Behaviour of influenza A viruses differentially expressing segment 2 gene products in vitro and in vivo

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The influenza A virus genome comprises eight segments of negative-sense RNA that encode up to 12 proteins. RNA segment 2 encodes three proteins, PB1, PB1-F2 and N40, that are translated from the same mRNA by ribosomal leaky scanning and reinitiation. PB1 is a subunit of the trimeric viral RNA polymerase. PB1-F2 has been reported to be a potential virulence factor, and has been shown to be involved in a number of activities including induction of apoptosis, regulation of virus replication and modulation of the immune response. No function has yet been ascribed to N40, which represents an N-terminally deleted form of PB1. Previous studies on PB1-F2 function mainly used viruses genetically engineered to prevent PB1-F2 expression by mutation of the PB1-F2 start codon. However, ablation of the start codon was shown to increase the expression level of the downstream protein N40. In the present study, we generated recombinant A/WSN/33 viruses carrying different combinations of PB1-F2- and N40-knockout mutations. Overexpression of N40 in a PB1-F2-deficient background had a detrimental effect on virus growth in vitro and in vivo. However, ablation of PB1-F2 or N40 expression individually was not disadvantageous for the virus. Primer-extension analyses revealed an increase in vRNA production by viruses that overexpressed N40. Our data suggest that the observed attenuation of mutant viruses in vitro and in vivo results from these changes in transcription and replication.

INTRODUCTION

Influenza A viruses remain a major health and economic burden worldwide. Seasonal influenza epidemics affect millions of individuals each year, while sporadic influenza pandemics result in millions of human deaths across the world (Medina & Garcia-Sastre, 2011). The genome of influenza A viruses comprises eight segments of negative-sense ssRNA that encode 11 or 12 proteins. RNA segment 2 in influenza A viruses comprises eight segments of negative-sense RNA that encode up to 12 proteins. RNA segment 2 encodes three proteins, PB1, PB1-F2 and N40, that are translated from the same mRNA by ribosomal leaky scanning and reinitiation. PB1 is a subunit of the trimeric viral RNA polymerase. PB1-F2 has been reported to be a potential virulence factor, and has been shown to be involved in a number of activities including induction of apoptosis, regulation of virus replication and modulation of the immune response. No function has yet been ascribed to N40, which represents an N-terminally deleted form of PB1. Previous studies on PB1-F2 function mainly used viruses genetically engineered to prevent PB1-F2 expression by mutation of the PB1-F2 start codon. However, ablation of the start codon was shown to increase the expression level of the downstream protein N40. In the present study, we generated recombinant A/WSN/33 viruses carrying different combinations of PB1-F2- and N40-knockout mutations. Overexpression of N40 in a PB1-F2-deficient background had a detrimental effect on virus growth in vitro and in vivo. However, ablation of PB1-F2 or N40 expression individually was not disadvantageous for the virus. Primer-extension analyses revealed an increase in vRNA production by viruses that overexpressed N40. Our data suggest that the observed attenuation of mutant viruses in vitro and in vivo results from these changes in transcription and replication.
distribution of the protein, PB1-F2 was also found in the nucleus of infected cells, where it interacts with the polymerase subunit PB1 (Mazur et al., 2008); viruses lacking PB1-F2 showed a decrease in polymerase activity in a minigenome system. However, recent data show that the ability to enhance polymerase activity is strain- and cell type-specific (Chen et al., 2010; McAuley et al., 2010b).

Despite these observations, the importance of PB1-F2 remains unclear, as introduction of a full-length protein into the 2009 H1N1 pandemic influenza virus did not enhance virulence significantly in mice or ferret models (Hai et al., 2010).

In 2009, a twelfth protein expressed by influenza A viruses was found. This protein, named PB1-N40 or N40, is an N-terminally deleted version of PB1, lacking the first 39 aa of PB1 that are responsible for binding the polymerase subunit PA, and its translation is initiated from the fifth AUG codon (Wise et al., 2009). N40 has a predominantly cytoplasmic localization and is not transcriptionally active. Two short ORFs (sORFs), initiated at AUG codons 2 and 3, are also present on segment 2, and sORF2 regulates expression of PB1-F2 and N40. In particular, sORF2 upregulates N40 by a reinitiation mechanism that allows skipping of the PB1-F2 AUG (Wise et al., 2011). The role of N40 in the virus life cycle remains unknown. Expression of all three proteins is interdependent, and deletion of the PB1-F2 start codon increased levels of N40, whereas removal of the N40 start codon influenced the levels of PB1 (Wise et al., 2009).

