Identification of Genetic Mutations Associated With Attenuation and Changes in Tropism of Urabe Mumps Virus

Dion Shah,1 Silvia Vidal,2 Malen A. Link,3 Steven A. Rubin,3 and Kathryn E. Wright1*

1Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, Ontario, Canada
2Department of Human Genetics, McGill University, Montreal, Quebec, Canada
3Office of Vaccines Research and Review, Center for Biologics Evaluation and Research, FDA, Bethesda, Maryland

Although several effective mumps virus vaccines have been developed, almost nothing is known about the genetic changes responsible for loss of virulence. One vaccine, Urabe AM9, was withdrawn from the market because of insufficient attenuation. The vaccine was found to contain a mixture of viruses that could be distinguished based on the sequence of the hemagglutinin-neuraminidase gene (HN). Viruses containing lysine at HN amino acid position 335 were isolated from cases of post-vaccination parotitis or meningitis whereas viruses containing glutamic acid at this position were not associated with post-vaccination disease. Using a rat based model of mumps neurovirulence, we demonstrate that this latter virus is significantly attenuated compared to a virus isolated from a patient with post-vaccination meningitis. Complete sequence analysis of the genomes of the two viruses identified sixteen genetic differences, some or all of which must be responsible for differences in virulence. These same genetic differences also account for changes in tropism in cell culture. J. Med. Virol. 81:130–138, 2009. © 2008 Wiley-Liss, Inc.

KEY WORDS: Mumps virus; attenuation; vaccine; mutations

INTRODUCTION

Mumps virus is a member of the family Paramyxoviridae, subfamily Paramyxovirinae, and in the genus Rubulavirus. Prior to the introduction of a vaccine in the 1970s, mumps virus infections were a major cause of viral meningitis and unilateral hearing loss in children [reviewed in Wright 2006]. Widespread use of live attenuated mumps vaccines has been responsible for a dramatic decline in mumps disease [Bakshi and Cooper, 1990]. Despite this success, the frequency of mumps virus infections continues to be high worldwide, as vaccination is unavailable or voluntary in many countries, and large outbreaks occur even in countries where vaccination is routine [Centers for Disease Control and Prevention, 2006].

Full genome sequences of four mumps vaccines have been determined, Jeryl Lynn (JL) [Clarke et al., 2000], Miyahara (Okazaki et al., accession number NC002200), L-Zagreb [Ivancic et al., 2005], and Urabe AM9 [Kimura et al., 1996; Amexis et al., 2001], but in no case has it been possible to compare the sequences to a parent wild-type virus. Consequently, very little is known about the genetic basis of attenuation of mumps viruses, and because of this lack of molecular markers for attenuation and virulence, concerns about vaccine safety increase. The Urabe AM9 vaccine is an example of a vaccine that was insufficiently attenuated, and hence was withdrawn from use in several countries [Balraj and Miller, 1995]. Specifically, the Urabe AM9 component of trivalent measles, mumps and rubella vaccines was associated with meningitis and parotitis at rates higher than observed with vaccines containing JL mumps [Forsey et al., 1990; Brown et al., 1991].

The Urabe AM9 vaccine was developed in Japan and was originally attenuated by six passages of a wild isolate in chicken amniotic cavity, after which the virus underwent two plaque purifications in quail fibroblasts. The ninth plaque purified clone was named AM9 [Yamanishi et al., 1970]. After testing in humans, the isolate of AM9 with the highest seroconversion rate and with no side effects was further passaged 4 times in...
chick amniotic cavity to produce a seed stock [Yamanishi et al., 1973]. Analysis of several vaccine lots demonstrated that there was a mixture of closely related viruses in the vaccine that could be differentiated based on the sequence of the hemagglutinin-neuraminidase (HN) gene at nt 1,081 (nt 7,616 of the genome) [Brown et al., 1996]. The consensus sequence of all mumps strains is an adenosine (A) at this site [Yates et al., 1996] while some viruses in the vaccine, “G” viruses, possessed a guanine at nt 7,616, resulting in a lysine to glutamic acid change at HN335. We and others showed that viruses associated with post-vaccination disease all possessed the consensus lysine [Brown et al., 1996; Afzal et al., 1998; Wright et al., 2000], while a virus with the mutation has been isolated from a recently vaccinated individual who showed no symptoms [Afzal et al., 1998]. These observations suggest that the “G” viruses are amongst the most attenuated viruses in the vaccine.

