

Isolation and characterization of a transfectant influenza B virus altered in RNA segment 6

Kate V. Rowley, Ruth Harvey and Wendy S. Barclay

School of Animal and Microbial Sciences, University of Reading, Whiteknights, PO Box 228, Reading RG6 6AJ, UK

This report describes the successful generation of an influenza B transfectant virus altered in RNA segment 6, which encodes the neuraminidase (NA) protein. The procedure for selection of the transfectant virus relies on the use of strain-specific anti-NA monoclonal antibodies to inhibit growth of the helper virus within the system. A transfectant virus has been engineered which has a coding change in the NA protein. This change resulted in attenuated growth *in vitro* that could be rescued by addition of exogenous bacterial NA. The mutant virus-associated NA activity was unstable as a result of the engineered changes. The ability to genetically manipulate influenza B virus segment 6 will allow us to assess the function of both NA and the small protein NB, also coded from this RNA, within the context of the virus infectious cycle.

Introduction

Influenza B viruses have a negative-strand RNA genome of eight RNA segments complexed with nucleoprotein (NP) and polymerase subunits PB1, PB2 and PA. These ribonucleoprotein (RNP) complexes are packaged within a host cell-derived lipid envelope which displays three virally encoded glycoproteins, haemagglutinin (HA), neuraminidase (NA) and a small protein of unassigned function, NB (Lamb & Krug, 1996).

It is possible to introduce specific changes into the RNA genome of influenza virus (Enami *et al.*, 1990). In this reverse genetics technique, synthetic RNP complexes are transfected into cells previously infected with helper virus. The procedure relies on a stringent selection system to isolate viruses containing the altered RNA segment from a background of helper viruses. A number of selection strategies have been employed, including host restriction (Enami *et al.*, 1990; Subbarao *et al.*, 1993; Grassauer *et al.*, 1998), temperature sensitivity (Enami & Palese, 1991; Li *et al.*, 1995; Egorov *et al.*, 1998), antibody-mediated negative selection (Li *et al.*, 1992; Barclay & Palese, 1995) and antibody-mediated virus trapping (Horimoto & Kawaoka, 1994). The ability to genetically manipulate the influenza virus genome is a powerful method that allows valuable insight into the function of the individual virus genes.

Reverse genetics has been extensively employed to study the influenza A virus NA gene, encoded on RNA segment 6 (Bilsel *et al.*, 1993; Castrucci & Kawaoka 1993; Li *et al.*, 1993; Luo *et al.*, 1992, 1993; Muster *et al.*, 1991; Percy *et al.*, 1994; Zheng *et al.*, 1996). In contrast, studies of the influenza B virus NA protein have been restricted to the analysis of temperature-sensitive mutants (Shibata *et al.*, 1993; Yamamoto-Goshima *et al.*, 1994) or mutants generated by passage of virus in the presence of NA-specific inhibitory compounds (Staschke *et al.*, 1995). Influenza B virus NA is also encoded by segment 6 RNA, and an additional overlapping open reading frame within this segment gives rise to the NB protein. NB is a component of the influenza B virion (Betakova *et al.*, 1996; Brassard *et al.*, 1996) and has been shown to conduct ions (Sunstrom *et al.*, 1996; Chizmakov *et al.*, 1998). These observations suggest that NB is the functional homologue of the influenza A virus protein, M2. However, since no natural mutants with lesions within the NB coding region are described, this hypothesis awaits confirmation. Here we report the first use of reverse genetics to obtain an influenza B virus altered in segment 6. This offers a new strategy for studying the functions of both NA and NB in the virus replicative cycle.

Methods

■ **Generation of plasmids.** Primers used were BNA3', 5' TCTAGA-TCTGAAGACTTAGCAGAAGCAGAGC 3', for RT (complementary to the 3' terminal 14 nucleotides of RNA) and BNA3' in combination with 5'T3H3, 5' GCGCGCAAGCTTAATTAACCCTCACTAAAAG-

Author for correspondence: Wendy Barclay.

