Creation of Matrix Protein Gene Variants of Two Serotypes of Vesicular Stomatitis Virus as Prime-Boost Vaccine Vectors

Gyoung Nyoun Kim, Kunyu Wu, Jiho Patrick Hong, Zain Awamleh, C. Yong Kang

Department of Microbiology and Immunology, Schulich School of Medicine and Dentistry, The University of Western Ontario, London, Ontario, Canada

ABSTRACT

To take advantage of live recombinant vesicular stomatitis viruses (rVSVs) as vaccine vectors for their high yield and for their induction of strong and long-lasting immune responses, it is necessary to make live vaccine vectors safe for use without losing their immunogenicity. We have generated safer and highly efficient recombinant VSV vaccine vectors by combining the M51R mutation in the *M* gene of serotype VSV-Indiana (VSV_{Ind}) with a temperature-sensitive mutation (*tsO23*) of the VSV_{Ind} Orsay strain. In addition, we have generated two new serotype VSV-New Jersey (VSV_{NJ}) vaccine vectors by combining M48R and M51R mutations with G22E and L110F mutations in the *M* gene, rVSV_{NJ}(G22E M48R M51R) [rVSV_{NJ}(GMM)] and VSV_{NJ}(G22E M48R M51R L110F) [rVSV_{NJ}(GMML)]. The combined mutations G21E, M51R, and L111F in the *M* protein of VSV_{Ind} significantly reduced the burst size of the virus by up to 10,000-fold at 37°C without affecting the level of protein expression. BHK₂₁ cells and SH-SY5Y human neuroblastoma cells infected with rVSV_{Ind}(GML), rVSV_{NJ}(GMM), and rVSV_{NJ}(GMML) showed significantly reduced cytopathic effects *in vitro* at 37°C, and mice injected with 1 million infectious virus particles of these mutants into the brain showed no neurological dysfunctions or any other adverse effects. In order to increase the stability of the temperature-sensitive mutant, we have replaced the phenylalanine with alanine. This will change all three nucleotides from UUG (leucine) to GCA (alanine). The resulting L111A mutant showed the temperature-sensitive phenotype of rVSV_{Ind}(GML) and increased stability. Twenty consecutive passages of rVSV_{Ind}(GML) with an L111A mutation did not convert back to leucine (UUG) at position 111 in the *M* protein gene.

IMPORTANCE

Recombinant vesicular stomatitis viruses as live vaccine vectors are very effective in expressing foreign genes and inducing adaptive T cell and B cell immune responses. As with any other live viruses in humans or animals, the use of live rVSVs as vaccine vectors demands the utmost safety. Our strategy to attenuate rVSV_{Ind} by utilizing a temperature-sensitive assembly-defective mutation of L111A and combining it with an M51R mutation in the *M* protein of rVSV_{Ind} significantly reduced the pathogenicity of the virus while maintaining highly effective virus production. We believe our new temperature-sensitive *M* gene mutant of rVSV_{Ind}(GML) and *M* gene mutants of rVSV_{NJ}(GMM) and rVSV_{NJ}(GMML) add excellent vaccine vectors to the pool of live viral vectors.

Vesicular stomatitis virus (VSV) is a rapidly replicating virus. Eventually humoral and cellular immune responses against VSV are elicited in the animal host, like any other viral vectors (1–3). Animals infected with VSV develop immune responses within 2 weeks and start to neutralize the VSV (1). This hinders the efficacy of boost immunizations for vaccination with the same vector. Serotype-specific antibodies against the viral surface glycoprotein G neutralize VSV. Two different serotypes of VSV, VSV-Indiana (VSV_{Ind}) and VSV-New Jersey (VSV_{NJ}), show 50% amino acid homologies in glycoprotein G (4). Antibodies raised against one serotype of VSV do not neutralize the other serotype of VSV (5). Accordingly, other investigators have used VSV_{Ind} with its own surface glycoprotein G and VSV_{Ind} carrying the G gene from other serotypes as a vaccine vector (6, 7).

VSV causes self-limiting disease in animals such as pigs, cows, and horses, whereby vesicular lesions on the mouth, nose, teats, and hooves are cleared in a couple of weeks after the onset (8). Although it is very rare, human infections with VSV have been reported mostly in animal care workers, veterinarians, and laboratory personnel who were in close contact with the diseased animals or with the viruses (9–11).

Safety concerns have been raised on the usage of VSV as a vaccine vector in humans because of the neurovirulence shown in

nonhuman primates and young mice after intracerebral or intranasal inoculation (12–15). Any live viral vaccines for human use must pass the neurovirulence test in nonhuman primates (16–19). In order to take advantage of live rVSVs as a vaccine vector for their high yield and for their induction of strong and long-lasting immune responses, it is necessary to make the live vaccine vector safe to use in humans without losing its immunogenicity.

Efforts have been made to attenuate rVSV vectors to generate safer vaccine vectors. rVSVs with the G gene deleted (Δ G) and with single-cycle replication in regular cells and in the animal host were developed, and the Δ G rVSV was amplified in the VSV G

Received 26 January 2015 Accepted 30 March 2015

Accepted manuscript posted online 8 April 2015

Citation Kim GN, Wu K, Hong JP, Awamleh Z, Kang CY. 2015. Creation of matrix protein gene variants of two serotypes of vesicular stomatitis virus as prime-boost vaccine vectors. *J Virol* 89:6338–6351. doi:10.1128/JVI.00222-15.

Editor: D. S. Lyles

Address correspondence to C. Yong Kang, cykang@uwo.ca.

Copyright © 2015, American Society for Microbiology. All Rights Reserved.

doi:10.1128/JVI.00222-15

protein-expressing cell line (20–22). This approach renders approximately a 2-log reduction of virus production, and it may produce inadequate quantities of virus for the preparation of live recombinant virus vaccines, should the vaccination require a high dose of virus. Another type of attenuated rVSV was generated by combining the deletion of the cytoplasmic tail of the G protein, gene translocation (especially the *N* gene to the 4th position), and noncytopathic *M* gene mutations with M33A and M51R (23, 24). These mutations affected high-level virus production, since the attenuations were made by reducing replication efficiency and formation of virions.

VSV *M* protein induces cell death by blocking host cellular protein synthesis. Amino acid changes from methionine at position 51 (Met51) to arginine (Arg) in the VSV_{Ind} *M* protein and changes of Met48 and Met51 to Arg in the VSV_{NJ} *M* protein make the host cellular protein synthesis near normal (25, 26). While VSVs with Met-to-Arg changes in the *M* protein have significantly reduced cytopathic effects (CPE), these viruses still replicate and produce progeny viruses in the cells and in the infected animal, maintaining their neurovirulence.

We sought a way to attenuate VSVs to maintain normal efficiency of viral replication in preparing a high-titer viral stock. We looked into temperature-sensitive (*ts*) mutants of VSVs, which cannot assemble or release infectious particles properly at a non-permissive temperature (39°C) and also at a semipermissive temperature (37°C). One of the temperature-sensitive *M* gene mutants of the VSV_{Ind} Orsay strain, the *ts*O23 mutant, has shown limited replication in a mouse glial cell line at 37°C and 39°C (27). It has been demonstrated that the temperature sensitivity of the *ts*O23 mutant was the result of improper assembly or lack of initiation in viral assembly, with the characteristic bullet-shaped structure at 39°C (28, 29). In addition, the original *ts*O23 mutant lost its neurovirulence in mice, as demonstrated by direct inoculation of the virus into the brain (27).

Thus, we combined the *M* gene mutations, noncytopathic mutation with assembly-defective mutation *ts*O23 to attenuate the VSV_{Ind} strain without negating viral production efficiency. We characterized some newly generated *M* gene mutants and found newly attenuated *M* gene mutants, VSV_{Ind}(G21E M51R L111F) [rVSV_{Ind}(GML)] or VSV_{Ind}(G21E M51R L111A) [rVSV_{Ind}(GML-New)] and two new *M* mutants of VSV_{NJ}, rVSV_{NJ}(G22E M48R M51R) [rVSV_{NJ}(GMM)] and rVSV_{NJ}(G22E M48R M51R L110F) [rVSV_{NJ}(GMM-L)]. These new VSV *M* mutants were attenuated significantly and lost their neurovirulence but could still be used for induction of robust adaptive immune responses against inserted gene products.

MATERIALS AND METHODS

Cells. Baby hamster kidney cells of the BHK₂₁ line (ATCC, CCL-10) were grown in Dulbecco's minimal essential medium (DMEM) (Invitrogen) containing 5% fetal bovine serum (FBS). Cells of the human neuroblastoma cell line SH-SY5Y (ATCC, CRL-2266) were maintained in a 1:1 mixture of Eagle's minimal essential medium (EMEM) (Sigma-Aldrich) and Ham's F-12 medium (Invitrogen) containing 10% FBS. Green monkey kidney cells of the Vero E6 line (ATCC, CRL-1586) were maintained in MEM (Invitrogen) containing 10% FBS and 1 mM sodium pyruvate. BSR T7/5 cells (30) were obtained from K. K. Conzelmann. The BHK₂₁ cells constitutively expressing bacteriophage T7 RNA polymerase were grown in DMEM containing 5% FBS and 500 µg/ml G418 (Invitrogen).