Recently, a rodent model has been developed that is predictive of neurovirulence of mumps isolates in humans [Rubin et al., 2000, 2005]. In this system, the severity of hydrocephalus developing in brains of neonatal rats after intracranial (i.c.) inoculation of virus correlates with the virulence of mumps strains for humans. In order to test the hypothesis that a Urabe “G” virus, Gw7, is more attenuated than a Urabe virus from a case of post-vaccination meningitis, 1004-10/2, the neurovirulence of both viruses was assayed in the rat-based model. The genomes of both viruses were sequenced, and thus the genetic basis of differences in virulence was determined.

**MATERIALS AND METHODS**

**Cells and Viruses**

Monolayers of A549, HeLaT4 (NIH AIDS Research and Reference Resource Program) and Vero cells were grown in DMEM supplemented with 7% fetal bovine serum (FBS) and antibiotics (penicillin/streptomycin). Urabe Gw7 was isolated from the Urabe/PM vaccine (Institut Pasteur Merieux, France) by three plaque purification steps in Vero cells [Wright et al., 2000]. The stocks used in all experiments had been passaged no more than 5 times in Vero cells. Stock of the Urabe meningitis isolate, 87-1004 was originally received from the Bureau of Biologics, Health Canada. 87-1004 was originally received from the same stock of 87-1004, but no more than 5 times in Vero cells. Stock of the Urabe virus, Gw7, is more attenuated than a Urabe virus that could be differentiated based on the mutation has been isolated from a recently vaccinated individual who showed no symptoms [Afzal et al., 1998]. These observations suggest that the “G” viruses are amongst the most attenuated viruses in the vaccine.

**Rats and Virus Inoculation**

Lewis rats were inoculated intracerebrally within 24 hr of birth with 100 pfu of virus in a volume of 0.02 ml. At 30 days post-infection, animals were euthanized, brains were removed, divided into two hemispheres and fixed in 10% neutral buffered formalin. Fixed brains were processed, embedded in paraffin and sectioned sagittally at 8 μm. Two sections from each rat brain taken at anatomical midline were stained with hematoxylin and eosin and assessed for neurovirulence as described previously [Rubin et al., 2000]. Briefly, the percentage of the total brain cross sectional area (excluding cerebellum) occupied by the lateral ventricle was determined for each pair of brain sections for each rat using Image Pro Plus™ image analysis software (Media Cybernetics, Silver Spring, MD). The mean value of these measurements is the neurovirulence score for the animal. The Mann–Whitney Rank Sum test was used to compare neurovirulence scores.

**Cloning and Sequencing**

Using Trizol reagent (Invitrogen, Burlington, ON), total RNA was isolated from Vero cells infected 2–4 days previously at an MOI of 0.1, and reverse transcribed using SuperScript II polymerase (Invitrogen) and random hexamer primers. Using a published sequence for Urabe mumps (AF314560), primer pairs were designed to generate 13 overlapping amplicons ranging in size from 1,019 to 1,724 bp in length. PCR was carried out using Taq polymerase (Clontech, Mountainview, CA). Products were purified with QIAEX II Gel Extraction Kit (Qiagen, Mississauga, ON) and ligated into pBluescript KS (Stratagene, La Jolla, CA) previously digested with EcoRV and treated with Taq polymerase. A minimum of three clones was sequenced for each genome fragment. Cycle sequencing reactions were performed using ABI Prism (PE Biosystems, Foster City, CA) according to the manufacturer's instructions, with universal M13 primers labeled with IRDye™ 700 and IRDye™ 800 infrared dyes. Sequencing fragments were analyzed on a Licor IR2 DNA analyzer (Li-COR Biosciences, Lincoln, NE). Nucleotide and amino acid sequence analysis of each clone and sequence assembly was performed using DNAsis MAC v2.0 software (Hitachi Software Engineering, San Francisco, CA). Accession numbers of sequences included in comparisons are AF314560 (87-1004), AF314562 (87-1005), AB000386 (AM9-A),
Binding and Fusion Assays

To assess virus binding, triplicate tubes with equal numbers of cells were centrifuged, and pellets were resuspended in 500 μl of media containing either Gw7 or 1004-10/2 at MOI = 0.1. After incubation for 1 hr on ice, cells were washed 3 times with PBS, and lysed by a freeze/thaw cycle prior to determination of infectious virus by plaque assay.

Fusion activity mediated by transient expression of HN and F proteins in HeLaT4 cells was measured as described by Nussbaum et al. [1994]. Briefly, using Lipofectin (Invitrogen), monolayers of HeLaT4 cells were transfected with 500 ng of HN containing plasmids (pDrive, Qiagen) and either 167 or 50 ng of F plasmids to produce a fusion activity, obtained from B. Moss [Fuerst et al., 1986]. After 24 hr, HeLaT4 cells that had been transfected with a plasmid containing the β-galactosidase gene under control of T7 promoter and infected with wild-type vaccinia virus were overlaid onto the cells expressing the viral glycoproteins. After a 3 hr incubation period, the monolayers were gently washed and lysed. Samples were assayed for β-galactosidase activity over a 50 min time period, using the substrate chlorophenol red-β-d-galactopyranoside (CPRG, Roche, Canada, Mississauga, ON). The slopes of activity were calculated to represent relative fusion mediated by each sample.

