Single amino acid changes in the mumps virus haemagglutinin–neuraminidase and polymerase proteins are associated with neuroattenuation

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It has previously been shown that three amino acid changes, one each in the fusion (F; Ala/Thr-91→Thr), haemagglutinin–neuraminidase (HN; Ser-466→Asn) and polymerase (L; Ile-736→Val) proteins, are associated with attenuation of a neurovirulent clinical isolate of mumps virus (88-1961) following serial passage in vitro. Here, using full-length cDNA plasmid clones and site-directed mutagenesis, it was shown that the single amino acid change in the HN protein and to a lesser extent, the change in the L protein, resulted in neuroattenuation, as assessed in rats. The combination of both amino acid changes caused neuroattenuation of the virus to levels previously reported for the clinical isolate following attenuation in vitro. The amino acid change in the F protein, despite having a dramatic effect on protein function in vitro, was previously shown to not be involved in the observed neuroattenuation, highlighting the importance of conducting confirmatory in vivo studies. This report provides additional supporting evidence for the role of the HN protein as a virulence factor and, as far as is known, is the first report to associate an amino acid change in the L protein with mumps virus neuroattenuation.

INTRODUCTION

Mumps virus (MuV) is a non-segmented, negative-stranded, enveloped RNA virus in the family *Paramyxoviridae*. The 15.4 kb genome encodes open reading frames (ORFs) for a nucleoprotein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), small hydrophobic protein (SH), haemagglutinin–neuraminidase protein (HN) and polymerase protein (L) (Carbone & Rubin, 2007; Elango et al., 1988). In addition, the P gene synthesizes the V and I proteins through an RNA-editing mechanism (Paterson & Lamb, 1990).

MuV causes an acute infectious disease characterized by fever, parotitis and a number of more serious acute inflammatory reactions including meningitis and encephalitis (Aygun et al., 2001; Beghi et al., 1984; Carbone & Rubin, 2007; Rotbart, 2000). Based on findings of cerebrospinal fluid pleocytosis, MuV invades the central nervous system in more than 50% of cases (Bang & Bang, 1943). Given the neurotropism and neurovirulence potential of wild-type MuV, candidate live-attenuated vaccine virus strains must be assessed for neuroattenuation. A rat-based investigational pre-clinical neurovirulence safety test developed at the US Food and Drug Administration appears to accurately and reproducibly assess the human neurovirulence potential of MuV (Rubin et al., 2000, 2005). Using this assay coupled with sequencing of the virus genome, we previously reported the identification of three amino acid changes associated with neuroattenuation of the highly neurovirulent MuV clinical isolate 88-1961: one each in the F (Ala/Thr-91→Thr), HN (Ser-466→Asn) and L (Ile-736→Val) proteins (Rubin et al., 2003). In vitro experiments using cells transfected with plasmids expressing the F, HN or L protein of wild-type or neuroattenuated 88-1961, or using cells infected separately with the two viruses, showed that each of the single amino acid changes affected protein function. The amino acid selection in the F protein resulted in a dramatic decrease in fusion activity, whilst the amino acid substitutions in the HN and L proteins resulted in moderate decreases in receptor-binding/neuraminidase activities and increased replicative/transcriptional activity, respectively (Malik et al., 2007b). Because the amino acid substitution (Ala to Thr) in the F protein had the most dramatic effect in vitro, we subsequently constructed a full-length cDNA plasmid clone representative of the 88-1961 clinical isolate containing an Ala at aa 91 in the F protein and modified this clone by site-directed mutagenesis to produce a cDNA clone expressing a Thr at this position, and tested these two virus variants in rats (Malik et al., 2007a). Surprisingly, this change had no effect on virus neurovirulence, suggesting that the amino acid substitution in either the HN or L, or in both proteins, was responsible for the previously observed neuroattenuation. To confirm this hypothesis,
we have now constructed and tested three additional full-length cDNA plasmid clones, one expressing an Asn at aa 466 in the HN protein, a second expressing an Ile at aa 736 in the L protein and a third expressing both amino acid substitutions. All three of these newly constructed cDNA clones contained a Thr at aa 91 of the F protein to represent the attenuated variant of 88-1961. Here, we have shown that the individual amino acid changes in HN and L proteins were each associated with some degree of in vivo neuroattenuation and that the combination of both amino acid changes neuroattenuated the virus in rats to levels previously reported for the clinical isolate following passage in vitro (which resulted in the three mutations). This is the first report to describe the association of an amino acid change in the polymerase with MuV neuroattenuation. Interestingly, despite the decreased neurovirulence of the two mutant viruses relative to the parental attenuation. Interestingly, despite the decreased neurovirulence of the two mutant viruses relative to the parental attenuation.

