JVI Accepts, published online ahead of print on 13 August 2014
J. Virol. doi:10.1128/JVI.01542-14

The N-terminus of the influenza B virus nucleoprotein is essential for virus viability, nuclear localization and optimal transcription and replication of the viral genome.

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Abstract: 250 words
Main text word count: 7337 words
Figures: 8

Running title: Influenza B virus viability requires the NP N-terminus

Abstract

The nucleoprotein (NP) of influenza viruses is a multifunctional protein with essential roles throughout viral replication. Despite influenza A and B viruses belonging to separate genera of the *Orthomyxoviridae* family, their NP proteins share a relatively high level of sequence conservation. However NP of influenza B viruses (BNP) contains an evolutionarily conserved N-terminal 50 amino acid extension that is absent from NP of influenza A viruses. There is conflicting evidence as to the functions of the BNP N-terminal extension, however this has never been assessed in the context of viral infection. We have used reverse genetics to assess the significance of this region on the functions of BNP and virus viability. Truncation of more than three amino acids prevented virus recovery suggesting that the N-terminal extension is essential for virus viability. Mutational analysis indicated that multiple regions of the protein are involved in nuclear localization of BNP with the entire N-terminal extension required for this to function efficiently. Viruses containing mutations in the first ten residues of BNP demonstrated little differences in nuclear localization, however the viruses exhibited significant reductions in viral mRNA transcription and genome replication resulting in significantly attenuated phenotypes. Mutations introduced to ablate a previously reported nuclear localization signal also resulted in a significant decrease in mRNA production during early stages of viral replication. Overall our results demonstrate that the N-terminal extension of BNP is essential to virus viability not only for directing nuclear localization of BNP, but also for regulating viral mRNA transcription and genome replication.

Importance

The multifunctional nucleoprotein (NP) of influenza viruses has roles throughout the viral replication cycle and is therefore essential for virus viability. Despite high levels of homology between the NP proteins of influenza A and B viruses the NP of influenza B virus (BNP) contains an evolutionarily conserved 50 amino acid N-terminal extension that is absent from the NP of influenza A viruses. In this study we show that this N-terminal extension is essential for virus viability and we confirm and expand upon recent findings that this region of BNP is required for nuclear localization of the protein. Furthermore we demonstrate for the first time that the N-terminus of BNP is involved in regulating viral mRNA transcription and replication of the viral genome. As the NP of influenza A virus lacks this N-terminal extension it suggests that these viruses have evolved separate mechanisms to regulate these processes.

Introduction

Influenza viruses are the cause of significant morbidity and mortality worldwide with seasonal epidemics causing between 250,000 and 500,000 annual fatalities. Therefore continued effort is required to understand influenza virus biology for development of improved antiviral drugs and vaccines. Much of this effort is focused on studying influenza A viruses due to their ability to cause worldwide pandemics (1). However, although influenza B viruses are strictly human pathogens and as such do not have the potential to create such pandemic strains, they are responsible for significant seasonal epidemics (1). In fact influenza B viruses are the predominant circulating strain of influenza virus in one of every three years (2), making these viruses no less significant than their influenza A virus counterparts.

Despite being classified within distinct genera of the family *Orthomyxoviridae*, influenza A and B viruses share many characteristics in terms of virion structure, genetic coding strategies and protein function (3). Both types of viruses are segmented negative sense RNA viruses that contain eight segments of genomic viral RNA (vRNA) encoding up to 13 proteins. Each segment of vRNA is in the form of a viral ribonucleoprotein (vRNP) complex in which the RNA is encapsidated by nucleoprotein (NP) and associated with a polymerase complex (3). Each genome segment of influenza A and B viruses encodes one or more proteins that share similar structure and function between the different viruses, however genetic sequences are highly divergent at both the nucleotide and amino acid level. Our understanding of the biology of these viruses has improved significantly since the advent of plasmid-based reverse genetics techniques in the last 14 years (4-7). Although attempts to generate chimeric influenza A/B viruses have so far proven unsuccessful, reverse genetics has allowed the study of characteristics that are

unique to each virus, such as the NB and BM2 proteins of influenza B viruses (8-11). The results of such studies and those based on other traditional approaches (12-14) are beginning to explain the incompatibility of genetic components derived from each virus.

The NP of influenza viruses is an indispensable multifunctional protein involved in many stages of influenza virus replication (3). It is the major viral protein found within viral vRNP complexes in which it encapsidates the vRNA, with each monomer of NP binding approximately 24 nucleotides of vRNA (15). NP can homo-oligomerize (16), which adds a high-order structure to the vRNPs. Once released from the incoming virus particles the vRNPs are transported into the nucleus through a process that utilizes cellular importin proteins (17-19) with the nuclear localization sequences (NLS) in NP necessary and sufficient for this process (17, 18). Early in infection newly synthesized NP utilizes these NLS sequences to localize in the nucleus where it has essential roles in transcription and replication of the viral genome. Later in infection NP is found predominantly in the cytoplasm in the form of newly synthesized vRNPs, a process in which NLS sequences are masked by the binding of newly synthesized matrix protein M1 (20), ready for packaging into progeny virus particles.

NP is one of the most highly conserved proteins between influenza A and B viruses exhibiting up to 38% amino acid conservation. Despite these proteins having similar functions during viral replication there are obvious differences at the amino acid level, including differences in regions of known function. An example of this is the position of nuclear localization signals (NLS) within NP, as the NP of influenza A viruses (ANP) contains at least two regions previously shown to exhibit NLS activity (17, 21, 22), however these regions are absent from the NP of influenza B virus (BNP). One of the

striking differences between the NP of these viruses is that the first 69 residues of BNP show no homology with those of ANP and there is an evolutionarily conserved 50 amino acid extension on the N-terminus of BNP that is absent from ANP (14). The molecular structure of BNP has recently been solved and interestingly the entire N-terminal extension is missing from this structure due to lack of electron density, suggesting that this region of BNP is highly flexible (23). Previous work reported that this N-terminal extension is not essential for nuclear accumulation of BNP or for the protein to function in an *in vitro* transcription/replication assay (14). More recent studies have reported conflicting evidence demonstrating that multiple regions of the N-terminal extension are required for efficient nuclear import of BNP (24, 25).

None of the previous studies have assessed the role of the BNP N-terminal extension in the context of viral infection as previous work has been based on expressed recombinant proteins. Here we report the attempts to create mutant influenza B viruses using reverse genetics in which the BNP N-terminal extension has been deleted, truncated or mutated to assess the requirements of this region of BNP for the production of viable virus. The results demonstrate that the N-terminal extension is essential for virus production and truncation of more than three amino acids rendered the virus non-viable. We show that the 50 amino acid extension in its entirety is required for optimal nuclear import and that all sequences able to act as an NLS are located within the first 80 amino acids of BNP. Furthermore we show for the first time, to our knowledge, that various regions of the BNP N-terminal extension have significant roles in the processes of viral mRNA transcription and genome replication.

