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Changes in mumps virus neurovirulence phenotype associated with quasispecies heterogeneity

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Abstract

Mumps virus is a highly neurotropic virus with evidence of central nervous system invasion (CNS) in approximately half of all cases of infection. In countries where live attenuated mumps virus vaccines were introduced, the number of mumps cases declined dramatically; however, recently, the safety of some vaccine strains has been questioned. For example, one of the most widely used vaccines, the Urabe AM9 strain, was causally associated with meningitis, leading to the withdrawal of this product from the market in several countries. This highlights the need for a better understanding of the attenuation process and the identification of markers of attenuation. To this end, we further attenuated the Urabe AM9 strain by serial passage in cell culture and compared the complete nucleotide sequences of the parental and passaged viruses. Interestingly, despite a dramatic decrease in virus virulence (as assayed in rats), the only genomic changes were in the form of changes in the level of genetic heterogeneity at specific genome sites, i.e., either selection of one nucleotide variant at positions where the starting material exhibited nucleotide heterogeneity or the evolution of an additional nucleotide to create a heterogenic site. This finding suggests that changes in the level of genetic heterogeneity at specific genome sites can have profound neurovirulence phenotypic consequences and, therefore, caution should be exercised when evaluating genetic markers of virulence or attenuation based only on a consensus sequence.
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Keywords: Mumps virus; Vaccine; Urabe AM9; Central nervous system; Neurovirulence; Neuroattenuation; Quasispecies; Rat model; Genetic heterogeneity

Introduction

Mumps virus is a member of the genus *Rubulavirus* within the family *Paramyxoviridae* (order *Mononegavirales*). Its single-stranded negative sense RNA genome consists of 15,384 nucleotides containing seven tandemly linked transcription units that encode seven open reading frames: the gene order is NP (nucleoprotein), V/P (phosphoprotein), M (matrix), F (fusion), SH (small hydrophobic), HN (hemagglutinin-neuraminidase) and L (large). Each open reading frame encodes the indicated protein, with the exception of the P open reading frame, which encodes three proteins, P, V and I via an RNA editing process (Paterson and Lamb, 1990; Elliott et al., 1990).

Mumps is an acute, communicable disease transmitted via the respiratory route. Mumps virus infection often results in viremia with dissemination of virus to a number of organ systems, including the central nervous system (CNS). The predilection of the virus for the CNS was suggested in a study by Bang and Bang (1943) demonstrating cerebrospinal fluid pleocytosis in 65% of mumps cases. Later, epidemiological studies would reveal mumps virus as a leading cause of virus-induced aseptic meningitis and encephalitis in the United States and Europe (Carbone and Wolinsky, 2001).

With the advent of live attenuated mumps virus vaccines, the incidence of mumps dropped dramatically in countries where universal vaccination was implemented, demonstrating an overall high efficiency of these vaccines. However, some vaccines, including the Urabe AM9, Leningrad–Zagreb and Sofia-6, have been found to cause excessive neurological adverse reactions, including aseptic meningitis (Odisseev and

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Gacheva, 1994; Arruda and Kondageski, 2001; da Silveira et al., 2002; Azzopardi, 1988; Furesz, 2002). Such problems have resulted in vaccine withdrawal, removal of mumps vaccine from national immunization programs and public resistance to vaccination. One of the best studied of these strains is the Urabe AM9 vaccine. That rates of meningitis after vaccination with the Urabe AM9 strain differed not only by manufacturer but also by formulation (reviewed in (Plotkin and Wharton, 1999)) and that Urabe AM9 exhibits substantial genetic variability (Brown and Wright, 1998; Brown et al., 1996; Afzal et al., 1998; Mori et al., 1997; Amexis et al., 2001), strongly suggests the role of virus strain variants in disease-inducing potential. Previous studies of the causal association between Urabe AM9 sequence differences and virulence are difficult to interpret due to the fact that genetic analyses were limited to a subset of the virus genes (mostly the HN gene) and relied on passive reporting of adverse events (Brown et al., 1996; Amexis et al., 2001; Afzal et al., 1998). To overcome these limitations, we developed two neuroattenuated variants of the Urabe AM9 vaccine strain (through serial passages in cell culture) and fully sequenced all three viruses. The neurovirulence of these viruses was assessed in rats, a system in which the relative human neurovirulence potential of mumps virus strains can be quantitatively assessed (Rubin et al., 2000; Rubin et al., 2005). Genomic changes associated with neuroattenuation were then identified. Interestingly, the only genomic changes associated with attenuation were in form of changes in the level of genetic heterogeneity at specific genome sites, i.e., disappearance and evolution of nucleotide heterogeneity at various positions. Expectedly, many of the changes associated with attenuation were specific to the cell line used, however, in at least one instance, an identical mutation independently evolved in both passaged viruses. The possible relevance of the identified changes with respect to neurovirulence phenotype is discussed.

