Borna disease virus (BDV) is a neurotropic virus with a negative-stranded RNA genome that has adopted a noncytolytic replication cycle (2, 11). It preferentially infects neurons of the central nervous system in experimentally infected rodents as well as in natural hosts, such as horses and sheep (6, 15). We recently established a procedure for generating BDV from cloned cDNA (12, 14) that can be used to introduce defined mutations into the virus genome. It remained unclear, however, whether the compact genome of BDV has sufficient flexibility to accommodate foreign genes.

To clarify this question, we inserted a gene cassette encoding a green fluorescent protein (GFP) at four different sites of the BDV genome. The GFP gene was flanked by short sequences derived from the BDV genome that comprise transcriptional start (S1 or S3) and termination (T2 or T4) signals as indicated in Fig. 1A. Plasmids expressing the corresponding full-length viral antigenomes under the control of an RNA polymerase II promoter were transfected into human 293T cells along with expression plasmids encoding the BDV proteins N, P, and L as previously described (7). The transfected cells were subsequently cocultured with Vero cells for several weeks until newly generated virus had infected the majority of Vero cells. Successful virus propagation in Vero cells was monitored by direct observation of GFP-derived fluorescence.

Several independent attempts to generate recombinant BDV carrying the GFP cassette near the 3' end of the virus genome from construct i were not successful. Similarly, no virus carrying the GFP cassette between the X/P and the M genes could be rescued from construct ii. However, virus was successfully generated when the GFP cassette was inserted near the 5' end of the virus genome (Fig. 1A). Interestingly, virus rescue was possible only when the entire 5' untranslated region (UTR) of the BDV genome remained intact at the 5' end of the recombinant genome as in construct iv. No virus was rescued if only the terminal 56 nucleotides following the T4 signal remained in the UTR as in construct iii (Fig. 1A). Analysis of polymerase II-mediated transcription from constructs i, ii, and iv suggested that reduced transcription from the trans- fected plasmids is not responsible for the inability of constructs i through iii to support virus rescue (Fig. 1B). Furthermore, alignment of the BDV leader and trailer sequences did not provide evidence for a paramyxovirus-like B-box sequence that might regulate BDV transcription and replication. Thus, the lack of rescue from constructs i and iii can probably not be explained by accidental disruption of this putative regulatory element near the ends of the BDV genome. Northern blot analysis revealed normal levels of the various viral RNAs (Fig. 1C, left and middle) and easily detectable levels of GFP RNA (Fig. 1C, right) in Vero cells infected with the GFP-expressing virus (BDV-GFP) obtained from construct iv. We further observed bright fluorescence in cultured primary rat neurons and Vero cells infected with BDV-GFP (Fig. 1D). Careful analysis of viral growth in Vero cells showed severely reduced propagation of BDV-GFP compared to that for wild-type virus (Fig. 2A). Surprisingly, the growth kinetics of BDV and BDV-GFP in Vero cells infected at a high multiplicity of infection (MOI; 0.25) did not reveal different levels of viral transcripts at 66 h postinfection (Fig. 2B). Since BDV spreads only very slowly in cell culture, synthesis of viral RNA under these experimental conditions is probably restricted to cells infected with the inoculum. Thus, attenuation of BDV-GFP appears to be mainly a consequence of impaired viral cell-to-cell spread rather than impaired viral genome transcription and replication. It should be noted that we cannot exclude the possibility that although too subtle for detection by our assay, small differences in RNA synthesis can result in delayed propagation of BDV-GFP if the cells are infected at a low MOI (Fig. 2A).

Injection of 2,000 focus-forming units of BDV-GFP into the brains of 4-week-old Lewis rats did not result in neurological disease within 47 days of infection (Table 1). Immunohistochemical analysis (10) revealed only a few virus-positive cells in the brains of these animals (data not shown). By contrast, injection of the same dose of recombinant wild-type virus resulted in fulminate virus growth in rat brains (data not shown) and neurological disease within 20 to 21 days postinfection (Table 1). These results demonstrated that the GFP-expressing virus is strongly attenuated.

During an independent study, which will be published else-
where, we observed that two mutations in the L gene greatly enhance the growth kinetics of BDV. The mutant L protein (designated LRD) carries an arginine residue instead of leucine at position 1116 and aspartic acid instead of asparagine at position 1398. To determine whether these growth-promoting mutations would also enhance the propagation of BDV-GFP, we introduced these mutations into the L gene of construct iv as shown in Fig. 1. Transfection of the modified iv construct yielded a replication-competent virus, termed BDV-LRD-GFP, which replicated in Vero cells almost as efficiently as wild-type BDV-LRD virus (Fig. 3A), indicating the clearly improved propagation efficacy of this virus. The finding that a more active polymerase drastically improved the fitness of BDV-GFP is not in conflict with the growth kinetics presented in Fig. 2B. In fact, a scenario in which impaired cell-to-cell spread of BDV-GFP is overcome once the polymerase activity reaches a certain threshold level provides a plausible explanation for the fact that propagation of BDV-GFP is more
strongly impaired than that of BDV-LRD-GFP in comparison to those of their respective wild-type counterparts.

