

Discrimination of Mumps Virus Small Hydrophobic Gene Deletion Effects from Gene Translation Effects on Virus Virulence[∇]

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Deletion of the small hydrophobic (SH) protein of certain paramyxoviruses has been found to result in attenuation, suggesting that the SH protein is a virulence factor. To investigate the role of the mumps virus (MuV) SH protein in virulence, multiple stop codons were introduced into the open reading frame (ORF) of a MuV molecular clone (r88-1961_{SHstop}), preserving genome structure but precluding production of the SH protein. No differences in neurovirulence were seen between the wild-type and the SH_{stop} viruses. In contrast, upon deletion of the SH gene, significant neuroattenuation was observed. These data indicate that the MuV SH protein is not a neurovirulence factor and highlight the importance of distinguishing gene deletion effects from protein-specific effects.

Mumps virus (MuV), a rubulavirus of the *Paramyxoviridae* family, contains tandemly linked transcription units encoding the nucleo- (N), V/phospho-I (V/P/I), matrix (M), fusion (F), small hydrophobic (SH), hemagglutinin-neuraminidase (HN), and large (L) protein genes (4, 6). The MuV SH protein is a 57-amino-acid type III integral membrane protein. Certain MuV strains contain a point mutation in the F gene polyadenylation signal that results in an F-SH bicistronic mRNA of which only the upstream cistron (F) is translated (12, 22, 23), demonstrating that the SH protein is not essential for virus replication, a conclusion supported by studies of related paramyxoviruses (2, 3, 8).

The amino acid sequence of the MuV SH protein is the most hypervariable in the genome, with differences between some strains exceeding 20% (23), further indicating its nonessential role in the life cycle of the virus. Nonessential proteins typically serve a luxury function, and in the case of the MuV SH protein, this appears to be the blocking of apoptosis in certain cell types (9, 13, 26). Multiple reports have also suggested that the paramyxovirus SH proteins may play an ancillary role in virus-mediated cell fusion (10, 21), aid virus transport through the secretory pathway (19), or act as a viroporin, a group of small, highly hydrophobic proteins that can oligomerize and form ion channels at the cell membrane to modulate membrane permeability (18). *In vivo* studies of SH gene deletion mutants of related viruses, such as human respiratory syncytial virus (hRSV) and avian metapneumovirus, have been interpreted to indicate a role of the protein in replication, virulence, and immunogenicity (3, 11, 14, 24, 25). However, in these studies,

because the SH mutants were produced by deletion of the gene, it was not possible to discern if the observed altered phenotypes are related to the absence of the SH protein itself or if they are a consequence of altering the genome structure (e.g., a decrease in genome length, an altered untranslated region, a change in transcription gradient of downstream genes, or other yet-to-be-identified effects).

To investigate the role of the MuV SH protein in virus neurovirulence, we generated two modified versions of a molecular clone of the highly neurovirulent wild-type MuV clinical isolate 88-1961_{WT}, one wherein the SH gene was deleted (r88-1961_{ΔSH}) and one wherein three stop codons were introduced in the SH open reading frame (ORF) (r88-1961_{SHstop}) to abrogate SH protein expression without altering the structure of the genome.

The plasmid p88-1961_{WT} (15), which encodes the 15,384-nucleotide positive-strand (antigenomic) RNA sequence of 88-1961_{WT}, was modified by site-directed mutagenesis (SDM) to introduce the MluI and AscI restriction sites in the intergenic regions flanking the SH gene, resulting in plasmid p88-1961_{AscI/MluI} (Fig. 1). The restriction sites in p88-1961_{AscI/MluI} were subsequently used to introduce a linker, comprising only the 5' and 3' SH intergenic sequences, into the 88-1961 genome in place of the ORF, resulting in plasmid p88-1961_{ΔSH} (Fig. 1). Deletion of the SH ORF (174 nucleotides) maintained the genome length at an even multiple of six. The detailed cloning strategy for this construct and for those described below can be obtained from the authors upon request. Plasmid p88-1961_{ΔSH} was used to produce three independently rescued virus stocks of r88-1961_{ΔSH} from BHK BSR-T7/5 cells (15), which were amplified once on Vero cells. The genetic identities of the cDNA-derived r88-1961_{ΔSH} viruses were confirmed by sequencing a 1,366-bp region encompassing the deleted SH ORF. The restriction sites in p88-1961_{AscI/MluI} were also used to introduce a modified SH ORF containing three translational stop codons at positions 13, 73, and 115 of the SH ORF (genome positions 6279, 6341, and 6381) into the 88-1961