As many previous studies on PB1-F2 involved mutating its start codon, the level of N40 expression would have been increased inadvertently. This makes it difficult or even impossible to ascribe the observed effects to loss of PB1-F2 as opposed to an increase in N40 expression. Therefore, we created recombinant A/WSN/33 viruses lacking PB1-F2 that differed in their expression level of N40. Additionally, viruses lacking N40 or both PB1-F2 and N40 were rescued. By comparing the replication features of all viruses in vitro and in vivo, we observed a negative impact of over-expressed N40, whereas loss of either PB1-F2 or N40 alone was not apparently disadvantageous for the virus.

RESULTS

Characterization of A/WSN/33 PB1-F2 deletion viruses

In previous reports describing influenza A viruses that did not express any PB1-F2, it was found to be necessary, in addition to mutating the start codon of the PB1-F2 ORF, to mutate downstream in-frame AUG codons or to introduce downstream stop codons, to prevent synthesis of C-terminal products (Le Goffic et al., 2010; Mazur et al., 2008; Zamarin et al., 2006). Therefore, to ablate PB1-F2 expression in influenza A/WSN/33 (WSN) virus, we made three mutations (T120C, C153G and G291A) in the segment 2 cDNA clone (Fig. 1b). The modified plasmid was used in the 12-plasmid reverse-genetics protocol (Neumann et al., 1999) and the resulting virus was called rWSNΔAUG. In addition, we created a second virus in which only the two stop codons (C153G and G291A) were introduced into the PB1-F2 ORF, and the rescued virus was named rWSN-F2-11 (Fig. 1b). This virus could potentially express an 11 aa peptide representing the N terminus of PB1-F2. None of these mutations altered the amino acid sequence of the PB1 or N40 protein.

We compared the proteins synthesized by the mutant viruses with those made by wild-type virus (rWSN WT). As seen in Fig. 1(c), expression of PB1 was not altered in either of the mutant viruses, and was similar to that of rWSN WT. No PB1-F2 was detected in cells infected with either of the mutant viruses rWSNΔAUG or rWSN-F2-11. However, a striking difference was noted in the expression levels of N40. In rWSNΔAUG-infected cells, the level of N40 was markedly increased, as recorded previously for other influenza A virus strains carrying analogous mutations (Wise et al., 2009, 2011). However, in cells infected with rWSN-F2-11, the level of N40 was similar to that in cells infected with rWSN WT.

Consistent with a previous publication on the PB8 strain of influenza virus (Mazur et al., 2008), we observed a small-plaque phenotype for rWSNΔAUG in Madin–Darby canine kidney (MDCK) cells (Fig. 1d). However, the plaques produced by rWSN-F2-11 were similar in size to those produced by rWSN WT. Thus the difference in plaque size correlated with increased expression of N40 in combination with the lack of PB1-F2, rather than with loss of PB1-F2 alone.

Characterization of A/WSN/33 N40 deletion mutants

To investigate a possible role of N40 in the virus life cycle, we generated two N40-deletion viruses. We mutated the start codon of N40 (A142T) in segment 2 cDNA and the rescued virus was named rWSNΔN40. This mutation led to an amino acid change in PB1, Met40Leu (Fig. 2a). The second mutant was designed to ablate expression of both N40 and PB1-F2, and contained two mutations, G144A and G291A; the rescued virus was called rWSNAF2/ΔN40, and carries a Met40Ile change in PB1 (Fig. 2b). No N40 expression could be detected in cells infected with either mutant (Fig. 2c). The amount of PB1-F2 in cells infected with rWSNΔN40 was similar to that in cells infected with rWSN WT, whereas no PB1-F2 could be detected in cells infected with the double mutant. No change in plaque size compared with wild-type virus was observed for either N40-deletion virus (Fig. 2d); notably, loss of PB1-F2 did not change the size of the plaques of rWSNAF2/ΔN40.