Results

Attenuation of Urabe P-AM9 by passage in Vero and CEF cells

CEF and monkey kidney cells have proved to be suitable for attenuation of wild type mumps virus isolates (Plotkin and Wharton, 1999). Therefore, Urabe P-AM9 virus was passaged six times on CEF and Vero cells to create two virus stocks, Urabe-P6-CEF and Urabe-P6-Vero. The in vitro growth curves obtained during passaging showed that in both cell lines viral titers dropped during the first three passages but then gradually increased over the subsequent three passages, suggesting that adaptation to the respective cell lines occurred (Fig. 1). Notably, however, infectious virus titers did not recover to initial levels. To analyze changes in the neurovirulence potential of Urabe P-AM9 passaged in Vero and CEF cells, parental Urabe P-AM9, Urabe-P6-Vero and Urabe-P6-CEF were tested in the newborn rat neurovirulence model. In comparison to the parental Urabe P-AM9, the neurovirulence scores for Urabe-P6-Vero and Urabe-P6-CEF were reduced by 81% and 86%, respectively (Fig. 2). These differences were statistically significant ($P =< 0.001$, Mann–Whitney Rank Sum Test). Thus, using this quantitative disease model, we could demonstrate that passaging of Urabe P-AM9 in both cell lines led to a significant reduction in neurovirulence. Of note, the mean neurovirulence score of Urabe P-AM9 (10.7) is less than typical neurovirulence scores of wild type viruses (usually ranging from 15 to 25), but substantially greater than neurovirulence scores typical of attenuated vaccine strains (usually ranging from 0 to 5) (Rubin et al., 1998, 2000, 2003, 2005).

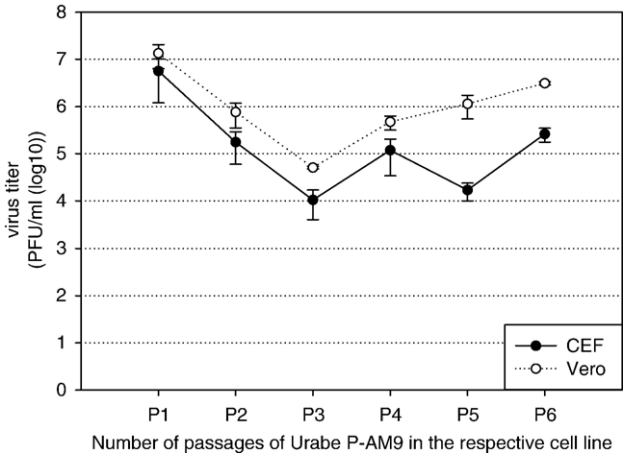

Fig. 1. Amount of Urabe P-AM9 recovered from Vero and CEF cells after repeated passaging. Urabe P-AM9 was passaged six times in Vero and CEF cell lines. Each infection was done at an m.o.i. of 0.05 or lower (see Materials and methods for further information). Virus was harvested when more than 90% of cells were lysed, or, in the absence of detectable cell lysis, at day 7 p.i. Virus titers after each passage were determined at least twice independently. Error bars represent standard error of the mean.

Reduction in neurovirulence phenotype is associated with only a slight decreased ability to replicate in the brain

To investigate if the neurovirulent and neuroattenuated viruses differed in their ability to replicate in the brain, we compared Urabe P-AM9 and Urabe P6-CEF virus growth in rat brain over a 10-day period. As shown in Fig. 3, both viruses exhibited similar growth kinetics in rat brain: Virus titers (1) reached maximum values around day 3 p.i., (2) steadily declined until day 6 p.i., and (3) after day 6 p.i., both viruses appeared to stabilize in titer. However, while a two way analysis of variance (ANOVA) did not reveal a statistically significant difference among the two virus strains over the entire time course ($P = 0.055$), post hoc testing (Mann–Whitney rank sum test) revealed statistically significant differences between the two viruses at day 2 p.i. ($P = 0.023$), and day 3 p.i. ($P = 0.023$), where viral titers were approximately one log lower in brains of Urabe P6-CEF infected rats compared to Urabe P-AM9 infected rats.

Neuroattenuation is associated with changes in the level of genetic heterogeneity at specific genome sites

To associate the observed changes in neurovirulence between Urabe P-AM9, Urabe P6-Vero, and Urabe P6-CEF with changes in the viral genomes, the complete direct sequences of the three virus genomes were determined and are summarized in Table 1.

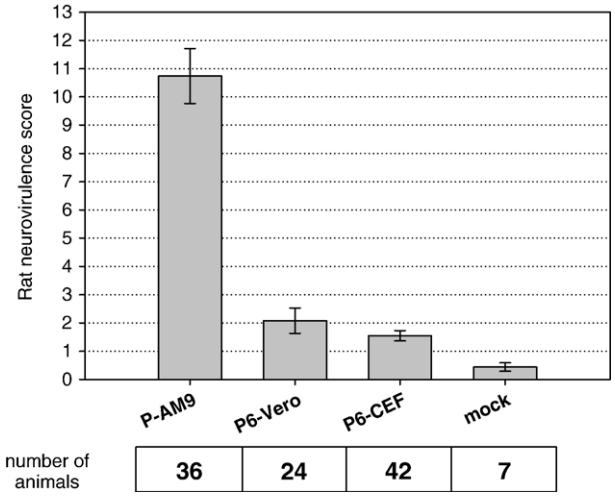

Fig. 2. Severity of hydrocephalus in rats inoculated with Urabe P-AM9, Urabe P6-Vero and Urabe P6-CEF. Newborn rats were inoculated i.c. with the respective viruses and euthanized 30 days later. Brains were processed for histology and the hydrocephalus score was calculated as described in materials and methods. The number of animals tested per different viruses is indicated. Mock: inoculation of rats with noninfectious cell culture supernatant. Error bars represent standard error of the mean.