Negative controls were untreated cells, cells infected with vTF7-3 in the absence of plasmids, and cells transfected with F plasmid alone. Prior to use, we confirmed that plasmid preparations were transfected at equivalent efficiencies by counting cells positive for expression of HN and F detected by indirect immunofluorescence. The surface expression of the glycoproteins was determined by measuring Mean Fluorescence Intensity after transfection of HeLaT4 cells with 300 ng of the relevant plasmid. For both assays, HN and F specific monoclonal antibodies were used (gifts from M. Afzal, National Institute for Biological Standards and Control, UK and C. Örvell, Karolinska Institute, Sweden). Flow cytometry was completed at the National HIV Laboratory, Ottawa, Canada.

RESULTS

Neurovirulence of Urabe Gw7 in Neonatal Rats

To determine if a “G” virus, Gw7, purified from the Urabe vaccine, is attenuated, the degree of hydrocephalus induced by this virus was assessed in the rat neurovirulence test (RNVT), and compared to that produced by 1004-10/2, a plaque purified virus from a case of meningitis resulting from immunization with the Urabe AM9 vaccine. This virus was plaque purified from a stock of 87-1004 virus which was heterogeneous at aa 464 of HN [Wright et al., 2000], and possessed a K at this site. It had previously been reported that the N→K change at aa 464 was observed in Urabe viruses from cases of post-vaccination meningitis but not in viruses isolated from cases of parotitis [Afzal et al., 1998; Wright et al., 2000]. Although individual variants of a virus may not be as virulent as the natural quasispecies that exist in nature [Vignuzzi et al., 2006], purified viruses were used in this study in order to correlate specific sequences with the properties of attenuation or virulence. Control viruses were the fully attenuated JL vaccine strain and the highly neurovirulent 88-1961 wild-type strain. As shown in Figure 1, Gw7 produced a hydrocephalus score of only 1.37% ± 0.50 (17 rats, 34 sections), which was statistically equivalent to the score in rats inoculated with JL, 1.81% ± 0.31 (8 rats, 16 sections). The wild-type virus, 88-1961, produced a hydrocephalus score of 11.47% ± 1.16 (18 rats, 36 sections), while 1004-10/2 produced an intermediate hydrocephalus score of 4.70% ± 0.77 (17 rats, 34 sections). The score of 1004-10/2 is statistically greater than the hydrocephalus score of the Gw7 virus and of the JL vaccine strain (P = 0.033). In a smaller experiment (not shown), Gw7 induced no measurable hydrocephalus (18 sections), whereas the hydrocephalus score induced by the parent stock of 87-1004 was 12% (34 sections), supporting the conclusion that Gw7 is a fully attenuated Urabe virus and the post vaccination isolate retains virulence. In the RNVT, there is no threshold value that discriminates a virulent virus from an attenuated virus, and the test is designed to compare the virulence or attenuation of one strain to another within a single experiment. Although the absolute scores determined for 1004-10/2 and the original stock of 87-1004 were different, the hydrocephalus score for the latter was shown to be intermediate when compared to 88-1961 and JL in a separate assay [Rubin et al., 2000].

Sequence Comparison of Gw7 and 1004-10/2

To identify genetic changes associated with the differences in neurovirulence, the complete genomes of both Gw7 and 1004-10/2 were sequenced (accession FJ375177, FJ37517). The Urabe mumps virus genome is
15,384 nt in length with seven open reading frames (ORF) encoding 9 polypeptides, nucleoprotein (NP), phosphoprotein (P), matrix (M), fusion (F), small hydrophobic (SH), hemagglutinin-neuraminidase (HN), L (polymerase), and V and I. When compared to each other, 16 differences were found between the viruses (Table I). Five were non-coding; nt 1,880 in the intergenic region between NP and P located 85 nt downstream of the NP stop and 99 nt upstream of the P start; nt 1,387 in the NP gene; nt 7,573 in the HN gene; nt 10,529 in the L gene, and the last at nt 15,328, in the trailer region 46 nt from the genome end. The 11 coding differences were located in 4 genes, F, SH, HN, and L. No differences were found in either of the genome ends. The differences at HN$^{335}$ and HN$^{464}$ were previously described [Afzal et al., 1998; Wright et al., 2000], while HN$^{526}$ was unexpected. There was one conservative difference at F$^{91}$ in F2, and another non-conservative difference at F$^{120}$, which would fall within the fusion peptide in F1. There was a single non-conservative difference at SH$^{40}$, while the last 5 coding differences occurred in the viral polymerase, at L$^{163}$, L$^{320}$, L$^{512}$, L$^{1085}$, and L$^{1871}$.