Virus stocks prepared from Vero cells infected with BDV-LRD-GFP exhibited normal infectivities, and infection of 4-week-old Lewis rats with BDV-LRD-GFP induced neurological disease in six of seven animals (Table 1). The time between infection and onset of disease was slightly longer (average, 26 days) than for control animals infected with BDV-LRD lacking a foreign expression cassette (average, 18 days). Analysis of brains from diseased animals for cells expressing GFP revealed particularly strong staining of neurons in the dentate gyrus and CA3 region of the hippocampus formation (Fig. 3B), which are known to contain high concentrations of viral antigen after natural and experimental infections of animals with BDV (1).

This study demonstrated that foreign genes can be inserted into the genome of BDV and that a foreign gene was expressed well in neurons of infected rats. It suggests that BDV vectors can be used to study the effects of therapeutic genes in neurons of rodents. BDV vectors may represent a good alternative to classical transgenic approaches for mice and can be used in place of other viral vectors with no intrinsic preference for neurons.

The potential use of BDV as vector for experimental gene delivery is complicated by the fact that this virus can induce neurological disease in rodents (4). However, since BDV-in-

![FIG. 2](image-url)

**FIG. 2.** Growth characteristics of wild-type (wt) and GFP-expressing BDV in Vero cells. (A) Cultures infected with either virus at an MOI of 0.01 per cell were split twice weekly, and the proportion of infected cells was determined by immunostaining for the BDV N protein as described previously (7). (B) Vero cells (4 × 10^3) were infected at an MOI of 0.25 per cell with the indicated virus for 2 h at 37°C. The cells were then washed with 1 ml of phosphate-buffered saline, and fresh growth medium was added. At the indicated hours postinfection (h.p.i.), total RNA was extracted and 10-μg samples were subjected to Northern blotting. Viral RNA was detected using a DNA probe corresponding to nucleotides 976 to 1749 of the BDV antigenome (X/P gene). The identities of major viral transcripts are indicated. For a loading control, we show the ethidium bromide stain of the cellular 18S rRNA. n.i., not infected.

![TABLE 1](image-url)

**TABLE 1.** Frequency of disease in 4-week-old Lewis rats infected with different BDV variants

<table>
<thead>
<tr>
<th>Virus</th>
<th>Frequency of disease (no. infected/no. ill)</th>
<th>Days of onset of disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDV (wild type)</td>
<td>4/4</td>
<td>20, 20, 21, 21</td>
</tr>
<tr>
<td>BDV-GFP</td>
<td>3/0</td>
<td>&gt;47³, &gt;47³, &gt;47³</td>
</tr>
<tr>
<td>BDV-LRD</td>
<td>4/4</td>
<td>15, 18, 19, 19</td>
</tr>
<tr>
<td>BDV-LRD-GFP</td>
<td>7/6</td>
<td>26, 26, 26, 27, 28, &gt;42²</td>
</tr>
</tbody>
</table>

³ Only a few virus-infected cells were detected in the brains of these nondiseased animals upon analysis by immunohistochemical methods.

![FIG. 3](image-url)

**FIG. 3.** A BDV mutant carrying two mutations in the L gene and the GFP cassette near the 5’ end of the genome (LRD-GFP) replicates well in cultured cells and rat hippocampus neurons. (A) Growth analysis of Vero cells was done as described in the legend to Fig. 1. (B) Four-week-old Lewis rats were infected by the intracerebral route with 2,000 focus-forming units of BDV-LRD-GFP. After signs of neurological disease appeared at around 26 days postinfection, the animals were killed and cryosections of the brain were examined by fluorescence analysis.
duced disease is mediated by CD8 T cells (16), disease can be prevented by several means, including suppression of the antiviral immune response by appropriate drugs and the use of animals that lack CD8 T cells. BDV vectors may be considered for use in mice because this problem is less severe in mice than in rats. Most mouse strains fail to mount an efficient antiviral response against BDV and, consequently, remain healthy in spite of viral persistence in the central nervous system (5).

Out of three insertion sites analyzed, only the insertion site close to the 5′ end of the BDV genome yielded a replication-competent viral vector. Interestingly, the insertion of GFP at this site was tolerated only when the complete UTR remained intact at the 5′ end of the BDV genome, indicating that the sequence between the termination codon of the L gene and T4 might be important for the regulation of viral replication. It remains unclear why the insertion of the foreign gene at the two other positions failed to yield replication-competent virus. A simple interpretation of these results is that both the region preceding the first gene and the third intergenic region contain unrecognized regulatory elements that may not be destroyed. Alternatively, the relative expression levels of the various genes of BDV may be in a delicate balance, which is lost if an additional transcription unit is inserted. Evidence in favor of this view comes from previous observations that nonbalanced expression of the N and P genes has drastic negative effects on BDV polymerase activity (3, 8, 13). We noted that insertion of the GFP gene even at the tolerated site near the 5′ end of the virus genome resulted in strong virus attenuation. Interestingly, the attenuating effect of foreign gene insertion was much less pronounced in a BDV mutant with intrinsically more active polymerase. Although the molecular basis of this observation remains unclear, it has important practical consequences. Unlike wild-type virus, the mutant represents a robust vector for transgene delivery approaches in rodents.

Altogether, our study is the basis for future work aimed at developing BDV into a vector system for experimental gene therapy approaches in rodents. It further paves the way for new genetic approaches to answering questions related to the complex biology of BDV. We demonstrate in the accompanying paper by Poenisch and coworkers (9) that the essential functions of the BDV-X gene can be complemented by ectopic expression of the X protein with the BDV vector system.

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