All sequence differences identified among the Urabe P-AM9 parent and the two passaged viruses were in the form of changes in genetic heterogeneity, i.e., either selection of one nucleotide variant at positions where the starting material exhibited genetic heterogeneity or the evolution of an additional nucleotide to create a heterogenic site, i.e., without the loss of the original nucleotide sequence. Subcloning and sequencing of selected PCR fragments confirmed heterogeneous sites identified in the electropherograms (Table 1).

Fifteen heterogeneous sites were identified in parental Urabe P-AM9, all of which became monotypic in the neuroattenuated Urabe P6-CEF strain (i.e., only one of the two nucleotides at heterogeneous sites in the parental virus were present in the passaged virus). At roughly half of these sites, selection took place in favor of the nucleotide that predominated in Urabe P-AM9, while at four sites selection for the less frequent parental nucleotide occurred. In the remaining sites where parental heterogeneity was lost, the ratio of nucleotides was found at approximately 1:1 in the parent virus. In addition to loss of heterogeneity, at five sites there was an apparent "gain" in heterogeneity, i.e., sites that were formally monotypic in the parental virus became heterogeneous upon passage in CEF cells. At two of these sites, the nucleotide present in the parental virus predominated at the new heterogeneous site in the passaged virus whereas at two other sites the newly evolved nucleotide predominated. Amino acid changes were predicted for three out of the five sites of new heterogeneity (one each in the NP, F and HN proteins).

In comparing the parental Urabe P-AM9 to the neuroattenuated Urabe P6-Vero, seven of the 15 heterogeneous sites in the parental virus became monotypic. Selection at all seven positions took place in favor of the nucleotide that predominated in the parental virus. Of the eight heterogeneous sites that were preserved in the passage process, nucleotide predominance was maintained at three sites and was reversed at four sites. For the remaining site, the ratio of the two nucleotides in the parental virus was approximately 1:1. In addition to loss of heterogeneity, at eight sites there was an apparent gain in heterogeneity upon passage in Vero cells. At all eight sites, the parental nucleotide either predominated or existed at an equivalent proportion to the newly evolved nucleotide. The changes observed at seven out of these eight positions were coding changes (two each in the F and HN genes and three in the L gene. The remaining position was identified in the nontranslated intergenic region between the NP and V/P genes.

Sequencing of the genome ends also revealed sequence heterogeneity. In the case of the 3′ end, up to seven different variants were detected among the three viruses (Urabe AM9, Urabe-P6-Vero and Urabe-P6-CEF) as shown in Fig. 4, with genotype "a" predominating in all cases when the RACE system was used. Interestingly, when the RNA self-ligation method was used, unique genotypes ("c" and "d") were additionally observed. Of note, upon occasion long stretches of A residues were identified upstream the 3′ end of genotype "d" sequences. Whether or not this is an artifact of the method is not clear, though inconsistent results using RNA self-ligation as compared to 3′RACE have been reported previously (Pleschka et al., 2001; Rosario et al., 2005). In the case of the 5′ end, the sequences of all three viruses were identical to the published sequence of the vaccine, with the exception of a truncated sequence seen in one out of eight clones of the Urabe-P6-CEF virus (Fig. 4).

Notably, outside of differences in heterogeneous sites, the nucleotide sequence of Urabe P-AM9 was identical to that of the published sequence of Urabe AM9 SB, the original vaccine strain from which Urabe P-AM9 was derived for the present studies (Amexis et al., 2001; Afzal et al., 1998; Mori et al., 1997; Brown et al., 1996).

Discussion

Wild type mumps viruses are highly neurotropic, with evidence of CNS infection in greater than half of all cases (Bang

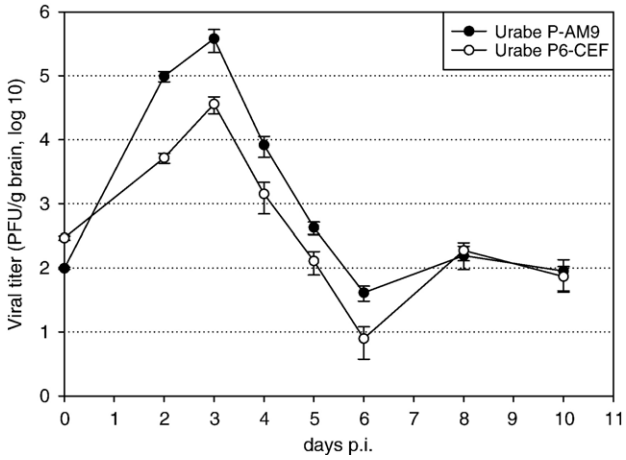

Fig. 3. Virus growth in rat brains inoculated with Urabe P-AM9 and Urabe P6-CEF. For Urabe P6-CEF, five rats were analyzed per time point. In case of Urabe P-AM9, nine rats each were analyzed for days 2, 3, 4 and 6 p.i.; five rats for day 5 p.i. and four rats for day 10 p.i. Error bars represent standard errors of the mean.