RT-PCR and sequencing. Reverse transcription reactions were performed with an appropriate gene-specific primer and Superscript II reverse transcriptase (Invitrogen) in accordance with the manufacturer’s protocol. All PCRs were performed with Pyr polymerase (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 were analysed by using the Chromas (Technelysium Pty) and Jellyfish (LabVelocity) software packages.

Construction of plasmids

Expression plasmids. Construction of the plasmids expressing the 88-1961 N, P and L gene products has been described previously (Malik et al., 2007b).

Full-length molecular clones. Construction of the 88-1961 infectious cDNA clone p88-1961A27, has been described elsewhere (Malik et al., 2007a). This clone is referred to here as p88-1961WT and was used in this study as the wild-type control for both the in vitro and the in vivo assays and as a reagent for construction of three additional clones: p88-1961HN-ATT, p88-1961L-ATT and p88-1961HN/L-ATT.

The p88-1961HN-ATT cDNA clone was constructed by first introducing a G→A nucleotide substitution in plasmid pBlunt-A564bp-M-FA271-HN-L70bp (Malik et al., 2007a) at position 1397 in the HN ORF using site-directed mutagenesis (QuickChange II XL dite-directed mutagenesis kit; Stratagene). The nucleotide substitution was introduced by using the complementary primer pair 5’-CTCCTGTTCTAGGCAAACCTGATGGTTGAAAATGTGTGCCC-3’ and 5’-CGAGGAGAAAATCAAATATCATCATTATTGAGCC-3’; the altered nucleotides are underlined. The nucleotide substitution and the absence of undesirable mutations in the resulting plasmid (pBlunt-A564bp-M-FA271-HN1397L70bp) were verified by sequencing the insert. This modified plasmid was digested with BssHI and Apal, and a 4.7 kb fragment encompassing the altered HN gene was cloned into the BssHI and Apal sites of pBlue-A170bp-L5’ (Malik et al., 2007a). This intermediate vector (pBlue-A564bp-M-F-HN1397-L5’) was digested with BssHI and NrdI, and a 9.7 kb fragment encompassing the HN gene was cloned into p88-1961WT pre-digested with BssHI and NrdI to yield p88-1961HN-ATT.

The p88-1961L-ATT cDNA clone was constructed by first introducing an A→G nucleotide substitution in pTM1-L4772 (Malik et al., 2007a) at position 2206 in the L ORF using site-directed mutagenesis. The nucleotide substitution was introduced by using the complementary primer pairs 5’-GCTCTCAGATGGATATTTATATATTTTCTCCTTCG-3’ and 5’-CGAGGAGAAAATCAAATATCATCATTATTGAGCC-3’; the altered nucleotides are underlined. The nucleotide substitution and the absence of undesirable mutations in the resulting plasmid (pTM1-L4772-5’) were verified by sequencing the insert. This plasmid was digested with Apal and BamHI, and a 5.8 kb fragment encompassing the modified L gene was cloned into the Apal and BamHI sites of pBlue-A564bp-M-F-HN-L5’ (Malik et al., 2007a). The resulting vector (pBlue-A564bp-M-F-HN-L2206-5’) was digested with BssHI and NrdI, and a 9.7 kb fragment encompassing the L gene was cloned into p88-1961WT pre-digested with BssHI and NrdI to yield p88-1961L-ATT.

