Gene order rearrangement of the M gene in the rabies virus leads to slower replication

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Received: 22 November 2013 / Accepted: 7 May 2014 / Published online: 7 June 2014
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Abstract The matrix protein (M) is one of only five genes in the RV genome and is an important multifunctional protein. Besides to allow for the release of newly replicated virions pairing with G, the M protein also functions in virus replication, pathogenicity, and host cell apoptosis. The goal of present study is to generate recombinant viruses with M gene rearranged, thus laying the foundation for further exploring what will happen when the gene for M is relocated on the RV single-strand RNA. We used rHEP-Flury, an attenuated virus that remains virulent for less than 3 days in sucking mice, to reshuffle the M gene, using an approach that leaves the other viral nucleotide sequence intact. Two viruses with translocated M genes (N1M2 and N1M4) were recovered from each of the rearranged cDNAs, whose gene order is 3'-N-M-P-G-L-5' and 3'-N-P-G-M-L-5' respectively. The growth dynamics of these viruses showed slower replication than the wild-type virus in multiple-step growth curves, but they can

grow to a comparable titer in tests of single-step growth curves. Further experimentation with these rearranged viruses will provide insights into the relationships between genome structure and virus phenotypes.

Keywords Gene rearrangement · Matrix protein · Rabies virus · Reverse genetics

Introduction

Rabies is a neurological disease which causes acute encephalitis in warm-blooded animals including humans. Mortality is near 100 % if patients are not treated before clinical symptoms such as fever, slobbering, anxiety and hydrophobia and so on have developed. Rabies remains a potent threat to the public health in developing countries. According to the World Health Organization (WHO), rabies causes ca. 55,000 human deaths, with most of the fatalities occurring in Asia and Africa [22].

The causal agent rabies virus (RV) is the type species in the genus *Lyssavirus*, a close relative to *Vesicular stomatitis virus* (VSV) in the genus *Vesiculovirus*. These genera both belong to the *Rhabdoviridae* family [27] and have a single strand, non-segmented, negative sense RNA genome that encodes five structural proteins nucleoprotein (N); phosphoprotein (P); matrix protein (M); glycoprotein (G); and large protein (L) (3'-N-P-M-G-L-5') [1, 8]. The RNA is encapsidated by the N, P and polymerase protein L, forming a complex called nucleocapsid (RNP), which serves as the functional template for transcription and replication [2, 3]. The rescue system for RV is comprised of a full-length genomic cDNA plasmid and three helper plasmids expressing N, P and L proteins. Since the infectious RV was first successfully rescued in 1994 [26],

Electronic supplementary material The online version of this article (doi:10.1007/s13337-014-0220-1) contains supplementary material, which is available to authorized users.

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the RV reverse genetics system has been well studied [16–18, 32].

The RV reverse genetics system has been employed to dissect the relationship between genome elements or its structure and function. Among this research, the most attention has been paid to the G. The G gene is associated with virulence [19, 35] and a single amino acid mutation at position 333 can lead highly attenuated RV strains to revert to virulence [9, 28]. Overexpression of the RV G seems to result in enhancement of apoptosis and antiviral immune response [10]. Wirblich et al. [31] showed that G expression levels are not critical for pathogenicity of RV, suggesting that the function of the G gene may be complicated. Other studies have found that each gene is vitally important for RV, including the M. The RV M protein regulates the balance of virus transcription and replication [11, 13] and can induce apoptosis by a TRAIL-dependent mechanism [21]. Meanwhile, the virulence and the spread of RV is also under the control of the M protein, which may be achieved by the M–G or the M–host proteins interactions to regulate viral replication and spread between cells [25, 27]. Thus, the RV M protein is obviously an important multifunctional protein and requires more study.

Gene expression of the viruses in *Rhabdoviridae* is controlled by the order of genes relative to the single transcriptional promoter. Genes proximal to the 3' promoter are transcribed at higher levels than those at promoter-distal positions [20]. The structural protein genes of VSV have been extensively reshuffled with many observed character changes, including gene expression, kinetics, pathogenicity, and immune reaction occurring in the recombinant virus [4, 14, 15, 30]. However, there is little information available on gene rearrangement in RV. To our knowledge, the only study on RV gene relocation involved the translocation of the G gene with the M gene [32]. Given the fact that the pathogenicity can be attenuated by gene rearrangement in VSV [14, 15, 30], together, in view of no evidence of recombination was found in lyssavirus evolution [4], we expect a less virulent live attenuated vaccine can be generated by M gene rearrangement based on the attenuated parental virus rHEP-Flury. In the present study we explored the effects of two rearrangements of the M gene in RV.