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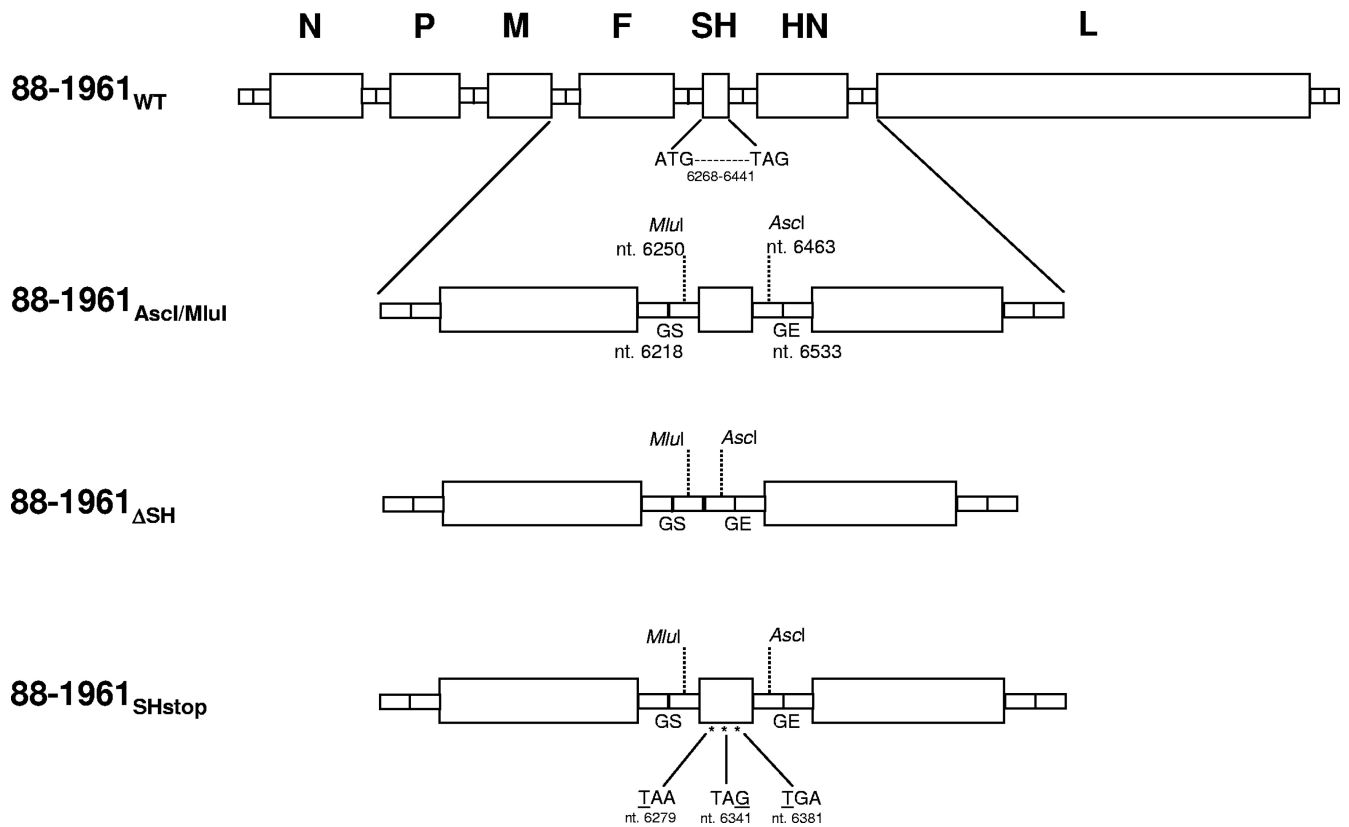


FIG. 1. Construction of 88-1961 variant plasmids to abrogate SH protein expression. Schematic diagram showing stepwise construction of p88-1961_{AscI/MluI}, p88-1961_{ΔSH}, and p88-1961_{SHstop} from p88-1961_{WT}. Nucleotide numbers are in genome sense.

genome, resulting in plasmid p88-1961_{SHstop} (Fig. 1), which was used to produce two independently rescued virus stocks of r88-1961_{SHstop} from BHK BSR-T7/5 cells, amplified once on Vero cells. The genetic identities of the cDNA-derived r88-1961_{SHstop} viruses were confirmed by sequencing a 1,540-bp region encompassing the 173-bp SH ORF containing the stop codons and all sequences in the plasmid that had been produced by PCR. No mutations were identified other than those specifically introduced by SDM.

