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# A single nucleotide change in the mumps virus F gene affects virus fusogenicity *in vitro* and virulence *in vivo*

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**Mumps virus is highly neurotropic, with evidence of infection of the central nervous system in more than half of clinical cases. In the prevaccine era, mumps was a major cause of viral meningitis in most developed countries. Despite efforts to attenuate the virus, some mumps vaccines have retained virulence properties and have caused aseptic meningitis in vaccinees, resulting in public resistance to vaccination in some countries. Ensuring the safety of mumps vaccines is an important public health objective, as the need for robust immunization programs has been made clear by the recent resurgence of mumps outbreaks worldwide, including the United States, which in 2006 experienced its largest mumps outbreak in 20 years. To better understand the molecular basis of mumps virus attenuation, the authors developed two infectious full-length cDNA clones for a highly neurovirulent strain of mumps virus. The clones differed at only one site, possessing either an A or G at nucleotide position 271 in the F gene, to represent the heterogeneity identified in the original virulent clinical isolate. In comparison to the clinical isolate, virus rescued from the A-variant cDNA clone grew to higher cumulative titers *in vitro* but exhibited similar cytopathic effects *in vitro* and virulence *in vivo*. In contrast, virus rescued from the G-variant cDNA clone, in comparison to the clinical isolate and the A-variant, was more fusogenic *in vitro* but replicated to lower cumulative titers and was less neurovirulent *in vivo*. These data suggest that nucleotide position 271 in the F gene plays a significant role in virus pathogenesis. This infectious clone system will serve as a key tool for further examination of the molecular basis for mumps virus neurovirulence and neuroattenuation.** *Journal of NeuroVirology* (2007) **13,** 513–521.

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## Introduction

Mumps virus (MuV), a member of the paramyxovirus family, causes an acute infectious disease mainly in children and young adults. The most prominent clinical manifestation is parotitis, but there is evidence of central nervous system (CNS) infection in greater than 50% of mumps cases (Bang and Bang, 1943). The natural tropism of MuVs for the CNS is highlighted by the difficulty in virus neuroattenuation. This has been most clearly demonstrated through clinical use of a number of MuV vaccines, including the Urabe AM9 (Sugiura and Yamada, 1991; Brown *et al*, 1991; Miller *et al*, 1993) and Leningrad-Zagreb (Arruda and Kondageski, 2001; Tesovic and Lesnikar, 2006) strains, which caused meningitis in

vaccinees despite considerable *in vitro* attenuation efforts. The continued need for robust immunization programs and public confidence in mumps vaccines is highlighted by the recent resurgence of mumps outbreaks worldwide (Centers for Disease Control and Prevention [CDC], 2006a, 2006b; Weibel *et al*, 1979; Szomor *et al*, 2007; Kojouharova *et al*, 2007; Spanaki *et al*, 2007; Castilla *et al*, 2007; Schmid *et al*, 2006).

As wild-type MuVs are neurovirulent, candidate live attenuated vaccine virus strains must be assessed for attenuation. This has historically been evaluated in monkeys; however, published reports suggest that the monkey test does not reliably discriminate neurovirulent from nonneurovirulent MuV strains (Rubin *et al*, 1999; Afzal *et al*, 1999). An alternative neurovirulence safety test using rats has been subsequently developed at the U.S. Food and Drug Administration (FDA) and appears to accurately assess the human neurovirulence potential of MuVs (Rubin *et al*, 2000, 2005). Formal validation studies are currently underway to support the use of this test in a regulatory setting, i.e., as a preclinical assay for assessing the neurological safety of candidate MuV vaccines. However, to facilitate use of the rat neurovirulence assay for investigating the pathogenesis of MuV infection of the CNS and to examine possible molecular markers of MuV neurovirulence and neuroattenuation, development of a neurovirulent infectious MuV cDNA clone is required. Through gene replacement and targeted site-directed mutagenesis of the cDNA clone, such a complementary tool will permit detailed investigations of the contribution of specific virus genes and gene regions on virus neurovirulence.

The MuV genome encodes open reading frames (ORFs) for the three core proteins: the nucleoprotein (N), the phosphoprotein (P), and the large multifunctional polymerase (L) protein; the fusion (F) and the hemagglutinin-neuraminidase (HN) surface glycoproteins; and the membrane associated matrix (M) and small hydrophobic (SH) proteins (Carbone and Rubin, 2007; Elango *et al*, 1988). Additionally, the P gene, like that of other paramyxoviruses, has the capacity to encode multiple proteins, i.e. the P, V, and I proteins (Paterson and Lamb, 1990). A reverse genetics system for the highly attenuated MuV vaccine strain Jeryl Lynn was established by Clarke *et al* several years ago (Clarke *et al*, 2000); however, given that we are interested in the study of the process of attenuation, a virulent wild-type virus strain was needed. Thus, we constructed p88-$1961_{A271}$, a full-length cDNA plasmid clone derived from the highly neurovirulent clinical isolate 88-1961 (Amexis *et al*, 2003). Virus was rescued using a vaccinia virus-free system. Because the original clinical isolate contained a heterogeneous site where both A and G residues were identified at nucleotide position 271 in the F gene (Amexis *et al*, 2003), and which result in a predicted amino acid change, this site in p88-$1961_{A271}$ was altered by site-directed mutagenesis to create p88-$1961_{G271}$. Of note, heterogeneity was also reported at three other positions in the 88-1961 clinical isolate (Amexis *et al*, 2003). However, the different nucleotides at these positions were not predicted to result in amino acid changes and therefore were not considered in the construction of the cDNA clones described here. Our studies show that virus rescued from the G-variant cDNA clone, in comparison to the A-variant, replicated to lower cumulative titers and displayed greatly enhanced fusogenic potential *in vitro* but was less neurovirulent *in vivo*. Thus, nucleotide position 271 in the F gene appears to have a significant influence on virus pathogenesis. Further development and testing of molecular clone variants of mumps viruses will serve as a powerful tool in examining the molecular basis for mumps virus neurovirulence and neuroattenuation.