Materials and methods

Virus and cells

The RV rHEP-Flury strain was previously rescued through reverse genetics by our laboratory. BHK-21 (Baby hamster kidney) cells and BSR-T7/5 cells (a cell line derived from

BHK-21 that constitutively expresses T7 RNA polymerase) [6] were cultured with Dulbecco's modified Eagle's medium (DMEM) basic (1X), and SK-N-SH cells (Human neuroblastoma cell line) and maintained in RPMI Medium 1640 basic (1X). All of the cells were grown at 37 °C and supplemented with 10 % fetal calf serum (FCS, Gibco Life Technologies) under an atmosphere of 5 % CO₂.

Generation of complete genome cDNA clones with a rearranged matrix gene

The plasmid pHEP-3.0 containing the full-length genome cDNA of rHEP-Flury and the four helper plasmids pH-N, pH-P, pH-G, and pH-L were a kind gift of Dr. Kinjiro Morimoto from the NIH in Japan (for detailed information, please refer to Inoue et al., 2003) [17]. According to Ghanem et al. [16], in order to generate the exact 5' end of antigenomic viral RNA more efficiently, the last four nucleotides 5'-GGCG-3' of hepatitis delta virus ribozyme sequence in the pHEP-3.0, were replaced with 5'-AGCCA-3', resulting in the plasmid pHEP-3.0-Hdv-SC (Fig. 1a) which was used as a template for successive PCR amplifications. The Phusion High-Fidelity DNA Polymerase (F-530L, Thermo Scientific) was applied to all the PCR amplifications.

To avoid the introduction of any contaminant sequence, an efficient homologous-recombinant-based seamless cloning method was adopted according to the manufacturer's instructions (GB2001-24, Genebank Biosciences Inc). The genes targeted for relocation were first deleted by reverse PCR amplification with the pHEP-3.0-Hdv-SC plasmid, thus generating a linearized gene-deleted plasmid. Then the genes were amplified from the beginning of the transcription start site (AACA) to the transcription end signal (the polyA signal, AAAAAA). The end of each fragment was left with a 15 bp overhang, homologous to the junction fragment and the linearized plasmid. Finally, a recombinant reaction was carried out to generate the desired gene order by mixing the gene fragments and the linearized plasmid in the same centrifuge tube, according to the supplier's protocol. Thus two full-length cDNA clones with M gene in its unnatural orders pHEP-N1M2, and pHEP-N1M4 were generated. The resulting recombinant plasmids were transformed into competent cells (*E. coli* x-gold strains) by the standard technique. The plasmids were purified from the bacteria and sequenced to check for the correct rearrangement of the M gene and that no other mutations had occurred during recombination. The plenty of purified plasmids for transfection were prepared using the CompactPrep Plasmid Midi Kit (Qiagen, 12843) from the verified positive clone bacteria. The quality of the plasmids was detected by NANODROP 2000c Spectrophotometer (Thermo Scientific).

Fig. 1 Schematic diagram of full-length RV plasmids used in present study. **a** In order to improve the cut efficiency of the ribozyme, the last four nucleotides “GGCG” of Hdvz in pHEP-3.0 (*upper*) were replaced with “AGCAA”, resulting in the plasmid pHEP-3.0-Hdv-SC (*lower*); **b** recombinant plasmids with the M gene rearranged from its

natural position at 3 to position 2, and 4 were constructed with pHEP-3.0-Hdv-SC by seamless cloning, which introduced no changes except the M gene order. The annealing sites and directions of the primers used in RT-PCR are shown by the *arrows*. Hhrz hammerhead hepatitis delta virus ribozyme; Hdvz hepatitis delta virus ribozyme