151 **Materials and Methods**

152 **Cell and Viruses**

153 293T and MDCK cells were maintained in Dulbecco's modified Eagle's medium (DMEM)
154 (Invitrogen) supplemented with 10% fetal calf serum (FCS) at 37 °C with 5% CO_2.
155 B/Yamanashi/98 wild-type (rBNP wt) and mutant viruses were generated using plasmid-
156 based reverse genetics as previously described (4, 5). Briefly, 293T cells were transfected
157 with eight genome-encoding bi-directional (pAB) plasmids using FuGENE 6 transfection
158 reagent (Promega) and at 16 h post-transfection the cells were co-cultured with MDCK
159 cells in serum-free DMEM containing 2.5 μg/mL N-acetyl trypsin (Sigma). Virus-containing
160 supernatant was harvested 4 days post-transfection, viruses propagated twice through
161 MDCK cells followed by plaque assay titration on MDCK cells. Viral RNA was extracted
162 using the QIAamp viral RNA kit (QIAGEN), followed by reverse-transcriptase PCR using
163 genome specific primers and the resultant DNA sequenced to confirm presence of the
164 desired mutations.

165

166 **Plasmids**

167 For protein expression experiments the B/Yamanashi/98 NP ORF was inserted into the
168 pCAGGS mammalian expression vector and an HA tag was fused to the C-terminus.
169 Mutations were introduced by site directed mutagenesis (primer sequences available on
170 request) and the presence of the desired mutations confirmed by DNA sequencing. For
171 virus recovery the pAB-NP plasmid was mutated by site-directed mutagenesis and
172 substituted into the reverse genetics system. To create the pHH-BNSren reporter plasmid
173 PCR primers were designed to amplify the renilla luciferase gene flanked by the non-
174 coding regions of the NS segment of the B/Yamanashi/98 virus (primer sequences
175 available on request). The resultant PCR product was cloned into the pHH-21 reverse-

176 genetics plasmid between the human RNA polymerase I promoter and terminator regions
177 using *Bsm*BI restriction sites.
178
179 **Luciferase-based mini-replicon assay**
180 293T cells in 12-well plates were transfected with 100 ng of pAB plasmids encoding PB1,
181 PB2, and PA, 100 ng of pCAGGS-NP (encoding wt or mutant NP proteins), 250 ng of
182 pHH-BNSren and 10 ng of pCMV-FF (which encodes firefly luciferase under the control of
183 the CMV promoter used to normalize variations in transfection efficiency). 24 hours post
184 transfection, cells were lysed and firefly and renilla luciferase activities were measured
185 using the dual luciferase reporter assay (Promega). For luciferase assays after virus
186 infection, 293T cells in 12-well plates were transfected with 250 ng of pHH-BNSren and
187 10 ng of pCMV-FF for 24 h. Cells were then infected with either rBNP wt, rBNP-
188 K44A/R45A, rBNP-M4 or rBNP-D7E virus at an MOI of 5 for 12 h. Cells were lysed and
189 firefly and renilla luciferase activities were measured using the dual luciferase reporter
190 assay. Results represent the average of three independent experiments ± S.D.
191
192 **Immunoblotting**
193 293T cells in 12-well plates were transfected with 500 ng of pCAGGS-NP constructs and
194 at 24 hours post-transfection cells were lysed in 2x disruption buffer (6 M urea, 2 M β-
195 mercaptoethanol, 4% sodium dodecyl sulphate). Proteins were separated by SDS-PAGE
196 and transferred to Immobilon-FL polyvinylidene difluoride membranes (Millipore).
197 Membranes were blocked in blocking buffer (PBS, 0.1% Tween 20, 5% dried milk) and
198 incubated with anti-HA (Sigma) or anti-actin (Sigma) monoclonal antibodies followed by
199 IRDye 680- or IRDye 800-conjugated secondary antibodies (Licor). Protein detection was

performed using an Odyssey CLx near infrared scanner (Licor), images were collected using ImageStudio (Licor) and processed using Photoshop CS5 software (Adobe).

Nuclear / cytoplasmic fractionation

Confluent 293T cells in 25 cm^2 flasks were transfected with 3 μg of pCAGGS-NP (encoding wt or mutant NP proteins) using FuGENE 6. At 24 h post-transfection cells were washed in PBS before cellular fractionation as previously described (26). Briefly, cells were resuspended in DMEM containing 10% FCS and pelleted by centrifugation at 150 x g for 5 mins. Cells were washed with PBS and resuspended in 500 µl DMEM containing 20 µg/ml digitonin (Sigma) followed by incubation on ice for 10 min. Following centrifugation (2000 x g / 4°C / 10 mins) the supernatant (cytoplasmic fraction) was collected and the pellet (nuclear fraction) was resuspended in 100 µl 1% NP-40 in PBS, followed by the addition of 100 µl 2x disruption buffer. Equivalent proportions of cytoplasmic and nuclear fractions were analyzed by immunoblotting. The purity of cytoplasmic and nuclear fractions was analyzed using an anti-tubulin antibody and an anti-B23 (nucleolar phosphoprotein B23; nucleophosmin [NPM]) antibody respectively. BNP was detected using an anti-B/Hong Kong/73 polyclonal antisera. Protein detection was performed using IRDye 680- or IRDye 800-conjugated secondary antibodies (Licor) on an Odyssey CLx near infrared scanner (Licor), images were collected and protein band intensities were quantified using ImageStudio (Licor). For virus infected cells, MDCK cells were infected with viruses at an MOI 5, cells were collected at 4, 6, 8 and 10 h post-infection and cellular fractionation was performed as described above.

Immunofluorescence

293T cells were transfected with 100 ng of the pCAGGS-NP constructs for 24 hours followed by fixation in 5% formaldehyde and permeabilization in PBS/0.5% Triton X-100/0.5% NP40. Cells were blocked in PBN blocking buffer (PBS/1% BSA/0.02% sodium azide) for 1 h at room temperature followed by incubation with either an anti-influenza B NP monoclonal antibody (Abcam) or a rabbit polyclonal anti-HA antibody (Sigma). Cells were then incubated with either a Texas Red-conjugated goat anti-mouse (Abcam) or Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody (Life Technologies), followed by incubation with 1 µg/ml 4',6-diamidino-2-phenylindole (DAPI) for 5 min. Images were captured at either 20x or 63x magnification using an Axioplan 2 epi-fluorescent microscope (Zeiss), data analyzed using Axiovision 4.8.1 software (Zeiss) and processed using Photoshop CS5 software (Adobe). For immunofluorescence analysis of virus-infected cells, MDCK cells were infected with either rBNP wt, rBNP-K44A/R45A, rBNP-M4 or rBNP-D7E virus at an MOI of 5. At various times post-infection cells were fixed in 5% formaldehyde and processed as above.