## Results

*Construction and recovery of 88-1961 from cDNA*

The plasmid p88-$1961_{A271}$ encoding the 15,384-nucleotide positive-strand (antigenomic) RNA sequence was constructed from four cDNA fragments and cloned contiguously into a modified pBluescript SK(+) vector (Clarke *et al*, 2000) (Figure 1) as described in Materials and Methods. The 88-1961 clinical isolate contains a heterogenous nucleotide population (A/G) at position 271 of the F gene (analogous to position 4816 of the genome) (Amexis *et al*, 2003). Therefore p88-$1961_{A271}$, containing an A at this position, was subsequently modified by site directed mutagenesis to yield a second 88-1961 clone, p88-$1961_{G271}$. Each of the resultant 88-1961 full-length cDNA clones were thus a faithful copy of the wild-type genomic sequence (GenBank no. AF467767), except for the silent T to C nucleotide substitution at position 5772 of the L gene (genome position 14161) introduced as a genetic tag for confirming virus identity following virus recovery. This silent mutation did not affect the transcriptional/translational activities of the viruses based on assessment of CAT activity using our previously described minigenome assay (data not shown) (Malik *et al*, 2007).

The cDNA-derived 88-1961 viruses were rescued from BHK BSR-T7/5 cells as described in Materials and Methods and subsequently amplified once on Vero cell monolayers for the production of single virus stocks suitable for all foreseeable studies. Approximately $3 \times 10^6$ plaque-forming units (pfu)/ml of 88-$1961_{A271}$ and $2 \times 10^7$ pfu/ml of 88-$1961_{G271}$ virus was recovered per $1 \times 10^7$ Vero cells in a total volume of 20 ml. The genetic identity of these cDNA-derived 88-1961 viruses was confirmed by reverse transcriptase–polymerase chain reaction (RT-PCR) and sequencing of regions encompassing nucleotide position 271 in the F gene and the genetic tag in the L ORF (data not shown).

