Effects of a point mutation in the 3’ end of the S genome segment of naturally occurring and engineered Bunyamwera viruses

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Received 7 November 2002
Accepted 17 December 2002

The genome of Bunyamwera virus (BUN) consists of three segments of single-stranded RNA of negative polarity. The smallest segment, S, encodes the N protein and a nonstructural protein called NSs. We recently described a mutant virus (BUNdelNSs) that does not express NSs but overexpresses N and grows to lower titres than wild-type (wt) BUN. Here we report a BUNdelNSs variant that expresses lower levels of N protein and grows to higher titres. Sequencing of the 3’ and 5’ termini of the BUNdelNSs S RNA segment and analysis using a minireplicon system show that the N overexpressing phenotype results from a single nucleotide substitution at position 16 in the 3’ terminus. This mutation could also be detected in wtBUN populations, and was isolated by plaquing a ‘wt’ variant carrying the mutation. This variant was found to express increased N and NSs levels, and grew to lower titres than wtBUN.

Bunyamwera virus (BUN) is the prototype of the Bunyaviridae, a family of mainly arthropod-borne viruses. Bunyaviruses have become increasingly recognized as posing a threat to human health as examples of the so-called ‘emerging infections’. The BUN genome consists of three segments of single-stranded RNA of negative polarity. The largest segment, L, codes for an RNA-dependent RNA polymerase (L protein), the medium segment (M) for two glycoproteins (G1 and G2) and a nonstructural protein (NSm), whereas the smallest segment codes for the nucleoprotein N and a second nonstructural protein called NSs (Elliott, 1996, 1997). Virus replication takes place in the cytoplasm, while maturation and budding occur at the Golgi apparatus (Bishop, 1996).

Following infection viral mRNAs are transcribed from genomic RNAs that contain a non-templated, host-derived capped primer at the 5’ end and are truncated but not polyadenylated at the 3’ end (Bouloy et al., 1990; Jin & Elliott, 1993, Patterson & Kolakofsky, 1984). For replication, the negative-stranded genome RNAs serve as template for the synthesis of full-length, positive-sense RNAs called antigenomes, which in turn serve as templates for synthesis of progeny negative-stranded genomes. Genomes and antigenomes are encapsidated by the N protein to form biologically active structures called the viral ribonucleoproteins or RNPs. The 3’ and 5’ genome termini are complementary and interact to form a ‘panhandle’ structure (Hacker & Kolakofsky, 1991). Reverse genetics systems developed for BUN and other members of the Bunyaviridae (Dunn et al., 1995; Lopez et al., 1995; Flick & Pettersson, 2001) have shown that transcription and replication of artificial minigenomes require only two viral proteins, the polymerase (L) and the N protein.

For bunyaviruses the N and NSs proteins are translated from a single mRNA but use different start codons and are encoded in different open reading frames (ORF), the NSs ORF being located within the N ORF. Previously we described recovery of infectious bunyavirus entirely from cDNA (Bridgen & Elliott, 1996) and subsequently we reported the creation of BUNdelNSs, a BUN virus that no longer expresses NSs (Bridgen et al., 2001). Point mutations were introduced into the S segment to ablate the NSs ORF but maintain the N ORF intact (Fig. 1A). BUNdelNSs showed increased levels of N expression, an impaired ability to shut-off host protein synthesis, a small plaque phenotype and slower growth in tissue culture. BUNdelNSs, as opposed to wtBUN, was also found to be an inducer of the interferon-β promoter (Bridgen et al., 2001).

It was not clear why BUNdelNSs expressed higher levels of N than wt virus, and our initial thoughts were that this was...
a translational effect due to the mutations around the two AUG codons at the start of the NSs ORF (Fig. 1A). Possibly changes in secondary structure could slow scanning ribosomes to allow more time for initiation at the upstream AUG (reviewed by Kozak, 2002) for N protein that is in a poor sequence context. Previously we confirmed the sequence of the S segment coding region, which revealed no mutations other than those introduced to destroy the NSs ORF (Bridgen et al., 2001). In this study we determined the sequences of the 3′ and 5′ noncoding regions of the S segment by employing RACE technology (rapid amplification of cDNA ends; Roche) using viral antigenome RNA from BHK-21 cells infected with BUNdelNSs isolate 9a (Bridgen et al., 2001) as template. No differences from wt virus were detected in the 5′ region of BUNdelNSs. However, the S segment of BUNdelNSs 9a virus appeared to contain heterogeneity at position 16 of the 3′ end of the genome, as two peaks were detected on the electropherogram (Fig. 1B), indicating both the expected U residue and a G residue. BUNdelNSs 1a, an independently isolated virus from the rescue experiment (Bridgen et al., 2001), gave an identical result (data not shown). Another virus recovered from cDNA in a separate experiment (using a different plasmid to specify S segment RNA) was also analysed. This virus (BUN132CT) was created to carry a C-to-U mutation in position 132 in the S segment, resulting in a lysine-to-phenylalanine change in NSs and a threonine-to-isoleucine change in the N protein, both proteins being expressed. Analysis of the 3′ end of the S segment of BUN132CT showed that this virus stock also contained a mix of mutant and wt sequence (data not shown). The S-segment-encoding plasmids used to rescue the viruses described above were also sequenced but no evidence of a G residue at position 16 was detected, suggesting that the mutation in the genomic RNA could have arisen during the rescue process.