**Sequence Comparisons With Other Urabe Viruses**

To determine whether the differences observed between Gw7 and 1004-10/2 were present in other

<table>
<thead>
<tr>
<th>Gene/AA</th>
<th>Gw7</th>
<th>1004-10/2</th>
<th>Consensus/87-1004</th>
<th>87-1005</th>
<th>AM9</th>
</tr>
</thead>
<tbody>
<tr>
<td>1387/NP</td>
<td>a</td>
<td>g</td>
<td>g</td>
<td>g</td>
<td>g</td>
</tr>
<tr>
<td>1580/NP-P</td>
<td>c</td>
<td>t</td>
<td>c</td>
<td>c</td>
<td>c</td>
</tr>
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<td>4817/F91</td>
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<td>c-Ala</td>
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<td>c-Ala</td>
</tr>
<tr>
<td>4903/F120</td>
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<td>c-Gln</td>
<td>c-Gln</td>
<td>c-Gln</td>
<td>c/Gln/Glu</td>
</tr>
<tr>
<td>6385/SH40</td>
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<td>c-His</td>
<td>a-Asn</td>
<td>c-His</td>
</tr>
<tr>
<td>7573</td>
<td>t</td>
<td>c</td>
<td>t</td>
<td>c</td>
<td>t</td>
</tr>
<tr>
<td>7616/HN335</td>
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<td>a-Lys</td>
<td>a-Lys</td>
<td>a-Lys</td>
<td>a/g-Lys/Glu</td>
</tr>
<tr>
<td>8005/HN464</td>
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<td>a-Lys</td>
<td>a-Lys</td>
<td>a-Lys</td>
<td>a/Lys</td>
</tr>
<tr>
<td>8191/HN526</td>
<td>a-Lys</td>
<td>c-Asn</td>
<td>c-Asn</td>
<td>c-Asn</td>
<td>a-Lys</td>
</tr>
<tr>
<td>8925/L163</td>
<td>a-Asn</td>
<td>c-Thr</td>
<td>c-Thr</td>
<td>c-Thr</td>
<td>a-Thr</td>
</tr>
<tr>
<td>9397/L320</td>
<td>g-Met</td>
<td>a-Ile</td>
<td>a-Ile</td>
<td>a-Ile</td>
<td>a-Ile</td>
</tr>
<tr>
<td>9972/L512</td>
<td>c-Ser</td>
<td>t-Phe</td>
<td>c-Ser</td>
<td>t-Phe</td>
<td>c/t-Ser/Phe</td>
</tr>
<tr>
<td>10529/L</td>
<td>c</td>
<td>t</td>
<td>t</td>
<td>t</td>
<td>t</td>
</tr>
<tr>
<td>11692/L1085</td>
<td>g-Leu</td>
<td>t-Phe</td>
<td>g-Leu</td>
<td>t-Phe</td>
<td>g/t-Leu/Phe</td>
</tr>
<tr>
<td>14049/L1871</td>
<td>c-Ala</td>
<td>t-Val</td>
<td>c-Ala</td>
<td>t-Val</td>
<td>c-Ala</td>
</tr>
<tr>
<td>15328</td>
<td>a</td>
<td>g</td>
<td>g</td>
<td>g</td>
<td>g</td>
</tr>
</tbody>
</table>

Residues that are unique to Gw7 or 1004-10/2 are bolded. Residues shared between 1004-10/2 and other Urabe viruses are on a shaded background. AM9 sequence from Sauder et al. [2006].