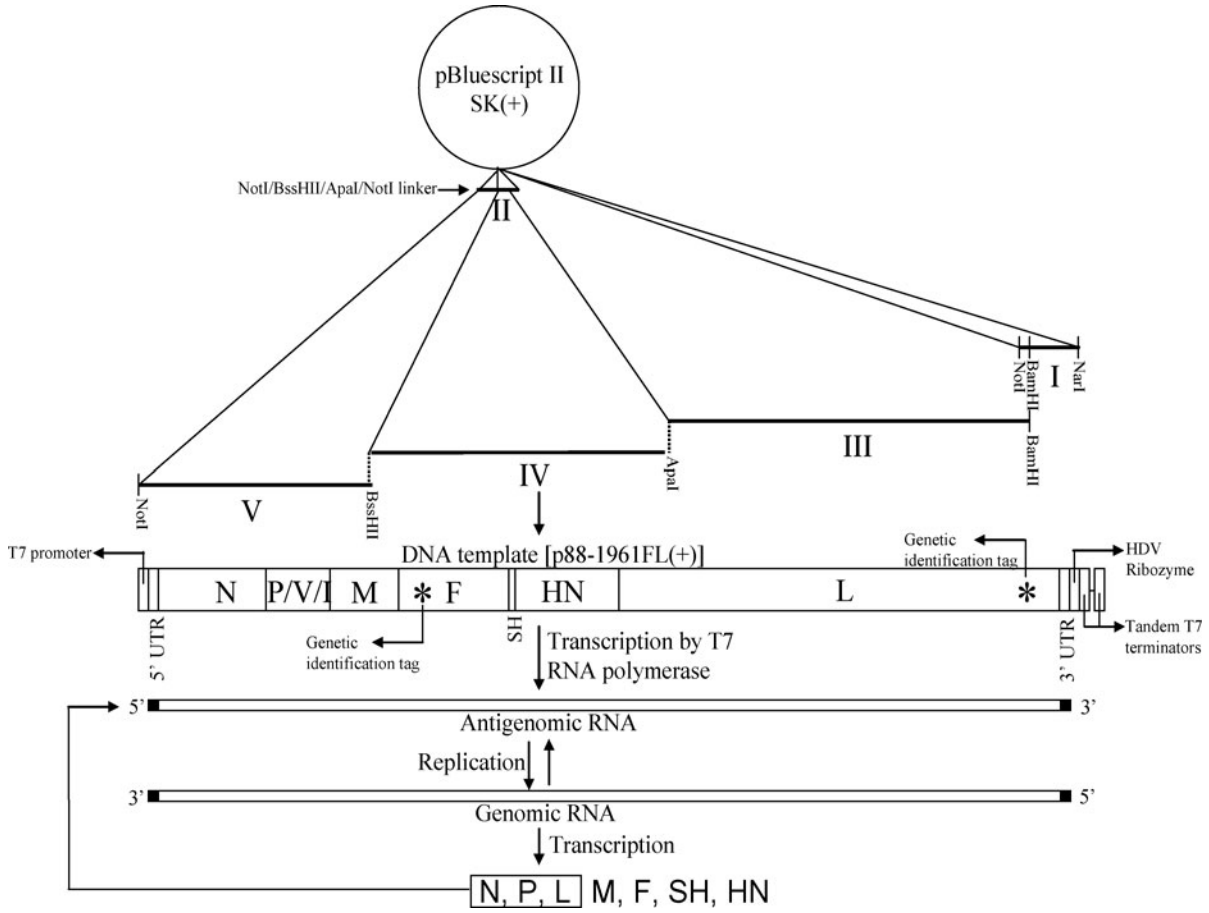


**Figure 1** Construction of the full-length 88-1961 genomic plasmid, p88-1961$_{A271}$, and generation of antigenomic and genomic RNA. The 15,384-nucleotide 88-1961 genome was amplified as four cDNA fragments and cloned into the pBluescript II SK (+) vector, in the positive-sense orientation, as described in Materials and Methods. Roman numerals indicate the step-wise order (right to left) of fragment insertion into pBluescript II SK (+). Fragment II, a linker, was used to introduce the *Apa*I and *Bss*HII restriction sites into the vector to allow contiguous cloning of fragments III, IV, and V. The 88-1961 genome was positioned between a T7 promoter (introduced by fragment V) and a hepatitis delta virus (HDV) ribozyme (partially introduced by fragment I) and tandem T7 terminators (already present in the modified pBluescript II SK (+) vector). Transcription from the T7 promoter resulted in the production of an antigenomic (+) sense RNA transcript with the exact 5'-genomic terminal nucleotide, and the HDV ribozyme generated the precise 3'-terminal nucleotide. This antigenomic RNA served as template for viral replication to generate both genomic and antigenomic RNA species. The positions of the two genetic tags (*) used subsequently for the identification of the recovered virus are indicated.

*Characterization of the* in vitro *growth kinetics and cytopathogenicity of rescued 88-1961 viruses*

To analyze the *in vitro* growth characteristics of the rescued viruses relative to the 88-1961 clinical isolate, cumulative virus production during the first 10 days post infection (p.i.) (Figure 2) and cytopathogenicity (data not shown) on Vero cells was determined for each virus. Although a two-way analysis of variance (ANOVA) did indicate statistically significant cumulative titer differences between the clinical isolate and 88-1961$_{A271}$ strains ($F(1, 35) = 12.727$, $P = .002$, Tukey test) during the 10 day time course (Figure 2), no differences in cytopathic effects were observed between these two virus strains. In contrast, 88-1961$_{G271}$ produced statistically lower cumulative titers ($F(1, 35) = 137.537$ and $F(1, 35) = 18.378$, all $P < .001$, Tukey test) (Figure 2) and was significantly more fusogenic relative to either the clinical isolate or 88-1961$_{A271}$ (data not shown), consistent with our previous findings of HeLa cells transfected with plasmids expressing the two different F variants (G271 and A271) (Malik *et al*, 2007).

*Characterization of the* in vivo *virulence of the rescued 88-1961 viruses in the newborn rat model*

Having shown that the rescued 88-1961$_{A271}$ and 88-1961$_{G271}$ viruses could both replicate effectively *in vitro*, we next determined their virulence *in vivo* using the previously described rat neurovirulence assay (Rubin *et al*, 2000). As shown in Figure 3, no significant differences were observed in the neurovirulence scores of rats inoculated with clinical isolate (19.542 $\pm$ 0.966) versus rescued 88-1961$_{A271}$ (18.021 $\pm$ 0.843, $P = .608$, Mann-Whitney rank sum test). In comparison, the neurovirulence score of rats inoculated with 88-1961$_{G271}$ (14.398 $\pm$ 0.824) was significantly