Following passage of BUNdelNSs 9a virus in BALB/c mice (Bridgen et al., 2001) it was observed that the plaque sizes were not homogeneous (data not shown). For further analysis, one virus displaying a larger plaque phenotype (subsequently called BUNdelNSs vL) was plaque purified, and the 3′ end of the S segment RNA was sequenced using RACE. As shown in Fig. 1(B), this virus had a sequence identical to wtBUN in its 3′ S segment terminus (i.e. U at position 16), but still maintained the mutations that abrogated the NSs ORF.

To analyse protein expression profiles of these viruses, CV1 cells were infected at an m.o.i. of 1 and labelled for 3 h at 16 h post-infection with 50 μCi [35S]methionine per dish. Equal amounts of cell extracts were analysed by 10 % SDS-PAGE (Fig. 2A), and quantification of labelled proteins was carried out by phosphorimager analysis (Bio-Rad; Quantity One software). The G1 glycoprotein band was used to normalize radioactive protein levels. The levels of N expressed by BUNdelNSs vL were similar to wtBUN, whereas N levels of BUNdelNSs 9a were 2-fold higher than wtBUN. N levels expressed in BUN132CT-infected

Fig. 1. (A) Sequences of pT7riboBUNS and pT7riboBUNN plasmids showing N and NSs start codons (underlined) as well as mutations made to ablate the NSs open reading frame (stop codon indicated by asterisk). (B) RACE analysis of 3′ and 5′ termini of BUNdelNSs 9a and BUNdelNSs vL. Represented is a portion of the 3′ end sequence cDNA. A polymorphism at position 16, shown by two peaks in the electropherogram of the cDNA obtained from BUNdelNSs 9a, as well the corresponding cDNA sequence for BUNdelNSs vL are shown. UTR, untranslated region.
cells were 1·6-fold higher than those of wtBUN. Virus yields from Vero cells infected by these BUN viruses were measured by plaque assay on BHK-21 cells at 72 h post-infection as previously described (Bridgen et al., 1996). BUNdelNSs vL gave titres only slightly lower than those of wtBUN (8 × 10^6 p.f.u. ml⁻¹ vs 11 × 10^6 p.f.u. ml⁻¹), and significantly higher than the titre achieved by BUNdelNSs 9a (1 × 10^6 p.f.u. ml⁻¹). Virus yields measured at earlier time-points showed a similar pattern.

To determine if the U-to-G mutation at position 16 could be found in a wild-type population, a stock of wtBUN was plated out on BHK-21 cells and individual plaques were picked and amplified. The S segment 3' end was sequenced using RACE. Out of six randomly picked plaques, two were identified that carried the mutation at position 16; one of these (called v10BUN) was amplified. Protein profiles were analysed in CV1 cells infected at an m.o.i. of 1 by metabolic labelling with ^35S)methionine as described above (Fig. 2B).

Equal amounts of cell extracts were analysed by 10 % SDS-PAGE, and protein levels compared by phosphorimag- er analysis. Interestingly, v10BUN virus showed 2-fold increased levels of both N and NSs proteins compared to authentic wtBUN. This suggests that the mutation affected the level of both proteins encoded by the same mRNA. To explore their growth characteristics, wtBUN and v10BUN yields at 72 h post-infection from CV1 cells (m.o.i. 1) were determined. We found that v10BUN grew to slightly lower titres than wtBUN (9·75 ± 1·8 × 10^6 vs 1·73 ± 0·3 × 10^6 p.f.u. ml⁻¹). This 2-fold difference in titre was also seen in samples taken at 48 h post-infection. The mutation at position 16 of the S segment can thus be isolated from a wild-type population and again leads to overexpression of S segment gene products.