Construction of plasmids. The full-length cDNA clone of VSV_{Ind} was constructed from a VSV_{Ind} heat-resistant (HR) strain. The cDNA from the leader region to the end of the *P* gene was synthesized by reverse

transcription (RT) using primer Ind I (5'-ACGAAGACAAACCAACCAT TATT-3'). The double-stranded DNA from the leader region to the end of the *P* gene was synthesized by a two-step PCR. The PCR product with the restriction enzyme *SapI* site in part of the pTV vector (31) and the beginning of the leader region of the VSV_{Ind} genome was first synthesized by using the primers Ind-I (5'-AATGATAATAATGGTTTGTTCCTCG TCCTATAGTGAGTCGTATTAATTT-3') and TV-*SapI* (5'-CGGGCGG CCGCAAGCGGAAGAGCGCCAA-3'). The first PCR product was used as a megaprimer for the amplification of the *NP* region of the VSV_{Ind} genome together with the primer Ind P(R) (5'-CGGGCGGC CGCCGACCGTTAATTAACACAGAGAATATTTGACTCTCGCT-3'). The amplified PCR products were cloned into pBluescript II KS (pBKS) vector (Stratagene) at the *NotI* site (pBKS-Ind-*NP*). The cDNAs from the intergenic junction in front of the *L* gene, the *L* gene, and the trailer region were also synthesized by reverse transcription using primer Ind-L(F) (5'-CGGGCGGCGCGGTTTAATTAACCTCAATCCT GCACAACAGA-3'), and the double-stranded DNA was synthesized by using primers Ind-L(F) and Ind *RsrII*(R) (5'-CGGGCGGCGCGGAC CGCGAGGAGGTGGAGATGCCATGCCGACCCACGAAGACCA CAAAACAGATAAA-3'). The PCR products were cloned into a pBKS vector at the *NotI* site (pBKS-Ind-*Lt*). The *NP* region was cut from pBKS-Ind-*NP* and cloned into pTV vector by using the *SapI* and *RsrII* sites in pTV (pTV-Ind-*NP*). The *Lt* region was cut from pBKS-Ind-*Lt* by using restriction enzymes *PacI* and *RsrII* and cloned into pTV-Ind-*NP* to construct the plasmid with the *NP* and *Lt* regions of the VSV_{Ind} genome (pTV-Ind-*NP/Lt*). The cDNAs of the *M* gene and *G* gene region of VSV_{Ind} were synthesized by reverse transcription, using the primer Ind M(F) (5'-TCCCCGCGGTTAATTAACCTATGAAAAAAGTAACA GATATCACACTAAGTGTTATCCCAATCCATTCATCATGAGT TCCTTAAAGAAGATTCTC-3') and was used for the synthesis of each double-stranded DNA of the *M* gene and *G* gene. The Ind *M* gene region was amplified by the PCR product using the primers Ind M(F) and Ind M(R) (5'-ATAGTTAGCGGCCGCTAGACTAGCTCATTTGAAGTGG CTGACAGAAAT-3'). The PCR product was cloned into pBKS by using *SacII* and *NotI* sites (pBKS-Ind *M*). The double-stranded DNA of the *G* gene region of VSV_{Ind} was amplified by PCR using Ind G(F) (5'-ATAAGAATGCGGCCGCACTAGTTTCTATCCCTATGAAAAAAT TAACAGAGATCGATCTGTTTCCTTGACACCATGAAGTGCCTTTT GTACTAGCTT-3') and Ind G(R) (5'-GCTCTAGATTAATTAAC TAGTGGTACCTTACTTCCCAAGTCGGTTCATCT-3'). The PCR product of the *G* gene region was cloned into the pBKS vector by using the *NotI* site and *XbaI* site (pBKS-Ind *G*). The *M* gene region and *G* gene region were combined in the pBKS vector using the *NotI* and *XbaI* sites (pBKS-Ind *MG*). *PacI* sites were introduced into the beginning and the end of the *M* and *G* gene regions, and a *NotI* site was introduced between the *M* gene and *G* gene. The combined *M* and *G* gene region was cut from the pBKS-Ind *MG* with the restriction enzyme *PacI* and cloned into the pTV-Ind-*NP/Lt* to construct the full-length DNA clone of VSV_{Ind} (prVSV_{Ind}). More unique restriction enzyme sites *PmeI*, *XhoI*, and *MluI* were added into the untranslated region at the end of the *G* gene in prVSV_{Ind} by inserting a double-stranded DNA linker of Ind-PXM(1) (5'-TAAGTTTAAAGCCTCGAGATACGCGTTTAAT-3') and Ind-PXM(2) (5'-TAAACGCGTATCTCGAGGCGTTTAACTTAAT-3') into the *PacI* site. The resulting plasmid was named prVSV_{Ind}(WT). Mutations in the VSV_{Ind} *M* gene were introduced by the megaprimer PCR method. The double-stranded DNA megaprimer with mutation was synthesized by the PCR using prVSV_{Ind}(WT) as a template and as a forward primer IMM(F) (5'-GGGCTCGAGTACAAAAAGTTGTACAATCAGGCGAG-3') and one of the mutagenic primers of IM(M51R) (5'-CATGAGTGTC CCTCTCGTCAACT-3') or IM(G21E) (5'-GGTGCATTTCTAATTTCT TAGATTTC-3'). The first PCR product was used together with the primer IMM(R) (5'-CTTCATGGTGTCAAGGAAACAG-3') to synthesize the full-length rVSV_{Ind} *M* gene with the G21E mutation or M51R mutation. The PCR product was cut with *PacI* and *NotI* and replaced the *M* gene of prVSV_{Ind}. The resulting plasmids were prVSV_{Ind}(G21E) and

prVSV_{Ind}(M51R). The mutagenic primer IM(M51R) and the template prVSV_{Ind}(G21E) were used to construct prVSV_{Ind}(G21E M51R) [prVSV_{Ind}(GM)]. The mutagenic primer IM(L111F) (5'-TAGAAGAAGCAAAAAAGCCAAG-3') was used for the PCR with the template prVSV_{Ind}(G21E) to construct prVSV_{Ind}(G21E L111F) [prVSV_{Ind}(GL)]. prVSV_{Ind}(G21E M51R L111F) [prVSV_{Ind}(GML)] was generated by introducing mutations with the mutagenic primer IM(L111F) and prVSV_{Ind}(GM) as a template. prVSV_{Ind}(GML)-New was generated by a two-step cloning process. First, the codon for arginine at 51R in the M protein of the rVSV_{Ind}(GML) was changed to CGA. In order to change the codon to CGA, we used the forward primer IMM(F) and the mutagenic primer IM(M51R-CGA) (5'-CATGAGTGTCTCGCTCGTCAACT-3') and prVSV_{Ind}(GML) as a template for the first PCR product. The first PCR product was used as a megaprimer together with the reverse primer IMM(R) to synthesize the full-length M gene with the codon change at 51R. The second codon change at F111 was introduced by changing phenylalanine (TTT) to alanine (GCA). The F111A mutation in the M gene was generated by using the forward primer IMM(F), the reverse primer IMM(R), and the mutagenic primer IM(L111A) (5'-AGAAGAAGCTGCAAAAGCCAAGATC-3').

We added more unique restriction enzyme sites at the junction of the G gene and L gene of prVSV_{NJ}(WT) (26). Unique restriction enzyme sites FseI, PmeI, XhoI, and MluI were added by inserting the double-stranded DNA linker of NJ-FPXM(1) (5'-CGGCCGCGCATGTTTAAACCACTCAGATACGCGTGGTAC-3') and NJ-FPXM(2) (5'-CAGCGTATCTCAGTGGTTTAAACATGGCCGCGCGGTAC-3') into the G gene and L gene junction at the KpnI site of prVSV_{NJ}(WT). The mutation G22E was introduced into the M gene of VSV_{NJ}(WT) by the megaprimer PCR method using the forward primer NJMM(F) (5'-GGGCTCGAGAGACTTGATGGGAGCAATCATCATG-3') and mutagenic primer NJM(G22E) (5'-GAGGTGGCAATTCTAGTTTCTTTGAT-3'). The full-length DNA of the VSV_{NJ} M gene with the G22E mutation was synthesized by using the first PCR product with the mutation and reverse primer NJMM(R) (5'-CACCATTGCAAGAATTAGATAAGACAA-3'). The PCR product with the mutation was cut with PacI and NotI and replaced the wild-type (WT) M gene in the prVSV_{NJ}(WT) to generate prVSV_{NJ}(G22E). By the same method, we generated prVSV_{NJ}(G22E M48R M51R) [prVSV_{NJ}(GMM)], prVSV_{NJ}(G22E L110F) [prVSV_{NJ}(GL)], and prVSV_{NJ}(G22E M48R M51R L110F) [prVSV_{NJ}(GML)]. prVSV_{NJ}(GMM) was constructed by using prVSV_{NJ}(M48R M51R) as a template and the mutagenic primer NJM(G22E). prVSV_{NJ}(GL) was constructed by using prVSV_{NJ}(G22E) as a template and NJM(L110F) (5'-GGAGCCAATAAAGCAATTATC-3') as a mutagenic primer. prVSV_{NJ}(GML) was constructed by using prVSV_{NJ}(GMM) as a template and NJM(L110F) as a mutagenic primer. prVSV_{NJ}(GMM)-New was constructed by using prVSV_{NJ}(GMM) as a template and the mutagenic primer NJM(M48R M51R-CGT) (5'-TATCATATAATCTCGATCCTCTCGTCCGAAGAAGTCA-3').

