Borna disease virus (BDV) uses a non-cytolytic replication strategy that results in virus persistence in cultured cells and infected animals (de la Torre, 2002; Staeheli et al., 2000). Its negative-stranded RNA genome encodes six viral proteins (Briese et al., 1994) of which the nucleoprotein (N), the polymerase cofactor (P) and the RNA-dependent RNA polymerase (L) form the viral polymerase complex (Perez et al., 2003; Schneider et al., 2003; Yanai et al., 2006). The matrix protein (M) and the glycoprotein (G) mediate particle assembly and virus attachment, respectively. The sixth protein, termed X, is an 87 aa polypeptide that is abundantly present in infected cells but absent in viral particles (Schwardt et al., 2005). Although X has been shown to strongly inhibit viral RNA synthesis from an artificial BDV minigenome through interaction with P (Perez et al., 2003; Poenisch et al., 2004; Schneider et al., 2003), we recently demonstrated that a functional X gene is indispensable for rescuing recombinant virus from cDNA (Poenisch et al., 2007). Published protocols for viral minigenome amplification (Perez et al., 2003; Schneider et al., 2003; Yanai et al., 2006) and generation of recombinant BDV (Martin et al., 2006; Schneider et al., 2005) rely on the reconstitution of the BDV polymerase complex by co-transfection of three helper plasmids encoding L, N and P. However, these systems reconstitute the early phase of BDV infection inadequately because, in BDV-infected cells, P is expressed from a bicistronic 0.8 kb viral mRNA (Fig. 1a) that encodes both X and P (Schneemann et al., 1994). To analyse whether the tight coupling of X and P protein expression is of functional relevance for the establishment of BDV infection (Schneider, 2005), we sought to generate a functional 0.8 kb expression vector in order to analyse its efficacy in BDV rescue experiments.

The 0.8 kb mRNA contained an exceptionally long 5’ non-coding region (NCR) consisting of 48 nt (Fig. 1a). The NCR harboured a short open reading frame (ORF) that initiated at position −26 and terminated at the first of two adjacent stop codons, which overlapped the initiation codon of the X gene (ORF1, Fig. 1a). In addition, the intergenic region between the N and the X gene contained a second, highly conserved AUG triplet starting at position −50 that defined an additional ORF (ORF2, Fig. 1a) in the region preceding the X gene. To acknowledge a possible function of the NCR and ORF2 in the regulation of X and P protein expression in infected cells, we generated three 0.8 kb expression constructs that contained either no NCR (Fig. 1b, pCA-X/P), the original 0.8 kb NCR (Fig. 1b, pCA-0.8) or the 0.8 kb NCR extended by an AT dinucleotide required to generate ORF2 (Fig. 1b, pCA-at0.8). The corresponding DNA fragments were amplified by PCR (primer sequences are available from the authors upon request) from vector pBRPol II-HrBDVc (Martin et al., 2006) and inserted into vector pCA-P (Schneider et al., 2003) using the unique NolI and BgII restriction sites (Fig. 1b). This cloning strategy resulted in the presence of approximately 80 plasmid-derived nucleotides up- and downstream of the inserted viral sequences in the mRNAs transcribed from these vectors. It should be noted that no additional ATG triplets were present upstream from those indicated in Fig. 1(b).

To assess the potential of these vectors to support the generation of recombinant BDV from cDNA, 293T cells were transfected essentially as described previously (Martin et al., 2006) but using either 50 ng pCA-P or 100 ng of the various 0.8 kb expression vectors to supply P. In three independent experiments, we found that co-transfection of construct pCA-at0.8 increased the number of BDV-positive cells in the cultures at 14 days post-transfection by approximately eightfold compared with cells receiving pCA-P (Fig. 2a). Interestingly, constructs pCA-X/P and...
pCA-0.8 completely failed to support BDV rescue (Fig. 2a).

To evaluate whether differential X and P protein expression from the various vectors was responsible for this unexpected result, we transfected 293T cells in 25 mm dishes with 1 μg of the indicated constructs (Fig. 2b) and harvested the protein 24 h later. Viral protein expression was analysed by SDS-PAGE and Western blotting using a polyclonal rabbit antiserum recognizing both X and P (Poenisch et al., 2007). Quantification of the X and P signals (data not shown) showed that P expression from all 0.8 kb vectors was
comparable and only weakly affected by the presence of regulatory viral sequences upstream of the X ORF (Fig. 2b).