Effects of PB1-F2 and N40 deletion on virus replication in vitro

The growth kinetics of the recombinant viruses were monitored in MDCK cells infected at a low m.o.i. of 0.001.
Supernatant fluids were collected at the indicated time points and released virus was titrated by plaque assay (Fig. 3). Loss of PB1-F2 alone (rWSN-F2-11) caused a small decrease in virus titre compared with wild-type virus (Fig. 3a) but, when combined with increased expression of N40 (rWSN*ΔAUG), virus replication was more obviously attenuated. Viruses lacking a functional N40 ORF showed a slight reduction in virus growth compared with wild-type virus, but this was independent of PB1-F2 expression (Fig. 3b). These results indicate that loss of neither PB1-F2 nor N40 had a dramatic negative effect on virus replication, but increased levels of N40 in a PB1-F2-deficient background attenuated virus growth in MDCK cells.

**Effects of PB1-F2 and N40 in a minigenome assay**

We thought that the observed reduction in virus replication in cultured cells might be reflected by an impaired production of viral RNAs or proteins. To test the former, we performed minigenome assays with the different PB1 mutants. Therefore, 293FT cells were transfected with plasmids expressing the viral proteins PB2, PA and NP, together with wild-type or mutant PB1-expressing constructs, together with the minigenome-expressing plasmid [comprising the Renilla luciferase gene, in a negative-sense orientation, flanked by influenza segment UTRs (Lutz *et al.*, 2005)]. A plasmid expressing the firefly luciferase gene served as an internal transfection control. The amount of *Renilla* luciferase expressed is a measure of the activity of the viral polymerase. We first compared minigenome activity in cells expressing all segment 2 gene products (i.e. from the wild-type PB1-expressing plasmid) with those in cells expressing the various mutant segment 2 cDNAs (Fig. 4a). Whilst loss of PB1-F2 did not affect minigenome activity markedly (ΔAUG or F2-11), the absence of N40 had a negative
effect on the viral polymerase. This decrease in activity was exacerbated when PB1-F2 was also not expressed (ΔF2/ΔN40). Expression of just N40 did not support minigene activity, indicating that full-length PB1 is needed. Additional expression of PB1-F2 in WT PB1- or PB1-F2-deficient minigene assays had no significant effect on viral polymerase activity, and, while expressing extra N40 stimulated the WT activity mildly, it had no effect in the PB1-F2-deficient minigene assay (Fig. 4b). Additional expression of N40 in ΔN40-transfected cells increased minigene activity slightly, although this change was not statistically significant (Fig. 4c). Expression of N40 or PB1-F2 alone in ΔF2/ΔN40-transfected cells also resulted in only a slight increase in activity. However, when both N40 and PB1-F2 were supplied into cells programmed with the ΔF2/ΔN40 construct, a significant increase in minigene activity was seen (Fig. 4c).

**Fig. 2.** Deletion of the N40 ORF. (a) Deletion of N40 by introduction of a single point mutation. This causes an amino acid change in the PB1 protein (Met40 Leu). (b) Deletion of both PB1-F2 and N40 ORFs. Two point mutations were introduced (G144A, G291A). The first mutation removes the start codon of N40 and introduces a stop codon into PB1-F2 ORF after residue 8. The change also alters residue 40 in PB1 (Met40Ile). The second point mutation is silent in the PB1 ORF, and introduces a second stop codon into the PB1-F2 ORF to abolish possible expression of the C terminus of PB1-F2. (c) Western blot analysis of cells infected with the panel of recombinant viruses. MDCK cells were infected at an m.o.i. of 3 and cell lysates were analysed for the accumulation of the indicated proteins with specific antibodies. (d) Plaque size. Confluent MDCK cell monolayers were infected with the panel of viruses and plaques were allowed to develop under a semi-solid overlay (1.2 % Avicel). Mean ± SEM plaque sizes at 72 h p.i. are plotted (P<0.05) on the graph below.