Table 1
Comparison of the nucleotide sequences of Urabe P-AM9, Urabe P6-CEF and Urabe P6-Vero

Nucleotide	Gene (aa position)	Urabe P-AM9 [a]		Urabe P6-CEF [a]		Urabe P6-Vero [a]	
46	3′ Leader	A/G (A ≥ G; 6/6)		A		A/G (A ≥ G; 8/3)	
195	NP (17)	T	Leu	T/A (T ≤ A; 9/5)	Leu/His	T	Leu
1880	Intergenic region	C		C		C/A (C > A; 6/2)	
2842	P (289)	T/C (T > C; 5/1)	Leu/Pro	T	Leu	T	Leu
2843	P (289)	T/C (T > C; 5/1)	Leu	T	Leu	T	Leu
4903	F (120)	C/G (C ≥ G; 2/3)	Gln/Glu	C	Gln	C/G (C > G; 5/3)	Gln/Glu
5414	F (290)	A/T (A > T; 3/2)	Tyr/Phe	A	Tyr	A	Tyr
5653	F (370)	A	Thr	A/G (A < G; 3/4)	Thr/Ala	A/G (A ≤ G; 7/5)	Thr/Ala
5723	F (393)	C	Pro	C	Pro	C/T (C > T; 10/2)	Pro/Leu
7365	HN (251)	A	Asp	A	Asp	A/G (A > G; 9/1)	Asp/Gly
7407	HN (265)	C/A (C > A; 7/2)	Thr/Asn	C	Thr	C	Thr
7498	HN (295)	T/C (T > C; 8/2)	Thr	T	Thr	T	Thr
7616	HN (335)	A/G (A = G; 7/7)	Lys/Glu	A	Lys	A/G (A > G; 8/2)	Lys/Glu
8005	HN (464)	C/A (C ≥ A; 12/7)	Asn/Lys	A	Lys	C/A (C < A; 4/7)	Asn/Lys
8015	HN (468)	G/A (G > A; 17/2)	Glu/Lys	G	Glu	G/A (G > A; 10/1)	Glu/Lys
8088	HN (492)	A	Gln	A/G (A < G; 6/3)	Gln/Arg	A	Gln
8174	HN (521)	G	Ala	G	Ala	G/A (G > A; 9/2)	Ala/Thr
9918	L (494)	G	Arg	G	Arg	G/A (G = A; 5/3)	Arg/Lys
9972	L (512)	C/T (C > T; 10/1)	Ser/Phe	T	Phe	C/T (C < T; 2/6)	Ser/Phe
10529	L (698)	C/T (C > T; 4/2)	Leu	T	Leu	C/T (C < T; 1/5)	Leu
11107	L (890)	G/A (G > A; 3/3)	Glu	G	Glu	G	Glu
11521	L (1028)	G	Met	G	Met	G/A (G > A; 5/4)	Met/Ile
11665	L (1076)	C	Gly	T/C (T ≥ C; 5/1)	Gly	C	Gly
11692	L (1085)	G/T (G > T; 5/1)	Leu/Phe	T	Phe	G/T (G < T; 1/8)	Leu/Phe
12291	L (1285)	A/G (A > G; 3/1)	Gln/Arg	A	Gln	A	Gln
12690	L (1418)	A	Glu	A	Glu	A/G (A > G; 9/1)	Glu/Gly
12781	L (1448)	C	His	T/C (T > C; 5/1)	His	C	His

[a] Parentheses indicate the estimated frequency of the two nucleotides at the respective position based on a qualitative evaluation of electropherograms. The numbers shown after the semicolon correspond to the numbers of subcloned and sequenced PCR fragments that possess the respective nucleotides.

and Bang, 1943; Brown et al., 1948). The most common neurological complication of mumps is aseptic meningitis, though more severe consequences including permanent deafness and fatal encephalitis can occur (Koskiniemi et al., 1983; Modlin et al., 1975; Timmons and Johnson, 1970; Ogata et al., 1992; Nussinovitch et al., 1992; Venketasubramanian, 1997; Cohen et al., 1992; Ichiba et al., 1988; Bitnun et al., 1986).

Attenuation of such pathogenic characteristics is a prerequisite for use of a live mumps virus vaccine. However, the

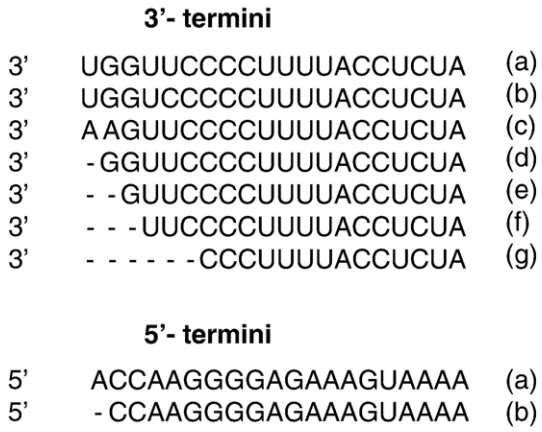

Fig. 4. Sequence heterogeneity observed in Urabe AM9 3′ and 5′ termini. Summary of the different sequences of 3′ and 5′ termini identified in Urabe P-AM9, Urabe P6-Vero and Urabe P6-CEF, as determined by 3′ RACE, 5′ RACE or RNA self-ligation. A total of 15 clones of the 3′ region and 14 clones of the 5′ region were sequenced using the RACE kits. In addition, 15 subcloned RT-PCR fragments that were obtained using the RNA self-ligation method, were sequenced. The sequences designated "a" are the predominant sequences observed. Sequences designated "c" and "d" were only observed using the RNA self-ligation method.

mechanisms for virus neuroattenuation are not understood. As new strains of mumps vaccine continue to be licensed for clinical use, identifying the molecular basis of mumps virus neurovirulence will be of growing value for support of vaccine safety.