Virus replication kinetics

For multiple cycle growth kinetics analysis MDCK cells were infected with either rBNP wt, rBNP-K44A/R45A, rBNP-M4 or rBNP-D7E virus at an MOI of 0.001 and supernatant samples harvested every 12 h until 72 hours post-infection. For single cycle replication analysis cells were infected with each of the viruses at an MOI of 5 and supernatant samples harvested every 3 h until 24 hours post-infection. All infections were performed in triplicate. The infectivity of the samples was determined by titration on MDCK cells by plaque assay. Results represent the average of three independent experiments ± S.D.

Plaque assays

MDCK cells in six-well plates were infected with serial 10-fold dilutions of each virus in serum-free DMEM for 1 h at 37 °C. Cells were overlaid with DMEM-1% agarose supplemented with 2 µg/ml N-acetyl trypsin and incubated at 37 °C for 72 h. Cells were fixed in 5% formaldehyde and plaques visualized by staining with 1% crystal violet.

Quantitative reverse transcription-PCR (qRT-PCR)

Renilla mRNA, vRNA and cRNA were quantified in samples used in the luciferase-based mini-replicon assay by qRT-PCR. Total cellular RNA was extracted from transfected 293T cells using the RNeasy Kit (QIAGEN). Total mRNA was reverse transcribed using an Oligo(dT) primer (Promega) and renilla vRNA or cRNA was reverse transcribed using renilla vRNA or cRNA gene-specific primers, using RevertAid Premium Reverse Transcriptase (Thermo Scientific). For qPCR renilla gene-specific primers (sequences available on request) were designed to amplify a 150 nt fragment of DNA and various concentrations of primers were optimised against each other by qPCR using various concentrations of pHH-BNSren as a standardized template. cDNAs generated by reverse transcription were then assayed by qPCR using serial four-fold dilutions of cDNA and 1µM renilla gene-specific primers using Precision Mastermix (Primer Design) on a Stratagene Mx3005P real-time PCR thermocycler. A standard curve was generated using serial 10-fold dilutions of pHH-BNSren and used to convert ct values of cDNA samples into DNA concentrations. The cDNA samples were also analysed for actin mRNA content using actin gene-specific primers to normalize for cell number. cDNA samples were then also analysed for firefly luciferase (FFluc) mRNA content using FFluc gene-specific primers to normalize for transfection efficiency. After renilla cDNA values were normalised

for cell number and transfection efficiency the data was expressed as a percentage of the BNP wt transfected cells. All results show the average of triplicate qPCR analyses ± SD.

For quantification of viral mRNA, vRNA and cRNA levels MDCK cells were infected with rBNP wt or mutant viruses at an MOI of 5 and at various times post-infection total cellular mRNA was extracted using the RNeasy kit. Total mRNA was reverse transcribed using an Oligo(dT) primer and viral vRNA/cRNA (HA gene segment) was reverse transcribed using either an HA vRNA or HA cRNA gene-specific primer, using RevertAid Premium Reverse Transcriptase. For qPCR HA gene-specific primers (sequences available on request) were designed to amplify a 150 nt fragment of DNA, primers were optimised and used in qPCR as above using serial four-fold dilutions of cDNA and pAB-HA plasmid as a DNA standard. The cDNA samples were also analysed for actin mRNA content using actin gene-specific primers to normalize for cell number. After actin normalization the data was expressed as a percentage of the rBNP wt virus-infected cells at each time point. All results show the average of triplicate qPCR analyses ± SD.

299 **Results**

300 **The influenza B virus NP N-terminal extension is required for optimal replication of**
301 **a viral-like RNA.**

302 To determine whether the N-terminal extension of the influenza B virus NP (BNP) is
303 required for optimal viral replication constructs were created that expressed serially
304 truncated versions of BNP (Fig. 1A). The BNP ORF was inserted into the pCAGGS
305 mammalian expression plasmid and the ATG initiation codons at positions one and four
306 were removed by site-directed mutagenesis to create the pCAGGS-BNP-ΔATG plasmid.
307 ATG initiation codons were then introduced into the BNP ORF at codon positions 11, 21,
308 31, 41 and 51 and an HA tag was introduced at the 3' end of the ORF. We also identified
309 residues K44 and R45 as part of a putative nuclear localization signal (NLS), which has
310 since been reported by Wanitchang *et al.,* (2013). Therefore the pCAGGS-BNP-
311 K44A/R45A construct was created in which residues 44 and 45 were mutated to alanine
312 (Fig. 1A). The panel of mutant constructs were then used in a mini-replicon assay to
313 assess the ability of the mutant BNP proteins to support replication of a viral-like RNA. For
314 this purpose a mini-replicon plasmid (pHH-BNSren) was created in which the renilla
315 luciferase gene was inserted between the non-coding regions of the influenza B virus
316 vRNA segment eight and inserted into a plasmid in a negative sense orientation under the
317 control of the human RNA polymerase I promoter. The pHH-BNSren plasmid was
318 transfected into 293-T cells alongside plasmids expressing the viral polymerase subunits
319 and the panel of BNP mutants and 24 hours post-transfection (h.p.t.) the luciferase
320 activity was measured. Despite residues 44 and 45 appearing to be part of a NLS,
321 mutation of these residues did not significantly affect the ability of BNP to support
322 replication of a viral-like RNA as luciferase levels were similar to those of wild-type (wt)
323 BNP (Fig. 1B). However as the 50 amino acid extension was serially truncated by 10

amino acids at a time the luciferase activity was successively reduced such that complete removal of the first 50 residues of BNP (BNP-P51M) resulted in less than 40% luciferase activity when compared to wt BNP. This reduction was not due to lower expression or instability of the truncated BNP proteins as expression levels were similar for each construct (Fig. 1C).

