Identification of viral genomic elements responsible for rabies virus neuroinvasiveness

Milosz Faber†, Rojjanaporn Pulmanausahakul†, Kazuhiko Nagao†, Mikhail Prosnik†, Amy B. Rice†, Hilary Koprowski†, Matthias J. Schnell†, and Bernhard Dietzschold§

Departments of †Microbiology and Immunology and ‡Biochemistry and Molecular Pharmacology, Thomas Jefferson University, 1020 Locust Street, Philadelphia, PA 19107

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Attenuated tissue culture-adapted and natural street rabies virus (RV) strains differ greatly in their neuroinvasiveness. To identify the elements responsible for the ability of an RV to enter the CNS from a peripheral site and to cause lethal neurological disease, we constructed a full-length cDNA clone of silver-haired bat-associated RV (SHBRV) strain 18 and exchanged the genes encoding RV proteins and genomic sequences of this highly neuroinvasive RV strain with those of a highly attenuated nonneuroinvasive RV vaccine strain (SN0). Analysis of the recombinant RV (SB0), which was recovered from SHBRV-18 cDNA, indicated that this RV is phenotypically indistinguishable from WT SHBRV-18. Characterization of the chimeric viruses revealed that in addition to the RV glycoprotein, which plays a predominant role in the ability of an RV to invade the CNS from a peripheral site, viral elements such as the trailer sequence, the RV polymerase, and the pseudogene contribute to RV neuroinvasiveness. Analyses also revealed that neuroinvasiveness of an RV correlates inversely with the time necessary for internalization of RV virions and with the capacity of the virus to grow in neuroblastoma cells.

Rabies is a disease of the CNS that is almost invariably fatal (1). The causative agent is rabies virus (RV), a negative-stranded RNA virus of the rhabdovirus family. RV has a relatively simple genome of 12 kb that encodes five proteins: a nucleoprotein (N), a phosphoprotein (P), a matrix protein (M), a single external surface glycoprotein (G), and an RNA-dependent RNA polymerase (L).

Neuroinvasiveness is the major defining characteristic of a classical RV infection. Attenuated (fixed) RV strains differ significantly from street RV strains in this characteristic, with attenuated RV strains having either no or only limited ability to invade the CNS from a peripheral site, whereas street RV strains are highly neuroinvasive (2). Among the street RV strains, the silver-haired bat-associated RV (SHBRV) is particularly pathogenic (2). It has been suggested that an epizootic in the silver-haired bat population might reflect adaptation of the virus to this species by either increasing its neuroinvasiveness or altering its tissue tropism, enabling transmission of disease by only a low number of virus particles (3). Such changes in the pathogenicity phenotype, in particular increased neuroinvasiveness, are likely to have public health implications: In fact, SHBRV has been identified as the etiological agent of 16 of the 26 indigenous human rabies cases that occurred from 1994 to the present in the United States (4). None of these 16 cases has been related to any known exposure, consistent with the hypothesis that neuroinvasive properties of SHBRV are distinct from those of the more common canine-associated RVs.

The current understanding of RV pathogenesis is based only on either descriptive studies with street RV strains that provide limited insight into mechanisms underlying neurological disease or experiments using cell culture-adapted RV strains with questionable significance for the natural history of rabies. Several studies indicate that the viral G protein is a major contributor to RV pathogenicity (5–9), and some G-associated pathogenic mechanisms have been identified: (i) G must interact effectively with cell surface molecules that can mediate rapid virus uptake (10); (ii) G must interact optimally with the RNA-N-P-M complex for efficient virus budding (7, 11, 12); and (iii) expression levels of G of a particular RV must be tightly regulated to prevent functional impairment of the infected neuron (8). However, the G protein is not the sole determinant of neuroinvasiveness of that virus. Phenotypic analyses of recombinant RVs in which the G gene of a nonneuroinvasive and less neurotropic strain was replaced with that obtained from highly neuroinvasive and neurotropic strains revealed that the pathogenicity of the recombinant viruses was, in every case, markedly lower than that of the WT viruses (7), suggesting that RV pathogenesis, like that of influenza virus (13–15), is a multigenic trait. Regulation of gene expression, especially expression of the L gene, is also likely to play a role in RV pathogenesis (16). Although gene order is a major factor in transcriptional regulation of nonsegmented negative-strand RNA viruses (17, 18), specific gene border sequences that contribute considerably to the regulation of RV gene expression have also been implicated in viral pathogenesis (16).