The classical approach to attenuation of wild type viruses to produce live virus vaccines is serial passage of the virus in vitro. However, the specific changes responsible for attenuation via this process are not understood, and empiric use of in vitro adaptation to reduce vaccine virus virulence can produce variable outcomes. Taking advantage of a newly developed rat model for quantitative assessment of mumps virus neurovirulence, here, we associate the genomic changes of in vitro passaged viruses with neuroattenuation. Interestingly, despite a dramatic decrease in virus virulence, the only genomic changes were in form of changes in the level of genetic heterogeneity at specific genome sites, i.e., either selection of one nucleotide variant at positions where the starting material exhibited genetic heterogeneity or the evolution of an additional nucleotide to create a heterogenic site. It should be noted that the growth kinetics of the virulent (Urabe P-AM9) and attenuated (Urabe P6-CEF) viruses in brain were similar, suggesting that the observed neuroattenuation is not due to an impaired ability of the attenuated variant viruses to replicate. Nonetheless, the peak titer of the neuroattenuated variant in rat brain was approximately one log lower than that of the more neurovirulent parental strain. Whether or not this relatively small and transient difference in viral load is biologically significant requires further study. It is interesting to note that work with other mumps virus strains showed that inoculation of rats with one log less virus (10 pfu) or one log higher virus (1000 pfu) did not significantly affect the severity of hydrocephalus, suggesting that viral load per se is not responsible for disease severity (unpublished data). Further, in a recent report, where neurovirulent and neuroattenuated variants of a different mumps virus strain (88–1961) were studied, differences in the extent of virus infection, cell type tropism, spread or distribution in rat brain were not observed (Rubin et al., 2003). This suggests that the severity of hydrocephalus (as a measure of neurovirulence) is more of a qualitative, rather than a quantitative, effect of the virus on infected cells. For example, it is conceivable that upon infection by Urabe P-AM9, ependymal cells (the major target of mumps virus infection and the cell type responsible for maintaining the homeostasis of cerebrospinal fluid flow in the ventricular system) rapidly become functionally disabled whereas Urabe P6-CEF infection of these cells may be more sparing. This, and a number of other possibilities, including lytic versus semi-persistent infection, requires further study.

Determination of the complete nucleotide sequence of the parental virus (Urabe P-AM9) and of the two neuroattenuated variants (Urabe P6-CEF and Urabe P6-Vero) revealed limited genomic changes at the nucleotide level. These changes consisted solely of losses and gains in genomic heterogeneity of the passaged viruses relative to the parental strain. In no case did the new mutation in the passaged virus completely replace the nucleotide seen in the parent virus.

In cases where the site-specific heterogeneity observed in the parental virus was lost both in CEF and Vero cell passaged viruses, there was a clear tendency towards selection of the same nucleotide. While in most cases of heterogeneity loss, the predominant nucleotide seen in the parental virus was selected for, there were several instances of a strong selection for the minority nucleotide. It is tempting to speculate that the tendency to select for the same nucleotides in the neuroattenuated CEF and Vero cell passaged viruses indicates a relevance of these changes to the neuroattenuation phenotype.

In addition to loss of heterogeneous sites at some positions, there was an apparent induction of heterogeneity at others. In all such cases, one of the two nucleotides identified in these heterogenic positions was present in the parental virus. Intriguingly, we observed identical, but independent induction of heterogeneity at position 5653 of the F-gene in Urabe P6-Vero and Urabe P6-CEF, again suggesting a relevance of this change to the neuroattenuation phenotype. Because our ability to detect heterogeneous sites was limited to those sites with differences of more than 10% between the detected nucleotides in the consensus sequence, whether or not this "induction" of heterogeneity is the result of the creation of de novo mutations at these positions or is the selection of pre-existing sequences within the minority quasispecies cannot be ascertained.

As to which genes may influence the neuroattenuation phenotype, these results suggest a combination of changes of various genes may be important. Passaging of Urabe P-AM9 in CEF cells and Vero cells led to multiple predicted amino acid changes in the NP, P, F, HN and L proteins. As the NP, P and L proteins are involved in replication/transcription and the HN and F proteins are involved in receptor recognition, fusion events and nascent virus release from infected cells, it is quite plausible that any or all predicted amino acid changes

in these proteins, individually or in combination, could be responsible for the observed attenuation. In addition, non-coding changes occurred at one position in the untranslated 3′ leader region upon passage in CEF cells and at one intergenic position upon passage in Vero cells. As untranslated regions have a regulatory role in genome transcription and replication, it is quite possible that changes in these regions may also affect virus phenotype.

The precise role of these changes on virus function and ultimately on virus virulence will require further study, however, a few of the changes are worthy of discussion here. As depicted in Fig. 5, all of the predicted amino acid changes in