Fax +44 1189 316671. e-mail w.s.barclay@reading.ac.uk

TAGTAACAAGAGCATTTC 3' (complementary to the 5' terminal 22 nucleotides of cRNA and including a T3 promoter site), for PCR. The *Bgl*II/*Hind*III-digested PCR product was cloned into *Bam*HI/*Hind*III-digested pUC19 to generate the plasmid, pT3BYNA6. A *Bbs*I site introduced upstream of the virus sequence allowed linearization of plasmid such that T3 transcription produced RNA with authentic termini. We next generated a derivative of pT3BYNA6 containing mutations within the NA open reading frame at nucleotide positions 1229–1231 (numbering with respect to positive-sense segment 6 RNA) which introduced a *Sac*I site as a genetic tag. Two PCR products were produced from pT3BYNA6 using primers *Sac*I+ sense 5' CCATGGACTGACAGTGAGCTCCTT 3' with primer 5'T3H3, and *Sac*I- antisense 5' AAGGAGCTCACTGTCAGTCCATGG 3', with primer NA2 corresponding to nucleotides 987–1007 of positive-sense segment 6 RNA, 5' GCTGAAATAAGATTGATGTGC 3'. Overlapping PCR joined them into a 568 bp product, which was cloned into pT3NA6 using restriction enzymes *Nco*I and *Hind*III to generate the plasmid pT3BYNASaI.

■ **RT-PCR and restriction enzyme digests of PCR products.** pT3BYNA6 transfectants were screened using primers BNA3' and NBSTOP, 5' AATATTGAATTCGCTCGACTAATTAGGAGTGCTTCTG 3' (complementary to nucleotides 330–350 of positive-sense segment 6 RNA). The PCR products were digested with *Hpa*I since this site is not present in the cDNA of B/Lee/40 segment 6 but exists at nucleotides 77–82 within the B/Yamagata/88 sequence. To screen for pT3BYNASaI transfectants, we used PCR primers NA2 and 5'T3H3, which flanked the genetic tag. These PCR products were restricted with *Sac*I.

■ **Viruses and cells.** Influenza B virus strains 2X (Barclay & Palese, 1995) and B/Yamagata/88 were grown by inoculation into 10-day-old hen eggs followed by incubation at 34 °C for 2 days. Infectivity assays were performed by plaquing in MDCK cells. Briefly, viruses diluted in serum-free DMEM were inoculated onto confluent monolayers of cells in 12-well tissue culture plates. Following 1 h incubation at 34 °C, inoculum was removed and cells were overlaid with DMEM containing 0.6% Oxoid agar (Biowhitacker). Following 3 days incubation inverted at 34 °C, remaining cells were visualized by staining with crystal violet and the plaques were counted. MDBK cells for RNP transfection were grown in DMEM containing 10% FCS.

■ **Haemagglutination assay.** Viruses were diluted twofold across 96-well V-bottomed microtitre plates in PBS in a total volume of 50 µl. Fifty µl 0.5% freshly prepared chicken red blood cells were added and plates were incubated at 4 °C for 1 h.

■ **Neuraminidase assay.** An adaptation of the method of Potier *et al.* (1979) was used. Purified viruses were diluted in 0.1 M KPO₄ pH 5.9 and 0.5 mM substrate 2'-(4-methylumbelliferyl)- α -D-N-acetylneuraminic acid (4-MUNANA, Sigma) was added. Assays were performed in U-bottomed microtitre plates in a total volume of 10 µl. Plates were incubated in a water bath at 37 °C for 30 min unless otherwise stated, and reactions were stopped by addition of 200 µl 0.1 M glycine pH 10.7 containing 25% ethanol. Fluorescence was read on a Biolumin 960 fluorescence spectrophotometer (Molecular Dynamics) with excitation wavelength of 360 nm and emission wavelength of 460 nm. Protein assays were performed using BCA protein assay reagent (Pierce).