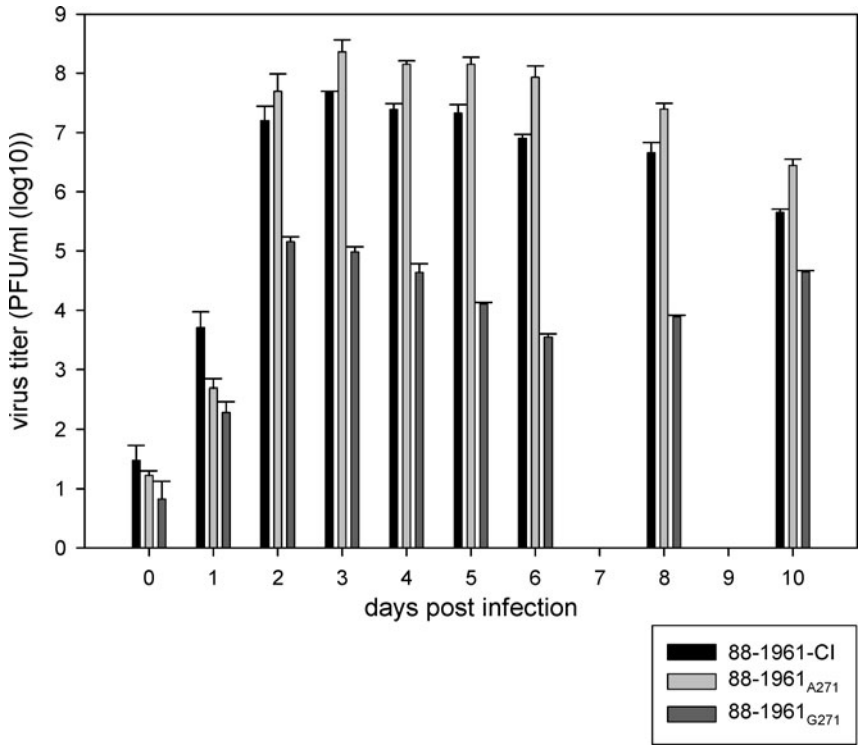


**Figure 2** Comparison of the *in vitro* growth kinetics of the clinical isolate and the rescued 88-1961 viruses. Confluent monolayers of Vero cells were infected with the clinical isolate (881961ci) or the rescued 88-1961 viruses (88-1961$_{A271}$ or 88-1961$_{G271}$) at an m.o.i. of 0.05. Cumulative virus production over a time period of 10 days (days 7 and 9 p.i. were not evaluated) was determined as described in Materials and Methods. The data represents mean values ± SEM from two independent experiments. Limit of detection of the assay = 1 pfu/ml.

lower compared to both the clinical isolate ($P \leq 0.001$, Mann-Whitney rank sum test) and 88-1961$_{A271}$ ($P = 0.005$, *t* test). There was little to no evidence of neurovirulence in rats inoculated with the recombinant Jeryl Lynn cDNA clone (0.838 ± 0.186), consistent with earlier reports that used the Jeryl Lynn vaccine (Rubin *et al*, 2000).

## Discussion

In this report, we describe the development of two infectious cDNA full-length clones p88-1961$_{A271}$ and p88-1961$_{G271}$ for a highly neurovirulent strain of MuV (88-1961). The two clones differed at only one site (nt 271 in the F gene) to represent the heterogeneity (A/G) reported in the clinical isolate (Amexis *et al*, 2003). $F_{A271}$ is predicted to result in a threonine residue at amino acid position 91 in the F protein, whereas $F_{G271}$ is predicted to result in an alanine residue. Recovery of the viruses was achieved by transfection of the T7 driven p88-1961 and the T7 driven helper plasmids expressing N, P, and L into BHK-BSR-T7/5 cells.

To determine whether the cDNA-derived 88-1961 viruses were comparable to the clinical isolate, we analyzed the phenotype of the rescued viruses *in vitro*. Relative to the clinical isolate, 88-1961$_{A271}$ grew to statistically significant higher cumulative titers but displayed equivalent fusogenicity in Vero cells. In contrast, 88-1961$_{G271}$ grew to statistically lower titers and was dramatically more fusogenic than either the clinical isolate or 88-1961$_{A271}$. It is likely that the increased fusogenicity displayed by 88-1961$_{G271}$ led directly to the reduction in cumulative titers due to a much more extensive and early destruction of the infected cell monolayer and thus a lack of viable cells for further virus production. The increased fusogenicity of 88-1961$_{G271}$ versus that of 88-1961$_{A271}$correlates with our previous finding of greater fusogenicity in cells following transfection with $F_{G271}$ versus $F_{A271}$ expression plasmids (Malik *et al*, 2007). This difference was associated with an additional *N*-linked glycosylation site in $F_{A271}$.

The *in vitro* differences between the two viruses were also reflected *in vivo* using a rat-based assay shown to be predictive of the human neurovirulence potential of MuVs (Rubin *et al*, 2000, 2005). Whereas 88-1961$_{A271}$ was of equivalent high neurovirulence in rats as compared to the original clinical isolate, 88-1961$_{G271}$ exhibited a reduced neurovirulence phenotype. Virus rescued from the Jeryl Lynn cDNA clone (representative of the major component of the Jeryl Lynn vaccine and kindly provided by Wyeth Vaccines), which was also highly fusogenic *in vitro*, was demonstrated to be completely attenuated