**Fig. 3.** Growth properties of PB1-F2 and N40 mutant viruses. Confluent MDCK cells were infected at an m.o.i. of 0.001 for 48 h with rWSN WT and (a) PB1-F2-deficient viruses (rWSNΔAUG and rWSN-F2-11) or (b) N40-deficient viruses (ΔN40 and ΔF2/ΔN40). Supernatants were collected at indicated time points and virus titres were determined by plaque assay. Mean ± SEM virus titres are plotted (*P<0.05).

**Effects of PB1-F2 and N40 on viral RNA accumulation**

As minigene systems are a rather indirect way of assessing viral RNA synthesis, we investigated genome replication and transcription in infected cells more directly by using primer-extension analysis. A549 cells were infected at an m.o.i. of 3 and total cellular RNA was extracted at 8 h post-infection (p.i.). Specific [γ-32P]ATP-labelled primers were used to amplify mRNA, cRNA or vRNA from genome segment 1, or cellular 5S rRNA as a control (Fig. 5a). The amounts of radiolabelled virus-specific products from RNA obtained at 8 h p.i. were normalized with respect to the 5S rRNA product, and the levels of viral RNAs detected in rWSN WT-infected cells were set to 100 %. Comparison of N40 and PB1-F2 mutant viruses with WT showed only minor differences (Fig. 5b). Although the absolute amounts of the produced RNAs varied compared with WT virus, the ratios between the different RNA species remained similar, except for rWSN ΔAUG virus. In this case, production of vRNA was remarkably increased compared with mRNA levels. To compare the relative amounts of transcription and
replication, the ratio between mRNA and cRNA was calculated. As seen in Fig. 5(c), the ratio was strongly shifted towards replication in ΔAUG virus-infected cells. Primer-extension analyses were also performed on several other viral genome segments and all exhibited the same trend of relatively increased amounts of vRNA for ΔAUG virus (data not shown). However, none of these differences in RNA levels resulted in change in titre of virus released from the same cell monolayers (Fig. 5d).

**Imbalanced replication/transcription correlates with attenuation of rWSN ΔAUG in vivo**

Previous studies had reported PB1-F2 to have an influence on virus pathogenicity and virulence in a mouse model (Le Goffic et al., 2011; Zamarin et al., 2006). Here, we infected 6-week-old female BALB/c mice intranasally with 5 × 10^3 p.f.u. of the mutant viruses. The body weight of the mice was observed over 7 days and titres of virus in the lungs were measured on days 4 and 7 p.i. Mice infected with the recombinant WSN WT virus dramatically lost up to 25% body weight in 1 week. Mice infected with ΔAUG virus showed no loss in weight, which had previously been associated with lack of PB1-F2 expression (Le Goffic et al., 2011; Zamarin et al., 2006). However, mice infected with rWSN-F2-11 virus showed a similar weight loss to WT virus-infected mice (Fig. 6a), suggesting that the increased level of N40 correlates with the reduced virulence rather than the loss of PB1-F2. Virus titres support these findings, as mice infected with either the WT or F2-11 viruses had similar titres in the lung, whereas ΔAUG-infected mice showed decreased virus titres on both tested days (Fig. 6c). Viruses not expressing N40 in a PB1-F2-expressing background showed no difference from WT pathogenicity (Fig. 6b, d). An intermediate phenotype for viruses carrying the double mutation was observed. In these mice, weight loss was less pronounced and mice seemed to start to regain weight at day 6. This was also supported by the virus titres in the lung, as they were similar to WT at day 4 but were much lower at day 7, suggesting a more efficient clearance of virus (Fig. 6d).