The p88-1961HN/L-ATT cDNA clone was constructed by digestion of pBlue-A564bp-M-F-HN-L2206-5’ with Apal and BamHI, and the resulting 5.8 kb fragment encoding the modified L gene was cloned into pBlue-A564bp-M-F-HN1397-L5’ pre-digested with Apal and BamHI. The vector, pBlue-A564bp-M-F-HN1397-L2206-5’, was digested with BssHI and NrdI, and a 9.7 kb fragment encompassing HN1397 and L2206 was cloned into p88-1961WT pre-digested with BssHI and NrdI to yield p88-1961HN/L-ATT. The p88-1961HN-ATT, p88-1961L-ATT and p88-1961HN/L-ATT plasmids and the intermediate constructs were all propagated in JM109 bacteria (Promega).

Rescue of the r88-1961 variant viruses from cDNA. To rescue the cDNA-derived r88-1961 viruses, BHK BSR-T7/5 cells were grown to 95 % confluency in a six-well plate in the absence of antibiotics and in a total volume of 2 ml growth medium. The cells were transfected with a mixture containing the p88-1961HN-ATT, p88-1961L-ATT or p88-1961HN/L-ATT full-length cDNA clone using Lipofectamine (Invitrogen) with the supporting N, P and L plasmids, as described previously (Malik et al., 2007a). When numerous syncytia became visible (day 9), the supernatant from a single well of the six-well plate was used to inoculate a confluent monolayer of Vero cells in a 225 flask for 1 h at 37 °C. The virus inoculum was removed 1 h post-infection and 20 ml fresh growth medium was added to the flask. The cells were mechanically detached 4 days later and along with the supernatant, were subjected to a single freeze–thaw and clarification by centrifugation at 2000 g for 10 min. Viral titre was determined by plaque assay on Vero cells (Malik et al., 2007a). Virus rescued from cultures transfected with p88-1961WT, p88-1961HN-ATT, p88-1961L-ATT and p88-1961HN/L-ATT were referred to here as r88-1961WT, r88-1961HN-ATT, r88-1961L-ATT and r88-1961HN/L-ATT, respectively.

Molecular characterization of the cDNA-derived 88-1961 viruses. Viral RNA from r88-1961HN-ATT, r88-1961L-ATT and r88-1961HN/L-ATT virus stocks was extracted by using a QIAamp minieute virus spin kit (Qiagen), as recommended by the manufacturer, and subjected to RT-PCR and sequencing to verify nucleotides specific to the HN (nt 1397) and L (nt 2206) genes, respectively. The primers used for amplification of a 425 bp region of the HN ORF were 5’-CCCTCACAATCAGGACGCTGAGTCGTC-3’ (forward primer; nt 1189–1217) and 5’-GGCAACAACCTCCTTGAAGTA-
CCATATGGG-3' (reverse primer; nt 1587–1614). The primers used for amplification of a 271 bp region of the L ORF were 5'-CCTACCTGTTGGAATGTGATTGACAATGGC-3' (forward primer; nt 2087–2116) and 5'-GGTGAATGGCATTGTTTGGTGCACC-3' (reverse primer; nt 2331–2358). 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 *vivo*, confluent monolayers of Vero cells in 12-well plates were inoculated with r88-1961WT, r88-1961HN-ATT, r88-1961L-ATT or r88-1961HN/L-ATT at an m.o.i. of 0.05 in growth medium. After incubation for 1 h at 37 °C, the inocula were removed and the monolayers were washed twice with PBS and overlaid with 2 ml growth medium. Every 24 h over a 10-day period, 200 μl cell culture supernatant was removed from three wells per virus treatment and immediately stored at −70 °C until determination of viral titres by plaque assay on Vero cells (Malik et al., 2007a). The removed volumes of medium were replaced with equal volumes of fresh growth medium.

