Identification of cis-acting packaging signals in the coding regions of the influenza B virus HA gene segment

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For influenza A and B viruses to be infectious, they require eight viral RNA (vRNA) genome segments to be packaged into virions. For efficient packaging, influenza A viruses utilize cis-acting vRNA sequences, containing both non-coding and protein coding regions of each segment. Whether influenza B viruses have similar packaging signals is unknown. Here we show that coding regions at the 3' and 5' ends of the influenza B virus vRNA segment 4 are required for genome packaging, with the first 30 nt at each end essential for this process. Synonymous mutation of these regions led to virus attenuation, an increase in defective particle production and a reduction in packaging of multiple vRNAs. Overall, our data suggest that the influenza B virus vRNA gene segments likely interact with each other during the packaging process, which is driven by cis-acting packaging signals that extend into protein coding regions of the vRNA.

INTRODUCTION

Influenza A and B viruses are members of the Orthomyxoviridae family, containing an eight-segmented negative sense RNA genome. Each segment encodes one or more proteins that show high levels of similarity in structure and function between the two types of viruses, despite significant differences in amino acid identity (Palese & Shaw, 2007). Influenza A and B virions also show high levels of structural similarity; however, a significant difference has been observed in genome arrangement within the core. The genome of both viruses is in the form of eight viral ribonucleoprotein complexes (vRNP) consisting of the viral RNA (vRNA) encapsidated by nucleoprotein and associated with a heterotrimeric polymerase complex. Electron microscopic analysis of influenza A virus particles demonstrated that the vRNPs are arranged in a highly ordered '7 + 1' orientation in which all segments align in a circular fashion with one segment in the centre (Noda et al., 2006). However, recent evidence suggests that this does not occur in influenza B viruses, as the vRNPs appear to twist around each other (Katz et al., 2014). This suggests that influenza A and B viruses potentially adopt different mechanisms for genome packaging.

It is now widely accepted that influenza A viruses utilize a selective packaging process by which a single copy of each of the vRNPs is packaged into progeny virions in a process likely driven by bipartite cis-acting sequences within the vRNAs (Gerber et al., 2014). Although early work suggested that the non-coding regions at the extreme termini of each vRNA were the minimal determinants of genome packaging (Luytjes et al., 1989), more recent studies have shown that the extreme 5' and 3' ends of the protein coding regions are required for optimal genome packaging (Hutchinson et al., 2010). These cis-acting sequences are hypothesized to be responsible not only for packaging each genome segment into the virion, but also for forming interactions between segments (Fournier et al., 2012) to create a complex of vRNPs, thereby acting as bundling signals to allow a single copy of each vRNP to be packaged (Goto et al., 2013). It is tempting to speculate that this may be responsible for the '7 + 1' genome arrangement, which questions whether influenza B viruses contain similar cis-acting packaging sequences due to the significantly different appearance of the vRNPs within the virions. Although mutagenesis of the non-coding regions of the influenza B virus vRNA segment 4 suggested these regions are necessary for genome packaging and may therefore act as cis-acting packaging signals (Barclay & Palese, 1995), whether these signals include protein coding regions, similar to their influenza A virus counterparts, is unknown.

Here we address this by analysing the role of the terminal protein coding regions of vRNA segment 4 of influenza B viruses in genome packaging. We show that the first and last 150 nt of the influenza B virus haemagglutinin (BHA) ORF are required for efficient packaging of a viral-like RNA into virus-like particles and that the first
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(a) 150 nt of the BHA ORF at the vRNA 3' end 150 nt of the BHA ORF at the vRNA 5' end

(b) HA(150)GFP(150) HA(0)GFP(0) HA(0)GFP(150)

(c) HA(150)GFP(150) HA(0)GFP(0) HA(0)GFP(150) HA(150)GFP(0)

(d) BHAGFP packaging levels (% of HA(150)GFP(150))

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30 nt of the BHA ORF at the 3’ end of the negative sense vRNA are essential for this function. Analysis of mutant viruses containing synonymous mutations in these regions demonstrated that mutations in the terminal 30 nt of the protein coding region at both the 3’ and 5’ ends of vRNA segment 4 led to virus attenuation, an increase in defective particle production and a reduction in packaging of multiple vRNA segments. Our findings give the first evidence of the role of influenza B virus protein coding regions in ensuring optimal genome packaging.