Recovery of rVSV by reverse genetics and the preparation of viral stocks. Recombinant VSVs were recovered by reverse genetics as described previously (26). The recombinant wild-type and M gene mutants of rVSV were recovered from the plasmids of full-length genomic clones of rVSVs: prVSV_{Ind}(WT), prVSV_{Ind}(M51R), prVSV_{Ind}(G21E), prVSV_{Ind}(GM), prVSV_{Ind}(GL), prVSV_{Ind}(GML), prVSV_{NJ}(WT), prVSV_{NJ}(M48R M51R), prVSV_{NJ}(G21E), prVSV_{NJ}(GMM), prVSV_{NJ}(GL), prVSV_{NJ}(GML), prVSV_{Ind}(GML)-New, and prVSV_{NJ}(GMM)-New. BSR T7/5 cells were cotransfected with the full-length genomic cDNA clones of rVSV and three other plasmids expressing the nucleocapsid protein (pBKS-IRES/N_{NJ}), phosphoprotein (pBKS-IRES/P_{NJ}), and VSV polymerase L protein (pBKS-IRES/L_{NJ}) in concentrations of 10 µg of pN, 10 µg of pP, 0.5 µg of pL, and 15 µg of prVSV.

The newly generated rVSVs were purified three consecutive times by plaque picking, and were amplified for a larger volume of stock viruses by infecting BHK₂₁ cells at a virus multiplicity of infection (MOI) of 0.1 at 31°C.

Viral preparation and titration. The plaque-picked viruses were first amplified in a small-scale 2-ml culture of BHK₂₁ cells, and the viruses were titrated using a monolayer culture of Vero E6 cells by plaque assay. The cells infected with the mutant viruses for the plaque assay were incubated at 31°C. For a larger viral stock, BHK₂₁ cells were infected with a virus MOI of 0.1 and incubated at 31°C, and the culture medium was harvested at 20 h postinfection.

Determination of protein expression. In order to examine the expression of VSV proteins, we analyzed 5 µg of cell lysate of infected cells by SDS-PAGE, and the proteins were transferred to the polyvinylidene difluoride (PVDF) membrane (Immobilon; Millipore). The VSV proteins were detected by Western blotting using ECL Plus enhanced chemiluminescence Western blotting detection reagents (Amersham Biosciences). VSV proteins were detected by using rabbit serum against total protein of VSV (32).

Determination of cell death. Death of the infected cells by rVSV infection was measured by detecting the release of lactate dehydrogenase (LDH) from the damaged cells using a cytotoxicity detection kit (Roche). The infected cells were maintained in an appropriate culture medium with 1% FBS to minimize the detection of LDH from the FBS. Culture medium from the infected cells was collected at 8 h postinfection, 100 µl of the culture medium was added to a 96-well plate, and then 100 µl of reaction mixture (diaphorase-NAD⁺ mixture with iodotetrazolium chloride and sodium lactate) was added. The mixture was incubated for 30 min at room temperature. The absorbance of the samples was measured at a 490-nm filter setting using an enzyme-linked immunosorbent assay (ELISA) reader (model 550; Bio-Rad).

Neuropathological study in mice. We used 7-week-old Swiss Webster mice from the Charles River Laboratory, which were implanted with an intralateral ventricular cannula (IVC) into the brain. The mice were anesthetized with ketamine (1.0 mg/10 g) and xylazine (0.1 mg/10 g) before injection. The mice were injected with viruses 1 week after arrival at the animal facility. The mice were injected with 1×10^3 or 1×10^6 PFU of viruses in 5-µl inoculums diluted with a serum-free DMEM. A sterile microsyringe with a 10-µl volume (Hamilton) was connected to a small piece of sterile polyethylene tubing (PE50; Becton Dickinson) to fit into the IVC. The syringe was mounted onto the manual stereotaxic injector (Stoelting), and the 5-µl volume of inoculum was injected into the brain over 15 min. The rVSV-injected mice were observed for neurological dysfunction and weighed every day for a month. Animal care and procedures were compliant with the guidelines of the Animal Care and Use Committee of the University of Western Ontario.

RT-PCR. Genomic RNA of the rVSV was isolated from the concentrated viruses by using the QIAamp viral RNA minikit (Qiagen). The cDNAs of the M gene of the old and new rVSV_{Ind}(GML) strains were synthesized by RT-PCR using primers IP2101F (5'-GAGGTAACGGA CGAATGTCT-3') and IL3160R (5'-AATCTGAGCTTGACGGGCAA-3'). The cDNAs of the M gene of the old and new rVSV_{NJ}(GMM) strains were synthesized by RT-PCR using primers H1F (5'-ACGAAG ACAAAAAAACCATTCTTAC-3') and H3100R (5'-AAGAGTATTAA AGCGGCCGCTT-3').

RESULTS

Addition of assembly-defective *ts* mutations into the M genes of rVSV_{Ind} and rVSV_{NJ}. There are three amino acid changes in the M protein of the VSV Orsay *ts*O23 mutant strain, which are the glycine-to-glutamic acid change at the 21st amino acid (G21E), the leucine-to-phenylalanine change at the 111th amino acid (L111F), and the histidine-to-tyrosine change at the 227th amino acid (H227Y) (33). The single-amino-acid reversion at F111 to L implied that F111 has a major role in the temperature sensitivity of the *ts*O23 mutant, resulting in retarded assembly and release of VSV particles at a nonpermissive temperature of 39°C (33). Therefore, we introduced the L111F mutation into the M gene of

FIG 1 Introduction of mutations in the *M* gene of rVSV. (A) Wild type and combined *M* gene mutants of rVSV_{Ind}. (B) Wild type and combined *M* gene mutants of rVSV_{NJ}. The mutations in the *M* gene were introduced by site-directed mutagenesis using the megaprimer PCR method, and the PCR products were cloned into PacI and NotI sites to replace the wild-type *M* gene in both rVSV_{Ind} and rVSV_{NJ}. The format used shows the original amino acid before the number followed by the mutated amino acid after the number. M; methionine, R; arginine, G; glycine, E; glutamic acid, L; leucine, F; phenylalanine.

rVSV_{Ind} together with the G21E mutation or G21E and M51R (Fig. 1A). The methionine-to-arginine change at the 51st amino acid (M51R) in the *M* protein, which reduces the inhibitory activity of the *M* protein on cellular protein synthesis, was combined with the G21E and L111F mutations in order to further attenuate the virulence of VSV_{Ind}.

The amino acids G21 and L111, which we changed to E and F, respectively, in the *M* protein of VSV_{Ind}, are not present at the exact positions in the *M* protein of VSV_{NJ} but there are G22 and L110, which are close to the G21 and L111 of rVSV_{Ind} *M*. Therefore, we changed G22 to E and L110 to F in the rVSV_{NJ} *M* protein. As with the VSV_{Ind} mutants, to further attenuate the virulence of VSV_{NJ}, we combined the mutations G22E and L110F with the M48R and M51R mutations of rVSV_{NJ}, which shows reduced inhibitory effects of the *M* protein on host cellular protein synthesis (Fig. 1B).

Wild-type and mutant rVSV_{Ind} and rVSV_{NJ} were recovered from the cDNA plasmids by reverse genetics, as described in Materials and Methods. The transfected BHK-T7 cells for the recovery of rVSV *ts M* mutants were incubated at 31°C, and the culture medium was collected when the CPE showed, which took approximately 4 to 5 days posttransfection.

The mutation L111F in the *M* protein significantly reduces the burst size of rVSV_{Ind} at a semipermissive temperature of 37°C. In order to examine the effect of L111F mutation on the release of the virus particles from the infected cells, we first checked the burst sizes of the new *M* mutants and compared them to that of the wild-type rVSV_{Ind} strain. First, the burst sizes of the viruses were determined in BHK₂₁ cells at 10 h postinfection with a virus MOI of 3 at both permissive (31°C) and semipermissive (37°C) temperatures. The rVSV_{Ind}(GL) and rVSV_{Ind}(GML) mutants produced 100-fold less infectious virus particles at 37°C than at 31°C, demonstrating the temperature sensitivity of the mutants

in the release of virus particles at 37°C (data not shown). Then we examined the kinetics of infectious virus production of the *M* mutants during the course of 10 h of incubation at 31°C and 37°C. The neurovirulence shown in mice by VSV injection intranasally, intracranially, or intravenously (27) prompted us to examine the replication of the mutant VSVs in the human neuroblastoma cell line SH-SY5Y. We infected BHK₂₁ cells and SH-SY5Y cells with wild-type and *M* mutant rVSVs at an MOI of 3 in order to infect every cell with at least one infectious particle. The infected cells were incubated at a permissive temperature (31°C) and at a semipermissive temperature (37°C), and culture medium from the infected cells was collected every 2 h until 10 h postinfection. The number of infectious viral particles in the culture medium was determined by plaque assay at 31°C using Vero E6 cells. The wild-type virus and all mutant viruses replicated equally well at 31°C and produced similar titers of infectious viruses throughout the 10 h of incubation (Fig. 2A and C). However, the *M* mutants of rVSV_{Ind} with L111F, rVSV_{Ind}(GL) and rVSV_{Ind}(GML), replicated with significantly lower titers—as much as 3 to 4 orders of magnitude lower during the course of infection than wild-type or M51R mutants of rVSV_{Ind} at 37°C (Fig. 2B and D). This demonstrated that the L111F mutation has a key role in impaired VSV particle production at 37°C. rVSV_{Ind}(GML) produced the smallest amount of infectious particles along the course of infection in both BHK₂₁ cells and SH-SY5Y cells at 37°C. The differences in production of infectious particles between the wild type and rVSV_{Ind}(GML) were as large as 4 logs at 6 h postinfection (Fig. 2B and D).