**Stability.** To determine the stability of r88-1961WT, r88-1961HN-ATT, r88-1961L-ATT and r88-1961HN/L-ATT, several microcentrifuge tubes containing 1 × 10⁸ p.f.u. of each virus in 100 μl Eagle’s minimum essential medium (EMEM) containing 5% FCS were prepared and incubated at 37 °C. On days 0–7, three microcentrifuge tubes of each virus preparation were removed from the incubator and immediately stored at −70 °C until determination of viral titres by plaque assay (Malik et al., 2007a).

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

**In vivo growth kinetics.** To determine the kinetics of virus production in *vivo*, newborn rats were inoculated intracerebrally with 20 μl EMEM containing 100 p.f.u. r88-1961WT, r88-1961HN-ATT, r88-1961L-ATT or r88-1961HN/L-ATT. On days 1–4, 6 and 8 post-inoculation (p.i.), brains were removed from three to five rats at each time point, weighed and immediately placed on dry ice prior to storage at −70 °C until the determination of viral titres by plaque assay (Malik et al., 2007a).

**Sequence confirmation of engineered mutations following virus replication in rat brain.** Viral RNA from homogenates of rat brain infected with r88-1961WT, r88-1961HN-ATT, r88-1961L-ATT and r88-1961HN/L-ATT (day 3 p.i.) was extracted by using a QIAamp MiniElute virus spin kit (Qiagen), as recommended by the manufacturer, and subjected to RT-PCR and sequencing to verify the presence of nucleotides specific to the HN (nt 1397) and L (nt 2206) genes, respectively, following virus replication in the brain. One primer pair was used for amplification of a 337 bp region of the HN ORF: 5'-GGGCGTGATGACCTCTCTATGAG-3' (forward primer; nt 1280–1302) and 5'-CGAGGCAACGTTCTCGATTACC-3' (reverse primer; nt 1594–1617). The primers used for amplification of a 246 bp region of the L ORF were 5'-GTATGGTATACCCACCCACGTGTTCAATGG-3' (forward primer; nt 2124–2151) and 5'-GCTCATACCTAGTGTGGCTGTCAGCCCTG-3' (reverse primer; nt 2341–2370). In each instance, the forward primer was also used for sequencing of the PCR product.

**Virus distribution in rat brain.** To evaluate the distribution of the virus variants in *vivo*, newborn rats were inoculated intracerebrally with 20 μl EMEM containing 100 p.f.u. r88-1961WT, r88-1961HN-ATT, r88-1961L-ATT or r88-1961HN/L-ATT. On day 4 p.i., brains were removed from three rats per virus group, divided sagittally and immersion-fixed in 4% paraformaldehyde at 4 °C overnight, followed by overnight incubations in PBS, 10% sucrose (w/v in PBS) and 20% sucrose (w/v in PBS). The brain hemispheres were then flash-frozen in 2-methylbutane and embedded in OCT compound. Using a cryostat, five 10 μm sagittal sections were obtained from each of three standard locations per brain (anatomical midline, midway through the hemispheres and the lateral aspect of the hemispheres) for a total of 13–15 sections per rat brain. All brain sections were stained for virus antigen detection by immunofluorescence assay. The brain sections were blocked overnight with 4% normal goat serum and 0.1% Triton X-100, incubated with a mouse anti-MuV N protein antibody (diluted 1:1000; Abcam) for 1 h, rinsed with PBS and finally incubated with a goat anti-mouse Cy3-conjugated IgG (diluted 1:200; Chemicon) for 1 h. The tissues sections were then covered with a coverslip, blinded by a second party and visualized under a fluorescent microscope. The extent of virus distribution and spread in each tissue section was graded on a scale of 0 to 4 as follows: 0, no virus staining; 1, occasional staining of cells of the ventricular system (ventricular and aqueductal ependymal cells, cells of the choroid plexus); 2, no evidence of virus spread beyond the ventricular system; 3, extensive staining of the ventricular system and evidence of virus staining at sites deeper in the brain parenchyma. After unblinding the slides for virus identity, a group mean score was calculated from the 39–45 brain sections (13–15 sections each from three rats) obtained from rats in each of the four virus treatment groups.