RESULTS AND DISCUSSION

A series of constructs were created for use in a mini-genome reporter assay (Sherry et al., 2014) to assess the importance of the terminal ends of the coding regions of influenza B virus vRNA segment 4 (which encodes the haemagglutinin protein, BHA) in genome packaging. To create the constructs, the GFP coding sequence was flanked by the vRNA segment four non-coding regions and the terminal 150 nt at both termini of the BHA coding region, followed by insertion into the pHK-21 plasmid in a 3′–5′ negative sense orientation, thereby allowing expression of a negative sense mini-genome viral-like RNAs. (b) GFP expression from each HA-GFP construct in a mini-genome replication assay. 293T cells were transfected with 1 μg of each mini-genome construct along with plasmids expressing the B/Yamanashi/98 virus polymerase complex and BNP proteins. At 24 h.p.t. GFP expression was observed at ×20 magnification. Cell nuclei were stained with DAPI and the total number of cells in each panel was determined by manual counting using ImageJ. GFP-positive cells were manually counted and the percentage of the total cell number positive for GFP was calculated. The percentages were calculated from three independent experiments and the mean percentage values are shown in the white boxes in each panel ± SD. (c, d) The packaging efficiency of each HA-GFP segment into VLPs. 293T cells were transfected with 1 μg of each mini-genome construct and at 24 h.p.t. infected with B/Yamanashi/98 virus at an m.o.i. of 10. Cells were monitored for GFP expression and at 16 h.p.i., all samples demonstrated GFP expression in 70–80% of cells. Supernatants were then harvested and N-acetyl trypsin-treated. Samples were serially twofold diluted and placed on MDCK cells for 10 h. At 10 h.p.i. cells were fixed, permeabilized and stained with an anti-BNP antibody followed by a Texas-red conjugated secondary antibody. Representative images of GFP and viral antigen staining by immunofluorescence at ×20 magnification are shown in (c) for four of the VLP-containing supernatants. Nuclei were stained with DAPI. (d) FACS analysis was performed to quantify the number of antigen-positive cells that were also expressing GFP. Five thousand cells were analysed per sample. The percentage of antigen-positive HA(150)GFP(150) VLP-infected cells that also expressed GFP was set at 100% packaging efficiency and the efficiency of all other constructs was determined as a percentage of this. Results represent the mean of two independent experiments ± SD.

