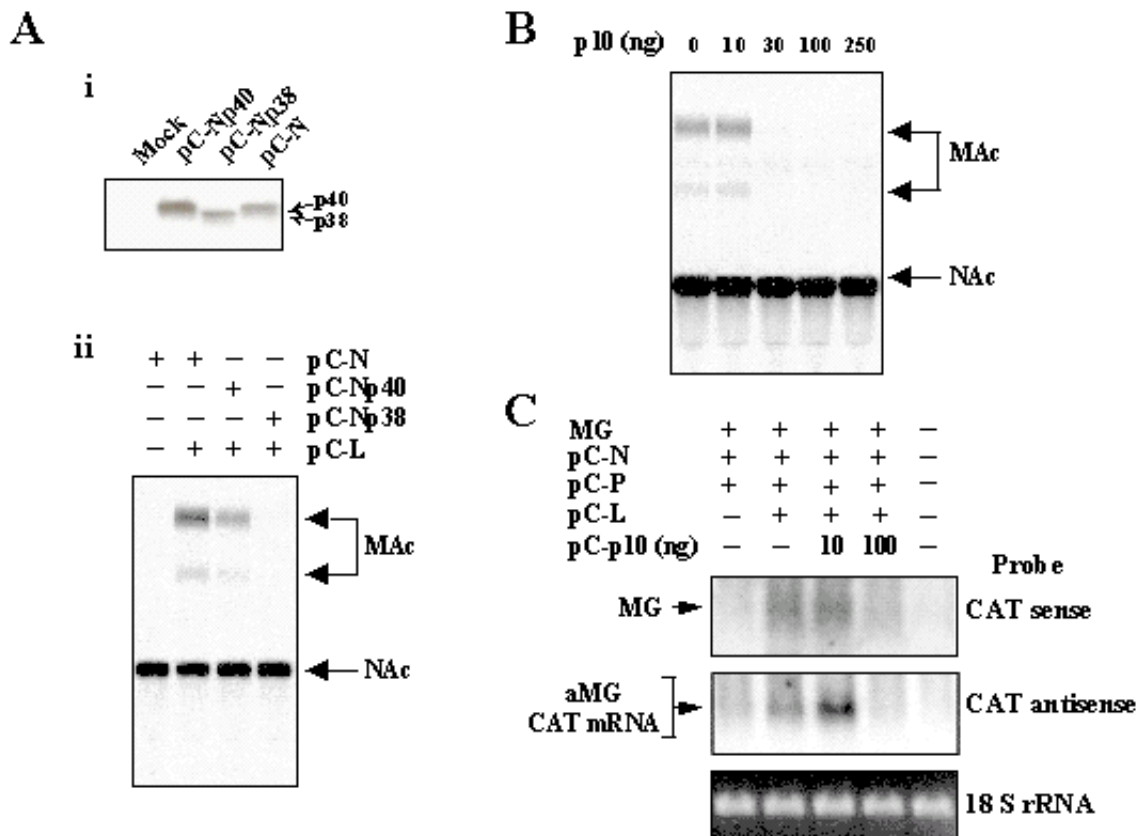
**Fig. 1.** Schematic of the pol-Ip-driven BDV minigenome (MG), showing also the transcription (subgenomic CAT mRNA) and replication (aMG) intermediate RNA species. The pol I-MG construct contains the following elements: the BDV 5' UTR, including the transcription termination/polyadenylation signal (GE4) for the BDV L gene, the CAT reporter gene in anti-sense polarity and the BDV 3' UTR, including the transcription initiation signal (GS1) for the BDV N gene. These elements are flanked by the murine RNA polymerase I promoter (pol-Ip) and terminator sequences (pol-It) of pRF42. Transcription of pol I-MG by cellular RNA pol I (primary transcription) generates the BDV MG RNA. Replication of MG (thick arrow) yields an aMG RNA, which serves as a template for synthesis of more MG RNA by the virus polymerase. Transcription (thin arrow) of MG RNA by the virus polymerase yields a subgenomic length CAT mRNA that initiates and terminates at GS1 and GE4, respectively. Subgenomic mRNA species have 5' end cap structures and are polyadenylated at their 3'-termini.