**Neurovirulence.** To evaluate the virus virulence in vivo, a rat neurovirulence test was performed as described previously (Rubin et al., 2000). Briefly, newborn rats were inoculated intracerebrally with 20 μl EMEM containing 100 p.f.u. r88-1961WT (ten litters; n = 91), r88-1961HN-ATT (five litters; n = 51), r88-1961L-ATT (five litters; n = 42) or r88-1961HN/L-ATT (six litters, n = 57). Animals were sacrificed at 1 month p.i. and the brains were removed, immersion-fixed in 10% neutral buffered formalin at 4 °C for 4–5 days and embedded in paraffin. From each brain hemisphere, one 10 μm sagittal section was selected at a standard distance from the anatomical midline and stained with haematoxylin and eosin. The neurovirulence score, which is a measure of the severity of hydrocephalus, was determined by examining the ventricular system with virus spread limited to the periventricular areas; 4, extensive staining of the ventricular system and evidence of virus staining at sites deeper in the brain parenchyma. After unblinding the slides for virus identity, a group mean score was calculated from the 39–45 brain sections (13–15 sections each from three rats) obtained from rats in each of the four virus treatment groups.

**RESULTS**

**Construction and recovery of 88-1961 variant viruses from cDNA**

To analyse the role of individual HN and L amino acid changes in neuroattenuation of 88-1961, the plasmid p88-1961WT encoding the wild-type positive-strand (anti-
Characterization of the stability and growth kinetics of the rescued 88-1961 viruses in vitro

The viability and replication competence of the rescued viruses was assessed in vitro to demonstrate that any differences we might observe in vivo could not simply be attributed to differences in variant virus stability or ability to replicate. Virus stability (loss of potency over time during storage at 37 °C) over a 7-day period is shown in Fig. 2(a) and cumulative virus production on Vero cells over a 10-day period is shown in Fig. 2(b). A two-way analysis of variance (ANOVA) did not indicate statistically significant differences in the stability of the four viruses during the study period [F (3, 28)=5.72, P=0.063], although a small residual amount of r88-1961 WT was detected at later time points when no virus could be titrated from vials containing the other three viruses. Statistically significant differences were also not apparent in the in vitro growth kinetics of the viruses, with the exception of r88-1961 L-ATT [F (1, 20)=72.28, P=0.014, two-way ANOVA], which displayed lower titres than the other viruses, but was nonetheless highly replication-competent. Of note, qualitative analyses showed that the lower titres displayed by r88-1961 L-ATT were probably due to enhanced and earlier cytopathic effects, as compared with r88-1961 WT, resulting in fewer viable cells for virus replication at later time points.

Characterization of the in vivo virulence, growth kinetics and spread of the rescued 88-1961 viruses in a newborn rat model

The virulence of the three variant viruses relative to the rescued wild-type virus (r88-1961 WT) was assessed in vivo using a previously described rat neurovirulence assay (Rubin et al., 2000). As shown in Fig. 3, there was a slight reduction in the neurovirulence scores of rats inoculated with r88-1961 L-ATT (15.70) compared with r88-1961 WT (17.61), but this was not statistically significant (P=0.21). In contrast, there was a statistically significant decrease in neurovirulence of r88-1961 HN-ATT (7.96) compared with r88-1961 WT (P<0.0001). In addition, a statistically significant decrease in neurovirulence of r88-1961 HN/L-ATT (11.81) compared with r88-1961 WT (P<0.0001) was observed (Fig. 3). All P values were determined by Student’s t-test.