Our ability to confirm the effectiveness of the three stop codons was hindered by the lack of an antibody against the MuV SH protein. Therefore, the SH_{WT} and SH_{STOP} sequences were isolated from p88-1961_{WT} and p88-1961_{SHstop}, respectively, and subcloned into the pCAGGS vector (kindly provided by J. C. de la Torre, La Jolla, CA) (17) in-frame with a hemagglutinin (HA) tag at the carboxyl terminus. Following transfection of cells, lysates were evaluated by Western blotting. Expression of HA-tagged SH protein was detected in cells transfected with pSH_{WT}-HA (Fig. 2, lane 3) but not in cells transfected with pSH_{stop}-HA (Fig. 2, lane 1), indicating, albeit indirectly, that the SH protein is not translated in the recombinant virus containing the three stop codons. Of note, insertion of the HA tag into the full-length recombinant viruses themselves was not performed, given the demonstration by others of HA tag interference with SH protein function (26).

To analyze the *in vitro* growth characteristics and to demonstrate the replication competence of r88-1961_{SHstop} and r88-1961_{ΔSH} relative to those of r88-1961_{WT}, we determined cu-

mulative virus production during the first 7 days postinfection (p.i.) for each virus on Vero cells as described previously (15) (Fig. 3). A one-way analysis of variance (ANOVA) did not reveal statistical differences between the replication kinetics of r88-1961_{WT} and r88-1961_{SHstop} ($P = 0.95$); however, differences between the replication kinetics of r88-1961_{ΔSH} and 88-

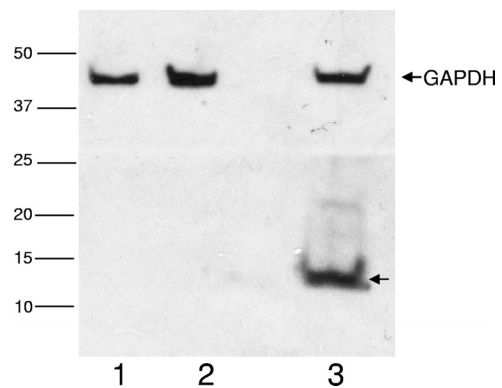


FIG. 2. Western blot analysis of SH_{WT} and SH_{STOP} expression. SH-HA expression (anti-HA antibody [1/1,000]; Roche, Indianapolis, IN) was observed in cells transfected with pSH_{WT}-HA (lane 3) but not in cells transfected with pSH_{stop}-HA (lane 1). Vero extracts isolated from cells transfected with the pCAGGS vector served as the assay control (lane 2). Expression of a cellular marker, glyceraldehyde-3-phosphate dehydrogenase (GAPDH; anti-GAPDH antibody [1/5,000]; Abcam, Cambridge, MA), was detectable in all samples.

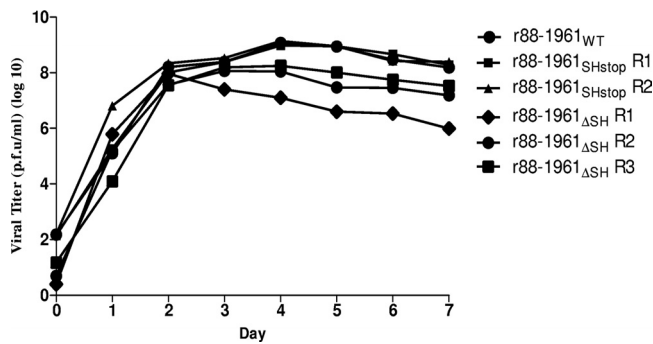


FIG. 3. Comparison of the *in vitro* growth kinetics of the 88-1961 variant virus strains. Confluent monolayers of Vero cells were infected with r88-1961_{WT}, r88-1961_{SHstop} (rescues 1 and 2), and r88-1961_{ΔSH} (rescues 1, 2, and 3) at a multiplicity of infection (MOI) of 0.05. Cumulative virus production over a period of 7 days was determined. The data represent mean values \pm standard errors of the means (SEMs) from two independent experiments. Note that the error bars, while present, are not resolvable on the figure at the scale presented. The limit of detection of the assay is 40 PFU/ml.