As a step in identifying viral elements involved in neuroinvasiveness, we constructed an infectious cDNA clone from the highly neuroinvasive SHBRV-18 strain and derived chimeric RV cDNAs with the cDNA of the attenuated nonneuroinvasive SN strain. After rescue from the corresponding cDNA clones, we analyzed the chimeric recombinant viruses for their ability to infect and grow in tissue cultures and to cause lethal encephalomyelitis in mice.

Materials and Methods

Mice. Four- to 6-week-old female Swiss–Webster mice or pregnant Swiss–Webster mice were purchased from Taconic Farms.

Cells and Viruses. BHK 21-derived BSR cells were grown in DMEM (Mediatech, Herndon, VA) supplemented with 10% FBS. Mouse neuroblastoma (NA) cells were grown in RPMI medium 1640 (Mediatech) supplemented with 10% FBS. The isolation of the SHBRV-18 strain and construction of the SN RV vaccine vector have been described (19, 20).

Isolation of Total RNA from Mouse Brains. Newborn mice were infected intracerebrally (i.c.) with 103 focus-forming units (ffu) of SHBRV-18. Four days after infection, mice were killed with

Abbreviations: ffu, focus-forming units; i.c., intracerebrally; NA, neuroblastoma; p.i., postinfection; RV, rabies virus; SHBRV, silver-haired bat-associated RV; TS, trailer sequence; VNA, virus-neutralizing antibody.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AY705373).

To whom correspondence should be addressed. E-mail: bernhard.dietzschold@jefferson.edu.

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CO₂, their brains were homogenized in an Ultraspec RNA isolation system (Biotex Laboratories, Houston), and total RNA was prepared according to the manufacturer’s instructions (Biotex Laboratories). Residual DNA contamination was removed by treating the RNA with RNase-free DNase I (Promega). Integrity of the RNA was assessed by electrophoresis on a 1.2% formaldehyde agarose gel.

Sequence Analysis of SHBRV-18 Genomic RNA. Nucleotide sequencing of the SHBRV RNA was carried out by primer walking with genomic antisense SHBRV RNA as a template. Briefly, total RNA from RV-infected brain (1 μg) was reverse-transcribed with the L-5 primer complementary to the conserved leader sequence of RV genomic RNA, and the cDNAs were used as templates for PCR amplification with primers N-5a and N589-3, which are homologous to a conserved region of the 5’ terminus of the RV N gene. All primer sequences can be found in Supporting Text, which is published as supporting information on the PNAS web site. The resulting 660-bp PCR product was purified (QIAquick PCR Purification Kit, Qiagen, Valencia, CA) and sequenced by using an automated sequencer (Model 377, PerkinElmer). The N gene fragment was used as the template for further sequencing of the entire genomic SHBRV RNA (see Fig. 1). Genomic sequences downstream of the N5a-N589-3 PCR fragment were cloned by using a 5’-RACE kit (GIBCO/BRL) and RV N gene-specific primers. Briefly, the SHBRV-18 genomic RNA was transcribed to single-stranded cDNA. In pSB0, the L gene and TS of SB0 were replaced by the corresponding genes of SN0. SB0, SN0, and chimeric RVs were rescued from full-length cDNA clones as described (21).