Results and Discussion

We applied a helper-dependent RNP transfection coupled with negative selection using NA strain-specific monoclonal antibodies. We first cloned segment 6 RNA of influenza B virus

strain B/Yamagata/88 by RT-PCR from vRNA using previously described methods (Stevens & Barclay, 1998) to generate plasmid pT3NA6. We next introduced nucleotide changes in the NA coding region by site-directed mutagenesis using PCR to create plasmid pT3NASaI (Fig. 1*a*). We screened anti-NA monoclonal antibodies and identified three which could inhibit growth of influenza B virus strain B/Lee/40 but not of B/Yamagata/88. They are known as 2D5, 3C6 and 1B9 and show different patterns of reactivity with a panel of strains of influenza B virus (data not shown). For RNP transfection, 10⁶ MDBK cells were infected with Md2X helper virus at a multiplicity of 1. The Md2X helper virus incorporates a B/Maryland/59 HA segment in the background of seven segments from B/Lee/40 (Barclay & Palese, 1995). RNP complexes derived from *in vitro* transcription of plasmids pT3BYNA6 or pT3BYNASaI were transfected into cells according to protocols described previously (Enami *et al.*, 1991; Barclay & Palese, 1995). Supernatants were passaged twice in MDCK cells in the presence of monoclonal antibodies 2D5 and 3C6 at immunoglobulin concentrations of 10 ng/ml and 470 ng/ml, respectively, then plaqued in the presence of the same concentration of antibodies. Individual plaques were screened immunohistochemically as follows: duplicate dishes of cells infected with each plaque were screened for reactivity with anti-influenza B virus NP monoclonal antibody MAS774b (Harlan Seralabs) or with monoclonal antibody 1B9, which had not been used in the selection. Binding of primary antibody was detected by a β -galactosidase-linked anti-mouse antibody and X-Gal substrate (Ward *et al.*, 1994). Reactivity with 1B9 indicated that the virus was a helper virus mutant which had escaped inhibition by 2D5 and 3C6 antibodies. In a typical experiment, approximately half of the viruses fell into this category. Viruses that reacted with MAS774b but failed to react with 1B9 were grown in embryonated hen's eggs. Viral RNA was extracted as previously described (Stevens & Barclay, 1998) and RT-PCR was performed to confirm the origin of segment 6 (Fig. 2).

We successfully isolated transfectant viruses derived from pT3BYNA6 and pT3BYNASaI. Of those viruses which failed to react with any of the three B/Lee/40-specific NA monoclonal antibodies, two out of three tested were transfectants (Fig. 2*a*, NA6 and NA7, and *b*, #15 and #16). The others were probably derivatives of helper virus which had acquired mutations in the 1B9 epitope. Their sequences were not determined. The stringency of the selection procedure may ultimately be improved by incorporating mutations which confer resistance to the NA-specific antiviral GG167 (Von Itzstein *et al.*, 1993; Staschke *et al.*, 1995) into pT3BYNA6, and using drug in addition to antibodies to inhibit helper virus.

The introduction of a *Sac*I restriction enzyme site at nucleotides 1229–1231 resulted in a change in amino acids 393 and 394 of the NA protein from aspartate-alanine to glutamate-leucine. These residues are conserved in all influenza B viruses sequenced to date except B/Hong Kong/73, which possesses