It was noted that a protein product with a lower molecular weight than wt BNP was expressed by the pCAGGS-BNP-ΔATG plasmid (Fig. 1C and D). In this construct the first ATG the ribosome would encounter is at codon 82 and is in frame with the BNP ORF. This protein product (hereafter termed BNP-Δ81) would be 479 amino acids in size with an approximate molecular weight of 48-50 kDa and is likely the protein product observed in Fig. 1C. As the BNP-Δ81 protein lacks the epitope for the BNP-specific antibody the protein could only be detected using the anti-HA antibody. Immunofluorescence analysis suggests that this protein is virtually entirely cytoplasmic (Fig. 1D), thereby confirming that the N-terminal extension is essential for nuclear localization of BNP. Nuclear localization was analysed for all of the mutant BNP proteins by immunofluorescence using a BNP-specific antibody. Increasing truncation appeared to increase the number of cells that contained predominant cytoplasmic localization (Fig. 2A). For each mutant nuclear versus cytoplasmic localization was determined by cellular fractionation and immunoblotting (Fig. 2B). The BNP-K44A/R45A mutation resulted in an increase in the cytoplasmic localization of BNP compared to BNP wt (Fig. 2C). If residues 44 and 45 are part of an NLS their removal must be compensated for by another region of the N-terminal extension. Furthermore the luciferase data (Fig. 1B) suggests that a significant proportion of BNP-K44A/R45A was nuclear as viral RNA transcription and replication occur exclusively in the nucleus.

Although the nuclear localization of the truncation mutants was altered, these differences did not appear great enough for mutants such as BNP-I21M and BNP-P41M to cause the reductions in luciferase levels observed in Fig. 1B. Therefore effects of the truncations on the ability of BNP to aid in transcription and replication of the renilla viral-like RNA were addressed. Total RNA was extracted from 293T cells transfected as in Fig. 1B and levels of renilla mRNA, vRNA and cRNA were analysed by quantitative reverse transcriptase PCR (qRT-PCR). As expected renilla mRNA levels mirrored the levels of luciferase activity in Fig 1B (data not shown), however differences were observed in renilla vRNA and cRNA levels (Fig. 3). Similar to the results of the luciferase activity assay the levels of vRNA in in BNP-K44A/R45A transfected cells were similar to those of BNP wt. Levels of vRNA in cells transfected with the truncation mutants were reduced, with increasing truncation resulting in slightly decreasing levels of vRNA. However all truncation mutants displayed vRNA and cRNA levels higher than those of the “no polymerase” negative control suggesting all mutants were able to aid in replication of vRNA, albeit to a lower level than BNP wt. The vRNA levels in the “no polymerase” control demonstrate the levels of vRNA driven from the pHH-BNSren plasmid only as the cRNA levels for this mutant suggest that no vRNA replication took place. The vRNA levels in the BNP-ΔATG sample were increased compared to the negative control and the cRNA levels suggest that this was due to a very low level of vRNA replication. Therefore despite the complete inactivity of the BNP-ΔATG mutant in the luciferase activity assay (due to the lack of mRNA synthesis) the BNP-Δ81 protein produced from this mutant construct was able to aid replication of vRNA, suggesting that the transcriptase activity of the polymerase was more significantly affected by the removal of the 81 N-terminal residues of BNP than its genome replicative ability.

In this assay system newly synthesized vRNA is able to act as a template for the production of mRNA, therefore decreases in vRNA synthesis should lead to decreases in mRNA levels. It is therefore possible that the reductions in renilla protein production were due not only to reductions in BNP nuclear localization but also to reductions in the levels of vRNA produced as a consequence of the BNP mutations.

Residues within the first 10 amino acids of BNP are required for optimal replication of a viral-like RNA and are essential for virus viability.

The panel of mutations was then introduced into the pAB-NP reverse genetics construct (4) in the attempt to recover mutant influenza B viruses containing these mutations. BNP-K44A/R45A was the only mutation that allowed the generation of infectious virus and as the BNP-T11M mutation prevented virus recovery it suggests the first ten residues of BNP are essential for virus viability. Therefore another panel of mutant pCAGGS-NP constructs were created in which individual amino acid truncations were introduced into the first ten codons of the BNP ORF (Fig. 4A). Residues 5-8 (DIDG) form a potential caspase cleavage site therefore residue 7 was mutated from aspartic acid to either alanine or glutamic acid in the attempt to ablate this function. The introduction of the initiating methionine at codon 5, 6 or 8 had a significant effect on the expression of the BNP protein and resulted in production of another protein product of a lower molecular weight (Fig. 4B). It is likely that these mutant forms of BNP are less stable and that the observed protein product is a cleaved form of BNP, as similar findings were reported by Wanitchang *et al.* (2013).

When the mutant constructs were used in the luciferase-based mini-replicon reporter assay all mutant BNP proteins displayed a reduced ability to support replication of a viral-like RNA, with only the BNP-M4 and BNP-D7E mutants reaching 80% luciferase activity when compared to wt BNP (Fig. 4C). The greatest reduction in luciferase activity was observed for the BNP-D5M mutant. It is possible that this may be due to the lower expression level of the full-length protein, an altered distribution within the cell or the ablation of an essential function of BNP during RNA transcription and replication. The expression level of BNP-I6M was reduced to a similar level as BNP-D5M (Fig. 4B) and although it displayed a reduced luciferase activity compared to BNP wt, this was not as significant a reduction as that of the BNP-D5M mutant. This suggests that although the reduction in BNP expression likely had a large influence on renilla mRNA expression in the BNP-D5M and BNP-I6M transfected cells, protein expression may not be solely responsible for the observed decrease in luciferase activity for the BNP-D5M mutant.

The panel of mutants was tested for cellular distribution of BNP. Mutants BNP-D7M, BNP-G8M, BNP-N10M and BNP-D7A displayed moderate increases in cytoplasmic localization compared to wt BNP, with increasing truncation leading to slight increases in cytoplasmic localization (Fig. 5A). The BNP-I6M mutant was the only BNP protein to show a considerable increase in cytoplasmic localization. The reason for this is unclear, however both the reduction in nuclear availability of this mutant and the decrease in protein levels likely resulted in the decrease in luciferase levels observed in Fig. 4C. The BNP-D5M mutant actually demonstrated a slightly enhanced nuclear localization when compared to BNP wt (Fig. 5A). Although this was not a considerable enhancement it demonstrates that the reduction in luciferase activity for the BNP-D5M mutant was likely not due to an alteration in cellular distribution of BNP. Therefore the most likely explanation for the

reduction in BNP-D5M-mediated luciferase levels was due to the mutation affecting an essential function of BNP during mRNA transcription/replication. To address this the levels of renilla mRNA, vRNA and cRNA in the mini-replicon samples (Fig. 4C) were assessed by qRT-PCR. As expected renilla mRNA levels mirrored the levels of luciferase activity in Fig 4C (data not shown), however differences were observed in renilla vRNA and cRNA levels (Fig. 5B). All mutant BNP proteins with the exception of BNP-D7E displayed reduced levels of renilla vRNA of between 55 and 80% that of BNP wt. This suggests that individual amino acid truncations in the first ten residues of BNP reduce the ability of BNP to aid in replication of a viral-like RNA and this region of BNP is essential for optimal genome replication. Interestingly, despite displaying the highest luciferase activity of any of the mutants the BNP-M4 mutant displayed a similar reduction in vRNA to the other truncation mutants. The reason for this is unclear, however it is plausible that the ability of the BNP-M4 protein to aid transcription of mRNA is affected to a lesser extent than the other mutants, which would result in higher levels of BNP-M4 mRNA compared to the other mutants, despite similar levels of vRNA production.