Recovery of infectious viruses

BHK-21 cells were grown overnight to about 90 % confluency ($\sim 2\text{--}3 \times 10^5$ cells per well) in 12-well plates in DMEM supplemented with 10 % FCS. Before transfection, the overnight medium was discarded and the cells were washed three times carefully with DMEM. Transfection used three different amounts (named large, medium and small, respectively) of plasmids with the same plasmid ratio (Table 1). For each well, 7.5 μl of SuperFect Transfection Reagent (Qiagen, 301305) in a final volume of 425 μl was used. 3 h after transfection, the transfecting medium was removed and the cells were washed once and maintained in DMEM supplemented with 10 % FCS, 1 % (v/v) Penicillin–Streptomycin mixture (Hyclone, 10,000 U/ml) for a further 6 days. The culture were centrifuged at $12,000 \times g$ for 10 min at 4 °C after three freeze–thaw cycles. The supernatant was inoculated into SK-N-SH cells and incubated for 4 days at 37 °C. The SK-N-SH cells were examined for the presence of rescued virus by immunofluorescence assay using fluorescein isothiocyanate (FITC)-labeled RV N protein-specific antibody (FDI, FUJIREBIO, USA). The supernatants were examined for positive RV signals and were then passaged on to SK-N-SH cells for propagation of virus stock for further experiments. The wild type rHEP-Flury possesses the gene order 3′-N-P-M-G-L-5′, the rescued viruses having the rearranged genomes 3′-N-

Table 1 Three different total plasmids amounts used in virus rescue for one well of a twelve wells plate

Systems	Full length plasmid	pH-N	pH-P	pH-G	pH-L	Total (μg)
Large	1.25	0.3125	0.15625	0.09375	0.0625	1.875
Medium	1	0.25	0.125	0.075	0.05	1.5
Small	0.75	0.1875	0.09375	0.05625	0.0375	1.125

M-P-G-L-5′ and 3′-N-P-G-M-L-5′ were designated N1M2 and N1M4 respectively.

Confirmation of virus rescue and gene order

RT-PCR was performed to detect genomic RNA of the rescued viruses from cloned cDNA. One set of primers was used to distinguish the rearranged genomes and the wild type genome. Primers were named based on the target genes (N, P, M, G, or L), the position in each target, and the direction (forward, f; reverse, r) and were designed as follows: primer pair N-1233f/M-337r was designed to differentiate rHEP-Flury and N1M2, N-1233f/G-174r was used to identify N1M4. The annealing sites and sequences of the primers are shown in Fig 1 and Table 1 of Electronic Supplementary Material respectively. PCR fragments of the expected size were sequenced to ensure their identity.

Passage and growth curves of rescued viruses

Serial passages of the rescued viruses in BSR cells were performed to examine the genetic stability of the recombinant virus. Monolayers of BSR cells were infected with each virus and the culture fluid was harvested at 4 d post infection (p.i.). This procedure was repeated 10 times. Virus sequences from the tenth passage were confirmed by sequencing RT-PCR fragments. The titer of each virus was examined by the direct fluorescent antibody test (FAT) as described previously [34]. Monolayers of BSR cells in 96-well plates were infected with serial 10-fold dilutions of virus suspensions and incubated at 34 °C. At 4 d p.i. cells were fixed in 80 % acetone at 4 °C for an hour and stained with FITC-labeled RV N protein-specific antibody. Foci were counted under a fluorescence microscope (EVOSf1, Advanced Microscope Group, USA) and calculated as focus forming units/ml (ffu/ml). All titrations were carried out in triplicate and the average used as the titer. BSR cells were infected at an MOI of 0.1 and 5 for the multiple-step and single-step growth curves respectively and samples of supernatant were taken at the indicated time points (24, 48, 72, 96, and 120 h p.i.). Infectious virus titers were determined as above.

Results

Construction of full-length cDNA plasmids with a rearranged matrix gene

With the RV full-length cDNA clone pHEP-3.0 of the rHEP-Flury strain and the four helper plasmids in hand,

we simply needed to relocate the M gene with the pHEP-3.0. The plasmids pHEP-N1M2, and pHEP-N1M4 were generated with rearranged M gene and different hepatitis delta virus ribozyme tail from the pHEP-3.0 (Fig. 1b). In contrast to traditional plasmid construction strategies, no enzyme sites were created nor bases changed in or between genes. There were no observed differences in the two recombinant plasmids or the parental plasmid pHEP-3.0-Hdv-SC except the gene order as verified by sequencing.

Rescue of RV from the respective plasmid

As shown by the specific fluorescence foci after the cells were stained with FITC-labeled RV N protein-specific antibody for viral antigen detection, N1M2 and N1M4 were successfully rescued with the medium plasmid and little plasmid treatments (Fig. 2). No rescue event occurred in N1M2 with the large plasmid treatment and only one out of twelve N1M4 wells were rescued under the same plasmid treatment. Although three attempts were made, the expected M1N2 (with the gene order 3'-M-N-P-G-L-5') was not acquired with any of the three plasmid treatments.