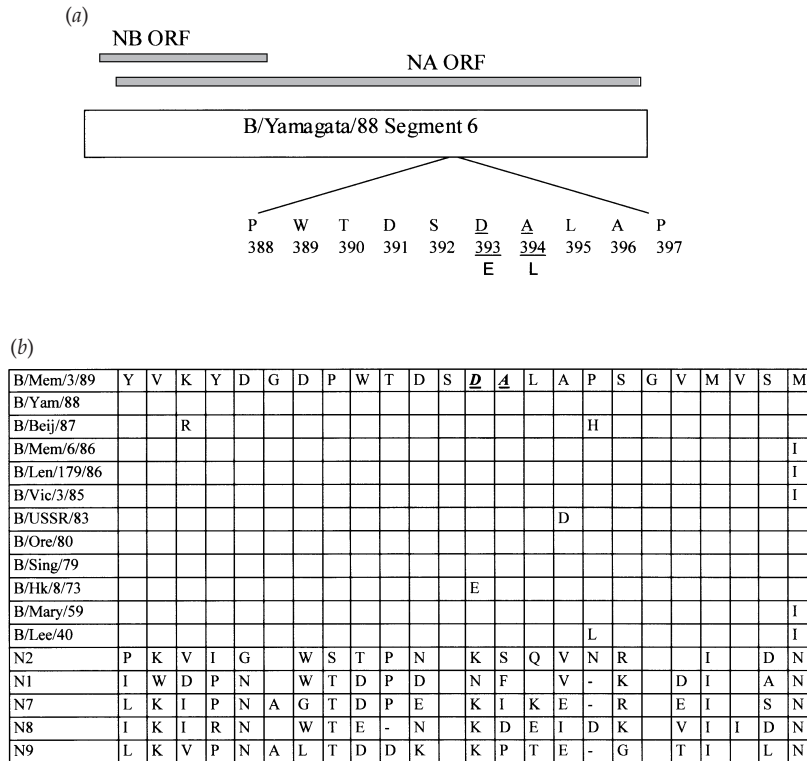


Fig. 1. Introduction of mutations into the influenza virus B/Yamagata/88 NA gene. (a) Schematic representation of segment 6 RNA of influenza B/Yamagata/88 virus. The two open reading frames, NA and NB are illustrated by grey bars. Numbers indicate amino acid residues within the NA protein. Coding changes introduced by the *SacI* mutation are underlined. (b) Comparison of influenza virus NA sequences surrounding amino acids 393 and 394. Amino acid residues for 11 influenza B virus NA proteins and five influenza A virus NA proteins are compared to the sequence of B/Memphis/3/89 NA. Residues 393 and 394 are shown in bold and underlined. Accession nos: B/Beijing/87, M54967; B/Yamagata/88, X67013; other influenza B viruses from Air *et al.* (1990) and influenza A viruses from Coleman (1989).

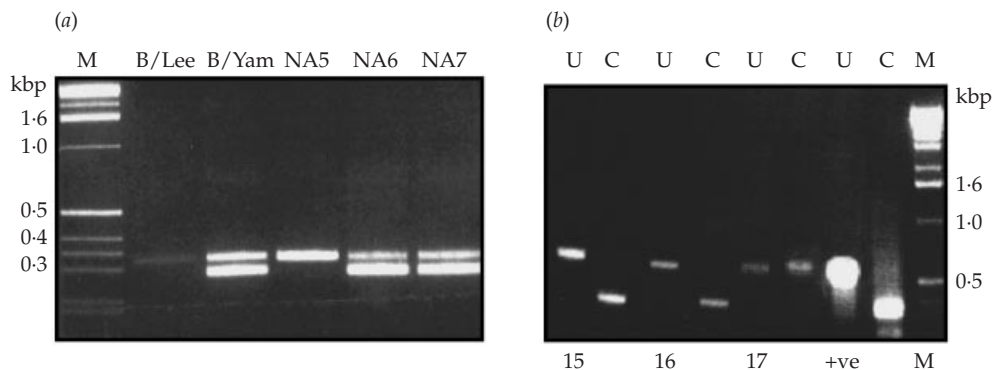


Fig. 2. Restriction enzyme analysis of PCR products generated by RT-PCR from vRNA of putative transfectant viruses. (a) RT-PCR was performed with primers which amplify the 3' region of the segment 6 vRNA. In the B/Yamagata/88 strain, this region contains an *HpaI* site which is not present in vRNA of B/Lee/40. PCR products digested with *HpaI* were analysed on a 2% agarose gel stained with ethidium bromide. Approximate sizes of DNA markers (M) are shown to the left. The 367 bp products from viruses with B/Yamagata/88 sequence were cut with *HpaI* to yield 271 bp and 96 bp fragments. Control viruses were B/Lee/40 and B/Yamagata/88. Results for three putative transfectant viruses, NA5, NA6 and NA7 are shown. (b) RT-PCR was performed with primers which amplify the 5' region of segment 6 vRNA. The 591 bp RT-PCR product digested with *SacI*, C, was run next to undigested PCR product, U, on a 2% agarose gel stained with ethidium bromide. Approximate sizes of DNA markers (M) are shown to the right. PCR product from plasmid pT3NAYS*SacI* DNA, which contains the genetic tag, was cut into 402 bp and 189 bp fragments (+ve). PCR products following RT from RNA of putative transfectant viruses isolates #15, #16 and #17 are shown.