The mutations were then introduced into the pAB-NP plasmid for virus recovery. Only the BNP-M4 and BNP-D7E mutations allowed recovery of viruses, suggesting that BNP can only withstand the truncation of 3 residues without compromising virus viability. Interestingly these were the only two mutant proteins able to support luciferase activity levels to over 80% of BNP wt, with other mutants showing reduced luciferase activity (Fig. 4C). These results suggest that the composition of residues in this region of BNP is essential for virus viability.

Mutations in the first ten residues of BNP lead to virus attenuation.

To analyse the growth kinetics of the recovered mutant viruses MDCK cells were infected with rBNP wt (wild-type virus), rBNP-K44A/R45A, rBNP-M4 and rBNP-D7E viruses at a multiplicity of infection (MOI) of 0.001, samples taken every 12 hours post-infection (h.p.i) and titrated by plaque assay. All three mutant viruses were attenuated compared to wt virus and reached their peak titer 12-24 hours after that of rBNP wt virus (Fig. 6A). Although the rBNP-K44A/R45A virus was attenuated 10-fold at early time points it reached a similar titer to rBNP wt. However the rBNP-M4 and rBNP-D7E mutants showed greater attenuation, especially at early time points, therefore it was plausible that the effects of the mutations were more prominent during the first few rounds of replication. Therefore the growth kinetics of the viruses were monitored through a single cycle of replication. MDCK cells were infected with the viruses at an MOI of 5, samples taken every 3 hours post-infection and titrated by plaque assay. Fig. 6B suggests that all virus infections began releasing infectious particles at the same time post-infection with only half a log difference in infectious titer at 9 h.p.i. Whereas rBNP-K44A/R45A retained wt-like titers throughout, the rBNP-M4 and rBNP-D7E viruses were both attenuated by nearly 2 orders of magnitude when infectivity began to tail off. No differences in particle-infectivity ratios were observed for any of the viruses (data not shown), which suggests that the attenuation was not due to mutant BNP-mediated vRNP packaging defects resulting in the overproduction of defective virus particles.

The reduced ability of mutant BNP proteins to support transcription and replication of a viral-like RNA in the context of infection does not correlate with cellular distribution.

To test the effects of the BNP mutations on viral transcription and replication during infection the mutant viruses were used in the luciferase-based mini-replicon assay. 293T cells were transfected with pHH-BNSren for 24 hours followed by infection with each virus at an MOI of 5. At 12 h.p.i cells were lysed and luciferase activity was measured. All three mutant viruses resulted in a significant reduction in luciferase activity compared to wt virus (Fig. 7A). The ability of the rBNP-K44A/R45A BNP protein to support transcription/replication of a viral RNA was reduced to 77% that of wt virus (p=0.02). An even greater reduction was observed for the rBNP-M4 and rBNP-D7E viruses, resulting in 37% (p=0.0005) and 51% (p=0.008) activity respectively. This mirrors the attenuation observed for these two mutants in Figs. 6A and B, which could be as a result of altered cellular distribution of the mutant BNP proteins during infection. To address this 293T cells were infected with each virus at an MOI of 5 and at various times post-infection BNP cellular distribution was analysed by immunofluorescence analysis (Fig. 7B) and cellular fractionation (Fig. 7C). Cellular distribution of BNP in rBNP-M4 and rBNP-D7E virus-infected cells was not significantly different to that in rBNP wt virus-infected cells at each time point (Fig. 7B and C). However at all times post-infection rBNP-K44A/R45A virus-infected cells displayed significant increases in cytoplasmic BNP localization (Fig. 7C), confirming the notion that these residues form part of a NLS. This may also indicate that the slight reduction in luciferase levels observed for the rBNP-K44A/R45A mutant in Fig. 7A were due to a decrease in the levels of nuclear BNP compared to those in rBNP wt virus-infected cells. However as there were no significant differences in cellular BNP distribution for the rBNP-M4 and rBNP-D7E mutants compared to rBNP wt the reduction

in luciferase activity observed for these mutants cannot be attributed to effects of the mutations on cellular localization of BNP.

Mutations in the N-terminal extension of BNP directly affect the transcription and replication of viral RNA species during infection.

The effects of the mutations on the ability of BNP to participate in both transcription of viral mRNA and genome replication were assessed. MDCK cells were infected with each virus at an MOI of 5 and at various times post-infection total cellular RNA was extracted for qRT-PCR analysis using primers specific for viral mRNA, vRNA and cRNA. At 4 h.p.i viral mRNA was reduced in all mutant virus-infected cells compared to rBNP wt virus-infected cells (Fig. 8A). By 8 h.p.i. mRNA levels in the rBNP-K44A/R45A mutant virus-infected cells were actually higher than in rBNP wt virus-infected cells, whereas the other mutants demonstrated significantly decreased levels of mRNA. Levels of mRNA in the rBNP-D7E virus-infected cells increased between 4 and 6 h.p.i., however this began to decrease and by 8 h.p.i. mRNA levels were back to those observed at 4 h.p.i., approximately 40% of rBNP wt mRNA. The mRNA levels in rBNP-M4 virus-infected cells remained at 40-50% those of rBNP wt throughout the experiment. These decreases in mRNA levels were likely responsible for the attenuation in the growth curves (Fig. 6) and the reduction in luciferase levels (Fig. 7A). The reduction in rBNP-K44A/R45A mRNA levels at early time points suggests that this mutant exhibits a delay in mRNA synthesis, which is likely responsible for the slight reduction in luciferase levels in Fig. 7, however as wt-like levels of mRNA are produced within the time frame of a single cycle of replication this explains the lack of attenuation of the mutant in the growth curve.

To determine whether the observed reduction in mRNA levels was a result of altered vRNA or cRNA levels produced during infection the samples were also analysed for vRNA/cRNA content by qRT-PCR. Interestingly the rBNP-K44A/R45A mutant demonstrated increased vRNA and cRNA levels at all time points when compared to rBNP wt (Fig. 8B and 8C), despite the reduction in mRNA levels at early time points. This may suggest that this mutant BNP protein exhibits a preference for genome replication over transcription, as it is not until vRNA levels reach 200% those of rBNP wt that the rBNP-K44A/R45A mRNA levels reach those of rBNP wt virus-infected cells. Both the rBNP-M4 and rBNP-D7E virus-infected cells contained strikingly reduced vRNA/cRNA levels when compared to rBNP wt, with the trend in vRNA/cRNA levels over time mirroring the mRNA levels in Fig. 8A. These results suggest that the mutations in the first ten residues of BNP significantly reduce the ability of the protein not only to function in mRNA transcription but also vRNA replication, which likely has a subsequent affect on secondary rounds of mRNA transcription, ultimately leading to viral attenuation. The results also suggest that in the context of viral infection the K44A/R45A mutations reduced the ability of BNP to aid in viral mRNA transcription, but did not negatively affect the function of the protein in viral genome replication.