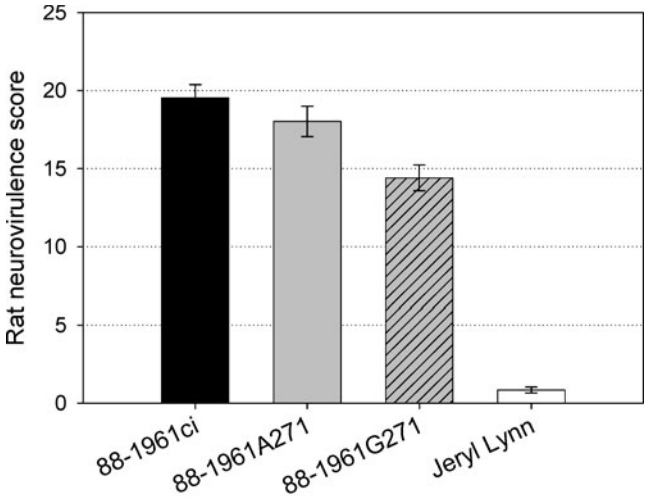


**Figure 3** Severity of hydrocephalus in rats inoculated with the 88-1961 clinical isolate (88-1961ci), the rescued 88-1961 viruses (88-1961$_{A271}$ or 88-1961$_{G271}$), or the rescued major component of the Jeryl Lynn vaccine strain (JL1). Newborn rats were inoculated i.c. with the respective viruses and euthanized 30 days later. Brains were processed for histology and the hydrocephalus score was calculated as described in Materials and Methods. The number of animals tested for each virus is indicated. Error bars represent SEM.

*in vivo*. Although these limited data suggest an inverse relationship between *in vitro* fusogenicity and *in vivo* virulence, others have reported an opposite association in studies of different MuV strains (Love *et al*, 1986; Lemon *et al*, 2007). On the other hand, it is possible that the observed lower growth potential of 88-1961$_{G271}$ *in vitro* may indicate restricted growth *in vivo*, which could explain this virus's reduced *in vivo* virulence. Whether or not this is the case cannot be determined because *in vivo* growth kinetics of these viruses were not examined in this study. Nonetheless, it is clear that the F gene can be a significant determinant in MuV neurovirulence as was recently demonstrated by Lemon *et al*, who replaced the F gene in a cDNA clone of the attenuated Jeryl Lynn MuV strain with the F gene from the highly neurovirulent Kilham MuV strain (Lemon *et al*, 2007). This resulted in a dramatic increase in the neurovirulence of the chimeric virus as determined in rats.

In summary, we successfully rescued two genotypic variants of a clinically isolated strain of MuV from positive sense transcripts of a cDNA clone, using a vaccinia virus-free system expressing T7 RNA polymerase. This is therefore the first report of the development of neurovirulent full-length infectious MuV cDNA clones of a wild-type clinical isolate. The two viruses differed only by the nucleotide residing at position 271 in the F gene; however, this was sufficient to significantly alter the virus's *in vitro* growth and *in vivo* virulence properties, suggesting that nucleotide position 271 in the F ORF plays a role in virus pathogenesis. The straightforward method of virus recovery described here will permit genetic manipulation of the entire genome to further examine the molecular basis of MuV neurovirulence.

## Materials and methods

*Cell lines and viruses*
Vero cells were maintained in Dulbecco's modified Eagle's medium (D-MEM) supplemented with 10% fetal calf serum (FCS). BHK-BSR-T7/5 cells (Buchholz *et al*, 1999), kindly provided by K. Conzelmann (Munich, Germany) and U. Buchholz (Bethesda, MD), were maintained in D-MEM supplemented with 10% FCS and 1 mg/ml Neomycin. The 88-1961 wild-type clinical isolate has been described previously (Amexis *et al*, 2003).

*RNA isolation, RT-PCR, and sequencing*
Total RNA was extracted from 88-1961 infected Vero cells using the RNAeasy kit in accordance with the manufacturer's instructions (Qiagen, Valencia, CA). Reverse transcription (RT) reactions were performed with an appropriate gene specific primer and Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's protocol. All PCR was performed with the Pfx enzyme (Invitrogen). Optimal PCR conditions were determined empirically for each primer set. PCR fragments and plasmids were sequenced on an ABI 3100 automated capillary DNA sequencer. Sequence data was analyzed with the Chromas (Technelysium, Tewantin, Australia) and Jellyfish (LabVelocity, Los Angeles, CA) software packages.

*Construction of plasmids*
*Expression plasmids:* The construction of the plasmids expressing the Jeryl Lynn and 88-1961 N, P, and L gene products has been described elsewhere (Clarke *et al*, 2000; Malik *et al*, 2007). In order to facilitate identification of the 88-1961 full-length molecular clone (see Results), the pTM1-L construct was modified using the QuikChange II XL Site-Directed mutagenesis kit according to the manufacturer's instructions (Stratagene, La Jolla, CA). The nucleotide substitution (T to C), adjacent to the *Bam*HI site within the L gene, was introduced by using a complementary primer pair: 5′-GGTAGGAGCCGATACATGTGCGTTGGTTCATG-TGG-3′ and 5′- CCACATGAACCAACGCACATGTAT-CGGCTCCTACC 3′. The altered nucleotide is underlined. The nucleotide substitution and the absence of undesirable mutations due to PCR-based

mutagenesis, in the resultant plasmid pTM1-$L_{t\text{-}c5772}$, was verified by resequencing the entire LORF.