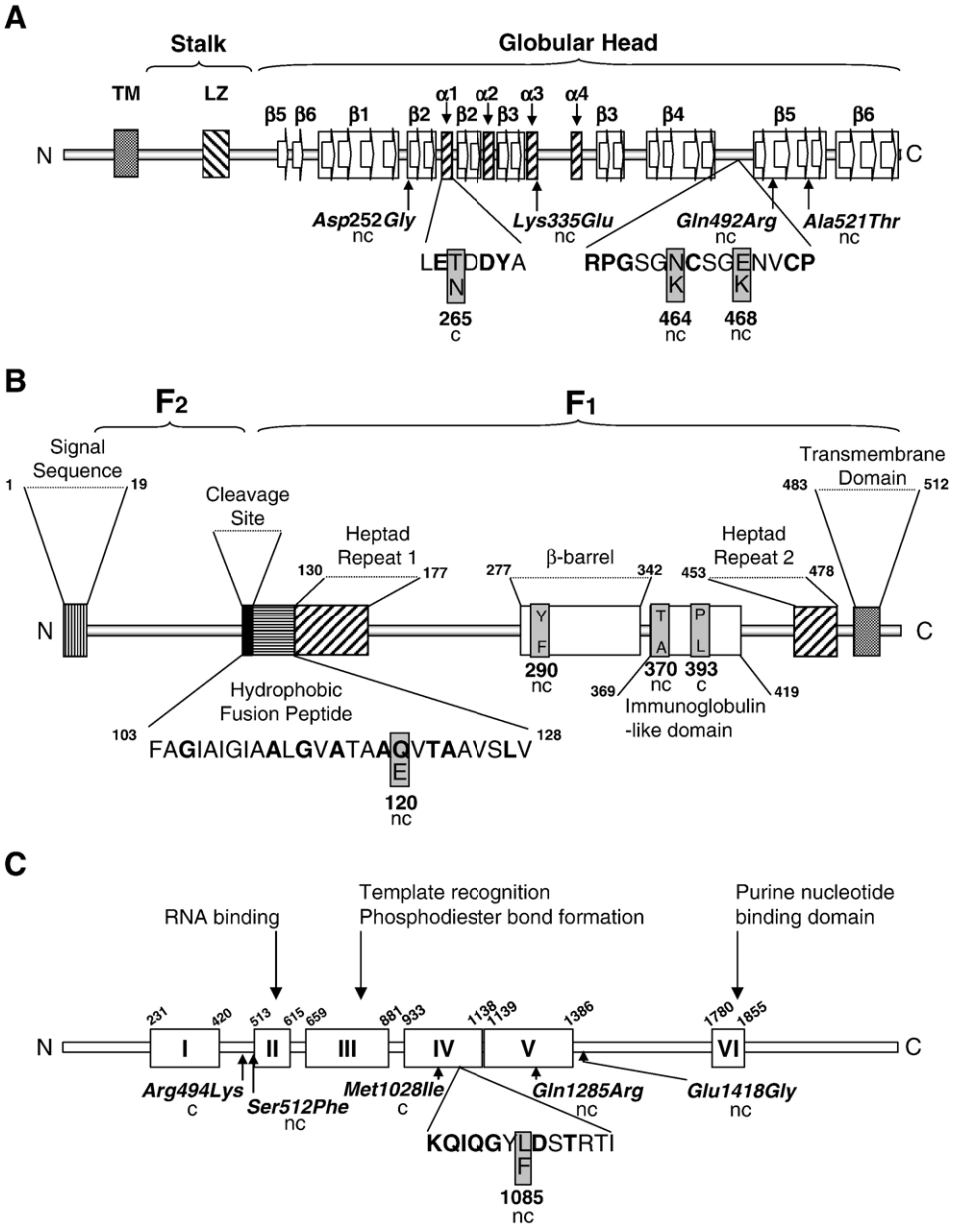

Fig. 5. Schematic diagrams of the HN, F and L mumps virus proteins and the location of identified amino acid heterogeneities. (A) HN protein structural elements are based on the crystal structure from Newcastle disease virus (NDV) (Crennell et al., 2000). TM: transmembrane domain, LZ: leucine zipper domain. Location and numbers of β-strands and α-helices are indicated. The positions of identified aa heterogeneities are indicated below the diagram. Letters in bold depict aa that are conserved among all paramyxoviruses. (B) Identification of F protein domains was based on published protein data for mumps virus F protein (Liu et al., 2004; Waxham et al., 1987), as well as based on sequence homologies to other paramyxoviruses, in particular NDV (Chen et al., 2001). The position of the proteolytic cleavage site as well as of the resulting F1 and F2 subunits is indicated. Small numbers refer to amino acid positions of the various domains (based on mumps virus Urabe AM9, GenBank accession number AF314559). Large numbers indicate aa positions of heterogeneous sites (see also Table 1). The affected aa in the latter are shown boxed. Letters in bold in the depicted sequence of the fusion peptide represent aa highly conserved among paramyxoviruses (see Russell et al., 2004). (C) L protein domains were identified based on (Sidhu et al., 1993). Positions of the domains as well as of identified aa heterogeneities are indicated. The sequence of a conserved motif in domain IV is shown. Letters in bold define aa conserved among rubulaviruses. Panels A–C: nc: non-conservative amino acid change; c: conservative amino acid change.

the HN gene are located in the terminal globular ectodomain, which includes the receptor recognition and neuraminidase active sites (Crennell et al., 2000; Zaitsev et al., 2004). Notably, single point mutations in viral surface proteins, including ones that lead to loss of N-linked glycosylation sites (as is predicted for the Asn464Lys change), can affect virus virulence (Guirakhoo et al., 2004; Hayasaka et al., 2004; Beasley et al., 2005; Panda et al., 2004). The HN amino acid change at position 335 is of particular interest as reports by others have debated the role of a Lys to a Glu substitution at this position with vaccine virulence (Brown and Wright, 1998; Brown et al., 1996; Cusi et al., 1998; Mori et al., 1997; Afzal et al., 1998; Amexis et al., 2001). In the present study, selection of Lys 335 was associated with attenuation.