Discussion

There are contradicting reports concerning the role of the N-terminal region of BNP in terms of its effect on BNP cellular distribution and the effect this likely has on the ability of BNP to support transcription and replication of a viral-like RNA (14, 24, 25). None of the previous studies were performed in the context of viral infection but by using protein expression methods in which intracellular conditions are significantly different from those during infection (27, 28). Therefore to gain a true understanding of the role of the BNP N-terminus we sought to study this in the context of viral infection through the use of reverse genetics.

Truncation of BNP to remove the 50 amino acid extension prevented virus recovery, which demonstrates that this region of BNP is essential for the production of viable virus. Successive truncations in groups of 10 residues at the BNP N-terminus also failed to result in viable virus, suggesting that the first 10 residues of BNP are critical for virus viability. Previous studies have indicated that packaging of influenza A virus vRNPs into progeny virions requires packaging signals in the coding regions of the vRNA segments (29). Such signals, although likely present in influenza B viruses, are currently unknown. However as no nucleotides were deleted from the BNP ORF in our experiments and only minimal nucleotide changes were made it is unlikely that the lack of virus recovery was due to defects in packaging signals.

Increasing the length of BNP truncation increased the extent of cytoplasmic localization of the protein, which correlated with a reduction in the ability of the mutant BNP proteins to support replication of a mini-genome reporter (Fig. 1B). This suggests that the truncated proteins were still able to function in transcription/replication if the protein was able to

localize in the nucleus. Despite the BNP-T11M mutant retaining over 60% functionality in this assay, it still did not result in the production of viable virus. This suggests that although these mutants are able to support transcription/replication either this is to an insufficient level to allow viable virus production or the truncation of BNP has affected another function of the protein. Our results are in conflict with those of Stevens and Barclay (1998) but corroborate those of Wanitchang *et al.,* (2013) in which they demonstrate that regions of the N-terminus of BNP affect nuclear import. We are unsure of the reason for the conflicting results of Stevens and Barclay, however we cannot rule out that this is a cell type-specific effect as they used canine kidney cells (MDCK) in their experiments whereas both this study and that of Wanitchang *et al.* used 293T cells, which are perhaps more relevant to influenza B virus being of human origin.

Even though all AUG initiation codons within the N-terminal extension of the BNP-ΔATG mutant were removed, a protein product (BNP-Δ81) was observed (Fig. 1), which is likely due to translational read-through. As the BNP-P51M mutant resulted in some nuclear localization of BNP but the BNP-Δ81 protein was virtually entirely cytoplasmic (Fig. 1D) this indicates that all NLS sequences in BNP are within the first 81 residues and that a region of BNP between residues 51 and 81 must contribute to nuclear localisation. We also noted that a particular sequence in BNP ($K_{44}RTR_{47}$) resembled an NLS, an observation that has since been reported to be functional by another group (Wanitchang, 2013). When this potential NLS was removed (BNP-K44A/R45A) cytoplasmic accumulation of BNP increased (Fig. 2B). Although Wanitchang *et al.* report that in the context of a full-length BNP protein this mutant was exclusively cytoplasmic our results suggest otherwise as 53% of mutant BNP protein was detected in the nuclear fraction of BNP-K44A/R45A transfected cells. Therefore although this sequence is involved it is not

595 essential for nuclear localization of BNP as i) this mutant was able to support
596 transcription/replication of a mini-replicon to similar levels to those of wt BNP (Fig. 1B);
597 and ii) a mutant virus bearing these mutations was viable. Wanitchang *et al.* indicated that
598 two regions of the BNP appear to be required for nuclear localization, residues 3-15 and
599 44/45. Despite our BNP-P51M mutant lacking both of these regions, 33% of BNP-P51M
600 protein was found in the nuclear fraction (Fig. 2C). Taken together with the fact that the
601 BNP-Δ81 protein was virtually entirely cytoplasmic this strengthens the hypothesis that
602 residues between positions 51 and 81 also participate in nuclear localization of BNP. It is
603 possible that residues 44/45 and others between 51 and 81 form a bipartite NLS. Overall
604 our results confirm that the N-terminal extension of BNP is essential for its nuclear import,
605 that multiple regions of this extension are involved and that the N-terminal region in its
606 entirety is required for BNP to function optimally in this capacity.
607
608 Reductions in nuclear localization of the N-terminally truncated BNP proteins was likely
609 partially responsible for the lack of virus recovery, however our results also demonstrate
610 that the effects of the truncations on the ability of BNP to aid in transcription and
611 replication of vRNA likely contributed to the failure to recover infectious virus. Although
612 the BNP-Δ81 protein was able to allow a low level of vRNA replication to occur it was
613 completely unable to aid in mRNA transcription. This possibly indicates that there are
614 separate mechanisms behind the roles of BNP in mRNA transcription and viral genome
615 replication and that the BNP N-terminus, although required for optimal levels of both, is
616 absolutely essential for viral mRNA transcription to occur. Further work is required to
617 dissect these mechanisms to understand the functional roles of BNP in these processes.
618

619 Our results suggest that residues 3-10 of BNP are essential for virus viability. Wanitchang
620 *et al.* (2013) reported that residues in the first 15 amino acids of BNP are required to
621 protect the N-terminus of BNP from proteolytic cleavage, with N-terminal truncations of 5
622 or more amino acids resulting in a cleaved BNP product. Interestingly in BNP-D5M
623 transfected cells we observed a protein that likely corresponds to this cleaved form of
624 BNP (Fig. 4B). However as this band is such a minor species or completely absent from
625 most of the mutants it is unlikely that the lack of virus recovery was due to the truncations
626 preventing BNP from inhibiting its own cleavage. Despite this, both this study and that of
627 Wanitchang *et al.* (2013) agree that residues in the first 15 of BNP are essential for BNP
628 to function efficiently and we show for the first time that they are essential for virus
629 viability.
630
631 BNP has been shown to contain two caspase cleavage motifs with one located in the N-
632 terminal 10 amino acids from residues 5-8 (D_5IDG_8) (30). ANP from human-derived
633 influenza viruses has been shown to contain a similar site ($E_{14}TDG_{17}$). When the aspartic
634 acid at position 16 (P1 position) of ANP is mutated to glycine, as is found naturally in
635 avian influenza A viruses, this site is no longer a substrate for caspase cleavage. It is
636 thought that this cleavage mechanism is involved in regulating apoptosis during viral
637 infection. Therefore we sought to test whether this sequence in BNP is required for virus
638 viability. For most caspases the aspartic acid at position P1 is essential, however caspase
639 3 and 7 can function with a glutamic acid at this position (31). When residue D7 was
640 mutated to glycine (data not shown), alanine or aspartic acid only the D7E mutation
641 allowed virus recovery. It is plausible that D7E is the only mutation that may have
642 maintained a functional caspase cleavage site contributing to the lack of recovery of other
643 viruses. Another plausible explanation is that the D7A and D7G mutations may have