Unexpectedly, BDV MG expression depended on a delicate balance of the amount of N- and P-expression plasmids. N : P ratios of 5 : 1 or lower resulted in abrogation of MG expression (Fig. 2B). Similar results were obtained in three independent experiments. This observation suggests that the intracellular stoichiometry of the components of the BDV ribonucleoprotein can influence virus polymerase activity dramatically. Consistent with this view, it has been documented that levels of N exceed those of P during the acute phase of BDV infection, while P is present in about eightfold molar excess over N during BDV persistence (Watanabe *et al.*, 2000). In addition, single viral nucleocapsid proteins have been reported to interfere with BDV replication in persistently infected cells (Geib *et al.*, 2003). Intriguingly, we found that in cells transfected with a plasmid N : P ratio of 5 : 1, the corresponding observed N : P protein ratio (Fig. 2C, panel i) was very similar to that seen in BDV-infected cells (m.o.i. of 0.5) within the first 72 h of infection (Fig. 2C, panel ii), a time during which we observed a linear phase of virus amplification. The reasons for this apparent contradiction are unknown. It

is possible that other viral or cellular proteins expressed in BDV-infected cells but which are not present in our MG rescue system might modulate the effect of P on virus polymerase-mediated RNA synthesis. In contrast to the delicate N : P balance required for MG expression, we observed in several independent experiments a wide range for the amount of L plasmid compatible with MG expression (Fig. 2D). Therefore, at least under our experimental conditions, the L : P ratio was not very critical for BDV polymerase activity.



**Fig. 2.** (A) Virus *trans*-acting factors required for BDV MG expression. BHK-21 cells ( $2 \times 10^5$  cells  $4.5 \text{ cm}^{-2}$ ) were transfected using lipofectamine (Lee *et al.*, 2002; Perez & de la Torre, 2003) with pol I-MG (0.5  $\mu\text{g}$ ) and the indicated combinations of pC-N (0.5  $\mu\text{g}$ ), pC-P (0.04  $\mu\text{g}$ ) and pC-L (0.5  $\mu\text{g}$ ). At 60 h after transfection, cell extracts were prepared and assayed for CAT activity, as described (Lee *et al.*, 2002; Perez & de la Torre, 2003). (B) Effect of N : P ratios on BDV MG expression. BHK-21 cells were transfected, as described in (A), with pol I-MG (0.5  $\mu\text{g}$ ), pC-N (0.5  $\mu\text{g}$ ), pC-L (0.5  $\mu\text{g}$ ) and the indicated amounts of pC-P. At 60 h after transfection, cell extracts were prepared and assayed for CAT activity, as described in (A). (C) Comparison of N : P ratios between transfected and BDV-infected cells. (Panel i) BHK-21 cells were transfected, as described in (A), with the indicated amounts of pC-N and pC-P. At 60 h later, whole cell extracts were prepared and analysed by Western blotting, as described (Pinschewer *et al.*, 2003), using rabbit sera specific to BDV N and P. (Panel ii) BHK-21 cells were infected with BDV (m.o.i. of 0.5) and, at the indicated time post-infection (h p.i.), whole cell extracts were prepared and analysed by Western blotting, as in panel (i). (D) Dose-response of L on BDV MG expression. BHK-21 cells were transfected, as described in (A), with pol I-MG (0.5  $\mu\text{g}$ ), pC-N (0.5  $\mu\text{g}$ ), pC-P (0.04  $\mu\text{g}$ ) and the indicated amounts of pC-L. At 60 h after transfection, cell extracts were prepared and assayed for CAT activity, as described in (A). MAc, Monoacetylated; NAc, nonacetylated.



**Fig. 3.** (A) Effect of Np40 and Np38 on BDV minigenome (MG) expression. (Panel i) BHK-21 cells were transfected with the indicated amounts of pC-N, pC-Np40 and pC-Np38. At 60 h later, whole cell extracts were prepared and analysed by Western blotting using a rabbit serum to BDV N. (Panel ii) BHK-21 cells were transfected, as described in Fig. 2(A), with pol I-MG (0.5  $\mu$ g), pC-P (0.04  $\mu$ g) and pC-L (0.5  $\mu$ g), and with either pC-N (0.5  $\mu$ g) or pC (0.5  $\mu$ g) expressing only Np40 (pC-Np40) or Np38 (pC-Np38). At 60 h later, cell extracts were prepared and assayed for CAT activity, as described in Fig. 2(A). (B) Effect of BDV p10 on BDV MG expression. BHK-21 cells were transfected, as described in Fig. 2(A), with pol I-MG (0.5  $\mu$ g), pC-N (0.5  $\mu$ g), pC-P (0.04  $\mu$ g), pC-L (0.5  $\mu$ g) and the indicated amounts of pC-p10. At 60 h after transfection, cell extracts were prepared and assayed for CAT activity, as described in Fig. 2(A). (C) Detection of BDV MG, aMG and CAT mRNA. Cells were transfected with the plasmid combinations indicated using lipofectamine. At 60 h later, total cellular RNA was isolated and equal amounts of each sample were analysed by Northern blot hybridization using either sense or anti-sense CAT riboprobes. MAc, Monoacetylated; NAc, nonacetylated.