As shown in Fig. 5, many of the predicted amino acid changes in the F protein (responsible for fusion of the host cell and viral membranes) and the L protein (part of the active RNA dependent RNA polymerase) are located within highly conserved motifs and domains (Morrison, 2003; Russell et al., 2004; Horvath and Lamb, 1992; Poch et al., 1990; Sidhu et al., 1993), suggesting a high potential for affect.

We also observed truncations of the genome ends, especially of the 3′ ends, in some of the subcloned PCR fragments derived from all three viruses. Such truncations may have potential implications for efficient genome and antigenome replication, which in the order mononegavirales is initiated at promoters at the very ends of the genome that exhibit complementarity (inverted terminal repeats). Truncations of genome ends has recently been suggested to represent a strategy to control replication (Schneider et al., 2005; Rosario et al., 2005). Moreover, truncated genome ends have been described for influenza virus (Szymkowiak et al., 2003) and members of the Hantaan virus family, such as Seoul virus, possibly playing a role in Hantaan virus persistence (Meyer and Schmaljohn, 2000). However, since the majority of genome ends in our study were complete and the observed deletions may also be due to RNA degradation, presence of defective interfering particles or a technical bias, the relevance of the observed terminal alterations is unclear.

In summary, our data add further information to the heterogeneous nature of the Urabe AM9 vaccine and demonstrate that changes in viral genetic heterogeneity may have dramatic impact on its neurovirulent potential in vivo. In particular, our data suggest that proportions of viral quasispecies, dynamic mixtures of viral particles whose genomic sequences are closely related but differ at one or more nucleotides (Holland et al., 1992; Domingo et al., 1996), may play as significant a role in virulence as de novo genetic mutations. The importance of genetic heterogeneity for viral neuropathogenesis has been highlighted in a recent publication, which demonstrated that cooperation/interdependence between different virus variants, rather then selection of individual viruses, is a determinant of pathogenesis. Accordingly, viral populations rather than individual variants are proposed to be target of evolutionary selection (Vignuzzi et al., 2005). Our data suggest a partial or complete loss of heterogeneity during passaging of Urabe AM9 in cell culture. It is therefore conceivable that the interplay between different variants present in the original virus seed, rather than one specific variant, is critical for neurovirulence.

Taken together, examining the relative proportions of heterogeneous sites of the whole genome, rather than solely relying on a consensus sequence is an important consideration in the study of the molecular basis of virus virulence.

Materials and methods

Cell lines

Vero (monkey kidney) and CEF (chicken embryo fibroblast) cells were employed for serial passaging the parental Urabe P-AM9 virus to obtain virus variants. Vero cells were cultivated in Eagle's minimum essential medium (E-MEM, Quality Biological, Gaithersburg, MD) supplemented with 2 mM L-glutamine and 9% fetal bovine serum (FBS, Quality Biological, Gaithersburg, MD). The CEF cells were cultivated in Dulbecco's modified Eagle's medium (Quality Biological, Gaithersburg, MD) supplemented with 4.5 g/l glucose, 2 mM L-glutamine, and 9% FBS.

Virus stock

The Urabe-AM9 vaccine strain (Pariorix), manufactured by SmithKline Beecham Biologicals, was kindly provided by Drs. Earl Brown and Kathryn Wright, University of Ottawa, Ontario, Canada. Vaccine stock was expanded by passage twice in Vero cells and stored at −70 °C. The resulting virus stock was designated Urabe P-AM9.

Plaque assay for virus titer

Virus was serially diluted 10-fold in E-MEM, and 0.3 ml of each dilution was incubated for 1 h 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 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 (without phenol red; Quality Biological) 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 subsequently added and incubated overnight, allowing for visualization of the plaques the following day. Virus was quantitated by counting the number of plaque forming units (pfu) and multiplying by the reciprocal of the dilution factor and volume plated.

Infection of cell lines and preparation of virus stocks

Confluent cell monolayers in 75 cm^2 tissue culture flasks were infected with Urabe P-AM9 at a multiplicity of infection (m.o.i.) of 0.05 in a total volume of 5 ml cell culture medium. Following incubation for 1 h at 37 °C, an additional 15 ml of cell culture medium was added and cells were incubated for 7

days (or earlier if cell lysis was evident). To harvest virus, cells were mechanically detached from the flasks, and, along with the supernatant, subjected to ultrasonic treatment and clarified by centrifugation at 1200 rpm for 10 min. Harvested virus was titered and used to infect additional cultures at an m.o.i. of 0.05. This procedure was repeated for a total of 6 passages. For some passages a lower m.o.i. had to be used due to low virus concentrations from the respective previous passages. Virus stocks obtained after 6 passages in Vero and CEF cells were designated Urabe P6-Vero and Urabe P6-CEF, respectively.

Determination of virus titers in brains

Litters of 1-day-old Lewis rats (Harlan, Indianapolis, IN) were inoculated intracerebrally with 20 μl of E-MEM containing 100 pfu of virus as previously described (Rubin et al., 1998). On days 2, 3, 4, 5, 6, 8 and 10 p.i., the brains from 4 to 9 rats from each virus group were removed aseptically. Brains were homogenized into a 20% (wt/vol) suspension in E-MEM with 2% FBS by dounce homogenization, followed by three brief pulses (10 s each) of ultrasonic treatment. Homogenates were clarified by centrifugation at 2000 × *g* at 4 °C for 10 min and stored at −70 °C until examination by plaque assay for virus content per gram of brain.