**DISCUSSION**

In 2001, Chen et al. discovered a novel influenza A virus protein, which was soon the focus of studies as a new viral virulence factor (Chen et al., 2001). PB1-F2 was described to have a number of functions, including induction of apoptosis and regulation of the viral polymerase (Chen et al., 2001; Krumbholz et al., 2011; Mazur et al., 2008; Zamarin et al., 2006). However, both activities were later reported to be strain- and cell type-specific (Chen et al., 2001; Chen et al., 2010; McAuley et al., 2010a, b). Most of the work on PB1-F2 used recombinant viruses deficient for PB1-F2 expression via mutation of the PB1-F2 start codon (Chen et al., 2001; Le Goffic et al., 2010, 2011; Mazur et al., 2008; McAuley et al., 2007, 2010a, b; Zamarin et al., 2006). However, this strategy was subsequently shown to increase expression levels of a third polypeptide deriving from segment 2, N40, an N-terminally truncated version of the polymerase subunit PB1, and expression of the three
proteins was shown to be interdependent (Wise et al., 2009, 2011). To address a possible role for N40 and to discriminate previously reported functions of PB1-F2, we created two PB1-F2-deletion viruses that differ in their N40 expression level (Fig. 1c). As described before, we also found a small-plaque phenotype for the rWSN ΔAUG virus (Fig. 1d), and this had previously been associated with the loss of PB1-F2 (Mazur et al., 2008). Consistent with attenuation in plaque formation, this virus also showed reduced growth properties in vitro (Fig. 3a). Other studies reported variable effects on replication, and these differences might be dependent on the virus strain, cell type and m.o.i. used in each study (McAuley et al., 2010b). In BALB/c mice, rWSNΔAUG virus is also attenuated compared with WT (Fig. 6a), in agreement with the results of Le Goffic et al. (2011), who used an analogous recombinant WSN virus deficient in PB1-F2 expression that would overexpress N40. However, Zamarin et al. (2006) reported that their version of WSN virus lacking PB1-F2, which again would overexpress N40, was as pathogenic as WT in C57BL/6 mice, and the authors concluded that, in this system, the contribution of PB1-F2 to pathogenicity might not be significant. These results highlight the effect that mouse strain and virus dose can have on the outcome of pathogenesis experiments.

Our results presented above show that a virus that lacks PB1-F2, but has a normal N40 expression level (F2-11), has no significant attenuation in plaque formation or growth properties. In addition, this virus behaves like WT virus in mice. This suggests that the reduction in virus replication is not due to the loss of PB1-F2 alone, but rather in combination with increased N40 expression. However, a virus lacking both PB1-F2 and N40 is attenuated in vivo, although there is no effect of deletion of N40 alone (Fig. 6). We also tested the different mutants in minigenome and primer-extension assays to evaluate possible roles of PB1-F2 and N40 in vRNA synthesis. Loss of N40 was disadvantageous for polymerase activity, which was even more pronounced if PB1-F2 was also lacking. On the other hand, no effect was observed if PB1-F2 alone was missing. The reduction in polymerase activity was at least partially restored if both proteins were added to the minigenome...

**Fig. 5.** Accumulation of vRNA species in infected A549 cells. Cells were infected with the panel of viruses at an m.o.i. of 3 and total RNA was extracted at 8 h.p.i. (a) Levels of mRNA, cRNA and vRNA derived from genome segment 1 and cellular 5S rRNA were detected by primer extension. Products were separated on a 6 % polyacrylamide/6 M urea gel and detected by autoradiography. (b) Quantification of mRNA, cRNA and vRNA. Values were normalized against cellular 5S rRNA and expressed as mean ± SEM percentages of WT NA RNA levels. Results are based on three independent experiments. (c) Ratios of quantified levels of mRNA and cRNA are shown. Error bars represent SEM based on three independent experiments. Asterisks indicate significant differences (*P<0.05; **P<0.01). (d) Supernatants from the same infected-cell monolayers were collected at 8 h.p.i. and virus titres were determined by plaque assay.
assay programmed with ΔF2/ΔN40. Whilst the data would support the hypothesis that lack of N40 and PB1-F2 might be responsible for this attenuation, a negative impact on the polymerase activity caused by the point mutations within the PB1 amino acid sequence in N40 deletion viruses cannot be ruled out. Minigenome systems are artificial systems in that other viral proteins are missing compared with a natural infection, which might affect the outcome of the experiment. In addition, others have shown that the cell type used to conduct minigenome assays can affect the outcome (McAuley et al., 2010b).