To determine the level of infectious virus present in the 293T cell supernatants, the samples were serially twofold diluted and used to infect MDCK cells. At 10 h post-infection (h.p.i.), cells were subjected to immunofluorescence analysis using an anti-BNP antibody to determine the number of virus antigen-positive cells. The dilution of supernatant that resulted in infection of 80–90% of cells was then placed onto fresh MDCK cells, and at 10 h.p.i. cells were subjected to immunofluorescence and FACS analysis. Fig. 1(c) shows representative GFP and viral antigen staining for cells infected with four of the supernatant samples. FACS analysis was performed to quantify the percentage of infected cells expressing GFP. 56% of virus-infected MDCK cells expressed the full-length HA(150)GFP(150) mini-genome vRNA segment, demonstrating the maximum packaging efficiency when using this approach, which is analogous to the level of packaging observed in a similar study using influenza A viruses (Fujii et al., 2005). The packaging levels of all other segments were expressed as a percentage of the full-length mini-genome segment (Fig. 1d). Removal of the 150 nt BHA coding region at the 3′ end of the vRNA reduced the packaging efficiency to less than 1%, which could only be increased if the first 30 nt of this region were retained. When the 150 nt BHA coding region at the 5′ end of the vRNA was removed, the packaging efficiency dropped to 9%, indicating that truncation at the 3′ end of the vRNA had a larger effect on packaging. However, when the terminal 30 nt of the BHA coding region at the 5′ end of the vRNA were replaced (HA(150)GFP(30)), packaging efficiency was increased to 73%. Overall, the data indicate that although the entire 150 nt regions at both ends of the BHA coding region are required for efficient genome packaging, the terminal 30 nt...
Fig. 2. Synonymous mutations in both the 3' and 5' coding regions of segment 4 vRNA lead to virus attenuation. (a) Schematic diagram of mutations inserted into B/Yamanashi/98 virus vRNA segment 4 by reverse genetics. The RNA sequence is shown in positive sense mRNA orientation to highlight the lack of protein coding changes. The first and last...
60 nt of the BHA coding region are shown (nucleotides 1–60 and 1706–1766, respectively) in the mRNA-sense orientation with the corresponding amino acid sequence indicated. Synonymous mutations were inserted into the 3’ end of the vRNA (i.e. the 5’ end of the mRNA) between nucleotides 1–30 (3m30 mutations) or 31–60 (3m60 mutations) of the BHA coding region, and into the 5’ end of the vRNA between nucleotides 1706–1736 (5m60 mutations) or 1737–1766 (5m30 mutations). Mutations are capitalised, highlighted and underlined. Wt indicates wt segment 4 sequence. (b) Multistep growth curve analysis of single site mutant viruses. MDCK cells were infected with wt and the four mutant viruses containing single site mutations (e.g. 3m30 mutations) at an m.o.i. of 0.001, samples harvested every 12 h until 72 h p.i. and infectious titres determined by plaque assay. Results show the mean of three independent experiments ± SD. (c) Multistep growth curve analysis of double site mutant viruses. MDCK cells were infected with wt and mutant viruses containing double site mutations and replication kinetics determined as in (b). (d) Particle to infectivity analysis of the mutant virus panel. Stocks of wt and mutant viruses were titrated by plaque assay and haemagglutination assay. Particle number was calculated based on 1 HA unit requiring approximately 10^6 virus particles (Donald & Isaacs, 1954). The particle to infectivity ratio (particles ml^-1 : p.f.u. ml^-1) was determined for each virus. (e) Particle to infectivity analysis of 3m30 mutation-containing viruses from growth curve samples in (b) and (c). Particle to infectivity ratios were determined for the 36 h and 48 h growth curve samples for wt and mutant viruses containing the 3m30 mutations. Results show the mean of three independent experiments ± SD.

at each end have a significant effect on this process, with those at the 3’ end having the greatest effect on packaging efficiency. These findings are similar to those observed for packaging of various influenza A virus segments (Fujii et al., 2003, 2005), suggesting that influenza B viruses may adopt a similar mechanism of genome packaging to their influenza A virus counterparts.

To assess the effects of the BHA coding regions on genome packaging in the context of infectious virus, a series of mutant viruses were created in which synonymous mutations were introduced into the first 30 nt of the BHA coding region at the 3’ end of the negative sense vRNA (3m30 mutations) or between nucleotides 31–60 (3m60 mutations) (Fig. 2a). Similar mutations were introduced into the BHA coding region at the 5’ end of the negative sense vRNA. Viruses were generated containing mutations at either one or both termini. The rBHA-3m30/5m30 virus took significantly longer to recover due to a propensity to generate defective particles; therefore this virus was plaque purified to remove these particles prior to experimentation. The only virus containing mutations at a single end of the gene that demonstrated attenuated replication kinetics compared to the rBHA wt virus was the rBHA-3m30 virus (Fig. 2b). Further attenuation was observed when combining the 3m30 mutation with mutations in the BHA coding region at the 5’ end of the vRNA, with the rBHA-3m30/5m30 virus demonstrating the largest degree of attenuation (Fig. 2c). This confirmed that the terminal 30 nt at both ends of the BHA coding region have a significant effect on virus replication. This is remarkably similar to the results of a previous study in which the packaging signals of the influenza A virus segment 8 were characterized (Fujii et al., 2005). In this study, synonymous mutations introduced into the 5’ end of the coding region did not alter viral replication, whereas mutations in the 3’ end of the coding region resulted in a slight attenuation, which was enhanced by introducing the mutations into both the 3’ and 5’ coding regions. The phenotype of the double mutant reported by Fujii et al. (2005) was therefore remarkably similar to the rBHA-3m30/5m30 virus, suggesting these sequences are essential for efficient viral replication of both influenza A and B viruses, possibly indicating similar functional requirements.