Passage and confirmation of recombinant viruses

Because some rescued viruses can't be passaged serially follow the original generation (unpublished data), we examined whether the acquired viruses can retain stability in passaging cells, the rescued N1M2 and N1M4 viruses were inoculated to monolayer BSR cultures and passaged blindly for ten passages. The FAT test imposed at the tenth

Fig. 2 BHK-21 cells were transfected with the recombinant full-length plasmids and four helper plasmids. Then the supernatants were transferred to SK-N-SH cells. 4 days after incubation, the SK-N-SH cells were fixed by 80 % acetone and subjected to a direct fluorescent

antibody test (FAT) with FITC-labeled rabies virus N protein-specific antibody. A large amount of fluorescence foci ($\times 400$) under the fluorescence microscope demonstrated successful rescue of N1M2 (a) and N1M4 (b)

generations suggested that the rescued viruses can propagate successfully and prolifically in the BSR cells.

To confirm that the two recovered viruses contain the expected gene arrangement, these viruses were subjected to RT-PCR using gene-position-specific primers (Fig. 1). Fragments with the expected size were successfully amplified for both viruses (Fig. 3) and gene sequencing confirmed that the sequences of the amplicons were consistent with that of their corresponding genes in the recombinant virus genome.

Growth kinetics of viruses in BSR

Since the RV matrix protein M not only functions in virus assembly/budding, but is also intimately associated with virus transcription and replication, and contributes to cell-to-cell spread of the virus [11, 25], we inferred that rearrangements of the M gene may lead to some differences in growth kinetics. Multiple-step growth curves suggested that when the M gene was translocated in front and behind its natural position, the virus replicated significantly more slowly than the wild type rHEP-Flury (Fig. 4a). With respect to the one-step growth curves, N1M2 and N1M4 grew more slowly than the wild type rHEP-Flury strain. N1M2 produced the smallest amount of virus during the early phase, but all three viruses eventually grew to the same titer (Fig. 4b).

Discussion

In previous work on the reverse genetics of RV, an enzyme-ligation strategy was used to insert or replace the original sequence, thus leading to sequence change in areas other than the target. These changes may affect the virus

phenotype in unknown ways. In this study we used a seamless clone-based method to construct the full-length cDNA clones of RV, resulting in sequences which are identical between the wild-type rHEP-Flury and its derivative variants, except for a rearranged gene order. The intergenic regions also remained the same as the wild type in terms of nucleotide composition and intergenic deployment order. It should be noted that in the nonsegmented negative-sense RNA virus Newcastle Disease Virus and the RV-closely related VSV, as well as RV itself, the intergenic sequences play a pivotal role in RNA transcription [5, 12, 33]. Therefore the seamless clone method eliminates the potential contributions of sequence changes and intergenic profiles to changes in viral phenotypes.

Two treatments were tested in order to improve the rescue efficiency. One employed optimized ribozyme sequences with an enhanced cleavage activity at the 3'-end of the full-length cDNA clone [16]. In a parallel rescue attempt, plasmids with the same quality and quantity were used and the one with the optimized ribozyme sequence was successful in rescuing the virus (10 wells out of 12 wells) while the attempt without the optimized ribozyme sequence failed to rescue anything (0 wells out of 12 wells). The other treatment involved a set of different amounts of plasmid. The results imply that incorporating large amounts of plasmid could have a negative effect on the rescue system (demonstrated by 1/24 wells successful rescue with the large amounts of plasmid versus 17/24 wells successful rescue with the medium amounts of plasmid). This may be because optimal binding of SuperFect-DNA complexes to the negatively charged groups on the cell surface requires a slightly positive net charge and too much negatively charged DNA impairs binding. Additionally, large amounts of plasmid with a high expression rate may increase plasmid mortality. Conversely, if insufficient plasmid with a low expression rate is used, transfection efficiency may be too low, so we recommend using a medium amount of plasmid.