Constitution of an SHBRV Full-Length cDNA Clone. Eight cDNA fragments (F1–F8, Fig. 1) spanning the entire SHBRV-18 genome were reverse-transcribed and amplified from total RNA isolated from SHBRV-18-infected mouse brain by using the ScriptPrep One-Step RT-PCR kit (Invitrogen) and the SHBRV-specific primers LD-F and Eco47III R (F1), Eco47III F and PpuMI R (F2), PpuMI F and BstEII R (F3), BstEII F and AgeI R (F4), AgeI F and PshAI R (F5), PshAI F and 3’ PacI-S (F6), 5’ PacI-L and 3’ NcoI-L (F7), and 5’ NcoI-L and TR-L-R (F8).

Constitution of Chimeric Viral cDNA Clones. Fig. 2 shows the full-length cDNA clones of parental and chimeric RVs. The SN
Clinical signs of rabies such as hind limb paralysis and tremors. 

Nemius muscle, with 3' focus inhibition test (RFFIT) as described (22). Mouse sera were heat-inactivated at 65°C for 30 min, and RV particles in 10 mice were infected i.m. with 100 ffu. Fragments of recovered viruses were confirmed by sequencing of RT-PCR.

Antibody (Centocor). Supernatants from RV-positive cell cultures were transferred onto BSR cells, and incubation was continued. Plasmids encoding RV-N, P, G, and L. After 3 days, supernatants from BSR-T7 cells were transfected by using a calcium phosphate method. SB2, SB3, SB4, and SB5 were rescued as described (21). Briefly, a gene and trailer sequence (TS) of SN was PCR-amplified from pSN0 by using primers 5'-MheI-SN-G and 3'-XmaI restriction site. Both fragments were digested with MheI and ligated; the ligation product was cloned into BstEI- and XmaI-predigested plasmid pSB2.

Clone pSB4 was constructed by PCR-amplifying a fragment containing part of the SB0 G gene, and a XmaI restriction site was introduced downstream by using primers BstEI- and SacI-P. A fragment containing the SN M gene was PCR-amplified, and a SacI restriction site was introduced upstream by using primers 5'-SacI-SN-M and 3'-XmaI-SN-M. Both fragments were digested with SacI and ligated, and the ligation product was cloned into BstEI- and MheI-predigested plasmid pSB3.

Clone pSB5 was constructed by PCR amplification of a fragment containing part of the SB P gene and introduction of a SacI restriction site downstream by using primers 5'-EcoRI-P and 3'-SacI+P. A fragment containing the SN M gene was PCR-amplified, and a SacI restriction site was introduced upstream by using primers 5'-SacI+SN-M and 3'-XmaI-SN-M. Both fragments were digested with SacI and ligated; the ligation product was cloned into EcoRI- and XmaI-predigested plasmid pSB4.

Sequences of the primers used to construct chimeric RVs are given in Supporting Text.

Virus Rescue from cDNA Clones. SB0, SN0, and chimeric viruses SB2, SB3, SB4 and SB5 were rescued as described (21). Briefly, BSR-T7 cells were transfected by using a calcium phosphate transfection kit (Stratagene) with full-length cDNA plasmid and plasmids encoding RV-N, P, G, and L. After 3 days, supernatants were transferred onto BSR cells, and incubation was continued for another 3 days. Cells were examined for rescued virus by immunofluorescence staining with FITC-labeled RV N-specific antibody (Centocor). Supernatants from RV-positive cell cultures were used to produce virus stock in BSR cells. Sequences of recovered viruses were confirmed by sequencing of RT-PCR fragments.

Infection of Mice. Adult mice were infected i.m., into the gastrocnemius muscle, with 3 x 10^5 infectious virus particles in 100 μl of PBS or i.c. under isofluorane anesthesia with 10^2 infectious RV particles in 10 μl of PBS. Mice were observed for 30 days for clinical signs of rabies such as hind limb paralysis and tremors.