As the 3m30/5m30 mutations may have reduced the efficiency of genome packaging, resulting in higher levels of defective virus production, the particle to infectivity ratio of all viral stocks was analysed. The only virus that demonstrated a marked increase in the number of defective particles was the rBHA-3m30/5m30 virus (Fig. 2d). The 36 and 48 h time point samples (Fig. 2a, b) of viruses containing the 3m30 mutations were analysed for particle to infectivity ratios. The only virus that demonstrated a significant increase in defective particles at both time points was the rBHA-3m30/5m30 virus (Fig. 2e). The level of defective virus particles increased for all viruses between the two time points; however, while most viruses demonstrated a two- to sevenfold increase, there was a 39-fold increase in rBHA-3m30/5m30 defective particles. The propensity for this virus to generate defective particles was likely responsible for its attenuation in the replication analysis.

Sequence analysis of vRNA segment 4 of multiple influenza B viruses isolated between 1940 and 2014 shows that the 3m30, 3m60, 5m30 and 5m60 regions of the genome are highly conserved, with the 3m30 region completely conserved in all analysed sequences (Fig. 3a). The lack of synonymous mutations in the 3m30 region over a 74-year time period may suggest that the sequence integrity of the RNA in this region is essential for virus viability, potentially through contributing to efficient genome packaging. This is not surprising, given that previous studies have shown that the areas of various influenza A virus vRNA segments that contain the highest levels of sequence conservation are found in the terminal ends of the coding regions that have been implicated as packaging signals (Gog et al., 2007; Marsh et al., 2007). As the mutated regions of the rBHA-3m30/5m30 virus potentially represent packaging signals, changes in RNA sequence or secondary structure may have reduced the efficiency of segment 4 packaging.
Influenza B virus genome packaging signals

(a) Influenza B virus genome packaging signals

(b) BHA wt BHA-3m30/5m30

(c) Protein levels (arbitrary units)

(d) BHA wt BHA-3m30/5m30

(e) Virion protein content (% of rBHA wt)

(f) Virion vRNA levels (% of rBHA wt)
**Fig. 3.** The 3m30/5m30 mutations do not affect mRNA synthesis/protein production but reduce the packaging efficiency of vRNA segment 4. (a) Sequence alignment of influenza B virus vRNA segment 4 sequences. The 3m30, 3m60, 5m30 and 5m60 regions of the influenza B virus vRNA segment 4 indicated in Fig. 2(a) (shown in positive sense orientation) were analysed in the published sequences of multiple viruses isolated between 1940 and 2014. Sequences were extracted from the NCBI Influenza Virus Sequence Database and aligned using Geneious software. Nucleotide differences from the consensus sequence are indicated with identical nucleotides shown as dots. (b) BHA protein expression is unaffected by the 3m30/5m30 mutations. **Fig. 3d.** A slight reduction in Coomassie staining. BHA was present in the trypsin-cleaved assay) were analysed for protein content by SDS-PAGE and of wt and mutant virions (determined by haemagglutination assessed. After purification of the viruses, an equal number the BNP and vRNA levels in wt and mutant virions were increased levels of defective particles shown in Fig. 2(d), reduced vRNA content in released virions, resulting in therefore did not affect BHA protein levels. The 3m30/5m30 mutations do not affect mRNA synthesis/protein production but reduce the packaging efficiency of the violins. This was confirmed by quantitative reverse-transcriptase-PCR (qRT-PCR) analysis (Fig. 3f). Levels of the virions. This was confirmed by quantitative reverse-transcriptase-PCR (qRT-PCR) analysis (Fig. 3f). Levels of three vRNA segments were present in the mutant virions at only 56–74 % of those of wt virus, which is a similar reduction to the levels of BNP observed in Fig. 3(e). This demonstrated a genome packaging deficiency in the mutant, and suggested that the deficiency in packaging segment 4 also reduced packaging of other segments. It has been suggested that intermolecular interactions form between influenza A virus vRNPs such that the full complement of vRNPs are present as a complex prior to packaging into virions (Goto et al., 2013). A reduction in packaging efficiency of a single segment could therefore reduce or even prevent packaging of other segments. It is possible that this is also true of influenza B virus vRNPs and the mutation of packaging signals in the rBHA-3m30/5m30 viruses could therefore reduce or even prevent packaging of other segments. This would explain the increased level of defective particles and virus attenuation observed in Fig. 2; however, whether the defective particles were semi-infectious as they lacked one or more vRNA segment, or lacked genome altogether, is unknown. The level of vRNA segment 8 was reduced to a larger extent than the other segments. Similar variations in packaging efficiencies between influenza A virus vRNPs have been described (Marsh et al., 2007), further suggesting that influenza B virus genome packaging may adopt similar mechanisms to that of influenza A viruses, potentially indicating the likelihood of direct interactions between specific vRNPs during influenza B virus packaging. It is not surprising that studies in recent years have identified coding regions in the influenza A virus vRNA as being