Urabe viruses, sequences at the 16 sites were compared to 8 published sequences of Urabe genomes, to a consensus sequence derived from these, to quasispecies described by direct sequencing of various Urabe vaccine lots, and to genomes of 2 other mumps viruses from Japan. The full genomes include representative sequences from SKB, Chiron and Biken Urabe vaccines [Amexis et al., 2001; Sauder et al., 2006], three sequences from a vaccine in Japan have been banked, AM9 parental, AM9-A, AM9-B (Genbank AB000388, AB000387, AB000386) and sequences from isolates associated with post-vaccination meningitis in Canada, 87-1004 and 87-1005 (Genbank AF314560, AF314562). All sequences are assumed to represent partially attenuated Urabe viruses, although we think 87-1004 and 87-1005 are among the most virulent viruses associated with the vaccine, and hence closer in sequence to wild-type Urabe virus. Gw7 was found to be unique at 5 of the 16 sites (bolded in Table I). One of these was the non-coding change in NP at nt 1,387, and the remaining differences predicted aa changes at F120, HN335, L163, and L320. Sequences generated from vaccines directly showed variation at two of these sites, HN335 and F120 [Amexis et al., 2001; Sauder et al., 2006]. At nt 15328, Gw7 had the same mutation as 87-1004, but this change has not been described for any other Urabe virus. The virulent virus, 1004-10/2, was unique at two sites, F91 and HN526. These mutations were verified by independent RT/PCR, so were not generated during the cloning process. At 8 sites, 1004-10/2 was identical to the published sequence for 1004-10/2, but unlike any other Urabe sequences (shaded in Table I). Variation at 4 of these sites, HN464, nt 10,529, L512, and L1085, has been detected in the SKB vaccine [Sauder et al., 2006]. Both viruses were identical to the short stretches of sequence available for wild-type Urabe P [Yamada et al., 1989] and the wild type SH gene was identical to that of Gw7 SH [Takeuchi et al., 1991]. The sequences of Gw7 and 1004-10/2 were also compared to Odake-1 virus, a closely related wild-type virus associated with a high incidence of meningitis in Japan [Saito et al., 1998], and to another vaccine strain of mumps, Miyahara, which is the same genotype as Urabe. At the 16 sites, neither virus displayed any of the sequences unique to Gw7 nor did they possess any of the sequences characterizing 1004-10/2.

**Table II. Restricted Replication of Gw7 in Human Cell Lines**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Vero</th>
<th>A549</th>
<th>HeLa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gw7</td>
<td>1.96 ± 0.89 x 10^6 (n=5)</td>
<td>1.43 ± 0.56 x 10^3 (n=6)</td>
<td>5.8 ± 4.19 x 10^3 (n=6)</td>
</tr>
<tr>
<td>1004-10/2</td>
<td>3.67 ± 1.97 x 10^2 (n=6)</td>
<td>1.5 ± 0.78 x 10^3 (n=5)</td>
<td>1.25 ± 0.92 x 10^2 (n=6)</td>
</tr>
<tr>
<td>1004-4/1</td>
<td>2.18 ± 1.03 x 10^8 (n=6)</td>
<td>1.50 ± 1.12 x 10^3 (n=6)</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

Confluent monolayers of cells were inoculated at MOI = 0.1. At day 4 p.i. supernatants were harvested, and yields of infectious virus were measured by standard plaque assay. n.d., not done.

**Gw7 and 1004-10/2 Have Different Tropisms in Tissue Culture Cells**

Previous work had found that in Vero cells the titer of “G” viruses was about 1 log lower than titers of viruses associated with post-vaccination meningitis [Brown et al., 1996]. When we tested the ability of Gw7 and 1004-10/2 to replicate in other cell types, specifically the human lung epithelial line A549, and HeLaT4 cells, growth of Gw7 was discovered to be severely restricted. In both human cells, titers of Gw7 were on average 3 logs lower than those in Vero cells 4 days post-infection, while growth of 1004-10/2 in the human cells was only slightly reduced compared to growth in Vero at the same time point (Table II). To ensure that the reduced titer of Gw7 in the human cells was not due to a shift in the kinetics of replication, growth of Gw7 was examined between days 1 and 4 p.i., and the titer of Gw7 was significantly reduced at all time points compared to titers in Vero cells (Fig. 2). Hence, the same constellation of sequence differences is responsible for the two phenotypes; virulence/attenuation in vivo, and tropism in vitro.

To determine the gene(s) responsible for the differential replication of the two viruses in human cells, we extended the growth studies to a virus plaque purified from the same stock as 1004-10/2. This virus, 1004-4, was selected as distinct from 1004-10/2 at HN464. Based on our sequencing of the HN, SH, and M genes, this virus is identical to the published sequence for 87-1004, so we have assumed that the remainder of the 87-1004 sequence applies to 1004-4. At all the sites where 1004-10/2 and Gw7 differ, 1004-4 has the consensus sequence, with the exception of nt 15,328, where 1004-4 is like Gw7. As 1004-4 yielded titers in A549 and Vero cells similar to those of 1004-10/2 (Table II), the ability to replicate well in A549 cells does not map to any of the sites where 1004-10/2 differs from 1004-4; F91, HN464, HN526, L512, L1085, L1871, and the non-coding mutations.

**Fig. 2.** Triplicate wells of Vero and A549 cells were inoculated with Gw7 at MOI of 0.1. At the indicated times post-infection, supernatants were collected and assayed for infectious virus by standard plaque assay. Each point is the mean from triplicate wells ± SD.
at nt1,880 and 10,529. We conclude from these results that the mutations in Gw7 account for the restricted growth of this virus in the human cells.