*Minireplicons*

The Jeryl Lynn (MUVCAT) (Clarke *et al*, 2000) and 88-1961 (p88-1961CAT) (Malik *et al*, 2007) minireplicons have been described previously.

*Full-length molecular clones*

*Jeryl Lynn.* The Jeryl Lynn–derived cDNA clone (pMUVFL) has been described elsewhere (Clarke *et al*, 2000) and was used in this study as a control in the rat assay.

*88-1961:* Primers 5′-CCTA**GCGGCCGC**GGATCCTGCGATCTTCATACTCCGACCC-3′ and 5′- ATCATT-**GGCGCC**AGCGAGGAGGCTGGGACCATGCCGGCCACCAAGGGGAGAAAGTAAAATC-3′ were used to amplify a 1-kb fragment (nucleotides [nt] 14366 to 15384 corresponding to the 5′ end of the genome) by RT-PCR using total RNA isolated from 88-1961–infected Vero cells. The first primer introduced a *Not*I (bold) restriction site and covered the *Bam*HI (underlined) restriction site within the L ORF. The second primer introduced the 5′ untranslated region (UTR), a partial hepatitis delta virus ribozyme (underlined) immediately following the 5′ end of the viral sequence, and a *Nar*I restriction site highlighted in bold. The PCR product was A-tailed using Taq polymerase (Promega, Madison, WI) and subcloned into the pGEM-T-Easy vector (Promega) according to the manufacturer's instructions. The viral insert in pGEM-T-Easy was sequenced, digested with *Not*I and *Nar*I and cloned into a modified pBluescript vector (MUVCAT) (Clarke *et al*, 2000) at the *Not*I and *Nar*I sites, thereby complementing the hepatitis delta virus ribozyme and juxtaposing the latter to two identical T7 RNA polymerase terminators derived from plasmid. This cloning step resulted in the loss of all previously cloned Jeryl Lynn and chloramphenicol acetyl transferase (CAT)-related sequence from MUVCAT. The resultant vector, pBlue-L-5′, was digested with *Not*I and a 44-bp double-stranded linker with *Not*I overhangs (see below) was inserted to introduce *Bss*HII and *Apa*I restriction sites.

5′-ggccgctaaactatgcgcgctaaactatgggccctaaactatgc-3′
3′-cgatttgatacgcgcgatttgatacccgggatttgatacgccgg-5′

We next inserted a 5.8-kb fragment of the viral genome (nt 8559 to 14366), corresponding to the majority of the 88-1961 L gene into pBlue-L-5′, using pTM1-$L_{t\text{-}c5772}$ as template at the *Apa*I and *Bam*HI restriction sites. The resultant vector, pBlue-$_{\Delta 170bp}$L-5′, was used for the following cloning step. A 4.7-kb cDNA fragment encoding the 3′ end of M, F, HN, and the 5' end of the L ORF (nt 3827 to 8559) was amplified by RT–PCR using total RNA isolated from 88-1961–infected Vero cells. Primer 1, 5′-GCAGCGAAGTCGACCGAGTTGCGCGC**CCATTGCTTGCTGC**-3′, covered the *Bss*HII restriction site (underlined) within the M ORF (bold) and primer 2, 5′-GCTAGTGGGCCC**AAGTCATCTGGCTCCAAATCATTTGGTAACTGGCC-**3′, covered the *Apa*I restriction site (underlined) within the 5′ end of the L ORF (bold). This blunt end PCR product was subcloned into the pCR-Blunt (Invitrogen) vector according to the manufacturer's instructions. Following sequencing, the vector (pBlunt-$_{\Delta 564bp}$M-$F_{A271}$-HN-$L_{170bp}$) was digested with *Bss*HII and *Apa*I and the insert cloned into the *Bss*HII and *Apa*I sites of pBlue-$_{\Delta 170bp}$L-5′. The final 3.7-kb fragment of the 3′ end of the viral genome encompassing coding sequences for N, P, the 5′ end of the M ORF, as well as the viral noncoding 3′ terminal sequence (nt 1 to 3827) was amplified by RT–PCR using total RNA isolated from 88-1961–infected Vero cells. Primer 1, 5′-AAGCTCG**GCGGCCGC**TTGTAATACGACTCACTATAACCAAGGGGAAAATGGAGATGGG-3′, introduced a *Not*I restriction site (bold) and the T7 promoter sequence (underlined). The second primer, 5′-GCAATGG**GCGCGC**AACTCGAAACTTGAGTGAGGCAGAACAAACTGTC-3′, covered the unique *Bss*HII site (bold) within the M ORF. This PCR product was A-tailed and subcloned into the pGEM-T-Easy vector. Following sequencing, the insert was digested with *Not*I and *Bss*HII and cloned contiguously into the*Not*I and*Bss*HII sites of pBlue-$_{\Delta 564bp}$ M-F-HN-L-5′ to complete the molecular clone p88-$1961_{A271}$. Of note, the consensus sequence at nucleotide position 271 in the F ORF of the clinical isolate was previously reported as heterogeneous (A/G) (Amexis *et al*, 2003). Therefore to construct an 88-1961 molecular clone representative of the G variant, pBlunt-$_{\Delta 564bp}$M-$F_{A271}$-HN-$L_{170bp}$ (described above) containing an A at position 271 in the F ORF, was modified by site directed mutagenesis (QuikChange II XL Site-Directed mutagenesis kit, Stratagene). The nucleotide substitution (A to G) was introduced by using the complementary primer pair: 5′-GCAGAGAATATAAACAATATTGCATCGCCCTCACC-3′ and 5′-GGTGAGGGCGATGCAATATTGTTTATATTCTCTGC-3'. The altered nucleotide is underlined. The nucleotide substitution and the absence of undesirable mutations in the resultant plasmid, pBlunt-$_{\Delta 564bp}$M-$F_{G271}$-HN-$L_{170bp}$, was verified by resequencing the insert. This vector was subsequently digested with *Bss*HII and *Pac*I and a 1.36-kb fragment encompassing $F_{G271}$ cloned into p88-$1961_{A271}$ predigested with *Bss*HII and *Pac*I. The resultant plasmid p88-$1961_{G271}$, p88-$1961_{A271}$ and the intermediate constructs were all propagated in JM109 bacteria (Promega).