However, it was also possible that such changes had affected the efficiency of mRNA transcription from the mutant vRNA, thereby leading to viral attenuation. To address this, vRNA segment 4 from the wt and rBHA-3m30/5m30 viruses was cloned into a plasmid under the control of the human RNA polymerase I promoter, allowing its expression in a mini-genome assay. The results showed similar levels of BHA for both wt and mutant segments (Fig. 3b, c). This demonstrated that the 3m30/5m30 mutations did not affect the ability of the polymerase to transcribe BHA mRNA and therefore did not affect BHA protein levels.

To analyse whether the BHA-3m30/5m30 mutations reduced vRNA content in released virions, resulting in increased levels of defective particles shown in Fig. 2(d), the BNP and vRNA levels in wt and mutant virions were assessed. After purification of the viruses, an equal number of wt and mutant virions (determined by haemagglutination assay) were analysed for protein content by SDS-PAGE and Coomassie staining. BHA was present in the trypsin-cleaved form of BHA1 and BHA2 (Fig. 3d). A slight reduction in overall viral protein levels was observed in the mutant virions compared to those of rBHA wt, suggesting the presence of fewer particles; however, a greater reduction was observed in BNP levels compared to other proteins. To accurately identify the viral proteins and to quantify their levels in both viruses, the viral proteins were analysed by immunoblotting. While BM1 and BHA levels were similar for both viruses, the BNP level in the mutant was only 63 % that of wt (Fig. 3e). As BNP is associated with the viral genome, this reduction likely indicated reduced vRNA content in the virions. This was confirmed by quantitative reverse-transcriptase-PCR (qRT-PCR) analysis (Fig. 3f). Levels of three vRNA segments were present in the mutant virions at only 56–74 % of those of wt virus, which is a similar reduction to the levels of BNP observed in Fig. 3(e). This demonstrated a genome packaging deficiency in the mutant, and suggested that the deficiency in packaging segment 4 also reduced packaging of other segments. It has been suggested that intermolecular interactions form between influenza A virus vRNPs such that the full complement of vRNPs are present as a complex prior to packaging into virions (Goto et al., 2013). A reduction in packaging efficiency of a single segment could therefore reduce or even prevent packaging of other segments. It is possible that this is also true of influenza B virus vRNPs and the mutation of packaging signals in the rBHA-3m30/5m30 viruses could therefore reduce or even prevent packaging of other segments. This would explain the increased level of defective particles and virus attenuation observed in Fig. 2; however, whether the defective particles were semi-infectious as they lacked one or more vRNA segment, or lacked genome altogether, is unknown. The level of vRNA segment 8 was reduced to a larger extent than the other segments. Similar variations in packaging efficiencies between influenza A virus vRNPs have been described (Marsh et al., 2007), further suggesting that influenza B virus genome packaging may adopt similar mechanisms to that of influenza A viruses, potentially indicating the likelihood of direct interactions between specific vRNPs during influenza B virus packaging. It is not surprising that studies in recent years have identified coding regions in the influenza A virus vRNA as being
important for efficient genome packaging, as prior work had shown that defective viruses containing truncated gene segments retained a full complement of eight vRNPs, with the defective segment retaining both the non-coding regions and a portion of the coding regions at either terminus (Duhaut & Dimmock, 1998; Duhaut & McCauley, 1996; Nayak et al., 1982), with these coding regions increasing the stability of the defective particles (Duhaut & Dimmock, 2000). It is plausible that short coding regions were retained as they contained the cis-acting packaging signals. Similar findings have been observed in defective influenza B viruses containing truncations in vRNA segment 4, with the first and last 150–200 bp of the BHA ORF retained (D. Jackson, unpublished results). This, along with the data shown above, suggests that influenza B viruses do contain similar genome packaging signals to those observed for influenza A viruses, and that these signals extend into the coding regions. Our results show that the extreme terminal 3’ and 5’ ends of the coding regions of the influenza B virus vRNA segment 4 are required for efficient genome packaging, with the first 30 nt at the 3’ end having the greatest influence on packaging efficiency, similar to findings in the context of influenza A virus segments 6 and 8 packaging (Fuji et al., 2003, 2005). It was previously shown through truncation analysis that the terminal 3’ and 5’ ends of the coding region of the influenza A virus segment 4 are required for efficient packaging to occur (Marsh et al., 2007; Watanabe et al., 2003). However, unlike the findings for influenza B virus segment 4, systematic synonymous mutagenesis in the influenza A virus segment revealed that a 15 nt stretch in the terminal 80 nt at the 5’ end of the coding region had the greatest influence on packaging efficiency (Marsh et al., 2007). Unfortunately, the studies of Marsh et al. (2007) did not include analysis of viruses containing synonymous mutations at both the 3’ and 5’ ends of the same segment; therefore, it is unknown whether this would have enhanced the attenuation of a recombinant virus, similar to the rBHA-3m30/5m30 virus in the current study. It is likely that these differences in segment 4 packaging sequence requirements between the two viruses are influenced by the mechanism of vRNA interaction between the individual segments, especially as it has been reported that vRNPs interact during transport to the site of assembly (Chou et al., 2013; Gavazzi et al., 2013; Lakdawala et al., 2014). Although our results suggest that the mechanism of genome packaging utilized by influenza A and B viruses is similar, the fact that the 3’ end of the coding region was more important in packaging of influenza B virus vRNA segment 4 compared to the 5’ end in influenza A viruses may suggest that there is a difference in the mechanism behind vRNA–vRNA interaction formation between the two viruses, or that the influenza B virus vRNA segment 4 interacts with different vRNPs compared to its influenza A virus counterpart. Further experiments using labelled vRNPs (Lakdawala et al., 2014) could address this. Furthermore, while electron microscopic comparisons of vRNP structural arrangement in influenza A and B viruses might suggest different mechanisms of genome packaging between the two viruses (Katz et al., 2014; Noda et al., 2006), our results suggest a similar mechanism of cis-acting RNA signal-mediated packaging is employed by both viruses. The difference in vRNP arrangement is likely due to vRNA–vRNA interactions differing between the two viruses, leading to the more twisted vRNP complex observed in influenza B viruses.