Recovery and titration of virus from rat brains showed that the growth kinetics of all three variant viruses in the brain tended to be lower than that of r88-1961 WT, particularly over the period of maximal titre values between days 2 and 4 (Fig. 4). Despite this trend, differences were not statistically significant [F (3, 40)=2.72, P=0.114, two-way ANOVA]. Similarly, no statistically significant differences were observed in the ability of the four viruses to spread in the brain at day 4 p.i. (all P>0.001, Student’s t-test) (Table 1). In all cases, the viruses were localized largely to the ventricular (e.g. choroid plexus) and periventricular regions. Selection of day 4 to study the ability of the virus variants to spread in the brain was based on previous experience showing that this time point coincides with

Fig. 1. Schematic diagram of the 88-1961 variant constructs. p88-1961 WT (carrying a Thr at aa 91 in the F protein) was modified, as described in Methods, to introduce nucleotide changes in the HN (a) and L (b) genes independently or in combination (c). The nucleotide substitutions and the resultant amino acid changes are indicated.
peak virus distribution (unpublished observations). Maintenance of the engineered mutations in r88-1961L-ATT, r88-1961HN-ATT and r88-1961HN/L-ATT in rat brain was confirmed by extraction of total RNA from brain homogenates at day 3 p.i. (time of maximal virus production), followed by RT-PCR and sequencing (data not shown).

**DISCUSSION**

We previously reported the identification of three amino acid changes associated with neuroattenuation of the
highly neuroviralent 88-1961 wild-type MuV strain: one each in the F (Ala/Thr-91→Thr), HN (Ser-466→Asn) and L (Ile-736→Val) proteins (Rubin et al., 2003). It is important to stress that, whilst we have postulated, using in vitro assays (Malik et al., 2007b), that the in vitro protein functional effects arising from these three amino acid changes may be responsible for virus neuroattenuation, this could not be tested until construction of full-length cDNA clones. We recently constructed cDNA clones of 88-1961 expressing the different F gene genotypes (Ala-91 and Thr-91) and, using a rat-based assay predictive of the human neurovirulence potential of MuV (Rubin et al., 2000, 2005), determined that the selection of a Thr in the F protein did not contribute to neuroattenuation of the virus (Malik et al., 2007a).

In the present study, we investigated the contribution of the amino acid changes in the HN and L proteins to neuroattenuation by modifying p88-1961 WT (cDNA clone containing a Thr at aa 91 in the F protein) to produce p88-1961L-ATT, p88-1961H-ATT and p88-1961H/L-ATT and assessed the in vitro and in vivo (in rats) phenotype of virus rescued from cells transfected with these plasmids. Whilst r88-1961L WT, r88-1961H-ATT and r88-1961H/L-ATT grew to similarly high titres in vitro, r88-1961L-ATT displayed lower titres, as evident by an approximate 1 log reduction in viral titre throughout the time course after the first 48 h of infection. Notably, this was not due to virus instability, as no differences were observed in potency losses between r88-1961L-ATT and r88-1961 WT when stocks of these viruses were held at 37 °C over this time period.

That the Ile-736→Val substitution affects protein function is consistent with our previous work using a mini-genome system demonstrating increased polymerase activity associated with a plasmid expressing the L protein containing Val-736 rather than Ile-736 when assessed at 48 h post-transfection (Malik et al., 2007b). In our present study using infectious virus, we clearly saw evidence of increased polymerase activity of r88-1961L-ATT (Val-736) compared with r88-1961 WT (Ile-736) when assessed at early time points, as indicated by greater virus titres on days 1 and 2 p.i. in cultures infected with r88-1961L-ATT. Although this trend reversed after day 2, with r88-1961L-ATT titres being tenfold lower than those of r88-1961 WT, we postulate that the decreased virus production during this time is due to elevated polymerase activity based on our observation of a much faster destruction of the infected cell monolayer by r88-1961L-ATT and hence a lack of viable cells for virus production relative to r88-1961 WT at 2 days p.i. Notably, whilst there was evidence of reduced growth of r88-1961L-ATT compared with r88-1961 WT in rat brain and of reduced neurovirulence by r88-1961L-ATT compared with r88-1961 WT, these differences were not statistically significant. Nonetheless, others have reported an association between polymerase activity and attenuation/virulence. Bankamp et al. (2002) reported increased polymerase activity as a marker of measles virus (a related paramyxovirus) attenuation and postulated that elevated RNA synthesis might lead to an enhanced antiviral state. This is supported by more recent studies that showed that the measles virus leader RNA binds to RIG-I and induces interferon (IFN)-β expression (Habjan et al., 2008; Plumet et al., 2007). RIG-I is a major intracellular recognition receptor for the genome of viruses of the order Mononegavirales (Habjan et al., 2008). However, this mechanism is not likely to be an explanation for our findings, as the most dramatic effects of the Ile-736→Val substitution in the L protein on virus growth were observed in Vero cells, which do not produce IFN.