We found no differences in the P or V proteins of Gw7 and 1004-10/2, which would have implicated a role for interferon (IFN) in tropism, as demonstrated for another Urabe “G” virus [Rosas-Murrieta et al., 2007]. The difference at nt 1,880 upstream of the P ORF, which could potentially alter transcription of P and V mRNA, was not responsible for differences in growth in A549 cells, as both 1004-4 and Gw7 have the same sequence at this site, but differences in growth. There are then three genes of Gw7 that contain unique mutations which could affect growth, HN, F, and L. To determine whether the mutation at HN<sup>316</sup> affected binding of Gw7 to A549 compared to Vero cells, infectious virus associated with equal numbers of each cell type was measured. In three experiments, there was no significant difference in binding of Gw7 to A549 cells compared to Vero cells (P = 0.1980), nor was there reduced binding of Gw7 to either cell type compared to binding of 1004-10/2. The results of one representative experiment are shown in Table III. Gw7 showed a trend to higher binding to both cell types than 1004-10/2, and for A549, the increase in binding was significant in all experiments (P = 0.0066). Thus, reduced binding was not a factor in the low replication of Gw7 in human cells. Replication in A549 could still be restricted if the ribonucleoprotein cannot enter the cell due to limited fusion of the viral envelope with the cell membrane. When the fusion activity of Gw7 HN/F proteins in human cells was measured in a quantitative assay, the fusion was not reduced compared to that mediated by the 1004-10/2 HN/F pair, as seen in a representative experiment in Figure 3. Surface expression of the HN from each virus was equivalent (n = 3), as determined by flow cytometry, while the surface expression of 1004-10/2 F was reduced by 30% compared to that of Gw7 (n = 5). When fusion activity was adjusted for this difference, Gw7 HN/F still fused as well or better than the HN/F pair of the other virus in all experiments conducted to date (>10). These results demonstrate that limited growth of Gw7 in the human cells was not due to reduced binding or reduced entry mediated by fusion, thus implicating the mutations in the polymerase of this virus in this phenotype.

**DISCUSSION**

Our results have established that a Urabe virus with a guanine at nt 7,616, Gw7, isolated directly from the AM9 vaccine, is attenuated in the RNVVT compared to a closely related virus, 1004-10/2, associated with meningitis after vaccination with the vaccine. More significantly, Gw7 is as attenuated as the JL vaccine, which is the gold standard for mumps vaccines. We identified 16 sequence differences, 11 resulting in coding changes in 4 proteins, which contribute to the observed differences in virulence between the viruses. We also demonstrated that these same sequence differences, specifically a subset of mutations in the viral polymerase, control the ability of Gw7 and 1004-10/2 to replicate in human cell lines.

We cannot conclude at this point that the decreased ability of Gw7 to replicate in A549 cells is correlated with attenuation, but the mutations in L controlling tropism may also be important for the in vivo phenotype. All of the sites identified by us in L, except the two characterizing Gw7, have been noted to vary or be mutated in either Urabe vaccines or in at least one other Urabe virus [Amexis et al., 2001; Sauder et al., 2006]. We believe that the mutations in the polymerase of Gw7 have not been generated by passage in culture since purification, as we have confirmed these mutations in a second “G” virus isolated independently from the same vaccine lot (unpublished results). None of the differences in L are within motifs associated with RNA-binding, with GDQN template recognition/phosphodiester bond-forming, or with purine nucleotide binding [Poch et al., 1990; Sidhu et al., 1993], nor are they in sites associated with methylation of mRNA caps [Grdzelishvili et al., 2006]. Three of the amino acids are

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**TABLE III. Binding of Gw7 and 1004-10/2 to A549 and Vero Cells**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Cell associated pfu Vero</th>
<th>Cell associated pfu A549</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gw7</td>
<td>7.00 × 10&lt;sup&gt;3&lt;/sup&gt; ± 1.73 × 10&lt;sup&gt;3&lt;/sup&gt; (n = 3)</td>
<td>6.00 × 10&lt;sup&gt;2&lt;/sup&gt; ± 4.02 × 10&lt;sup&gt;2&lt;/sup&gt; (n = 3)</td>
</tr>
<tr>
<td>1004-10/2</td>
<td>2.59 × 10&lt;sup&gt;3&lt;/sup&gt; ± 3.57 × 10&lt;sup&gt;2&lt;/sup&gt; (n = 3)</td>
<td>1.70 × 10&lt;sup&gt;2&lt;/sup&gt; ± 3.56 × 10&lt;sup&gt;2&lt;/sup&gt; (n = 3)</td>
</tr>
</tbody>
</table>

Equal numbers of Vero and A549 cells were incubated with virus at MOI = 0.1 for 1 hr on ice. After three washes with PBS, cells were lysed and assayed for infectious virus by plaque assay.