1961_{WT} were significant ($P = 0.01$). A *post hoc* Tukey's multiple-comparison test found differences between 88-1961_{WT} and r88-1961_{ΔSH} R1 and between 88-1961_{WT} and r88-1961_{ΔSH} R2 but not between 88-1961_{WT} and r88-1961_{ΔSH} R3. However, the three rescues of r88-1961_{ΔSH} replicated similarly to each other ($P = 0.09$; one-way ANOVA), and importantly, all were replication competent. Relative to that for r88-1961_{WT}, the onset of cytopathic effects (abnormal cell morphology, syncytial formation, and cell lysis) was more rapid in cells infected with r88-1961_{ΔSH} but not in cells infected with r88-1961_{SHstop} (data not shown). The faster destruction of the cell monolayer by r88-1961_{ΔSH} may account for the lower replication kinetics relative to that of r88-1961_{WT}. However, unpublished observations from our laboratory suggest that there is no correlation between the extent of virus fusogenicity or other *in vitro* cytopathic effects and virus virulence in our rat model.

Having confirmed that the modified 88-1961 viruses were replication competent, we compared the neurovirulence potentials of 88-1961_{ΔSH}, r88-1961_{SHstop}, and r88-1961_{WT} *in vivo* in a rat neurovirulence assay (20). No significant differences were apparent in the mean neurovirulence score of r88-1961_{WT} (17.6 ± 0.9) and that of r88-1961_{SHstop} (17.2 ± 0.7 ; $P = 0.72$; unpaired *t* test) (Fig. 4). Maintenance of the stop codons in the recombinant viruses from each rescue of r88-1961_{SHstop} following replication in rat brain was confirmed by extraction of total RNA from brain homogenates at day 3 p.i., the time of maximal virus production (16), followed by reverse transcription (RT)-PCR and sequencing (data not shown). In contrast, the mean neurovirulence score of 88-1961_{ΔSH} (9.6 ± 0.6) was significantly lower than that of r88-1961_{WT} (17.6 ± 0.9 ; $P < 0.0001$; unpaired *t* test). These data indicate the possibility that the attenuation observed by others may not be due to the absence of the SH protein *per se* but rather a consequence of disturbing the genome structure as a result of removing the SH transcription unit. The most likely cause of this would be an altered transcription/translation gradient, but this has proved difficult to assess. He et al. (8) and Wilson et al. (26) examined the effect of the removal of the SH gene from

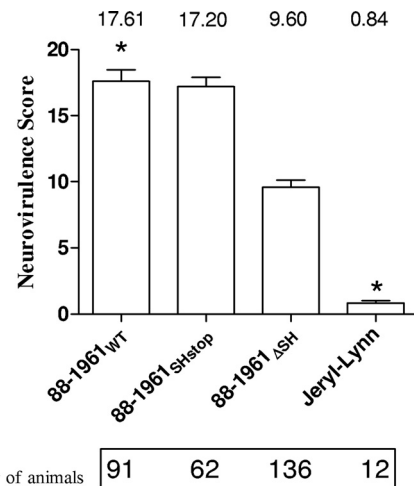


FIG. 4. Severity of hydrocephalus in rats inoculated with variant MuV strains. Newborn rats were inoculated intracerebrally (i.c.) with the indicated viruses and euthanized 30 days later. Brains were removed, and hydrocephalus scores were calculated. Error bars represent the SEM. An asterisk (*) denotes cumulative historic data from testing of the parental 88-1961_{WT} and Jeryl Lynn vaccine strains, respectively. Previously published data for the highly neuroattenuated Jeryl Lynn strain (15) are included to emphasize the ability of the assay to determine MuV neuroattenuation.

parainfluenza virus 5 (PIV-5) and were unable to reproducibly detect differences in levels of protein expression between the two viruses. Fuentes et al. attempted a similar analysis with an SH deletion mutant of hRSV and found variable results ranging from no effect to slight increases in expression levels of proteins both upstream and downstream of the deleted transcription unit (7). Thus, it is not clear from the literature if protein levels are affected by removal of the SH genes and, if they are, if such differences are detectable by Western blotting. While one could examine mRNA levels of the genes of interest, this has proven not to be predictive of protein levels (3). Whether altered levels of mRNA transcripts can affect virus phenotype without affecting protein levels is not known but seems unlikely. In the case of MuV, analyzing differences in gene-specific mRNA levels between two viruses is particularly complicated, given the abundance of polycistronic message species resulting in a laddering of transcripts that are not easily quantifiable (1, 5). Despite our inability to pinpoint the mechanism behind the observation that gene deletion alters virus phenotype, it is clear from our studies that the MuV SH protein *per se* is not a virulence factor in our animal model, although we cannot rule out that the MuV SH protein might be a virulence factor in a natural setting (i.e., respiratory infection in humans).

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