Two isoforms of BDV N (Np40 and Np38) are found in BDV-infected cells. These two isoforms have been proposed to be encoded by two different mRNA species (Pyper & Gartner, 1997) but differential usage of two in-frame AUG initiation codons present in the BDV N gene can be responsible also for the production of Np40 and Np38. The nuclear localization signal (NLS) of BDV N was mapped to the 13 N-terminal amino acids separating these two AUGs (Kobayashi *et al.*, 1998). Therefore, Np38 lacks a NLS signal and hence accumulates in the cytoplasm when expressed in the absence of other viral proteins (Kobayashi *et al.*, 1998). However, Np38 is translocated to the nucleus when expressed in the presence of P or Np40 (Kobayashi *et al.*, 1998). The biological implications of the existence of two isoforms of N

with different subcellular-targeting properties remain to be determined. To examine whether both isoforms were strictly required for BDV polymerase activity, we examined the activity of mutated forms of N that could express only the Np40 or Np38 isoforms (Fig. 3A, panel i) on our MG rescue assay (Fig. 3A, panel ii). High levels of CAT activity were obtained in cells transfected with pC-N or pC-Np40. In contrast, the Np38 isoform did not support BDV MG expression. We obtained similar results in three independent experiments. However, in cells co-transfected with P and L, nuclear levels of Np38 were similar to those seen with Np40 (data not shown). This finding suggests that the N terminus of N may play an important role, other than serving as a NLS, in the activity of the BDV polymerase complex.

We also examined the contribution of p10 to BDV MG expression. The p10 ORF encodes a polypeptide of 10 kDa present in BDV-infected cells (Wehner *et al.*, 1997). The p10 ORF starts within the same mRNA transcription unit, 49 nt upstream from P and overlaps, in a different frame, with the 71 N-terminal amino acids of P. In several independent experiments, co-transfection of 30 ng, or higher amounts, of p10-expressing plasmid resulted in a dramatic inhibition of BDV MG expression (Fig. 3B). In addition, RNA from cells co-transfected with 100 ng pC-p10 did not have detectable levels of BDV MG, CAT mRNA and aMG RNA species in Northern blotting (Fig. 3C). These findings suggest that p10 has a negative regulatory role in RNA synthesis mediated by the BDV polymerase. A similar inhibitory effect on viral RNA synthesis in a minireplicon system has been documented also for bunyamwera virus non-structural protein NSs and respiratory syncytial virus non-structural protein NS1 (Atreya *et al.*, 1998; Weber *et al.*, 2001). We did not observe a linear dose response for the inhibitory effect on BDV MG expression caused by 10 and 30 ng pC-p10. It is plausible that this inhibitory effect requires a threshold of p10 that is not achieved in cells co-transfected with 10 ng pC-p10. The p10 sequence contains both a characteristic leucine-rich nuclear export sequence (Wolff *et al.*, 2000) and an unusual importin-binding motif that promotes nuclear translocation of p10 into the nucleus (Wolff *et al.*, 2002). Whether p10 has an intrinsic nuclear export activity remains to be determined. The ability of p10 to modulate the subcellular distribution of P or N, or both, would be an attractive hypothesis to account for the inhibitory activity of p10 on BDV MG expression. However, in cells co-transfected with p10, in addition to MG+N+P+L, we did not observe differences in the subcellular distribution of N or P (data not shown). Several negative-stranded RNA viruses encode accessory proteins that are not strictly required for RNA synthesis mediated by the virus polymerase but they contribute to the regulation of a variety of steps in the virus life cycle (Neumann *et al.*, 2002). Interestingly, the accessory C proteins of the rhabdovirus vesicular stomatitis virus and several paramyxoviruses are encoded, as p10, by the P gene and are expressed via RNA editing or from additional ORFs that overlap the P gene (Nagai, 1999). These C proteins have been implicated in different activities, including virus assembly, virulence and virus countermeasures of the interferon-induced antiviral stage (Nagai, 1999; Neumann *et al.*, 2002). BDV p10 might have similar kinds of functions but they would have not been detected in the context of our BDV MG rescue assay. C proteins

have been documented also to have both positive and negative effects on RNA synthesis by the virus polymerase (Nagai, 1999; Neumann *et al.*, 2002). BDV p10 might also exert positive effects in viral RNA synthesis that were not recreated under the experimental conditions of our MG rescue system.

The BDV MG system described here opens the possibility of manipulating *cis*-acting RNA sequences within the BDV genome and *trans*-acting viral proteins. This, in turn, should facilitate future studies aimed at the investigation of the molecular mechanisms involved in the replication and gene expression of the BDV genome, as well as virus assembly and budding. Moreover, this system provides the foundations for the rescue of infectious BDV from plasmid DNA. The ability in the future to generate predetermined specific mutations within the BDV genome in order to analyse their phenotypic expression *in vivo* will contribute significantly to the elucidation of the molecular mechanisms underlying BDV–host interactions, including the bases of BDV persistence in the CNS and associated disease.

## ACKNOWLEDGEMENTS

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We thank Phi Lam for his excellent technical assistance and Juan Ortín for his very valuable and helpful suggestions. This work was supported entirely by NIH grant RO1 NS32355 to J. C. T.

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