Determination of Virus-Neutralizing Antibody (VNA). Groups of five mice were infected i.m. with 100 μl containing 3 x 10^6 ffu, and 7 or 8 days later, blood was collected from mice in each group. Mouse sera were heat-inactivated at 65°C for 30 min, and neutralizing activity was determined by using the rapid fluorescent focus inhibition test (RFFIT) as described (22).

Multicycle Growth Curve. Confluent NA cell monolayers grown in T25 culture flasks were infected with RV at a multiplicity of infection of 0.01. After incubation for 1 h at 37°C, inocula were removed and cells were washed three times with PBS. Cells were replenished with 5 ml of RPMI medium 1640 containing 0.2% BSA and incubated at 34°C. After infection, 100 ml of tissue culture supernatant was removed at the indicated time points, and virus was titrated in triplicate on NA cells.

Kinetics of Virus Internalization. Kinetics of RV uptake was analyzed as described (5). Briefly, monolayers of NA cells grown in 96-well plates were incubated with RV at a multiplicity of infection of 5 for various times, and noninternalized virus was neutralized by the addition of 2 units/ml of rabbit polyclonal antirabies serum. At 24 h postinfection (p.i.), the cells were fixed with 80% acetone, stained with FITC-labeled antirabies antibody, and assessed for percentage of rabies antigen-positive cells.

Results

Comparison of the Genomes of SB0 and SN0. Sequence analysis revealed only 81% nucleotide sequence identity between the genomes of SB0 and SN0. Homology in the individual genes was as follows: N = 84%, P = 80%, M = 81%, G = 80%, and L = 82%. The two RV Psi genes, comprising a 380-base nontranslated region of the G gene, showed the lowest nucleotide sequence homology (65%). SN0 and SB0 have identical intergenic regions between N/P, P/M, and M/G, but the G/L intergenic region differs substantially (33% homology). Significant nucleotide sequence differences between the two viral genomes were also found in the 3' leader sequence and the L 5' noncoding region (78% and 85% homology, respectively). Note that both of these
regions are conserved between SN and another attenuated RV strain, Pasteur Virus (23).

Pathogenicity of Recombinant RVs in Mice. As a step in determining the role of the various genomic elements in the neuroinvasiveness of RV, we compared the pathogenicity of chimeric viruses SB2, SB3, SB4 and SB5 with that of SHBRV-18, SB0, and SN0 in mice (Fig. 3). Although 100% of mice survived an i.m. infection with SN0 and SB5 without exhibiting neurological signs, all 10 mice infected i.m. with SHBRV-18 or SB0 developed hind limb paralysis and succumbed to the infection. Furthermore, 90% and 60% of the mice infected with SB2 and SB3, respectively, also developed hind limb paralysis and died from the infection (Fig. 3A and Table 1). The additional replacement of the G gene in SB with the corresponding G gene of SN0 (SB-4) markedly reduced the mortality and morbidity rate, and only 1 of 10 animals succumbed to rabies. In contrast to infection by the i.m. route, 100% of the mice inoculated i.c. succumbed to rabies regardless of the recombinant RV used for infection (Fig. 3B), indicating that all of the recombinant RVs were able to replicate in the CNS. However, as compared with SB0-infected mice, the time of death in SB2-, SB3-, SB4-, SB5-, or SN0-infected mice was delayed by 1–3 days. These data strongly suggest that in addition to the G and L genes, the Ψ gene and TS regions contribute to the ability of an RV to spread to and within the CNS.

Mice infected i.m. with SB0, SB2, or SB3 exhibited VNA titers of 6, 9, and 18 units, respectively at 7 or 8 days after infection, comparable to the titers detected at the same time p.i. in SB4-, SB5-, or SN0-infected mice (18, 18, and 9 units, respectively) (Table 1). These data suggest that, although SB4, SB5, and SN0 have only limited or no ability to invade the CNS, these viruses must have the capacity to replicate sufficiently at peripheral sites to produce the antigenic mass necessary for rapid and strong VNA response.