Neurovirulence phenotype determination

The neurovirulence phenotype of the three virus strains (Urabe P-AM9, Urabe P6-Vero and Urabe P6-CEF) was assessed using the newborn rat-based method previously described (Rubin et al., 2000, 2005). Briefly, on day 30 post-inoculation (p.i.), rats were sacrificed by CO_2 asphyxiation following the NIH Guidelines for the Care and Use of Laboratory Animals. Brains were removed, divided sagitally and fixed in 10% neutral buffered formalin at 4 °C for 4–5 days, followed by paraffin embedding. One 8–10 μm thick sagittal section was selected at a standard distance from either side of the anatomical midline and both were stained with hematoxylin and eosin. The neurovirulence score, which is a measure of the severity of mumps virus induced hydrocephalus, was determined by calculating the cross-sectional area of the brain (excluding the cerebellum) and that of the lateral ventricle on each tissue section 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 is the neurovirulence score for that particular brain. The neurovirulence score for a given virus variant is determined by the mean neurovirulence score for all brains within a treatment group. At least three litters (consisting of at least 7 animals each) were employed for a given virus variant.

Preparation of mumps virus RNA

RNA from mumps virus stocks was extracted with a QIAamp Minelute Virus Spin kit (Qiagen, Valencia, CA) as recommended by the manufacturer. To extract total RNA from mumps virus infected cells, cells were washed with PBS and subsequently lysed with 1 ml of TRIZOL reagent (Invitrogen, Carlsbad, CA). RNA then was prepared according to the manufacturer's instructions.

Mumps virus genome sequencing

Viral RNA extracted from 50 to 75 μl of virus stock was reverse transcribed using mumps virus specific primers. Reverse transcription (RT) was done either using a superscript II kit (Invitrogen) or a sensiscript RT kit (Qiagen) according to the manufacturer's instructions. PCR was done using the Expand high fidelity PCR system (Roche Diagnostics, Indianapolis, IN). Overlapping fragments of 300 bp up to 2000 bp in length were amplified. Sequences of primers used for RT-PCR can be obtained from the authors upon request. PCR fragments were subjected to direct sequencing. To verify presence of heterogeneous sites, selected PCR fragments were additionally subcloned into plasmid pCR2.1-TOPO using the TOPO TA cloning kit (Invitrogen). Four to 19 individual subclones were prepared from each PCR product and were sequenced. A summary of these results is shown in Table 1. DNA sequencing was done using ABI PRISM BigDye terminator sequencing chemistry (Applied Biosystems, Foster City, CA) and ABI 377 or 3100 sequencing technology. Sequence alignments were carried out using Jellyfish software (LabVelocity, Inc., Los Angeles, CA). To determine the 3′ ends of the viruses, RNA extracted from 50 to 100 μl of virus stock was subjected to tailing with A residues using poly-A polymerase (USB, Cleveland, Ohio). After incubation for 15 min at 37 °C, the RNA was extracted with phenol/chloroform and precipitated using ethanol/glycogen (Roche Diagnostics). A-tailed RNA was reverse transcribed with a poly-T adapter primer using a 3′ RACE system (Invitrogen). PCR was performed using 2.5 μl of cDNA, 10 pmol each of abridged universal amplification primer (3′ RACE kit, Invitrogen) and mumps virus specific primer NP437 (5′GAGCTTCAGGTGATCTATCTGC3′).

To determine the 5′ ends of Urabe P-AM9 and Urabe P6-CEF, total RNA was prepared from Vero cells or CEF cells 2 or 4 days after infection with Urabe P-AM9 or P6-CEF, respectively. 5 μg of RNA each were subjected to RT with Superscript II (5′ RACE system, Invitrogen) using 2.5 pmol of mumps virus specific primer 14.75 (5′GGGCCAATCATCAGATCATGACAC3′). Following RNase incubation, cDNA was column purified and 50% of the cDNA was tailed with C residues using terminal deoxynucleotidyl transferase (5′ RACE system, Invitrogen) as recommended by the manufacturer. First round PCR (35 cycles) was performed using 2.5 μl of tailed cDNA, 2.5 units of Taq DNA polymerase (Promega, Madison, WI), 20 pmol each of abridged anchor primer (5′ RACE kit) and of mumps virus specific primer 14.75. Nested PCR (35 cycles) was performed with 1 μl of first round PCR samples using 2.5 units of Taq DNA polymerase (Promega) and 10–15 pmol each of abridged universal amplification primer and mumps virus specific primer 15105 (5′AAGCCATTGGGTGTGTAATC3′).

Due to technical issues, the 5′ end sequence of Urabe P6-Vero was determined by self ligation. Briefly, RNA prepared from 50 μl of virus stock was self-ligated using T4 RNA ligase (NEB, Beverly, MA). After 1-h incubation at 37 °C, RNA was extracted with phenol/chloroform and precipitated using ethanol/glycogen. RNA was subjected to RT using the ThermoScript RT System (Invitrogen). Incubation was done for 1 h at 65 °C using mumps virus specific primer 15105. First round PCR (36 cycles) was carried out using 2.5 μl of primers 15105 and mu0rev (5′AATAACGAAGACTTT-GACTGCTGAC3′). Nested PCR (35 cycles) using 1 μl of first round product was done using primers 15272 (5′ CCAGCATCCAAATTCTTCTAGACC3′) and 117r (5′TCGA-CAATGAAAGGTGAGGATCG3′). 5′- and 3′ RACE, as well as RNA- ligation PCR products were both directly sequenced and subcloned into pCR2.1-TOPO and four to eight subclones each sequenced.

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