Despite several attempts to rescue virus with the full-length M1N2 cDNA plasmid, no recombinant virus was successfully acquired. Previous research demonstrated that gene expression levels progressively decrease based on the relative distance of a gene from the single 3'-terminal promoter for nonsegmented negative-strand RNA viruses [7, 30]. The enhanced G mRNA transcription was observed in the ERAgm (with the G gene switched with the M gene, gene order 3'-N-P-G-M-L-5') virus-infected BSR cells compared with parental ERA strain (gene order 3'-N-P-M-G-L-5') [32]. According to this principle, the hypothetical M1N2 would express the most M protein among the three M-rearranged viruses. The expressed M protein may be fatally deleterious to the hypothetical M1N2, given the poor growth potential presented by N1M2 in the growth

Fig. 3 Bands of the expected size were amplified by RT-PCR after being passaged in BSR cells for ten generations. **a** The primer pair N-1233f/M-337r was designed to detect N1M2. Lane1, molecular size marker; Lane2, rHEP-Flury; Lane3, N1M2; Lane4, pHEP-3.0; Lane5, positive control pHEP-3.0-N1M2; Lane6, negative control ddH₂O; **b** the primer pair N-1233f/G-174r was designed to detect N1M4. Lane1, molecular size marker; Lane2, ninth-generation N1M4; Lane3, tenth-generation N1M4; Lane4, positive control pHEP-3.0-N1M4; Lane5, pHEP-3.0; Lane6, negative control ddH₂O

Fig. 4 BSR cells were infected with each virus at an MOI of 0.1 (**a**, Multiple-step growth curves), or an MOI of 5 (**b**, single-step growth curves). The supernatant fluid of the cultured cells was harvested at the indicated time points (24, 48, 72, 96, and 120 h.p.i) and titrated in

BSR cells. Data are representative of the results of three independent experiments. Error bars indicate the standard error of the mean of three virus titer determinations

curve results. If M1N2 contained an abnormally plentiful M protein, it may harm the cells or it may be detected early by the cell's surveillance systems so that the virus does not have a chance to replicate. In fact, there is an association between the level of G protein cell surface expression and the extent of cell injury [10]. In addition, the genome of RV is so sophisticated and efficient that a small change may have a large influence on the survival of the virus [29]. Currently, the small difference between M1N2 and the corresponding region in rHEP-Flury (from the end of the leader sequence to the coding region), may also lead to the failure of M1N2's rescue.

The basic functions of the M protein were found to be crucial for the assembly and budding of bullet-shaped particles exuded by the cell. The M protein plays a role in cellular budding by interacting with the transmembrane spike glycoprotein G [24]. As RV research has continued more functions of the M protein have been discovered. Besides regulating the balance of virus RNA synthesis by shifting synthesis activity from transcription to replication, the M protein also plays a role in inhibiting translation in virus-infected cells through a protein-protein interaction with the cellular translation machinery [11, 13, 23]. Furthermore, pathogenicity and host cell apoptosis are also associated with the M protein. Replacing no other proteins than M protein in the attenuated Ni-CE with the corresponding proteins from Ni-CE's virulent parental Nishigahara leads to lower virus growth efficiency. This suggests that the M gene of the Nishigahara strain may be incompatible with other components of the Ni-CE genome, and the exchanged M protein may affect the interaction

with other molecules and lead to an unbalanced relationship among viral and host proteins [27]. Considering the variety of functions the M protein of RV is responsible for, we believe that recombinant viruses with a relocated M gene would present new characteristics. As the low virus density multiple-step growth curves demonstrated, the rearranged viruses N1M2 and N1M4 grew fairly slowly in relation to the wild type rHEP-Flury at all time points. In contrast, the single-step growth curves showed that although the recombinant viruses replicated more slowly than their parental virus at the same stage, the recombinant viruses eventually achieved a comparable titer. This may be explained by the initially high density virus compensating for growth deficiency in the cells. Similarly, the recombinant ERAgm grew as well as the wild type ERA RV suggested by the single-step growth curves [32]. In conclusion, it seems that no matter whether the M gene is rearranged in front or behind its normal position, the resulting recombinant virus is less viable than the wild-type virus. From an evolutionary perspective, the genetically engineered viruses may be somewhat deficient in reproduction, so the wild-type parent possesses the advantage.

The aim of this study is to provide insight into the relationship between genomic structure and phenotype of RV. Mice administration tests proved that the ERAgm virus was less virulent than the original ERA virus [32]. Based on the attenuated rHEP-Flury viruses, we expect the development of a recombinant virus with outstanding immunogenicity and less virulence by gene rearrangement. Comprehensive biological characteristics census of the two recombinant viruses will be the imminent work.

Acknowledgements This study was partially supported by a grant From the National Natural Science Foundation of China (Nos. 31172322) and Special Fund for Agro-scientific Research in the Public Interest (Nos. 201103032); The project of General Administration of Quality Supervision, Inspection and Quarantine of the People's Republic of China (Nos. 2012IK005).

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