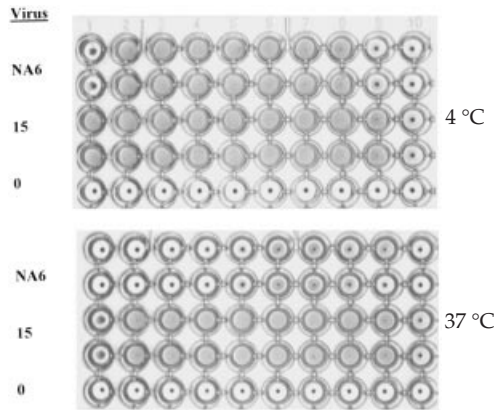


Fig. 3. Haemagglutination activity of transfectant virus. Loss of HA activity at 37 °C was compared for wild-type (NA6) and transfectant virus (#15). Viruses were diluted twofold across the plate from left to right and incubated with 50 μ l 0.5% suspension of freshly washed chicken red blood cells. A row of red blood cells without virus is shown at the bottom of each panel as a negative control. The upper panel shows HA activity following incubation at 4 °C. In the lower panel plates were shifted to 37 °C after the appearance of HA and monitored after a further 2 h.

glutamate at residue 393. There is also variation within the homologous regions of influenza A virus NA (Fig. 1*b*). We predicted that changes in this region might affect NA enzyme activity, since reference to the three-dimensional structure of influenza virus B/Beijing/87 NA protein revealed that these residues were situated in the head region of the protein near to the enzyme active site (Burmeister *et al.*, 1992). A crude measure of NA enzyme activity is the loss of red blood cell agglutination when plates are incubated at temperatures at which the NA is active. This is due to cleavage of sialic acid receptors from the erythrocytes. We performed haemagglutination assays with isolate #15 derived from pT3BYNASaI (Fig. 2*b*) and with the wild-type transfectant virus NA6 (Fig. 2*a*). After noting the HA titres, we shifted the plates to 37 °C and continued the incubation for a further 2 h. We noticed that whilst haemagglutination was lost from wells containing high concentrations of wild-type virus, this effect was not seen for the mutant, suggesting that the mutant virus NA was less active (Fig. 3).

Changes in NA enzyme activity may lead to attenuation of virus growth. Following low multiplicity infection of MDCK cells, the mutant virus #15 showed lower titres of released virus than the wild-type virus until 60 h post-infection, but at 72 h, titres of both viruses were comparable (Fig. 4*a*). The attenuation of mutant virus titre at early times was overcome by propagating the virus in the presence of 0.025 mg/ml exogenous bacterial NA (purified from *Clostridium perfringens*, Sigma) (Fig. 4*b*). Neuraminidase acts late in the virus life-cycle to aid release of progeny viruses from the cell (Palese *et al.*, 1974). When NA activity is decreased, HA on progeny viruses binds sialic acid residues at the infected cell surface, preventing release of virus. However, at late times after infection, or in the presence of exogenous bacterial NA, the amount of NA in the