Primer-extension assays were employed to investigate levels of viral RNA species produced by our panel of mutant viruses in infected lung epithelial cells (A549 cells). Cells infected with viruses deficient for PB1-F2 but expressing increased levels of N40 showed an increase in vRNA and cRNA relative to mRNA. This led us to hypothesize that N40 (and possibly also PB1-F2) is involved in the regulation of transcription and replication. Infection of immunocompetent cells with a virus that has limited mRNA-synthesis capability might have no effect if all cells are infected at the same time, i.e. using a high m.o.i. This is supported by the finding that viruses lacking PB1-F2 and overexpressing N40 were not attenuated in vitro when cells were infected at high m.o.i. (m.o.i. 3; Fig. 5d). Mice infected with high doses of virus also showed no attenuation compared with WT-infected mice (Le Goffic et al., 2011). However, infecting cells at a low m.o.i. requires the virus to interfere with the host innate immune response. This could be problematic if viral mRNA synthesis, including production of mRNAs encoding viral virulence factors, is impaired. This might explain the attenuation of the ΔAUG virus in certain in vitro experiments, as well as in vivo.

How might N40 and PB1-F2 be involved in vRNA synthesis? PB1 binds to itself, PA, PB2, PB1-F2 and a number of host factors including Hsp90 and Ebp1 (Bruns et al., 2007; González et al., 1996; Honda, 2008; Mazur et al., 2008; Naito et al., 2007). Most of the binding sites have yet to be mapped, but it is known that N40 lacks the binding site for PA, although the ability to interact with

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**Fig. 6.** Effects of PB1-F2 and N40 expression on pathogenicity in mice. Six-week-old BALB/c mice were infected intranasally with $5 \times 10^5$ p.f.u. of the different viruses. (a, b) Weight loss was measured over 7 days. (a) Infection with viruses lacking PB1-F2 ORF; (b) infection with viruses lacking N40 or both PB1-F2 and N40 ORFs. Data show mean ± SEM values for four mice per group. (c, d) Virus titres in the lungs. On days 4 and 7 p.i., lungs were removed from four mice per group and mean ± SEM virus lung titres were determined by plaque assay on MDCK cells. (c) Viruses lacking PB1-F2 ORF; (d) viruses lacking N40 and both PB1-F2 and N40 ORFs. Data are representative of two or three independent experiments.
PB2 remains (Wise et al., 2009). It is possible that N40 competes with full-length PB1 for binding factors involved in replication and transcription, e.g. PB2 or Hsp90 (Naito et al., 2007). Alternatively, N40 and PB1-F2 might interact with the polymerase complex itself and modulate its function. Clearly, further studies are warranted in order to understand the contribution of PB1-F2 and N40 to virus pathogenicity.

**METHODS**

**Cells and viruses.** MDCK, A549 and 293FT cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Gibco) supplemented with 10% FCS. Recombinant viruses (WT and mutant viruses) were rescued by the previously described 12-plasmid rescue system (Neumann et al., 1999). Briefly, 293FT cells were transfected with a mixture of eight genome-sense plasmids (cloned in pH21) containing cDNAs to A/WSN/33 genome segments, 0.5 µg each, plus four plasmids expressing viral polymerase proteins and nucleoprotein (cloned in pcDNA3.1; 1 µg each of PB2-, PB1- and NP-expressing plasmids and 0.2 µg PA-expressing plasmid) using FuGene 6 transfection reagent (Roche). Cells were incubated overnight in DMEM supplemented with 3% FCS, and then resuspended in DMEM/10% FCS and co-cultivated with MDCK cells for 6–8 h, before the medium was changed to serum-free DMEM supplemented with 2.5 µg N-acetylmethionin ml⁻¹ (Sigma). Rescued viruses were plaque-purified followed by a single propagation in MDCK cells before further studies.

**Generation of plasmids and viruses.** Point mutations were inserted into plasmid pHH-PB1 by QuikChange site-directed mutagenesis PCR (Stratagene). AAUG was generated as described previously (Zamarin et al., 2006) by changing the start codon of PB1-F2 (T120C) and creating two downstream stop codons (G115G; G239A). To generate mutant virus F2-11, only the latter two mutations were introduced. The mutation to delete N40 expression (A142T) was described previously (Wise et al., 2009). Another mutant PB1 (Met40Leu). To prevent both PB1-F2 and N40 expression, two mutations were introduced (G144A; G291A). The first resulted in an amino acid change in PB1 (Met40Ile), whereas the second nucleotide change was silent. Plasmids were sequenced to confirm that no additional changes were introduced before being used for virus rescue.