Fig. 3. Fusion mediated by HN/F pair from Gw7 and 1004-10/2. HeLaT4 cells were transfected with HN and F plasmids at the indicated ratios. Control wells received vT7-3 alone or F plasmid alone. At 24 hr post-transfection, fusion activity was assayed as described in Materials and Methods Section. Results are adjusted to surface expression of HN and F, as determined by flow cytometry, and are represented as relative to the highest value obtained. Each datum point is the mean of duplicate wells, and the results are representative of six experiments.

within domains defined by conserved sequences across polymerases from negative stranded RNA viruses [Poch et al., 1990]. L320 is within Domain I, in a region identified in the polymerases of Sendai virus and SV5 important for P/L interactions [Holmes and Moyer, 2002; Parks, 1994]. L512 is at the boundary of Domain II, which contains the RNA binding domain, and L1085 is within a highly conserved stretch of sequence in Domain IV. L163 is upstream of Domain I, within a region of Sendai virus L shown to mediate oligomerization [Cevik, 2007], while L1871 is immediately downstream of Domain VI. The 1004-10/2 sequences at L512 and L1085 do not impart virulence, as selection of the 1004-10/2 sequences over the Gw7 sequences at these residues was associated with increased attenuation of Urabe viruses [Sauder et al., 2006]. There is a single site, L1871, where 1004-10/2 and 87-1005 displayed unique sequence which could contribute to virulence in vivo, but this change is not required for growth in A549 cells as this mutation does not exist in 1004-4.

Our results point to the two mutations in Gw7 L as responsible for restricted growth in A549 cells. Changes in cell specific growth due to mutations in L have been described for measles virus (MV), where mutations in the amino half of the polymerase enhanced the ability of MV to grow in Vero cells but had no effect on growth in A549 cells [Tahara et al., 2005]. One of these mutations corresponds to L161 of the mumps polymerase, within 2 residues of the L163 mutation in Gw7. These findings imply that cell tropism can arise from differences in levels or sequences of host factors necessary for efficient functioning of the viral polymerase. For example, high levels of the heat shock protein 72 (hsp72) have been associated with increased replication and neurovirulence of MV [Vasconcelos et al., 1998; Carsillo et al., 2006], although disparity in the levels of hsp72 did not account for restricted replication of MV in mouse versus human cells [Vincent et al., 2002]. Other cellular proteins known to affect paramyxovirus transcription and replication, and which could differ between Vero and A549 cells, are the RNA-binding protein La, and hsp90, which enhances the function of negative stranded RNA virus polymerases by stabilizing L protein [Raha et al., 2004; Connor et al., 2007].

Of the other sites where the viruses differ, the three yielding non-coding differences in open reading frames are unlikely to be significant. The non-coding differences at nt 1,880 and 15,328 could alter transcription and replication, as the former is in an intergenic region, while the latter is within an enhancer element originally identified in the antigenome of SV5 [Keller and Parks, 2003]. The former site has been shown to be particularly susceptible to mutation after passage in Vero cells [Sauder et al., 2006], so there is a possibility that the mutation here in 1004-10/2 may have occurred after isolation of the virus from the patient. The mutation in Gw7 observed at nt 15,328 is also present in the published sequence for 87-1004, and a stock of this virus, like 1004-10/2, has been shown to have intermediate virulence in the RNV [Rubin et al., 2000], suggesting that this mutation on its own does not confer attenuation.

The remaining coding differences were located in the HN, F, and SH genes. The single amino acid difference in SH could be a factor in the differences in virulence if the H→N change affects the proposed anti-apoptotic function of this protein [Lin et al., 2003; Wilson et al., 2006]. On the other hand, a sequence for “wild-type” Urabe SH has been reported, and it is identical to the attenuated rather than the virulent viruses, suggesting that mutation at SH40 is not essential for the continued virulence of 1004-10/2.