*Assay for CAT activity*

BHK BSR-T7/5 cells were grown on 6-well plates to 70% confluency and cotransfected with p88-1961CAT and the three support N, P, and L

expression plasmids using lipofectamine (Invitrogen) according to the manufacturer's instructions. The previously optimized plasmid amounts were: 200 ng p88-1961CAT, 300 ng N, 50 ng P, and 200 ng L (Malik *et al*, 2007). Forty-eight hours post transfection, the cells were washed once in phosphate-buffered saline (PBS), lysed in 500 $\mu$l of lysis buffer (CAT Elisa kit [Roche Biochemicals, Indianapolis, IN]), and the protein content of each sample determined (BCA Protein Assay Kit; Pierce, Rockford, IL). CAT expression (used as an indicator of polymerase activity) of the normalized samples (2 $\mu$g) was measured in an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol (CAT Elisa kit).

*Rescue of Jeryl Lynn and 88-1961 from cDNA*
*Jeryl Lynn:* Rescue of infectious MuV encoding the major component (JL1) of the Jeryl Lynn vaccine strain was as described (Clarke *et al*, 2000), but with the following modifications. Briefly, BHK BSRT7/5 cells were grown to 95% confluency in 6-well dishes in the absence of antibiotics and a total volume of 2 ml D-MEM/9% FCS. Cells were transfected with a mixture containing 300 ng of pMUVNP, 50 ng of pMUVP, 200 ng of pMUVL, 6.95 $\mu$g of pMUVFL, and 30 $\mu$l of Lipofectamine 2000 (Invitrogen) in a total volume of 500 $\mu$l with Opti-MEM I medium (Invitrogen) as diluent. Cells were transferred to a 25-cm$^2$ flask 48 h post transfection and cultivated for 8 days when numerous syncytia became visible. The supernatant from this flask was used to inoculate a confluent monolayer of Vero cells in a T75 flask. The cells were mechanically detached from the flasks 4 days p.i. and, along with the supernatant, subjected to ultrasonic treatment and clarification by centrifugation at 1200 rpm for 10 min. Virus titer was determined by plaque assay.

*88-1961:* To rescue cDNA-derived 88-1961, BHK BSRT7/5 cells were grown to 95% confluency in a 6-well dish in the absence of antibiotics and a total volume of 2 ml D-MEM/10% FCS. The cells were transfected with a mixture containing 5.45 $\mu$g of p88-1961$_{A271}$ or p88-1961$_{G271}$, 300 ng pTM1-N, 50 ng pTM1-P, 200 ng pTM1-L, and 15 $\mu$l of Lipofectamine in a total volume of 830 $\mu$l with D-MEM as diluent, according to the manufacturer's instructions. The transfected cells were then cultivated in 2 ml D-MEM/10% FCS for 9 days when numerous syncytia became visible. The supernatant from a single well of the 6-well dish was subsequently used to inoculate a confluent monolayer of Vero cells in a T225 flask for 1 h at 37°C. The virus inoculum was removed 1 h post infection and the Vero cells cultivated in 20 ml fresh D-MEM/10% FCS. The cells were mechanically detached from the flask 4 days p.i. and, along with the supernatant, subjected to a single freeze thaw and clarification by centrifugation at 1200 rpm for 10 min. Virus titer was determined by plaque assay.