**Western blotting and antibodies.** Cell lysates were fractionated on 4–12% gradient gels (Invitrogen) or on 16% Tricine/SDS gels (Scha¨ gger, 2006), and the separated proteins were transferred by semi-dry blotting onto nitrocellulose membranes (Hybond C-Extra; GE Healthcare) for reaction with specific antibodies. Rabbit polyclonal anti-PB1 serum V19 raised against aa 50–370 of A/PR8/34 PB1 (Wise et al., 2009) was kindly provided by Paul Digard, University of Cambridge, UK. Rabbit polyclonal antisera to PB1-F2 of A/PR8/34 virus was described earlier (Henklein et al., 2009; Mitzner et al., 2005) and was kindly provided by David Mitzner (ViroLogik GmbH, Erlangen, Germany). Rabbit anti-tubulin antibody and HRP-conjugated anti-tubulin antibody were purchased from Sigma and anti-rabbit HRP-conjugated antibody was purchased from Cell Signaling Technology.

**Growth of viruses.** Virus titration by plaque assay was done in MDCK cells under an overlay comprising 1.2% Avicel in DMEM containing 2 µg N-acetyltrypsin ml⁻¹ (Matrosovich et al., 2006). Cells were fixed at 72 h p.i. with 4% formaldehyde and plaques were revealed by staining with crystal violet.

Virus replication in vitro was measured by infecting confluent MDCK cells at an m.o.i. of 0.001. Supernatants were collected at 6, 12, 24, 36 and 48 h p.i. and virus titres were determined by plaque assay.

For in vitro studies, 6-week-old female BALB/c mice (Harlan UK Ltd) were anaesthetized with Halothane (Rhone Merieux Ltd) and infected intranasally with 5 × 10³ p.f.u. Weight loss was observed over 7 days and virus lung titres were measured at day 4 and day 7.

**Minigenome assay for polymerase activity.** Confluent monolayers of 293FT cells in a 24-well plate were transfected with 50 ng pcDNA-PB2 and pcDNA-NP, 12.5 ng pcDNA-PA, 20 ng pH-H-20, 20 ng CMV-firely and 50 ng pcDNA-PB1 or mutant PB1-expressing plasmid. If necessary, 50 ng pcDNA-PB1-F2 and pcDNA-PB1 N40 plasmids were added. Empty pcDNA3.1 vector was added to adjust DNA amounts. Transfections were done using FuGene 6 reagent (Roche) according to the manufacturer’s instructions. At 24 h post-transfection, cells were lysed in 100 µl Passive Lysis Lysis Buffer (Promega), and firefly and Renilla luciferase activities were measured using the Dual-Luciferase Assay System (Promega) according to the manufacturer's protocol. Values were normalized against the internal firefly luciferase control and expressed as relative amounts compared with WT PB1.

**Primer-extension analysis.** A549 cells were infected with WT or mutant viruses at an m.o.i. of 3. After 8 h, total RNA was extracted from cells using an RNeasy Mini kit (Qiagen). To amplify mRNA, cRNA and vRNA in a reverse-transcription (RT) reaction, 2.5 µg RNA were mixed with ␥-[32P]ATP-labelled primers. Two segment 1-specific primers were used in the same RT reaction: 5’-TGCTAATTGGGCAAGGAGAC-3’ (to detect vRNA) and 5’-GCCATCATCCATTTCATCC-3’ (to detect mRNA and cRNA). A primer to detect 55 rRNA was used for detection of the internal control (5’-TCCAGGGGTTCTCCCATCC-3’). RT was done for 2 h at 42 °C using M-MLV (Moloney murine leukemia virus) reverse transcriptase (Promega) according to the manufacturer’s instructions. DNA products were separated on a 6% polyacrylamide gel containing 6 M urea (Geneflow; SequaGel 6) and detected by autoradiography. The expected sizes of the products were 143 nt (vRNA), 182 nt (cRNA) and 100 nt (5S rRNA). Quantification was performed using a Fujifilm FLA-5000 fluorescent image analyser.

**Statistical analysis.** Data of virus growth in vitro and in vivo, plaque size, weight loss and viral polymerase activity were analysed using Student’s t-test. P-values of <0.05 were considered as significantly different.

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