644 altered vRNP packaging signals, although it is unlikely that a single nucleotide change
645 would render such signals non-functional.
646
647 Of the mutant BNP constructs created only three led to the production of viable viruses
648 with two mutant viruses containing mutations in the first ten residues of BNP showing
649 significant attenuation. As replication analysis demonstrated that cells infected with any of
650 the four viruses began releasing infectious particles at the same time post infection (9
651 h.p.i.; Fig 6), the mutations therefore did not result in a lag in particle production. At this
652 time point the infectious titres of released virus were equivalent to one infectious particle
653 produced from every 10-50 cells infected, likely indicating that virus release had only just
654 begun. At this time post-infection it is likely that wt and mutant viruses had produced
655 enough protein/vRNPs to begin generating and releasing virus particles, therefore one
656 might not expect a large difference in released titres at this time point. However from 12
657 h.p.i. the rBNP-M4 and rBNP-D7E viruses were attenuated by 1.5-2 logs (Fig. 6B).
658 Despite the predominant nuclear localization of these mutant BNP proteins during
659 infection they demonstrated a reduced ability to support transcription and replication of a
660 luciferase-based mini-replicon (Fig. 7A) likely resulting from significant reductions in
661 mRNA and vRNA/cRNA synthesis over the time frame of a single cycle of replication (Fig.
662 8). It is plausible that after the initial virus release at 9 h.p.i. the higher levels of
663 transcription and replication of wt virus genome compared to those of the mutant viruses
664 resulted in higher levels of protein and RNA available for virion production. This potentially
665 generated higher levels of infectious wt virus over time, thereby resulting in the observed
666 attenuation of the mutant viruses after 9 h.p.i.
667

668 Interestingly although vRNA/cRNA levels in the context of viral infection were not affected
669 by the BNP-K44A/R45A mutations there was a significant decrease in mRNA levels prior
670 to 8 hours post-infection when compared to wt virus. This explains the decrease in
671 luciferase levels produced during infection of cells expressing a luciferase-based viral-like
672 RNA (Fig 7A). Although luciferase levels were reduced in this assay they were not
673 reduced in the transfection-based mini-replicon assay in Fig 1, however in the
674 transcription-based assay the mutant BNP mRNA was driven off a plasmid by RNA
675 polymerase II, which likely resulted in higher levels of mRNA. Overall these results might
676 suggest that the BNP-K44A/R45A mutations resulted in a preference for viral genome
677 replication over transcription. It is therefore likely that the majority of cytoplasmic BNP
678 observed in rBNP-K44A/R45A virus-infected cells from 10 h.p.i. represents BNP in the
679 form of newly synthesized vRNPs and explains the significant increase in cytoplasmic
680 BNP levels in rBNP-K44A/R45A virus-infected cells (Fig. 7). These findings indicate that
681 the N-terminal region of BNP may have a role in dictating whether the viral polymerase
682 undergoes transcription or genome replication in the early stages of viral infection. Further
683 work is required to confirm this and to elucidate the mechanism by which this occurs.
684
685 Overall our data demonstrates for the first time that the N-terminal extension of BNP is
686 essential for virus viability by controlling nuclear import of BNP and regulating viral mRNA
687 transcription and genome replication. Dis-regulation of these processes prevented virus
688 recovery. Although we agree with the previous reports that specific regions of BNP have a
689 strong influence on nuclear import we suggest that the entire N-terminal extension is
690 required for efficient BNP nuclear import and that sequences between residues 51 and 81
691 of BNP have a role in this process, potentially as part of a bipartite NLS. It is possible that
692 the mutations investigated in this study affected the structure of the protein such that

multiple functions of this region of BNP were affected. Unfortunately it is not possible to relate these functions to the structure of the protein as this region of BNP is highly flexible and therefore does not allow for X-ray crystallographic studies (23). The fact that the N-terminal extension of BNP is completely absent from the NP of influenza A viruses suggests that influenza A and B viruses have evolved different strategies for regulating transcription and replication of their genome. Further work is required to elucidate the mechanisms used by the N-terminal extension of BNP in these processes to enhance our understanding of the replication of influenza B viruses in an area that has significant potential for future vaccine/antiviral drug design.

Acknowledgements

The pAB plasmids were kindly provided by Dr Robert Webster (St Jude Children's Research Hospital, Memphis TN, USA). Lee Sherry is indebted to the University of St Andrews for a Ph.D. studentship through an MRC Doctoral Training Grant. We also gratefully acknowledge support by the University of St Andrews, which is a charity registered in Scotland (No.SC013532).

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Figure legends

Figure 1

Increasing truncations of the BNP N-terminus results in decreasing ability to support transcription of a viral-like RNA.

(A) Schematic diagram of the influenza A and B virus NP indicating the truncations introduced into the N-terminal 50 amino acids of BNP. The putative nuclear localisation signal (NLS) is located between residues 44-47 (KRTR) and is indicated as a black box in the wt and truncated BNP constructs. The two mutated residues (K44A and R45A) are underlined. (B) Luciferase-based mini-replicon assay measuring the ability of BNP proteins to support transcription of a viral-like RNA. 293T cells were transfected with mammalian expression plasmids encoding the influenza B virus polymerase subunits and pCAGGS-NP plasmids encoding wt or mutated forms of BNP. Cells were co-transfected with the pHH-BNSren reporter plasmid and a pCMV-FF expression plasmid for normalization against firefly luciferase. 24 hours post-transfection cells were lysed and renilla and firefly luciferase activity measured. Results are expressed as the average of three independent experiments ± S.D. (C) BNP protein expression from the pCAGGS-NP plasmids. 293T cells were transfected with the pCAGGS-NP plasmids encoding HA-tagged wt or mutant BNP proteins and lysed at 24 hours post-transfection, followed by immunoblotting with an anti-HA monoclonal antibody. Actin was detected as a loading control. (D) Immunofluorescence analysis of BNP wt and BNP-ΔATG protein expression. 293T cells were transfected with either pCAGGS-NP wt or pCAGGS-BNP-ΔATG for 24 h. Immunofluorescence was performed using an anti-BNP monoclonal antibody and a rabbit anti-HA polyclonal antibody, followed by anti-rabbit Alexa Fluor 488 and anti-mouse Texas Red conjugated antibodies. Nuclei were stained with DAPI. Images were taken at 63x magnification.