Mutations in the *M* protein of rVSV_{NJ} G22E and L110F are not temperature sensitive in viral production at 37°C. The temperature sensitivities of the newly generated *M* mutants of rVSV_{NJ} were examined by infecting BHK₂₁ cells with rVSV_{NJ} wild-type and *M* gene mutant viruses at an MOI of 3. Infected cells were

FIG 2 Growth kinetics and burst sizes of rVSVs at permissive (31°C) and semipermissive (37°C) temperatures. (A, B, C, and D) Growth kinetics of rVSV_{Ind}. We infected BHK₂₁ cells and SH-SY5Y cells with a virus MOI of 3. The infected cells were incubated at a permissive temperature of 31°C and at a semipermissive temperature of 37°C. Culture medium from the infected cells was collected every 2 h until 10 h postinfection. (E) Burst sizes of rVSV_{NJ}. We infected BHK₂₁ cells with the wild type and M mutants of rVSV_{NJ} at an MOI of 3 and incubated the cells at both 31 and 37°C. The culture medium from the infected cells was collected at 10 h postinfection. The number of infectious viral particles in the culture media was determined by plaque assay using Vero E6 cells. The error bar represents the standard deviation of the mean.

incubated at 37°C and 31°C, and the culture medium was harvested at 10 h postinfection to examine the burst size of the viruses. The wild type and all M mutants of rVSV_{NJ} replicated equally well at 31 and 37°C (Fig. 2E), indicating that the introduction of G22E and L110F mutations into the M protein of rVSV_{NJ} did not affect the assembly and release of the virus at 37°C. Although the mutations G22E and L110F did not make VSV_{NJ} temperature sensitive, the VSV_{NJ} mutant with the M48R and M51R mutations alone or together with G22E (GMM) and L110F (GMML) produced less infectious VSV_{NJ} by half a log to 1 log versus wild-type rVSV_{NJ}.

Assembly defectiveness at a semipermissive temperature by the L111F mutation in the M protein of rVSV_{Ind} does not affect the expression of VSV proteins. In order for the rVSV to be used as a vaccine vector, the virus should express genes of interest well,

and the temperature sensitivity of M gene mutants should not reduce gene expression. Because the mutations we introduced were intended to make VSV assembly defective at a semipermissive temperature without affecting genome replication or transcription of VSV, the levels of viral protein expression at both temperatures should be similar in cells infected with enough viruses to infect all cells. Therefore, we infected BHK₂₁ cells with both wild-type and M gene mutants of rVSV_{Ind} and rVSV_{NJ} at an MOI of 6. The infected cells were incubated at both 37°C and 31°C for 6 h. The infected cells were lysed at 6 h postinfection, 5 µg of total protein was analyzed using SDS-PAGE, and rVSV proteins were detected by Western blotting using rabbit antiserum against VSV_{Ind} or VSV_{NJ}. The result showed that in spite of the mutations that reduced the burst size of the rVSV_{Ind} at 37°C (L111F in the G21E L111F and G21E M51R L111F combined mutations), the

FIG 3 Protein expression of rVSV_{Ind} and rVSV_{NJ} at 37 and 31°C. BHK₂₁ cells were infected with rVSV_{Ind} and rVSV_{NJ} wild-type and M gene mutant strains at an MOI of 6. The infected cells were lysed at 6 h postinfection, 5 µg of total protein was loaded and electrophoresed on the SDS-PAGE gel, and rVSV proteins were detected by Western blotting using rabbit antiserum against VSV_{Ind} and VSV_{NJ} (1:5,000 dilution) separately.

level of VSV protein expression was the same as that of the wild-type rVSV_{Ind} (Fig. 3A) when each cell was infected with at least two infectious particles at an MOI of 6. The wild type and all M mutants of rVSV_{NJ} expressed their proteins at similar levels at both 31 and 37°C (Fig. 3B).

Combined mutations G21E M51R L111F in the M protein of rVSV_{Ind} and G22E M48R M51R and G22E M48R M51R L110F in the M protein of rVSV_{NJ} reduced cytopathogenicity significantly in BHK₂₁ cells and in SH-SY5Y human neuroblastoma cells. The typical cytopathic effects caused by the VSV are the rounding-up and lysis of infected cells (34). The mutations we introduced in rVSV made the rVSV defective in the assembly and release of the virus, and the defect would retard the spread of rVSV to the neighboring cells. The result is reduced overall cell death and damage to the cells over time. Extracellular lactate dehydrogenase (LDH) activity was measured to detect cell damage, which was released from the infected cells and induced by various causes (35–38). In order to examine the effects of mutations L111F and M51R of the M protein of rVSV_{Ind} on cytopathogenicity, we infected BHK₂₁ cells and SH-SY5Y cells with rVSV_{Ind} at an MOI of 6 and incubated them at 37°C. At 8 h postinfection, the culture medium was collected to measure the LDH activity, which indicates cellular damage caused by the rVSV infection. In BHK₂₁ cells, GL and GML mutants of rVSV_{Ind} caused the least cytolysis, in which LDH activity was slightly higher than that of the uninfected cell control (Fig. 4A). The GML mutant of rVSV_{Ind} showed the least cytolysis in SH-SY5Y cells (Fig. 4C), indicating that the combined mutations of M51R and L111F in the M protein of rVSV_{Ind} have the greatest effect on viral assembly, release of the viruses, and overall cell death.

All of the rVSV_{NJ} mutants showed reduced cytolysis of the infected cells compared to the wild-type rVSV_{NJ} in both BHK₂₁ cells and SH-SY5Y cells (Fig. 4B and D). Among the rVSV_{NJ} mutants, the GMM and GMML mutants caused the least cell damage in both BHK₂₁ and SH-SY5Y cells (Fig. 4B and D). In the SH-SY5Y cells, the rVSV_{NJ} GMM and GMML mutants caused cell damage similar to or less than that in the uninfected cells (Fig. 4D). The result of using rVSV_{NJ} mutants indicated that combined mutations of G22E and M48R M51R in the M protein made the virus less cytolytic in BHK₂₁ cells and SH-SY5Y cells.

In order to examine the effects of the mutations of L111F and M51R of the M protein of rVSV_{Ind} on virus release and subsequent overall cytopathic effects, we infected BHK₂₁ and SH-SY5Y cells with rVSV_{Ind} at an MOI of 0.1: this infected less than 1 in 10 cells (Fig. 5A and B). The cytopathic effects caused by the rVSV_{Ind} (GML) was compared with those of the other mutants of rVSV_{Ind}. At 20 h of infection, rVSV_{Ind}(GML) showed the most reduced cytopathic effects at 37°C, and the combination of mutations L111F and M51R (Fig. 5A–14 and B–12) further attenuated the cytopathogenicity of the virus versus the single mutation of each in both BHK₂₁ and SH-SY5Y cells.

In order to examine the effects of the mutations G22E, L110F, and M48R M51R of the M protein of rVSV_{NJ} on cytopathogenicity, we infected BHK₂₁ and SH-SY5Y cells with rVSV_{NJ} at an MOI of 0.1. At 20 h postinfection, we compared the cytopathic effects caused by the rVSV_{NJ} wild type and M mutants. The GMM and GMML mutant showed the most reduced cytopathic effects at 37°C, and the combination of the mutations G22E and M48R M51R further attenuated the cytopathogenicity of the rVSV_{NJ} in both BHK₂₁ (Fig. 6A–12 and A–14) and SH-SY5Y cells (Fig. 6B–12 and B–14).

rVSV_{Ind} with the mutations G21E M51R L111F and rVSV_{NJ} with the mutations G22E M48R M51R and G22E M48R M51R L110F in the M protein were attenuated to the degree that mice injected with these viruses into the brain showed no adverse neurological signs or symptoms. VSV does not show neurotropism in naturally infected animals through skin abrasion or sand fly or mosquito bites (8, 39). Nevertheless, when wild-type VSV is injected directly into the nose or brain of mice or monkeys, the animals demonstrate adverse neurological symptoms (12, 15, 27). In order to examine the neurovirulence of the new M mutants of VSVs, we injected 7-week-old Swiss Webster mice with 1×10^6 PFU of mutant VSV and 1×10^3 PFU of wild-type VSV into the intralateral ventricle of the brain. After viral injection, mice were observed for neurological dysfunctions and were weighed every 2 days for 4 weeks. The mice injected with 1×10^3 PFU of wild-type rVSV_{Ind} died within 4 days postinjection (Fig. 7A). Two mice injected with 1×10^6 PFU of rVSV_{Ind}(M51R) (Fig. 7C) lost about 20% of their body weight 6 days after injection. Their body weight bounced back to normal at about 14 days postinjection. One