To confirm that the U-to-G mutation at position 16 in the S segment 3' terminus influences N levels in infected cells, we used a minireplicon system (Weber et al., 2001). This consists of expression plasmids for N (pTM1-BUNN) and L (pTM1-BUNL) proteins as well as a plasmid, pT7riboBUNSREN(−), containing a reporter gene (Renilla luciferase) cloned in antisense direction between the 3' and 5' noncoding regions of the BUN S segment. Using site-directed mutagenesis, the mutation at position 16 was introduced into pT7riboBUNSREN(−)mut16. The plasmids were co-transfected into BHK-SinT7 cells (Agapov et al., 1998), together with an internal reporter encoding firefly luciferase (pTM1-FF-Luc) to measure transfection efficiency, and dual luciferase activities were measured as previously described (Weber et al., 2001). As shown in Fig. 3(A), Renilla luciferase activity obtained with pT7riboBUNSREN(−)mut16 was about 3-fold higher than with pT7riboBUNSREN(−). Firefly luciferase activities were similar in all assays, showing that transfection efficiencies were similar.

To test effects of the mutation on minigenome RNAs, the levels of genome, antigenome and mRNA were analysed by metabolic labelling with [3H]uridine (in the presence of actinomycin D) in BHK-21 cells (J. N. Barr and others, unpublished) transfected with pT7riboBUNSREN(−) or pT7riboBUNSREN(−)mut16. RNAs were separated by agarose gel electrophoresis (Fig. 3B), and RNA bands quantified by densitometry. The relative amounts (in %) of mRNAs and antigenomes were estimated compared to the amount of genome RNA in each lane. Cells transfected with pT7riboBUNSREN(−)mut16 showed increased levels of mRNA (97 % vs 73 %) and antigenome RNA (80 % vs 59 %) compared to cells transfected with pT7riboBUNSREN(−).

The effects of this mutation on the 'panhandle' structure formed by interaction of the non-encapsidated 3' and 5' ends of the genome on the predicted RNA secondary structures were determined using Mfold (Zucker et al., 1999) (Fig. 3C). The presented structures were formed using 25 bases of the S segment terminus in either the genomic (vRNA) or antigenomic (cRNA). Introduction of the mutation leads to a loop structure and a decrease in free energy for genome (mutant...
Our data further characterize BUNdelNSs, a genetically engineered BUN virus that does not express the NSs protein, described previously (Bridgen et al., 2001). Using RACE, we have found that this virus differed from wtBUN in that it carries a U-to-G mutation at position 16 (3’ end) of the viral genome. When introduced into a minireplicon system, this mutation lead to a 3-fold increase in reporter gene activity, and radioactive labelling of RNAs indicated an increase in antigenome and mRNAs levels.

These results suggest that the single U-to-G mutation at position 16 in the 3’ noncoding region is responsible for higher N levels in BUNdelNSs-infected cells as the result of increased transcription to make more S segment mRNA, and not to an increase in translatability of the viral mRNA. Indeed, the 3’ terminus of the BUNdelNSs vL S genome segment is identical to that of wtBUN, yet this virus also contains the mutations introduced around the NSs initiation codon(s). Moreover, we recovered a variant of wtBUN...
carrying the mutation at position 16 that overexpresses N. Increased N expression appears to be responsible for the lower titres obtained with those viruses. It has been shown for both BUN and Rift Valley fever virus that the relative molar rations of L and N proteins are critical for optimal viral RNA replication (Elliott, 1996; Lopez et al., 1995), and in the case of the viruses containing the mutation at position 16 described here the optimal ratio would be distorted.

The origin of the mutation in the S segment 3′ end remains unknown. It might have been introduced through selection or mutation during rescue of the virus from cDNA, and possibly the U-to-G mutation confers a short-term selective advantage. However, in the long term viruses that revert to the wild-type sequence appeared that make ‘normal’ levels of N. The same mutation was also identified in stocks of wtBUN, so some biological significance cannot be excluded, though because bunyaviruses that are diploid with regard to the S segment have been reported (see Pringle, 1996) the effect of the mutation might be masked in a population. The U-to-G mutation is predicted to give a more stable panhandle structure. In the case of La Crosse bunyavirus, it has been proposed that increased complementarity would lead to less mRNA synthesis because separating the paired ends would be more difficult (Hacker & Kolakofsky, 1991). However, for BUN virus we have found that increasing the complementarity of chimeric minireplicons containing L- and M-segment genome ends leads to an increase in minireplicon activity (E. Dunn & R. M. Elliott, in preparation).

For further experiments we will use BUNdelNSs vL as a virus lacking NSs since this expresses wild-type levels of N protein and has a wild-type 3′ genome end sequence.

ACKNOWLEDGEMENTS

We thank John Fazakerley (University of Edinburgh) for the mouse inoculations and Friedemann Weber (University of Freiburg, Germany) for fruitful discussion and helpful comments during the preparation of this manuscript. Work in R.M.E.’s laboratory was supported by Wellcome Trust grants 058366 and 065121.

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