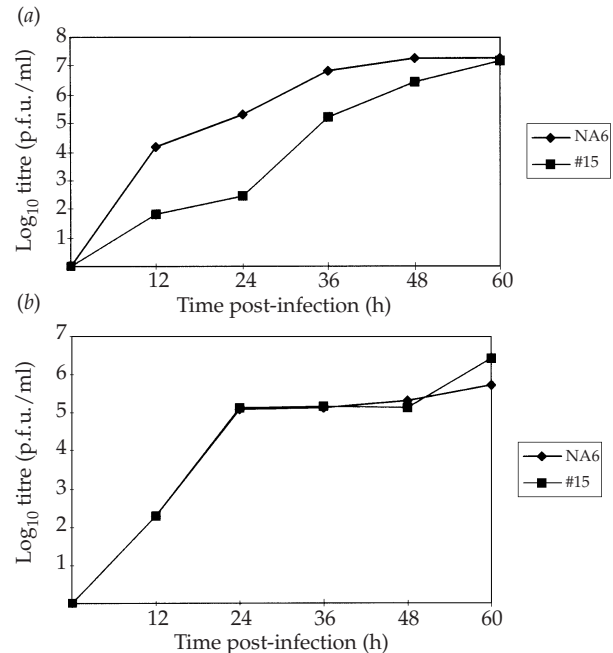


Fig. 4. Growth phenotype of transfectant virus. (a) Multistep growth analysis of transfectant virus. MDCK cells were infected with NA6 virus (wild-type) or isolate #15 (YNASaI) at an m.o.i. of 0.001 and overlaid with serum-free DMEM containing 2 μ g/ml trypsin. At 12 h intervals, supernatants were removed and released virus titrated by plaque assay in MDCK cells in the presence of 2 μ g/ml trypsin. (b) Multistep growth analysis of transfectant virus in the presence of exogenous NA. Growth analysis was repeated as in (a) in the presence of 0.025 μ g/ml endogenous bacterial NA from *Clostridium perfringens* (Sigma).

system has increased and this facilitates release of the mutant virus.

The complementation of virus growth by addition of exogenous NA suggested that the mutant phenotype was indeed due to a decrease of NA enzyme activity. To confirm this hypothesis, the activity of virion-associated NA was tested using 4-MUNANA (Potier *et al.*, 1979) as substrate. Both viruses were propagated to high titres in eggs and then concentrated by ultracentrifugation through a 30% sucrose cushion. Total protein assays combined with Coomassie gel analysis of virus preparations were used to standardize virus inputs to the NA assay. The mutant virus showed a slightly lower rate of substrate conversion and enzyme activity than wild-type (Fig. 5*a*). We next assessed the possibility that the engineered mutations have affected the stability of the NA enzyme. Indeed, mutations at the catalytic site of influenza A virus NA protein have been shown to decrease the stability of NA (McKimm-Breschkin *et al.*, 1996; Colacino *et al.*, 1997). We incubated equal concentrations of purified viruses overnight at 34 °C, 37 °C or 39 °C and tested the remaining NA activity. The activity of wild-type virus was relatively unaffected whereas that of the mutant was substantially decreased with the effect increasing at higher temperatures (Fig. 5*b*). Thus, when virus is incubated at growth temperatures of 34–37 °C the half-life of the NA enzyme is very short, leading to a lower

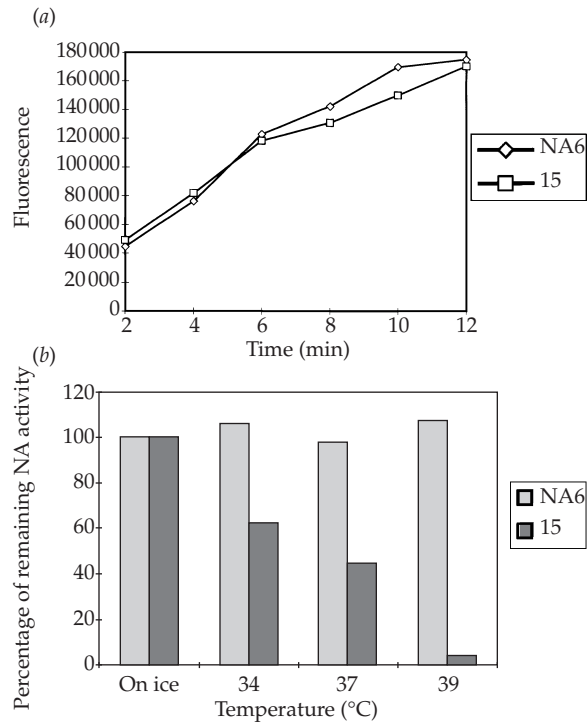


Fig. 5. Neuraminidase enzyme assay of transfectant virus. (a) Initial rates of activity. Viruses were grown in eggs and concentrated by centrifugation through 30% sucrose in 100 mM NaCl, 10 mM Tris-HCl (pH 7.8), 1 mM EDTA. Protein content was assayed by BCA kit (Pierce). Samples of 250 ng of each virus were incubated with 0.5 mM 4-MUNANA for 2 to 12 min. (b) NA activity of transfectant virus after overnight incubation at different temperatures. Aliquots of each virus were incubated overnight on ice or at 34 °C, 37 °C, 39 °C and then 250 ng was incubated with 0.5 mM 4-MUNANA for 30 min.