In contrast, the lack of the viral NCR in construct pCA-X/P resulted in an 8.5-fold enhanced X:P ratio compared with that obtained with the functional vector pCA-at0.8 (Fig. 2b). In the absence of a functional ORF2 in construct pCA-0.8, the X:P ratio was still enhanced 3.5-fold. These findings suggested that the NCR of the 0.8 kb mRNA is important for downregulating translation of X from the bicistronic mRNA and for generating an X:P protein ratio that is compatible with the formation of an active BDV polymerase complex. It is unclear at present why the presence of ORF2, which is not present on the virus-expressed 0.8 kb mRNA, was required in pCA-at0.8 for downregulation of X expression to a level that enhanced the rescue of recombinant BDV from cDNA. We favour the hypothesis that the presence of 80 plasmid-derived nucleotides upstream of the viral NCR sequence negatively influenced the regulatory potential of the NCR, which possibly adopts a defined secondary structure in order to display its full activity. The presence of ORF2 on mRNAs transcribed from pCA-at0.8 provides an artificial, but in the context of these pCA constructs essential, additional mechanism for downregulating translation of X. However, we cannot exclude the alternative possibility that the NCR is fully active in our construct but represents only one of several mechanisms to regulate X homeostasis in BDV-infected cells. Alternative mechanisms might involve the highly abundant nucleotides upstream of the viral NCR sequence negatively influenced the regulatory potential of the NCR, which possibly adopts a defined secondary structure in order to display its full activity. The presence of ORF2 on mRNAs transcribed from pCA-at0.8 provides an artificial, but in the context of these pCA constructs essential, additional mechanism for downregulating translation of X. However, we cannot exclude the alternative possibility that the NCR is fully active in our construct but represents only one of several mechanisms to regulate X homeostasis in BDV-infected cells. Alternative mechanisms might involve the highly abundant 1.9 kb viral RNA that encodes the N, X and P genes (Schneemann et al., 1994), which is the only subgenomic viral RNA that contains ORF2 in front of the X gene.

We next tested whether the enhanced rescue efficacy obtained by co-transfection of pCA-at0.8 was mediated by enhanced activity of the reconstituted viral polymerase complex. We transfected BSR-T7 cells with an artificial BDV minigenome and the required helper plasmids as described previously (Schneider et al., 2003), again using either 50 ng pCA-P or 100 ng of the indicated 0.8 kb expression vectors to supply P. Constructs pCA-X/P and pCA-0.8 failed to support expression of the chloramphenicol acetyltransferase (CAT) reporter gene from the minigenome, suggesting that the levels of X provided by these constructs resulted in strong inhibition of the polymerase complex (Fig. 3a). This was also true if lower amounts of these plasmids were used to reconstitute the polymerase complex (data not shown). In contrast, co-transfection of pCA-at0.8 resulted in formation of an active BDV polymerase complex, although this reached only approximately 50% of the activity of the polymerase complex reconstituted with pCA-P. This finding demonstrated that the enhanced rescue efficacy was not directly mediated by enhanced transcriptional activity of the polymerase, but most likely reflected an additional, as-yet-undescribed regulatory activity of X. We previously showed that the N:P stoichiometry strongly influences the activity of the BDV polymerase complex, resulting in optimal polymerase activity at a pCA-N:pCA-P plasmid ratio of 15:1 and almost complete inhibition at a plasmid ratio of 4:1 (Schneider et al., 2003). To test whether the presence of X reduced the inhibitory effect of increasing amounts of P, we tested different pCA-N:pCA-at0.8 ratios between 2.5:1 and 10:1 (Fig. 3b). We found that the polymerase complex reconstituted by co-transfection of pCA-at0.8 displayed a strongly reduced sensitivity to altered N:P ratios, resulting in only a slight reduction in CAT expression even at a pCA-N:pCA-at0.8 ratio of 2.5:1.

Our results indicate that, under appropriate conditions, X can stimulate rather than inhibit BDV replication.

![Image](326x451 to 525x715)

**Fig. 3.** The presence of X reduces the sensitivity of the BDV polymerase complex to inhibitory amounts of P. (a) BDV minireplicon assays were performed as described previously (Schneider et al., 2003). Briefly, subconfluent BSR-T7 cells in 25 mm dishes were transfected with 400 ng pT7-gmgA encoding the BDV minigenome, 500 ng pCA-N, 200 ng pCA-L and either 50 ng pCA-P or 100 ng of the indicated 0.8 kb constructs. In addition, we co-transfected 100 ng pBST7-luc, expressing firefly luciferase, to normalize CAT expression from the minireplicon for transfection efficiency. CAT expression was quantified by ELISA (Roche) by measuring A_{405}. The value obtained following transfection of pCA-P was set at 100%. The graph shows the means±SD of at least three independent experiments. (b) Minireplicon assays were performed as described above using the indicated amounts of pCA-at0.8 to supply P. The resulting pCA-N:pCA-at0.8 ratios are shown. The absorbance obtained following transfection of 100 ng pCA-at0.8 was set at 100%. The graph shows the means±SD of at least three independent experiments.
Although the mechanism remains speculative at present, our data suggest that the stimulating effect of X in BDV rescue might be mediated by sequestration of excessive P that would otherwise result in inhibition of the polymerase complex. Our findings indicate that the accessory X protein is not simply an inhibitor but rather serves as a regulator of BDV replication. A practical aspect of our work is that we introduced a modified protocol that greatly improved the efficacy of the BDV rescue technology.

Acknowledgements

We thank Andreas Ackermann and Sandra Wille for helpful comments on the manuscript. This work was supported by grant SCHN 765/1-5 from the Deutsche Forschungsgemeinschaft.

References