Overall, we propose that the 3’ and 5’ ends of the coding region of the influenza B virus vRNA segment 4 contains cis-acting packaging signals and that mutations in the first 30 nt of these regions reduce the efficiency of genome packaging into progeny virions by approximately 40%, similar to that observed for mutagenesis of packaging signals in the same segment of influenza A virus (Marsh et al., 2007). This reduction in segment 4 packaging subsequently reduces the packaging of other segments, resulting in an increase in the release of defective particles, thereby attenuating the virus. It is important to fully characterize the cis-acting packaging signals in influenza B viruses, as this could have significant consequences for vaccine production. Contemporary influenza B viruses replicate poorly in eggs and therefore introducing segments 4 and 6 (encoding the BHA and BNA proteins, respectively) into the background of a virus adapted for efficient growth in eggs by reverse genetics could offer significant advantages for vaccine production purposes (Hoffmann et al., 2002). However, natural mutations in the coding regions of these segments may reduce the efficiency of vRNA packaging, which has previously been shown to be a limiting factor in influenza A virus reassortment (Essere et al., 2013). Therefore, ensuring these signals are optimal for efficient recombinant virus generation could enhance vaccine seed production.

**METHODS**

**Cells and viruses.** 293T and MDCK cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen) supplemented with 10 % FCS at 37 °C with 5 % CO₂. B/Yamanashi/98 wt (rBHA wt) and mutant viruses were generated using plasmid-based reverse genetics as previously described (Hoffmann et al., 2002). Briefly, 293T cells were transfected with eight genome-encoding bi-directional (pAB) plasmids using FuGENE 6 transfection reagent (Promega), and at 16 h post-transfection, the cells were co-cultured with MDCK cells in serum-free DMEM containing 2.5 μg ml⁻¹ N-acetyl trypsin (Sigma). Supernatants were harvested 4 days post-transfection and viruses were propagated twice through MDCK cells followed by plaque assay titration on MDCK cells. Viral RNA was extracted using a QiAamp viral RNA kit (Qiagen), vRNA segment 4 of each virus was amplified by reverse-transcriptase PCR using genome specific primers and the resultant DNA was sequenced to confirm presence of the desired mutations.