FIG 4 Analysis of cytotoxicity in BHK₂₁ and SH-SY5Y cells by infection of M gene mutants of rVSV_{Ind} and rVSV_{NJ}. BHK₂₁ cells and SH-SY5Y cells were infected with rVSV_{Ind} and rVSV_{NJ} at an MOI of 6, and the infected cells were incubated at 37°C. At 8 h postinfection, the culture medium was collected to measure the released lactate dehydrogenase (LDH) activity, which indicates the cellular damage caused by the rVSV infection. (A and C) Cellular damage of BHK₂₁ and SH-SY5Y cells by the wild type and M gene mutants of rVSV_{Ind}. (B and D) Cellular damage of BHK₂₁ and SH-SY5Y cells by the wild type and M gene mutants of rVSV_{NJ}. *P* values (*, **, and ***, *P* < 0.05) were computed by using a two-sided independent sample *t* test. The error bars represent the standard deviation of the mean. The horizontal lines on the graph represent the background level of LDH activity from the uninfected cells.

mouse injected with the rVSV_{Ind}(M51R) mutant did not lose its body weight through the experiment. All three mice injected with rVSV_{Ind}(GML) showed no sign of illness and did not lose their body weight for 4 weeks (Fig. 7D), indicating the combination of mutations G21E, M51R, and L111F significantly attenuated rVSV_{Ind}, and it lost its neurovirulence in mice. One mouse injected with rVSV_{NJ}(WT) showed paralysis in both hind legs and was sacrificed on day 6 postinjection (Fig. 7E). Two other mice injected with rVSV_{NJ}(WT) and three mice injected with rVSV_{NJ}(M48R M51R), rVSV_{NJ}(GMM), or rVSV_{NJ}(GMML) showed no sign of illness or weight loss during the 4 weeks following injection (Fig. 7F, G, and H). These results show clearly that mutations in the rVSV_{Ind}(GML), rVSV_{NJ}(GMM), and rVSV_{NJ}(GMML) strains changed their neurovirulence to levels where there were no adverse neurological signs or symptoms.

Further nucleotide changes increase the stability of the temperature-sensitive mutations of L111F in the M gene of rVSV_{Ind} (GML) at 31 and 37°C. Temperature-sensitive mutants can be reverted to wild-type VSV when the infected cells are passaged many times at 37°C or 39°C. Our *ts* M mutants only had one or two nucleotide changes in the codon (underlined), such as from

GGG to GAA for G21E, from AUG to AGG for M51R, from UUG to UUU for L111F in the M gene of rVSV_{Ind}; from GGC to GAA for G22E, and from AUG to AGG for M48R and M51R in the M gene of rVSV_{NJ}. In order to prevent the possible reversion of the *ts* mutants, we changed all three nucleotides in the codons for M51R and L111F in the M gene of rVSV_{Ind}(GML) and all three codons for amino acid changes of M48R and M51R in the M gene of rVSV_{NJ}(GMM). The arginine codon, AGG, was further changed to CGU in M51R, and phenylalanine codon, UUU, was changed to GCA for alanine in L111F in the M gene of rVSV_{Ind}(GML). The arginine codon, AGG, was changed to another arginine codon, CGU, in the M48R and M51R mutations of rVSV_{NJ}(GMM) M protein. The *ts* mutants of the new rVSV_{Ind}(GML) and the new rVSV_{NJ}(GMM) mutant with all nucleotide changes were recovered from the DNA plasmid. First, the temperature sensitivity of the newly constructed rVSV_{Ind}(GMM) and rVSV_{NJ}(GMM) was examined by determining the growth kinetics of the viruses. BHK₂₁ cells were infected with a virus MOI of 3, and the culture medium was collected every 2 h until 10 h postinfection. Both old and new rVSV_{Ind}(GML) and rVSV_{NJ}(GMM) mutants replicated with the same growth kinetics, and the change of phenylalanine to

FIG 5 Comparison of cytopathic effects in BHK₂₁ cells (A) and in SH-SY5Y cells (B) caused by *M* gene mutants of rVSV_{Ind} at 31 and 37°C. We infected BHK₂₁ cells with rVSV_{Ind} at an MOI of 0.1. At 20 h postinfection, we compared the cytopathic effects caused by the rVSV_{Ind} wild type and *M* gene mutants. The images are at $\times 100$ magnification.

alanine in the L111A mutation of rVSV_{Ind} *M* maintained the temperature sensitivity of the new rVSV_{Ind}(GML) mutant (Fig. 8A).

We compared the stabilities of the mutations of the *ts M* gene mutants of rVSV_{Ind} and rVSV_{NJ}(GMM) between old and new viruses at 31 and 37°C. The stability of the mutation was examined after 20 consecutive passages of the viruses at 37°C. BHK₂₁ cells were initially infected with an MOI of 0.1 of old and new *M* gene mutants of rVSV_{Ind} and rVSV_{NJ}. After 18 h of incubation, the culture medium containing viruses was collected, and fresh

BHK₂₁ cells were infected with the collected viruses for additional viral passages at 31 and 37°C. At the 20th passage, the infectious viral titer was determined by plaque assay, and the fresh BHK₂₁ cells were infected with a virus MOI of 0.1 on the 20th passage of the old and new rVSV_{Ind}(GML) and rVSV_{NJ}(GMM) viruses. The rVSVs from 30 ml of the culture medium from the infected cell culture were concentrated and were resuspended in 1 ml of phosphate-buffered saline. The cDNAs of the *M* genes of the old and the new rVSV_{Ind}(GML) and rVSV_{NJ}(GMM) viruses were synthe-

FIG 6 Comparison of cytopathic effects in BHK₂₁ cells (A) and in SH-SY5Y cells (B) caused by *M* gene mutants of rVSV_{NJ} at 31°C and 37°C. We infected BHK₂₁ cells with rVSV_{NJ} at an MOI of 0.1. At 20 h postinfection, we compared the cytopathic effects caused by the rVSV_{NJ} wild type and *M* gene mutants. The images are in ×100 magnification.

sized by RT-PCR. Double-stranded DNA of the complete *M* gene was sequenced. There were no nucleotides that changed back to wild-type codons for G21E and M51R in the *M* genes of both the old and new rVSV_{Ind}(GML) mutants at both 31 and 37°C (Table 1). However, the phenylalanine codon, UUU, was changed back to CUU for leucine at L111F in the *M* gene of the old rVSV_{Ind}(GML) mutant after 20 consecutive passages of the viruses at 31°C and 37°C (Table 1). When the phenylalanine codon, UUU, was

changed to the alanine codon, GCA (L111A), the alanine was not changed back to leucine or any other amino acid (Table 1). These results demonstrate that all three nucleotide changes and an amino acid change from L111F to L111A kept the temperature-sensitive phenotype of rVSV_{Ind}(GML) at 37°C and increased the stability of the mutation L111A at both 31 and 37°C with continuous passages. Because the mutations in the *M* gene of rVSV_{NJ}(GMM) were not temperature sensitive, we expected

FIG 7 Neurovirulence measured by weight loss after direct intraventricular injection of rVSVs. We injected 7-week-old Swiss Webster mice with 1×10^6 PFU of *M* gene mutant rVSVs and 1×10^3 PFU of wild-type rVSV into the intralateral ventricle of the brain. After the viral injection, mice were observed for neurological signs and were weighed every 2 days for 4 weeks. The error bars represent the standard deviation of the mean.

no nucleotide changes of the *M* gene of rVSV_{NJ}(GMM) and rVSV_{NJ}(GMM)-New after 20 consecutive passages at 37°C (Table 2).

DISCUSSION

Since VSV was developed as a viral vaccine vector, it has been demonstrated that VSV could induce good cellular and humoral immune responses against foreign genes (6, 23, 40–42). Because of its ability to infect most mammalian cells and to induce immune responses, the use of the VSV as a vaccine vector for various hu-

man pathogens is increasing. However, the foremost concern for rVSV use as a vaccine vector is its safety. All attenuated live viral vaccines, including rVSVs, for human use have to go through stringent procedures to prove that they are safe to use in humans. Therefore, various strategies have been developed to attenuate the rVSVs to have reduced virulence. Attenuation of rVSV was accomplished by reducing the cytopathic effects of the *M* protein with mutations such as M51R in rVSV_{Ind} (41, 43) and M48R M51R in rVSV_{NJ} (26, 44). The M51R mutation alone in the *M* gene of rVSV_{Ind} reduced cytopathogenicity of VSV, but it was not

FIG 8 Growth kinetics of new *M* gene mutants of rVSV_{Ind}(GML) and of rVSV_{NJ}(GMM) with additional nucleotide changes. We infected BHK₂₁ cells with a virus MOI of 3. The infected cells were incubated at a permissive temperature of 31°C and a semipermissive temperature of 37°C. Culture medium from the infected cells was collected every 2 h until 10 h postinfection. The number of infectious viral particles in the culture medium was determined by plaque assay using Vero E6 cells. The error bar represents the standard deviation of the mean.