In the glycoproteins, the viruses differed at two sites in F, and three amino acids in HN. The two sites unique to 1004-10/2 and not found in 87-1005, F91, and HN526, may have arisen in the laboratory, as we do not know how many times this virus was passaged prior to our receipt of it. The F91 site has been shown to be susceptible to mutation in tissue culture [Rubin et al., 2003]. The mutations in Gw7 at HN335 and F120 and variation at HN464 have been found in other Urabe viruses [Afzal et al., 1998; Amexis et al., 2001; Sauder et al., 2006]. The residues in HN are all predicted to be on the globular head of the protein, but not within hydrophobic areas involved in dimer formation and fusion promotion, nor in sialic acid binding domains [Zaitsev et al., 2004; Crennell et al., 2000; Takimoto et al., 2002; Bousse et al., 2004]. Despite this, the K→E mutation at HN335 has been shown to shift the specificity of sialic acid binding from sialyl2,6 lactose towards a preference for sialyl2,3 linkages [Reyes-Leyva et al., 2007]. Within the F protein, F397 is at the end of a heptad repeat in F2 [Plemper and Compans, 2003], while F1320 is located at the carboxy end of the fusion peptide in F1. Although the differences in growth in A549 cells were not due to reduced binding or fusion, the differences in HN and F do appear to affect the functions of these proteins. Gw7 showed higher binding to both cell types compared to 1004-10/2, possibly due to the fact that human type II alveolar epithelial cells, such as the A549 line, and Vero cells express mainly 2,3-linked sialic acids [Ibricevic et al., 2006; Govorkova et al., 1996]. Additionally, fusion mediated by the HN/F pair from Gw7 was higher than that mediated by the glycoproteins from 1004-10/2, although not significantly so (P = 0.24). The lower surface expression of 1004-10/2/F contributed somewhat to this diminished function, but not totally. It has been shown for the 88-1961 strain of mumps that changes in the sequence at F391, from a mix of alanine/threonine to threonine alone, did reduce fusogenicity [Malik et al., 2007]. However, the mutation at F391 in 1004-10/2 is a more conservative change, from alanine to valine, so we cannot be sure that this mutation would reduce fusogenicity of this protein, nor can we rule out a role for the differences in HN affecting fusion.

Although the differences in HN and F did not control tropism in vitro, they are likely to affect in vivo virulence. High fusogenicity has been associated with attenuation of several paramyxoviruses, including mumps virus [Lemon et al., 2007], and we had
previously shown that Gw7 caused earlier fusion in Vero cells compared to 1004-10/2 and 87-1005 [Wright et al., 2000]. Virulence or attenuation of mumps strains in vivo has been more specifically mapped to mutations in F alone [Lemon et al., 2007], HN alone [Kövářenees et al., 1990], to changes in the degree of heterogeneity at sites within F and HN [Sauder et al., 2006], or to a combination of mutations in F, HN, and L [Rubin et al., 2003]. The two sites unique to 1004-10/2, F91, and HN526, were not found in 87-1005, another Urabe virus associated with meningitis, so the sequences at these sites may have functional implications but are not essential for virulence in humans. The presence of a lysine at HN464 is characteristic of Urabe viruses isolated from cases of vaccine associated meningitis, but not parotitis [Afzal et al., 1998; Wright et al., 2000], suggesting that this may be a marker for the ability of Urabe viruses to penetrate the CNS. However, attenuation of Urabe viruses by passage in CEF or Vero cells selected for the 1004-10/2 sequences at HN335, HN464, and F120 [Sauder et al., 2006]. Thus for the amino acids in HN and F, 1004-10/2 sequences are not essential for virulence, nor are the HN7 sequences necessary for attenuation as measured in the RNVVT. But this does not rule out a role for the observed HN and F mutations in the attenuation of Gw7. The Urabe virus selected in CEF cells, Urabe P6-CEF, was attenuated but did not display reduced replication in the brains of neonatal rats [Sauder et al., 2006], while others have shown that another “G” Urabe virus replicated significantly less well in neonatal rat brains than a virus without this mutation [Santos-López et al., 2006]. These findings indicate that the mechanism of attenuation of “G” viruses is different than that of the Urabe P6-CEF virus, and that at least the mutation at HN335 may play an important role in attenuation.

In this study, we defined a limited number of mutations accounting for increased attenuation of a “G” Urabe virus. We had hypothesized that the majority of sites characterizing the two viruses associated with meningitis, 1004-10/2 and 87-1005, were sequences retained from wild-type Urabe, but comparison with Urabe viruses subjected to passage through Vero cells or CEF indicate that some of these sequences have arisen during culture or otherwise do not contribute to the virulence of 1004-10/2 [Sauder et al., 2006]. The exception is the mutation at L1871. The mutations characterizing Gw7 could all contribute to the increased attenuation of this virus. To determine the relevance of differences at individual sites, these mutations can be introduced into the genome by reverse genetics, and rescued viruses assayed in the rat model and for growth in human cells. Another approach is to extend the type of analysis conducted in this study to the panel of naturally occurring variants which we have purified from the Urabe vaccine [Wright et al., 2000], and to additional Urabe viruses isolated from individuals with post-vaccination disease. Such studies will be beneficial in defining genetic markers of virulence and/or attenuation of the Urabe viruses in vivo and in tissue culture, and will have broader relevance for our understanding of mumps virus biology.

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