*Plaque assay*
Virus was serially diluted 10-fold in Eagle's minimum essential medium (E-MEM), and 0.3 ml of each dilution incubated for 1 hr at 37°C on Vero cell monolayers in 12-well plates. All virus dilutions were performed in triplicate. Viral inoculum was removed by aspiration and the cells were immediately overlayed with 1 ml of warm (42°C) 0.75% Difco agar noble (Becton, Dickinson and Company, Sparks, MD) in 1× minimum essential medium (MEM) (without phenol red) supplemented with 10 % FBS. Following incubation at 37°C for 5 days, a second layer of 0.75% Difco agar noble in 1× MEM containing 0.1% neutral red (Sigma-Aldrich, St. Louis, MD) was added and incubated overnight to allow for visualization of the plaques the following day. Virus was quantified by counting the number of plaque forming units (pfu) and multiplying by the reciprocal of the dilution factor and volume plated.

*Molecular characterization of the cDNA-derived 88-1961 viruses*
Viral RNA from rescued 88-1961$_{A271}$ and 88-1961$_{G271}$ supernatants (140 $\mu$l) was extracted with a QIAamp Mini-elute Virus Spin kit (Qiagen) as recommended by the manufacturer, and subjected to RT-PCR and sequencing to verify nucleotides specific to the F (nt 271) and L (nt 5772) genes, respectively. The primer pair used for amplification of a 341-bp region of the F ORF was forward primer (nt 4652 to 4786) 5′-GGCAACTAAGCTATTACTCACAAAGTTCAAGTTC-C-3′ and reverse primer (nt 4960 to 4993) 5′-CC-CGATTAGTTGCCTGTATTGAATTTTTCATCGC-3′.
The primers used for amplification of a 410-bp region of the L ORF were forward primer (nt 14042 to 14069) 5′-GGTATAGCAGCAGGAAATGGTGTAGTGC-3′ and reverse primer (nt 14420 to 14449) 5′-GGATGTA-GTGGGATCAACTGCTAATGTAGC-3′. In each instance, the forward primer was also used for sequencing of the PCR product.

In vitro *virus characterization of the cDNA-derived 88-1961 viruses*
*Growth kinetics:* To determine the kinetics of virus production *in vitro*, confluent monolayers of Vero cells in T-75 flasks or 12-well plates were inoculated with either wild-type, 88-1961$_{A271}$, or 88-1961$_{G271}$ at a multiplicity of infection of 0.05 pfu/cell in D-MEM/9% FCS. After incubation for 1 h at 37°C, inocula were removed, the monolayers washed twice in PBS, and then incubated with 20 or 2 ml, respectively, of D-MEM/9% FCS. For each time point, 10% of cell culture supernatant was removed and immediately stored at −70°C until determination of virus titers. The removed volumes of medium were replaced with equal volumes of fresh medium. This procedure was repeated every 24 h for a period of 10 days.

*Cytopathogenicity:* Confluent monolayers of Vero cells in T-25 flasks were inoculated with either

wild-type, 88-1961$_{A271}$, or 88-1961$_{G271}$ at a multiplicity of infection of 0.05 pfu/cell in D-MEM/10% FCS. Vero cells incubated with media alone served as control. After incubation for 1 h at 37°C, inocula were removed and the monolayers washed twice in PBS. The cell monolayers were cultivated in D-MEM/10% FCS and observed for cytopathogenicity.

In vivo *characterization of the cDNA-derived 88-1961 viruses*

To evaluate virus virulence *in vivo*, the rat neurovirulence test was performed essentially as described previously (Rubin *et al*, 2000). Briefly, newborn rats were inoculated intracerebrally with 20 $\mu$l of E-MEM containing 100 pfu of the 88-1961 clinical isolate (5 litters; $n = 46$), the molecularly derived 88-1961$_{A271}$ strain (6 litters; $n = 63$), the molecularly derived 88-1961$_{G271}$ strain (10 litters; $n = 93$), or the molecularly derived Jeryl Lynn strain (2 litters, $n = 12$), respectively. Animals were sacrificed at 1 month post inoculation and brains were removed for assessment of hydrocephalus severity (described below). Any rats showing signs of pain or distress prior to the planned 1-month end point were humanely euthanized immediately. Strict adherence to the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals was followed.

*Assessment of hydrocephalus severity*

The neurovirulence phenotype of the four virus strains (the clinical isolate, the two molecularly derived 88-1961 clones, and the molecularly derived Jeryl Lynn clone) was assessed in rats as described previously (Rubin *et al*, 2000). Briefly, brains from rats inoculated as newborns were collected at 1 month post inoculation and divided sagittally. The brain hemispheres were immersion fixed in 10% neutral-buffered formalin at 4°C for 4 to 5 days, followed by paraffin embedding. From each brain hemisphere, one 10 $\mu$m thick sagittal section was selected at a standard distance from the anatomical midline and stained with hematoxylin and eosin. The neurovirulence score, which is a measure of the severity of hydrocephalus, was determined by measuring the cross-sectional area of the brain (excluding the cerebellum) and that of the lateral ventricle on tissue sections from each pair of brain hemispheres per rat. Cross-sectional area measurements were performed using Image Pro Plus image analysis software (Media Cybernetics, Silver Spring, MD). The mean ratio (percentage) of these two measurements on each of the two tissue sections per rat brain was assigned as the neurovirulence score for that particular brain. The neurovirulence score for a given virus variant was determined by the mean neurovirulence score for all brains within a treatment group.

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