Table 1. In vitro growth and in vivo pathogenicity of parental and chimeric recombinant RVs

<table>
<thead>
<tr>
<th>Virus</th>
<th>Virus titer in NA cells, ffu/ml*</th>
<th>Hind limb paralysis after i.m. inoculation</th>
<th>Mortality after i.m. inoculation</th>
<th>Mortality after i.c. inoculation</th>
<th>Geometric mean VNA titer, units/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHBRV</td>
<td>1.2 × 10^5</td>
<td>6/10</td>
<td>10/10</td>
<td>10/10</td>
<td>ND</td>
</tr>
<tr>
<td>SB0</td>
<td>1.3 × 10^5</td>
<td>6/10</td>
<td>10/10</td>
<td>10/10</td>
<td>6.0*</td>
</tr>
<tr>
<td>SB2</td>
<td>9.4 × 10^4</td>
<td>8/10</td>
<td>9/10</td>
<td>10/10</td>
<td>9.3*</td>
</tr>
<tr>
<td>SB3</td>
<td>7.0 × 10^4</td>
<td>8/10</td>
<td>6/10</td>
<td>10/10</td>
<td>18.0*</td>
</tr>
<tr>
<td>SB4</td>
<td>1.2 × 10^6</td>
<td>3/10</td>
<td>1/10</td>
<td>10/10</td>
<td>18.0*</td>
</tr>
<tr>
<td>SB5</td>
<td>8.8 × 10^5</td>
<td>0/10</td>
<td>0/10</td>
<td>10/10</td>
<td>18.0*</td>
</tr>
<tr>
<td>SN0</td>
<td>5.0 × 10^7</td>
<td>0/10</td>
<td>0/10</td>
<td>10/10</td>
<td>9.3*</td>
</tr>
</tbody>
</table>

ND, not determined.

* Mice were infected i.m. with 3.3 × 10^2 ffu in 10 μl or i.c. with 3.3 × 10^2 ffu in 10 μl and observed for 30 days for clinical signs.

VNA response.

In Vitro Replication and Cell Entry of Recombinant Viruses. To determine whether the genetic manipulations described above affect virus replication, we compared the time course of parental and recombinant virus production in NA cells. Multistep growth kinetics analysis showed that SB0, SB2, and SB3 grow at similar rates in NA cells to SHBRV-18, but replicate to a lesser extent than SB4, SB5, and SN0 (Fig. 4). Although SHBRV-18, SB0, and SB4 reached maximum titers of 1.2 × 10^6, 13 × 10^9, and 7 × 10^6 ffu/ml, respectively, SB4, SB5, and SN0 reached maximum titers of 1.2 × 10^6, 8.8 × 10^5, and 5 × 10^7 ffu/ml, respectively.

Fig. 4. Multicycle replication growth curves of recombinant RVs. NA cells were infected with SB0, SN0, and chimeric recombinant RVs SB2, SB3, SB4, and SB5 at a multiplicity of infection of 0.01. Aliquots of culture supernatants were collected at the indicated time points p.i., and virus titers were determined in triplicate on NA cells. Data are mean ± SEM.

Fig. 5. The RV G protein determines the kinetics of virus internalization. Monolayers of NA cells grown in 96-well plates were infected with SB0, SB4, or SN0 at multiplicity of infection 5 and 2 units of RV. Neutralizing polyclonal antibody was added at the indicated times p.i. to neutralize noninternalized virus. At 20 h p.i., cells were examined for the presence of RV antigen by the fluorescent antibody technique, and the percentage of infected cells was determined. Values represent the mean (± SEM) from 12 individual wells.
To determine whether differential uptake of the recombinant RVs by cells might underlie differences in neuroinvasiveness, we tested for the presence of viral antigen in NA cell cultures incubated with polyclonal rabies VNA that showed identical neutralization kinetics with SN and SHBRV in rapid fluorescent focus inhibition test (RFFIT) (data not shown). When VNA was added at 5 min p.i., viral antigen was detected in 44% of SB0-infected cells but in only 5% and 8% of cells infected with SN0 or SB4, respectively (Fig. 5). The time necessary to infect 50% of cells (half entry time) was 7 min for SB0 and 26 and 30 min for SB4 and SN0, respectively. Thus, differences in the G protein, the only RV component involved in virus uptake by the host cell, might account for the different mortality rates in mice infected with the different recombinant RVs.