TABLE 1 Nucleotide codon changes in the amino acid mutations in the *M* gene of rVSV_{Ind}(GML) after 20 consecutive passages

rVSV strain	Nucleotide (amino acid) change from WT in <i>M</i> gene codon ^a					
	⁶¹ GGG ⁶³ (G21)		¹⁵¹ AUG ¹⁵³ (M51)		³³¹ UUG ³³³ (L111)	
	Mutation	37°C	Mutation	37°C	Mutation	37°C
rVSV _{Ind} (GML)	<u>GAA</u> (G21E)	<u>GAA</u> (G21E)	AGG (M51R)	<u>AGG</u> (M51R)	UUU (L111F)	<u>CUU</u> (L111L)
rVSV _{Ind} (GML)-New	<u>GAA</u> (G21E)	<u>GAA</u> (G21E)	<u>CGA</u> (M51R)	<u>CGA</u> (M51R)	<u>GCA</u> (L111A)	<u>GCA</u> (L111A)

^a Changes are underlined.

attenuated enough to be nonneurovirulent *in vitro* and in mice (24) (Fig. 4 and 5C). The *G* gene modifications in the rVSV genome, such as complete deletion of the gene (21) or partial deletion of the cytoplasmic tail region of the gene, were applied to attenuate the VSV (24, 45). Rearrangement of VSV genes, especially *N* gene translocation, was also used to attenuate the VSV (24, 46). These methods of VSV attenuation reduce the replication ability of rVSVs (24) and, inadvertently, the viral yields as well when viral stocks were prepared *in vitro*.

Because of the neurovirulence observed in animals injected intranasally or intracranially with rVSVs, researchers have been putting a lot of effort into making rVSVs as safer vaccine vectors. We have also been working on developing a safe rVSV as a vaccine vector. Our strategy was to use temperature-sensitive VSV strains that do not produce virus particles but have normal genome replication and gene transcription at a semipermissive temperature (37°C). In order to develop rVSVs where neurovirulence is attenuated at a normal body temperature but maintains productivity *in vitro*, we looked for *ts* mutants of the VSV *M* gene. One of the *ts* mutants was the *ts*O23 mutant of the Orsay strain of rVSV_{Ind}, which showed assembly defectiveness at semipermissive (37°C) and nonpermissive (39°C) temperatures (27, 33). We introduced mutations (single or combined mutations) of *ts*O23 into the *M* gene of our prVSV_{Ind} (HR strain) and prVSV_{NJ} Hazlehurst strain, as depicted in Fig. 1. The combined mutations G21E M51R L111F in the *M* gene of rVSV_{Ind} and G22E M48R M51R and G22E M48R M51R L110F in the *M* gene of rVSV_{NJ} showed the least cytopathic effects *in vitro* and nonneurovirulence in mice.

There have been several mutations found in the *M* gene of the *ts*O23 strain. It was suggested that the L111F mutation in the *ts*O23 strain was the key mutation that might have given the *ts*O23 mutant the phenotypic characteristics at a nonpermissive temperature (33). In both baby hamster kidney cells and human neuroblastoma cells, the rVSV_{Ind} strains with L111F mutation, rVSV_{Ind} (GL) and rVSV_{Ind}(GML), showed significantly lower viral production through the course of infection at 37°C (Fig. 2B and D). The rVSV_{Ind} strain with a G21E mutation showed the same growth kinetics as the wild type and the M51R mutant of rVSV_{Ind} at 31 and 37°C. However, when the L111F mutation was added to G21E to make the G21E L111F double mutant, the mutant be-

came temperature sensitive at a semipermissive temperature (37°C). Thus, this indicated that L111F is the key mutation for temperature sensitivity. In addition, when M51R was added to the G21E L111F mutation to generate rVSV_{Ind} with G21E M51R L111F mutations, the combined mutations reduced the growth rate of the rVSV_{Ind} by 5- to 10-fold (Fig. 2B and D). This is the same as the cytopathogenicity induced by the rVSV_{Ind} mutants, which is shown in Fig. 5A-13, A-14, B-11, and B-12. The addition of L111F and M51R mutations to G21E further decreased cytopathogenicity in BHK₂₁ cells and human neuroblastoma cell lines. The overall reduced cytopathogenicity *in vitro* was confirmed by the reduced LDH secretion from both BHK₂₁ cells and SH-SY5Y cells infected with rVSV_{Ind}(GML). Other attenuated rVSVs were generated by moving the *N* gene to other locations, by deleting various numbers of amino acids in the cytoplasmic tail of the *G* protein, and by placing a gene of interest as the first gene to be expressed (23, 24). These strategies of attenuation disrupted the stoichiometry of VSV proteins and assembly of the VSV, resulting in significant reduction of VSV production by 100-fold to 1,000-fold less than that of the wild-type VSV (24). An unwanted outcome of this strategy is significant reduction of the VSV yields *in vitro* for vaccine preparation. Our strategy to attenuate the VSV by introducing assembly-defective temperature-sensitive mutations in the *M* gene of VSV_{Ind} generated the vaccine vector without affecting genome replication and gene expression in the infected cells at both permissive (31°C) and semipermissive (37°C) temperatures. Importantly, the assembly of the VSV is not affected at 31°C, and the rVSV_{Ind}(GML) mutant could be prepared in a high titer *in vitro* at 31°C.

L111 in the VSV_{Ind} *M* protein is located at the alpha-helix region in which the amino acid is surrounded by hydrophobic amino acids (47). It has been suggested that the L111F mutation in the *M* protein of VSV_{Ind} aggregates *M* protein on the cytoplasmic membrane and prevents the initiation of nucleocapsid-*M* protein complex formation (28) for the assembly of VSV particles at a nonpermissive temperature (39°C). Leucine is not present exactly at the same 111th position in the *M* protein of VSV_{NJ}, but it is found at the 110th position. The *M* proteins of both VSV_{Ind} and VSV_{NJ} are 229 amino acids long. Because of the closeness of the leucine in the *M* protein of VSV_{NJ} to the L111 of the VSV_{Ind} *M*

TABLE 2 Nucleotide codon changes in the amino acid mutations in the *M* gene of rVSV_{NJ}(GMM) after 20 consecutive passages

rVSV strain	Nucleotide (amino acid) change from WT in <i>M</i> gene codon ^a					
	⁶⁴ GGC ⁶⁶ (G22)		¹⁴² AUG ¹⁴⁴ (M48)		¹⁵¹ AUG ¹⁵³ (M51)	
	Mutation	37°C	Mutation	37°C	Mutation	37°C
rVSV _{NJ} (GMM)	<u>GAA</u> (G22E)	<u>GAA</u> (G22E)	AGG (M48R)	<u>AGG</u> (M48R)	<u>AGG</u> (M51R)	<u>AGG</u> (M51R)
rVSV _{NJ} (GMM)-New	<u>GAA</u> (G22E)	<u>GAA</u> (G22E)	<u>CGU</u> (M48R)	<u>CGU</u> (M48R)	<u>CGU</u> (M51R)	<u>CGU</u> (M51R)

^a Changes are underlined.

protein, we changed the leucine to phenylalanine at the 110th position of the VSV_{NJ} M protein with the expectation of having the same temperature sensitivity of VSV_{NJ} as that of VSV_{Ind}. However, the L110F mutation in the rVSV_{NJ} M protein did not make the rVSV_{NJ} mutant temperature sensitive at a semipermissive temperature (37°C), and rVSV_{NJ} strains with the L110F mutation, such as rVSV_{NJ}(GL) and rVSV_{NJ}(GMML), produced similar levels of infectious rVSV_{NJ} at both 31 and 37°C (Fig. 2E). Therefore, the mutation is either irrelevant to the temperature sensitivity of virus particle assembly in VSV_{NJ} or the 111th isoleucine in the VSV_{NJ} M protein, which is as hydrophobic as leucine at the same position as the L111 of VSV_{Ind} M, may be important for the assembly of the VSV_{NJ}. The I111F mutation in the VSV_{NJ} M protein needs to be tested to see if it makes VSV_{NJ} assembly defective at 37 and 39°C. The G22E and L110F mutations in addition to the M48R M51R double mutation did not make rVSV_{NJ} assembly defective at 37°C. However, when we checked the cytopathogenicity of the mutants in BHK₂₁ cells and SH-SY5Y cells, the combined mutations G22E M48R M51R (GMM) and G22E M48R M51R L110F (GMML) reduced cytopathic effects most significantly at 37°C. The cytopathic effects *in vitro* by rVSV_{NJ}(GMM) and rVSV_{NJ}(GMML) were attenuated mainly by the presence of the M48R and M51R mutations in BHK₂₁ cells and SH-SY5Y cells (Fig. 6A-8 and B-8). The addition of the G22E mutation in rVSV_{NJ}(GMM) and the addition of G22E and L110F mutations in rVSV_{NJ}(GMML) to the M48R M51R combined mutation significantly reduced cytopathic effects (Fig. 6A-12, 6A-14, B-12, and B-14). The reduced cytopathogenicity in BHK₂₁ cells and SH-SY5Y cells by the rVSV_{NJ} mutants at 37°C was confirmed by the LDH assay (Fig. 4B and D). The M48R M51R mutant, GMM mutant, and GMML mutant of rVSV_{NJ} had less of a CPE in SH-SY5Y cells than in BHK₂₁ cells.