**Plasmids.** To create the pH-HA(150)GFP(150) mini-genome plasmid, the 3’ and 5’ non-coding regions of B/Yamanashi/98 vRNA segment 4 flanked by 150 nt of the BHA coding region at either end of the gene segment were amplified by PCR using the parental pKH-HA plasmid as a template. The eGFP coding sequence was amplified by

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PCR and inserted between the 3’ and 5’ end BHA PCR products by overlapping PCR. A translation termination codon was inserted after the GFP ORF. The entire HA(150)GFP(150) PCR product was inserted into the pHH-21 reverse genetics vector between BomBI restriction sites in a negative sense orientation, such that expression of the negative sense viral-like RNA was under the control of the human polymerase I promoter. To create the series of truncation mutant mini-genome constructs, truncated versions of the 150 nt BHA coding regions at either or both termini were amplified using specific primer sets. These PCR products were fused to the 3’ or 5’ non-coding region by overlapping PCR followed by insertion of the eGFP coding sequence by overlapping PCR. The resultant products were inserted into pHH-21 as above. All primer sequences are available upon request. For creation of mutant viruses, synonymous mutations were introduced into the BHA ORF within the pAB-HA reverse genetics plasmid by site-directed mutagenesis and the resultant plasmids substituted into the reverse genetics system. The presence of the desired mutations was confirmed by DNA sequencing. To generate the pHH-BHA wt and pHH-BHA-3m30/5m30 plasmids total RNA was extracted from rBHA wt or rBHA-3m30/5m30 virus and rRNA segment 4 was amplified by reverse-transcriptase PCR. The resultant cDNA was inserted into the pHH-21 plasmid in a negative sense orientation between the human RNA polymerase I promoter and terminator sequences using BomBI restriction site segments. Segment 4 sequences were confirmed by DNA sequencing.

**Mini-genome assay.** To test expression of GFP from the mini-genome plasmids, 293T cells in 12-well plates were transfected with 500 ng of pAB expression plasmids encoding PB1, PB2, PA and NP of B/Yamanashi/98 virus and 1 µg of each mini-genome construct. Twenty-four hours post-transfection (h p.t.), cells were fixed in 5 % formaldehyde and GFP expression was observed at ×20 magnification using a Nikon Microphot-FXA fluorescence microscope. Cell nuclei were stained with 1 µg ml⁻¹ DAPI and the total number of cells in each panel was determined by manual counting using ImageJ. GFP-positive cells were manually counted and the percentage of the total cell number positive for GFP was calculated. All samples were analysed in triplicate experiments.

**Virus-like particle (VLP) assay.** Transfections of 293T cells were performed with 1 µg of each mini-genome construct and at 24 h p.i. infected with B/Yamanashi/98 virus at an m.o.i. of 10. At 16 h p.i., supernatants were harvested, N-acetyl trypsin-treated at 37 °C for 30 min, serially twofold diluted and plated on MDCK cells. At 10 h p.i., cells were either analysed by immunofluorescence or FACS. For immunofluorescence analysis, cells were fixed in 5 % formaldehyde, permeabilized in PBS/0.5 % Triton X-100/0.5 % NP-40, blocked in PBN blocking buffer (PBS/1 % BSA/0.02 % sodium azide) for 1 h at room temperature and stained with an anti-BNP monoclonal antibody (Abcam) followed by an anti-mouse Texas-red conjugated secondary antibody. Cell nuclei were stained with DAPI and samples analysed at ×20 magnification using a Nikon Microphot-FXA fluorescence microscope. For FACS analysis, cells were treated with PBS/EDTA to gain single cell suspensions, fixed in 5 % formaldehyde, stained with the anti-BNP monoclonal antibody and anti-mouse Texas-red conjugated secondary antibody in suspension and subjected to FACS analysis to determine percentages of antigen-positive cells (red) that also expressed GFP. Five thousand cells were counted per sample and each sample was performed in duplicate.