In contrast, a much more dramatic reduction in neurovirulence was observed in rats inoculated with r-88-1961H-ATT and, to a greater extent, with r-88-1961H/L-ATT (both statistically significant). The double-mutant r-88-1961H/L-ATT approximated levels of neurovirulence reduction that have been observed following passage of the original 88-1961 wild-type clinical isolate in CEF cells (Rubin et al., 2003). These data indicate that neuroattenuation of the original 88-1961 wild-type clinical isolate is mostly a consequence of the Ser-466→Asn substitution in the HN protein. This is consistent with our previous work showing that the Ser-466→Asn substitution in the HN protein resulted in decreased binding and release activities of the HN protein. Interestingly, although we detected a trend towards reduction in r88-1961H-ATT growth in brain compared with r88-1961 WT, no in vivo growth differences were seen between r88-1961H-ATT and r88-1961H/L-ATT. Whilst we do not have an explanation for the apparent compensatory effect of the L mutation on the growth of r88-1961H-ATT in rat brain, given that the clinical outcome between r88-1961H-ATT and r88-1961H/L-ATT was different from that for r88-1961 WT and 88-1961 L-ATT, we postulated that the clinical outcome might be associated with virus ability to spread in the brain. However, no differences were observed among the three variant viruses with respect to their ability to spread in the brain relative to r88-1961 WT. All four viruses were localized largely to the periventricular regions (e.g. epithelial cells within the choroidplexus and ventricular ependymal cells). There was some evidence of spread to areas deeper in the brain parenchyma, but this was observed equally among all

### Table 1. Distribution of the 88-1961 variant viruses in rat brain

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>Mean grade ± SEM</th>
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<tbody>
<tr>
<td>Mock</td>
<td>–</td>
</tr>
<tr>
<td>r88-1961 WT</td>
<td>2.103 ± 0.072</td>
</tr>
<tr>
<td>r88-1961 HN-ATT</td>
<td>2.156 ± 0.101</td>
</tr>
<tr>
<td>r88-1961L-ATT</td>
<td>2.044 ± 0.055</td>
</tr>
<tr>
<td>r88-1961H/L-ATT</td>
<td>2.067 ± 0.067</td>
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four viruses. Of note, our previous studies also did not suggest a difference in virus spread between the original clinical isolate and the CEF-passaged variant (Rubin et al., 2003).

In summary, we successfully utilized the 88-1961 cDNA clone system to characterize molecular markers associated with neuroattenuation of the 88-1961 clinical isolate following passage in CEF cells. We showed that the HN protein, and to a lesser extent the L protein, play a role in MuV neuroattenuation. We wish to emphasize here that it is the altered in vitro function of these proteins and not the specific amino acid changes that might serve as markers of neuroattenuation. The Asn at aa 466 in the HN protein and the Val at aa 736 in the L protein are not themselves markers of virus neuroattenuation, as none of the many attenuated MuV strains for which sequence data are available in GenBank possess these sites. The precise mechanism by which the mutated HN and L proteins may affect MuV neuroattenuation remains to be elucidated.

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