Temperature-sensitive mutants can be reverted back to wild-type VSV when the viruses are passaged many times. The tsO23 mutant was the most temperature-sensitive strain among the assembly-defective *ts* mutants, and it showed the least neurovirulence in mice when the *ts* M gene mutants were inoculated directly into the brain (27, 33), which made the mutations in the tsO23 mutant preferable in our VSV vector for humans. When we injected mice intracranially with our rVSVs, the wild type and M gene mutants of rVSV_{Ind} and rVSV_{NJ}, mice injected with 1,000 infectious particles of rVSV_{Ind}(WT) died within 4 days of injection. The strain with the single mutation at M51R, rVSV_{Ind}(M51R), clearly more attenuated than the wild-type rVSV_{Ind}, and two of three mice injected with the virus showed signs of distress (more than 20% weight loss) without paralysis; however, all survived the experiment. Mice injected with rVSV_{Ind} with combined mutations M51R, G22E, and L111F in the M gene of rVSV_{Ind} did not show any neurological dysfunction (Fig. 7D). The combined mutations must have attenuated the VSV_{Ind} much more effectively than the L111F mutation alone (27) and resulted in the complete loss of neurovirulence by rVSV_{Ind}. Overall, the rVSV_{NJ} strains were less virulent than the rVSV_{Ind} strains in mice. Compared to rVSV_{Ind}(WT), 1,000 infectious virus particles of rVSV_{NJ}(WT) did not kill mice, but one mouse was paralyzed in both hind legs with injection of 1,000 infectious virus particles. None of the mutants of rVSV_{NJ}, the M48R M51R mutant, GMM mutant, or GMML mutant, caused any neurological dysfunctions or distress in the mice. Comparatively less neuropathogenicity of rVSV_{NJ} than rVSV_{Ind} may be linked to the intrinsic ability of rVSV_{NJ} to

induce interferons (48) and the induction of interferons in brain by the rVSV_{NJ} with M48R M51R mutations (49).

One of the successful human vaccines that has a temperature-sensitive phenotype is the poliomyelitis vaccine (50). Due to the sequential passages of the method used to develop the poliomyelitis vaccine, the genetic stabilities of the mutations were empirically proved. However, our *ts* M gene mutant, rVSV_{Ind}(GML), was generated by the use of site-directed mutagenesis. The initial mutations were introduced by changing one nucleotide in an amino acid codon, and our concerns about the reversion of the rVSV to the wild-type phenotype had to be resolved. The genetic stability of the attenuated *ts* mutant vaccines should be tested and confirmed for use in humans. Our hypothesis that increasing the number of nucleotide changes in a codon for an amino acid in a mutation increases the genetic stability of the *ts* mutation was tested by changing all three nucleotides in a codon for the amino acid change. After 20 consecutive passages of rVSV_{Ind}(GML)-New at 37°C, there were no nucleotide changes back to the wild-type amino acid codon (Table 1), proving that additional nucleotide changes increased the stability of the mutation *in vitro*. Although further testing for the genetic stability of the mutation in animal hosts is still required, our results are promising to pursue further vaccination studies with our new rVSV_{Ind}(GML) vector.

ACKNOWLEDGMENTS

This study was partially supported by an IRAP grant from the National Research Council of Canada and by a Research Contract from Sumagen Canada, Inc.

BSR T7/5 cells (30) constitutively expressing bacteriophage T7 RNA polymerase were a kind gift from K. K. Conzelmann. We are grateful to Rosanne Kang and Doris Hall for proofreading the manuscript.

REFERENCES

- Kalinke U, Bucher EM, Ernst B, Oxenius A, Roost HP, Geley S, Kofler R, Zinkernagel RM, Hengartner H. 1996. The role of somatic mutation in the generation of the protective humoral immune response against vesicular stomatitis virus. *Immunity* 5:639–652. [http://dx.doi.org/10.1016/S1074-7613\(00\)80277-0](http://dx.doi.org/10.1016/S1074-7613(00)80277-0).
- Puddington L, Bevan MJ, Rose JK, Lefrancois L. 1986. N protein is the predominant antigen recognized by vesicular stomatitis virus-specific cytotoxic T cells. *J Virol* 60:708–717.
- Yewdell JW, Bennink JR, Mackett M, Lefrancois L, Lyles DS, Moss B. 1986. Recognition of cloned vesicular stomatitis virus internal and external gene products by cytotoxic T lymphocytes. *J Exp Med* 163:1529–1538. <http://dx.doi.org/10.1084/jem.163.6.1529>.
- Gallione CJ, Rose JK. 1983. Nucleotide sequence of a cDNA clone encoding the entire glycoprotein from the New Jersey serotype of vesicular stomatitis virus. *J Virol* 46:162–169.
- Cartwright B, Brown F. 1972. Serological relationships between different strains of vesicular stomatitis virus. *J Gen Virol* 16:391–398. <http://dx.doi.org/10.1099/0022-1317-16-3-391>.
- Rose NF, Marx PA, Luckay A, Nixon DF, Moretto WJ, Donahoe SM, Montefiori D, Roberts A, Buonocore L, Rose JK. 2001. An effective AIDS vaccine based on live attenuated vesicular stomatitis virus recombinants. *Cell* 106:539–549. [http://dx.doi.org/10.1016/S0092-8674\(01\)00482-2](http://dx.doi.org/10.1016/S0092-8674(01)00482-2).
- Rose NF, Roberts A, Buonocore L, Rose JK. 2000. Glycoprotein exchange vectors based on vesicular stomatitis virus allow effective boosting and generation of neutralizing antibodies to a primary isolate of human immunodeficiency virus type 1. *J Virol* 74:10903–10910. <http://dx.doi.org/10.1128/JVI.74.23.10903-10910.2000>.
- Letchworth GJ, Rodriguez LL, Del Cbarrera J. 1999. Vesicular stomatitis. *Vet J* 157:239–260. <http://dx.doi.org/10.1053/tvj.1998.0303>.
- Brandly CA, Hanson RP. 1957. Epizootiology of vesicular stomatitis. *Am J Public Health Nations Health* 47:205–209. <http://dx.doi.org/10.2105/AJPH.47.2.205>.
- Brody JA, Fischer GF, Peralta PH. 1967. Vesicular stomatitis virus in