Discussion

Our previous data indicated that the pathogenicity of recombinant RVs in which the G gene of a nonneuroinvasive RV strain was replaced by G genes of highly neuroinvasive RV strains was significantly lower than that of the RV from which the G genes were derived (7). The incomplete restoration of the pathogenicity of a nonpathogenic RV by replacing its G gene with that of a highly pathogenic RV suggested that the pathogenicity of a virus likely depends on multiple factors, as shown for influenza viruses (13, 15). To analyze the role of the various RV proteins and regulatory elements in the neuroinvasiveness of RV, we sequenced and cloned an infectious cDNA (pSB0) derived from SHBRV-18, one of the most neuroinvasive RV strains (19). We found significant differences between SB0 and SN0, the latter derived from a nonneuroinvasive vaccine RV strain (20, 23) in the L protein, the 3′ leader sequence, and the L 5′ noncoding region.

To study the role of the different RV genome elements in viral pathogenesis, we constructed different chimeric RV cDNA clones between pSB0 and pSN0 and rescued the viruses from the corresponding cDNA clones. The ability to recover virus from pSB2, pSB3, pSB4, and pSB5, which contain at least the polymerase and 5′ end of SN0, indicates that SN proteins can support SB replication and transcription despite the sequence differences. Moreover, the SB0 antigenic promoter can be used by a chimeric SN/SB-polymerase complex composed of SN L and SB P proteins.

Characterization of the rescued parental viruses SB0 and SN0, and of the chimeric RVs SB2, SB3, SB4, and SB5, revealed the highest mortality rates in mice infected i.m. with SB0. Replacement of TS, L, and Ψ in SB0 with the corresponding elements of SN0 resulted in a moderate decrease in morbidity and mortality, and additional exchange of G or G plus M strongly reduced or completely abolished virus neuroinvasiveness. In NA cells, SB0 replicated at a similar rate to the original SHBRV-18, but almost 1,000 times lower than SN0; replacement of TS, L, and Ψ in SB0 with those of SN0 had little effect on virus growth kinetics, but additional replacement of G or G plus M of SB0 with the corresponding genes of SN0 resulted in a one-log increase in virus production. The kinetics of virus internalization into NA cells appeared to be determined by the RV G protein. These data have several important implications: (i) SB0 is phenotypically indistinguishable from the WT SHBRV-18; (ii) the G gene plays a predominant role in the ability of an RV to invade the CNS from a peripheral site, although several other viral elements such as the TS, L, and Ψ sequences likely contribute to the neuroinvasiveness; (iii) the pathogenicity of an RV correlates inversely with its ability to grow in tissue culture (e.g., NA cells); and (iv) the time necessary for virus uptake, which is largely regulated by the RV G protein, appears to be a critical factor in the neuronal spread of an RV to the CNS.

Our findings indicating that the neuroinvasiveness of a particular RV is determined by virus uptake and virus replication rates have significance for the natural history of rabies. For example, it is likely that the virus evades clearance by immune effectors such as macrophages through rapid internalization. On the other hand, the induction of adaptive immune effectors after infection, particularly VNA, does not appear to play a decisive role in the outcome of an RV infection. Furthermore, a lower replication rate may prevent induction of cell death, thereby maintaining the structural integrity of the neurons and enabling the virus to travel within axons to the CNS. This conclusion is consistent with previous findings demonstrating that the pathogenicity of a particular RV strain correlates inversely with its ability to trigger apoptosis in neuronal cells and that induction of apoptosis depends on RV G expression levels (7, 8, 24).

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