Figure 2

Increasing truncations of the BNP N-terminus results in increased cytoplasmic localization of BNP.

(A) Immunofluorescence analysis of BNP expression. 293T cells were transfected with pCAGGS-NP plasmids encoding wt or mutated BNP for 24 h. Immunofluorescence was performed using an anti-BNP specific monoclonal antibody and an anti-mouse Texas Red conjugated antibody. Nuclei were stained with DAPI. Images were taken at 20x magnification. The inset images in the BNP panel show cells at 80x magnification. (B) Analysis of nuclear and cytoplasmic localization of BNP. 293T cells were transfected as in (A), cells were lysed and separated into nuclear and cytoplasmic fractions. Samples were analysed by immunoblotting using an anti-B/Hong Kong/73 serum to detect BNP, an anti-tubulin antibody and an anti-B23 antibody to determine the purity of the cytoplasmic and nuclear fractions respectively. C = cytoplasmic fraction, N = nuclear fraction. (C) Quantitation of nuclear and cytoplasmic localization of BNP. The cytoplasmic and nuclear populations of BNP for each sample in (B) were quantified by densitometry and the percentage of BNP in the cytoplasmic fraction of each sample was determined.

Figure 3

Truncation of BNP leads to decreased levels of viral genome replication.

The effects of mutant BNP proteins on replication of a viral-like RNA. The samples used in the luciferase-based mini-replicon assay in Fig. 1B were analysed for renilla vRNA and cRNA content by qRT-PCR. Total RNA was isolated from transfected samples and renilla vRNA or cRNA was reverse transcribed using renilla gene-specific primers. Alternative renilla gene-specific primers were used to amplify a 150 nt DNA product by qPCR. Both vRNA and cRNA values were normalised against actin mRNA levels to control for cell

number and further normalised against firefly luciferase mRNA levels to control for transfection efficiency. Results are expressed as the percentage of BNP wt and are the average of triplicate samples ± SD.

Figure 4

Individual amino acid truncations and mutations in the first ten residues of the BNP N-terminus do not significantly alter the ability to support transcription of a viral-like RNA.

(A) Schematic diagram of BNP indicating the wt amino acid sequence and the individual truncations/mutations introduced into the N-terminal 10 amino acids. The translation initiation codon of each construct is highlighted in a box and the individual nucleotide mutations introduced are underlined and in bold. (B) BNP protein expression from the pCAGGS-NP plasmids. 293T cells were transfected with the pCAGGS-NP plasmids encoding HA-tagged wt or mutant BNP proteins and lysed at 24 hours post-transfection, followed by immunoblotting with an anti-HA monoclonal antibody. Actin was detected as a loading control. (C) Luciferase-based mini-replicon assay measuring the ability of BNP proteins to support transcription of a viral-like RNA. 293T cells were transfected as in Fig. 1B. 24 hours post-transfection cells were lysed and renilla and firefly luciferase activity measured. Results are expressed as the average of three independent experiments ± S.D.

Figure 5

Individual amino acid truncations and mutations in the first ten residues of the BNP N-terminus do not significantly alter cellular distribution of BNP but do affect vRNA replication.

(A) Quantitation of nuclear and cytoplasmic localization of BNP. 293T cells were transfected with the pCAGGS-NP constructs encoding wt or individual amino acid truncated/mutated BNP proteins. Analysis of nuclear and cytoplasmic localization of BNP was performed as in Fig. 2. (B) The samples used in the luciferase-based mini-replicon assay in Fig. 4C were analysed for renilla vRNA and cRNA content by qRT-PCR as in Fig. 3. Results are expressed as the percentage of BNP wt and are the average of triplicate samples ± SD.

Figure 6

Viruses containing mutations in the N-terminal region of BNP display varying levels of attenuation.

(A) Multiple cycle replication analysis. MDCK cells were infected with rBNP wt or mutant viruses at an MOI of 0.001. Samples were harvested every 12 h until 72 h.p.i. and titrated by plaque assay. Results represent the average of three independent experiments ± S.D. (B) Single cycle replication analysis. MDCK cells were infected with rBNP wt or mutant viruses at an MOI of 5. Samples were harvested every 3 h until 24 h.p.i. and titrated by plaque assay. Results represent the average of three independent experiments ± S.D.

Figure 7

BNP of mutant viruses displays a reduced ability to support transcription of a viral-like RNA and an altered cellular localization.

(A) Luciferase-based mini-replicon assay measuring the ability of virally encoded BNP proteins to support transcription of a viral-like RNA. 293T cells were transfected with the pHH-BNSren reporter plasmid and a pCMV-FF expression plasmid for normalization against firefly luciferase. 24 hours post-transfection cells were infected with rBNP wt or mutant viruses at an MOI of 5. At 12 h.p.i. cells were lysed and renilla and firefly luciferase activity measured. Results are expressed as the average of three independent experiments ± S.D. (B) Expression and cellular localization of BNP in virus-infected cells. MDCK cells were infected with rBNP wt or mutant viruses at an MOI of 5 and fixed at various times between 4 and 12 h.p.i. Immunofluorescence analysis was performed as in Fig. 2A. Images were taken at 40x magnification. The boxes in the bottom right of each panel show regions of the image at 80x magnification. (C) Quantitation of cytoplasmic localization of BNP. MDCK cells were infected with rBNP wt or mutant viruses at an MOI of 5 and at 6, 8 or 10 h.p.i. nuclear / cytoplasmic fractionation was performed as in Fig. 2B. Cytoplasmic levels of BNP were determined and expressed as a percentage of rBNP wt at each time point.

940 **Figure 8**

941 **Viruses with mutations in the BNP N-terminus display altered levels of viral**
942 **transcription and genome replication.**

943 The effects of mutant BNP proteins on transcription and replication of viral RNA species.
944 MDCK cells were infected with rBNP wt or mutant viruses at an MOI of 5 and at various
945 times post-infection cells were lysed and total cellular RNA extracted. Samples were
946 analysed for (A) viral mRNA, (B) vRNA and (C) cRNA content by qRT-PCR. mRNA was
947 reverse transcribed using Oligo(dT), whereas vRNA and cRNA were reverse transcribed
948 using an HA vRNA- or cRNA-specific primer. HA gene-specific primers were used to
949 amplify a 150 nt DNA product by qPCR. HA mRNA, vRNA and cRNA values were
950 normalised against actin mRNA levels to control for cell number. Results are expressed
951 as the percentage of BNP wt at each time point and are the average of three independent
952 experiments ± SD.

953