- Panama. Human serologic patterns in a cattle raising area. *Am J Epidemiol* 86:158–161.
11. Johnson KM, Vogel JE, Peralta PH. 1966. Clinical and serological response to laboratory-acquired human infection by Indiana type vesicular stomatitis virus (VSV). *Am J Trop Med Hyg* 15:244–246.
 12. Johnson JE, Nasar F, Coleman JW, Price RE, Javadian A, Draper K, Lee M, Reilly PA, Clarke DK, Hendry RM, Udem SA. 2007. Neurovirulence properties of recombinant vesicular stomatitis virus vectors in non-human primates. *Virology* 360:36–49. <http://dx.doi.org/10.1016/j.virol.2006.10.026>.
 13. Reiss CS, Plakhov IV, Komatsu T. 1998. Viral replication in olfactory receptor neurons and entry into the olfactory bulb and brain. *Ann N Y Acad Sci* 855:751–761. <http://dx.doi.org/10.1111/j.1749-6632.1998.tb10655.x>.
 14. Sur JH, Allende R, Doster AR. 2003. Vesicular stomatitis virus infection and neuropathogenesis in the murine model are associated with apoptosis. *Vet Pathol* 40:512–520. <http://dx.doi.org/10.1354/vp.40-5-512>.
 15. van den Pol AN, Dalton KP, Rose JK. 2002. Relative neurotropism of a recombinant rhabdovirus expressing a green fluorescent envelope glycoprotein. *J Virol* 76:1309–1327. <http://dx.doi.org/10.1128/JVI.76.3.1309-1327.2002>.
 16. Marchevsky RS, Freire MS, Coutinho ES, Galler R. 2003. Neurovirulence of yellow fever 17DD vaccine virus to rhesus monkeys. *Virology* 316:55–63. [http://dx.doi.org/10.1016/S0042-6822\(03\)00583-X](http://dx.doi.org/10.1016/S0042-6822(03)00583-X).
 17. Nathanson N, Horn SD. 1992. Neurovirulence tests of type 3 oral poliovirus vaccine manufactured by Lederle Laboratories, 1964–1988. *Vaccine* 10:469–474. [http://dx.doi.org/10.1016/0264-410X\(92\)90396-2](http://dx.doi.org/10.1016/0264-410X(92)90396-2).
 18. Rubin SA, Snoy PJ, Wright KE, Brown EG, Reeve P, Beeler JA, Carbone KM. 1999. The mumps virus neurovirulence safety test in rhesus monkeys: a comparison of mumps virus strains. *J Infect Dis* 180:521–525. <http://dx.doi.org/10.1086/314905>.
 19. Sharova OK, Rozina EE, Shteinberg LS, Gordienko NM, Kolyanova IS. 1979. Morphological characteristics of the pathological process in the central nervous system of monkeys infected with variants of measles virus strain L-16. *Acta Virol* 23:393–397.
 20. Kahn JS, Roberts A, Weibel C, Buonocore L, Rose JK. 2001. Replication-competent or attenuated, nonpropagating vesicular stomatitis viruses expressing respiratory syncytial virus (RSV) antigens protect mice against RSV challenge. *J Virol* 75:11079–11087. <http://dx.doi.org/10.1128/JVI.75.22.11079-11087.2001>.
 21. Schwartz JA, Buonocore L, Roberts A, Suguitan A, Jr, Kobasa D, Kobinger G, Feldmann H, Subbarao K, Rose JK. 2007. Vesicular stomatitis virus vectors expressing avian influenza H5 HA induce cross-neutralizing antibodies and long-term protection. *Virology* 366:166–173. <http://dx.doi.org/10.1016/j.virol.2007.04.021>.
 22. Schnell MJ, Johnson JE, Buonocore L, Rose JK. 1997. Construction of a novel virus that targets HIV-1-infected cells and controls HIV-1 infection. *Cell* 90:849–857. [http://dx.doi.org/10.1016/S0092-8674\(00\)80350-5](http://dx.doi.org/10.1016/S0092-8674(00)80350-5).
 23. Cooper D, Wright KJ, Calderon PC, Guo M, Nasar F, Johnson JE, Coleman JW, Lee M, Kotash C, Yurgelonis I, Natuk RJ, Hendry RM, Udem SA, Clarke DK. 2008. Attenuation of recombinant vesicular stomatitis virus-human immunodeficiency virus type 1 vaccine vectors by gene translocations and G gene truncation reduces neurovirulence and enhances immunogenicity in mice. *J Virol* 82:207–219. <http://dx.doi.org/10.1128/JVI.01515-07>.
 24. Clarke DK, Nasar F, Lee M, Johnson JE, Wright K, Calderon P, Guo M, Natuk R, Cooper D, Hendry RM, Udem SA. 2007. Synergistic attenuation of vesicular stomatitis virus by combination of specific G gene truncations and N gene translocations. *J Virol* 81:2056–2064. <http://dx.doi.org/10.1128/JVI.01911-06>.
 25. Jayakar HR, Whitt MA. 2002. Identification of two additional translation products from the matrix (M) gene that contribute to vesicular stomatitis virus cytopathology. *J Virol* 76:8011–8018. <http://dx.doi.org/10.1128/JVI.76.16.8011-8018.2002>.
 26. Kim GN, Kang CY. 2007. Matrix protein of VSV New Jersey serotype containing methionine to arginine substitutions at positions 48 and 51 allows near-normal host cell gene expression. *Virology* 357:41–53. <http://dx.doi.org/10.1016/j.virol.2006.07.022>.
 27. Rabinowitz SG, Huprikar J, Dal Canto MC. 1981. Comparative neurovirulence of selected vesicular stomatitis virus temperature-sensitive mutants of complementation groups II and III. *Infect Immun* 33:120–125.
 28. Flood EA, McKenzie MO, Lyles DS. 2000. Role of M protein aggregation in defective assembly of temperature-sensitive M protein mutants of vesicular stomatitis virus. *Virology* 278:520–533. <http://dx.doi.org/10.1006/viro.2000.0675>.
 29. Lyles DS, McKenzie MO, Kaptur PE, Grant KW, Jerome WG. 1996. Complementation of M gene mutants of vesicular stomatitis virus by plasmid-derived M protein converts spherical extracellular particles into native bullet shapes. *Virology* 217:76–87. <http://dx.doi.org/10.1006/viro.1996.0095>.
 30. Buchholz UJ, Finke S, Conzelmann KK. 1999. Generation of bovine respiratory syncytial virus (BRSV) from cDNA: BRSV NS2 is not essential for virus replication in tissue culture, and the human RSV leader region acts as a functional BRSV genome promoter. *J Virol* 73:251–259.
 31. Kim GN, Choi WY, Park M, Kang CY. 2002. Replication and transcription of viral RNAs by recombinant L proteins of New Jersey serotype of vesicular stomatitis virus. *Virus Res* 90:347–364. [http://dx.doi.org/10.1016/S0168-1702\(02\)00255-1](http://dx.doi.org/10.1016/S0168-1702(02)00255-1).
 32. Kim GN, Kang CY. 2005. Utilization of homotypic and heterotypic proteins of vesicular stomatitis virus by defective interfering particle genomes for RNA replication and virion assembly: implications for the mechanism of homologous viral interference. *J Virol* 79:9588–9596. <http://dx.doi.org/10.1128/JVI.79.15.9588-9596.2005>.
 33. Morita K, Vanderroef R, Lenard J. 1987. Phenotypic revertants of temperature-sensitive M protein mutants of vesicular stomatitis virus: sequence analysis and functional characterization. *J Virol* 61:256–263.
 34. Baxt B, Bablanian R. 1976. Mechanisms of vesicular stomatitis virus-induced cytopathic effects. I. Early morphologic changes induced by infectious and defective-interfering particles. *Virology* 72:370–382.
 35. Jurisic V, Spuzic I, Konjevic G. 1999. A comparison of the NK cell cytotoxicity with effects of TNF-alpha against K-562 cells, determined by LDH release assay. *Cancer Lett* 138:67–72. [http://dx.doi.org/10.1016/S0304-3835\(99\)00011-7](http://dx.doi.org/10.1016/S0304-3835(99)00011-7).
 36. Fotakis G, Timbrell JA. 2006. In vitro cytotoxicity assays: comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. *Toxicol Lett* 160:171–177. <http://dx.doi.org/10.1016/j.toxlet.2005.07.001>.
 37. Watanabe W, Sudo K, Asawa S, Konno K, Yokota T, Shigeta S. 1995. Use of lactate dehydrogenase to evaluate the anti-viral activity against influenza A virus. *J Virol Methods* 51:185–191. [http://dx.doi.org/10.1016/0166-0934\(94\)00103-N](http://dx.doi.org/10.1016/0166-0934(94)00103-N).
 38. Konishi E, Kitai Y, Kondo T. 2008. Utilization of complement-dependent cytotoxicity to measure low levels of antibodies: application to nonstructural protein 1 in a model of Japanese encephalitis virus. *Clin Vaccine Immunol* 15:88–94. <http://dx.doi.org/10.1128/CVI.00347-07>.
 39. Lyles DS, Rupprecht CE. 2007. Rhabdoviridae, p 1362–1408. In Knipe DM, Griffin DE, Lamb RA, Straus SE, Howley PM, Martin MA, Roizman B (ed), *Fields virology*, 5th ed, vol 1. Lippincott Williams & Wilkins, Philadelphia, PA.
 40. Egan MA, Chong SY, Rose NF, Megati S, Lopez KJ, Schadeck EB, Johnson JE, Masood A, Piacente P, Druilhet RE, Barras PW, Hasselschwert DL, Reilly P, Mishkin EM, Montefiori DC, Lewis MG, Clarke DK, Hendry RM, Marx PA, Eldridge JH, Udem SA, Israel ZR, Rose JK. 2004. Immunogenicity of attenuated vesicular stomatitis virus vectors expressing HIV type 1 Env and SIV Gag proteins: comparison of intranasal and intramuscular vaccination routes. *AIDS Res Hum Retroviruses* 20:989–1004. <http://dx.doi.org/10.1089/aid.2004.20.989>.
 41. Braxton CL, Puckett SH, Mizel SB, Lyles DS. 2010. Protection against lethal vaccinia virus challenge by using an attenuated matrix protein mutant vesicular stomatitis virus vaccine vector expressing poxvirus antigens. *J Virol* 84:3552–3561. <http://dx.doi.org/10.1128/JVI.01572-09>.
 42. Schwartz JA, Buonocore L, Suguitan A, Jr, Hunter M, Marx PA, Subbarao K, Rose JK. 2011. Vesicular stomatitis virus-based H5N1 avian influenza vaccines induce potent cross-clade neutralizing antibodies in rhesus macaques. *J Virol* 85:4602–4605. <http://dx.doi.org/10.1128/JVI.02491-10>.
 43. Ahmed M, Marino TR, Puckett S, Kock ND, Lyles DS. 2008. Immune response in the absence of neurovirulence in mice infected with M protein mutant vesicular stomatitis virus. *J Virol* 82:9273–9277. <http://dx.doi.org/10.1128/JVI.00915-08>.
 44. An HY, Kim GN, Wu K, Kang CY. 2013. Genetically modified VSV(NJ) vector is capable of accommodating a large foreign gene insert and allows high level gene expression. *Virus Res* 171:168–177. <http://dx.doi.org/10.1016/j.virusres.2012.11.007>.
 45. Roberts A, Kretzschmar E, Perkins AS, Forman J, Price R, Buonocore L, Kawaoka Y, Rose JK. 1998. Vaccination with a recombinant vesicular

- stomatitis virus expressing an influenza virus hemagglutinin provides complete protection from influenza virus challenge. *J Virol* 72:4704–4711.
46. Flanagan EB, Ball LA, Wertz GW. 2000. Moving the glycoprotein gene of vesicular stomatitis virus to promoter-proximal positions accelerates and enhances the protective immune response. *J Virol* 74:7895–7902. <http://dx.doi.org/10.1128/JVI.74.17.7895-7902.2000>.
 47. Gaudier M, Gaudin Y, Knossow M. 2002. Crystal structure of vesicular stomatitis virus matrix protein. *EMBO J* 21:2886–2892. <http://dx.doi.org/10.1093/emboj/cdf284>.
 48. Marcus PI, Sekellick MJ, Nichol ST. 1992. Interferon induction by viruses. XXI. Vesicular stomatitis virus: interferon inducibility as a phylogenetic marker. *J Interferon Res* 12:297–305.
 49. Trotter MD, Lyles DS, Reiss CS. 2007. Peripheral, but not central nervous system, type I interferon expression in mice in response to intranasal vesicular stomatitis virus infection. *J Neurovirol* 13:433–445. <http://dx.doi.org/10.1080/13550280701460565>.
 50. Almond JW. 1987. The attenuation of poliovirus neurovirulence. *Annu Rev Microbiol* 41:153–180. <http://dx.doi.org/10.1146/annurev.mi.41.100187.001101>.