release of virus until such time when NA is so abundant that a sufficient enzyme concentration is reached. We investigated the plaquing ability of virus #15 at 34 °C, 37 °C and 39 °C. Plaques were formed at all temperatures, but the appearance of the plaques became smaller and clearer as the temperature increased (data not shown). The wild-type virus also formed plaques at these temperatures but there was no obvious difference in their appearance. Recently the NA protein of an influenza A virus selected for resistance to GG167 was shown to be thermally unstable due to a coding change at the enzyme active site. The NA was found to exist as monomers instead of tetramers and the authors assumed that the monomers were either inactive or less active as enzymes. In that case a salt bridge between the altered residue and an amino acid at the intersubunit interface was destabilized (Colacino *et al.*, 1997). We have not assessed the oligomeric form of our mutant. However, by reference to the published structure of influenza virus B/Beijing NA (Burmeister *et al.*, 1992), we propose that alteration of amino acids 393 and 394 might also affect intersubunit interactions.

Several influenza virus mutants with decreased NA enzyme activity have recently been described in the literature (Staschke

et al., 1995; Blick *et al.*, 1995, 1998; Gubareva *et al.*, 1996, 1998; McKimm-Breschkin *et al.*, 1996, 1998; Tai *et al.*, 1998). This group of mutants have been isolated by passage in the presence of NA enzyme inhibitors and are resistant to the compounds. We tested whether our mutant influenza B virus showed resistance to the antiviral agent GG167 (zanamavir), which targets NA activity (Von Itzstein *et al.*, 1993). This was not the case. Both the wild-type and the mutant virus were equally sensitive to the compound and were equally inhibited at a range of drug concentrations from 0.3 to 0.0003 µg/ml (data not shown). Interestingly, we again noticed that for both viruses, the appearance of the plaques became smaller but clearer as drug concentration increased and NA activity concomitantly decreased (data not shown). It is still possible that the mutant virus could show a difference in drug sensitivity during growth in a different system. It has been reported that an influenza B virus which was resistant to GG167 *in vivo* did not display this phenotype when tested in MDCK cells *in vitro* (Gubareva *et al.*, 1998). Infection of ferrets with virus #15 in the presence of GG167 would be required to establish if this were the case for our mutant. The phenotype of our mutant virus clearly differs from that reported for the majority of influenza GG167-resistant viruses, which turn out to have acquired mutations in both NA and HA proteins that act synergistically to confer drug resistance (Staschke *et al.*, 1995; Blick *et al.*, 1995, 1998; Gubareva *et al.*, 1996; McKimm-Breschkin *et al.*, 1996, 1998). The lower affinity of the mutant HAs for the sialic acid receptor, which offsets the decreased activity of the NA enzyme during virus release, results in attenuation throughout multi-cycle replication because viruses bind and enter cells less well. Thus these viruses do not attain wild-type titres even at late times after infection, whereas our mutant #15 does (Fig. 4). In summary, using traditional methods for isolating NA mutants, concomitant changes in HA are likely to be selected. Indeed, it was found that HA mutants were the first to arise during GG167 selection (Blick *et al.*, 1998). In contrast, the reverse genetics procedure described here allows the introduction of changes in NA without additional changes in HA. Sequence comparisons of the entire HA open reading frames of virus NA6 and virus #15 have shown that there were no additional changes selected in the HA protein in the transfectant virus.

The mutations in the NA protein of BYNASaI at residues 393 and 394 are located in the head region of the enzyme (Burmeister *et al.*, 1992). Some natural isolates show sequence variation in this area. For example at amino acid 397 a histidine in B/Beijing is changed to proline in B/Yamagata NA (Fig. 1b) (Burmeister *et al.*, 1993). That this does not result in any loss of enzyme activity is not surprising since during natural evolution of influenza B virus strains, attenuating mutations are unlikely to be selected. However, it is likely that engineered mutations could affect the surrounding structure and compromise enzyme activity. Indeed, using site-directed mutagenesis of NA proteins expressed from recombinant SV40 viruses, Air *et al.*

(1990) showed that single amino acid changes at residues 251, 364 or 368 all decreased NA enzyme activity. The work presented here offers the ability to examine the effects of those and other mutations in NA on the phenotype of the virus. It will also enable the study of isolated mutations with the potential to confer resistance to NA inhibitors, as well as allowing insight into the role of the NB protein.

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