**Virus replication kinetics.** MDCK cells were infected with either rBHA wt or mutant viruses at an m.o.i. of 0.001 and supernatant samples harvested every 12 h until 72 h post-infection. The infectivity of the samples was determined by plaque assay titration on MDCK cells. Results represent the mean of three independent experiments ± S.D.

**Particle to infectivity ratio analysis.** The approximate number of virus particles in virus stocks was estimated by a haemagglutination assay. Virus samples were serially twofold diluted in PBS in rows of a V-bottomed 96-well plate in 50 µl volumes per well. Fifty microlitres of chicken red blood cells (1 % diluted in PBS) were added to each well, mixed and incubated at 4 °C until haemagglutination was observed. The reciprocal of the final dilution of virus to display haemagglutination was used to determine the HA titre ml⁻¹ of each virus. As approximately 1 × 10⁹ virus particles are required to achieve an HA titre of unity (Donald & Isaacs, 1954), the HA titres of the virus stocks were multiplied by 1 × 10³ to determine the number of virus particles ml⁻¹ in each sample. The infectious titre of each sample (p.f.u. ml⁻¹) was determined for each sample.

**Viral protein analysis by SDS-PAGE.** To analyse BHA mRNA transcription and protein expression levels from rBHA wt or rBHA-3m30/5m30 virus segments 4 post-transfection (3 h p.t.), cells were lysed in 2 × disruption buffer (6 M urea, 2 M β-mercaptoethanol, 4 % SDS), proteins separated by SDS-PAGE and transferred to Immobilon-FL polyvinylidene difluoride membranes (Millipore). Membranes were blocked in blocking buffer (PBS, 0.1 % Tween 20, 5 % dried milk) and incubated with an anti-B/ Hong Kong/73 polyclonal antisera (to detect viral BHA and BNP proteins) or an anti-actin monoclonal antibody. Protein detection was performed using IRDye 680- or IRDye 800-conjugated secondary antibodies (Liror) on an Odyssey CLx near infrared scanner (Liror), images were collected and protein band intensities were quantified using ImageStudio (Liror).

To analyse virion protein content, 500 000 HA units of rBHA wt and rBHA-3m30/5m30 viruses were purified by sucrose gradient ultracentrifugation. Virus samples were placed onto a 30 % sucrose cushion and subjected to ultracentrifugation at 112 000 g for 2.5 h at 4 °C. Pelleted viruses were resuspended in 500 µl NTE buffer (150 mM NaCl, 10 mM Tris/HCl pH 7.5, 1 mM EDTA) and placed onto a continuous 30–60 % sucrose gradient, followed by ultracentrifugation at 112 000 g for 2.5 h at 4 °C. Virus bands were extracted from the gradient and pelleted by ultracentrifugation at 112 000 g for 2 h at 4 °C. Purified virus pellets were resuspended in 50 µl NTE buffer. All virus purifications were performed in triplicate. Viral proteins in 5 µl of each virus sample were then separated by SDS-PAGE and analysed by Coomassie staining or immunoblotting using the anti-B/Hong Kong/73 polyclonal antisera as described above. Protein bands were detected and quantified for BHA, BNP and BM1.

**Analysis of virion rRNA content by qRT-PCR.** Total rRNA was extracted from three separate samples of rBHA wt or rBHA-3m30/5m30 virus (5.12 × 10⁹ particles for each virus as determined by haemagglutination assay). rRNA segments 4, 5 and 8 were reverse transcribed using gene-specific primers and RevertAid Premium Reverse Transcriptase (Thermo Scientific). For quantitative PCR (qPCR), viral gene-specific primers (sequences available on request) were designed to amplify a 150 nt fragment of DNA and various concentrations of primers were optimized against each other by qPCR using various concentrations of pAB-HA, pAB-NP or pAB-NS as standardized templates. cDNAs generated by reverse transcription were then assayed by qPCR using serial fourfold dilutions of cDNA and appropriate concentrations of gene-specific primers using Precision Mastermix (Primer Design) on a Stratagene Mx3005P real-time PCR thermocycler. A standard curve was generated using serial
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