Genetic elements regulating packaging of the Bunyamwera orthobunyavirus genome

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The genome of Bunyamwera virus (BUN; family Bunyaviridae, genus Orthobunyavirus) comprises three segments of negative-sense, single-stranded RNA. The RNA segments are encapsidated by the viral nucleocapsid (N) protein and form panhandle-like structures through interaction of complementary sequences at their 5′ and 3′ termini. Transcription and replication of a BUN genome analogue (minireplicon), comprising the viral non-coding sequences flanking a reporter gene, requires just the viral RNA polymerase (L protein) and N protein. Here, sequences of Bunyamwera serogroup M segment RNAs were compared and conserved elements within nt 20–33 of the 3′ and 5′ non-coding regions that can affect packaging of minireplicons into virions were identified. RNA-folding models suggest that a conserved sequence within nt 20–33 of the 5′ end of the genome segments maintains conserved structural features necessary for efficient transcription. Competitive packaging experiments using M, L and S segment-derived minireplicons that encode different reporter genes showed variable packaging efficiencies of the three segments. Packaging of a particular segment appeared to be independent of the presence of other segments and, for the S segment, packaging efficiency was unaffected by the inclusion of viral coding sequences in the minireplicon.

INTRODUCTION

The family Bunyaviridae comprises four genera of arthropod-borne viruses (Orthobunyavirus, Nairovirus, Phlebovirus and Tospovirus) and the genus Hantavirus of rodent-borne viruses. Bunyamwera virus (BUN) is the prototype of both the genus Orthobunyavirus and the family. The BUN genome consists of three segments of single-stranded RNA of negative polarity. The largest segment, L, encodes an RNA-dependent RNA polymerase (L protein). The medium segment, M, encodes a precursor to the two virion glycoproteins, Gn and Gc, and a non-structural protein termed NSm. The smallest segment, S, encodes the nucleocapsid protein N and a second non-structural protein called NSs; N and NSs are translated from the same mRNA, but in different open reading frames (ORFs). The N protein associates with the genomic and antigenomic (replicative intermediate) RNA segments to form helical ribonucleoprotein complexes termed nucleocapsids or RNPs. Genome replication and transcription take place in the cytoplasm; virus budding generally occurs at the Golgi apparatus (Elliott, 1996, 1997; Schmaljohn & Hooper, 2001).

BUN replication and transcription in both mammalian and mosquito cells have been investigated by the use of minireplicon systems, based on a Renilla luciferase reporter gene cloned in an antisense orientation between the 5′ and 3′ non-coding terminal sequences of the L, M or S segments (Kohl et al., 2004b; Weber et al., 2001). The viral L and N proteins, which are necessary and sufficient for transcription and replication, are expressed transiently from co-transfected plasmids. The minireplicon mimics a viral genomic RNA and its encapsidation, transcription and replication are monitored by measuring Renilla luciferase or direct labelling of minireplicon RNAs (Barr et al., 2003; Weber et al., 2001). Analogous minireplicon systems have been described for other bunyaviruses, such as La Crosse virus, Uukuniemi virus, Hantaan virus, Rift Valley fever virus and Crimean-Congo hemorrhagic fever virus (Blakqori et al., 2003; Flick & Pettersson, 2001; Flick et al., 2003a, b; Lopez et al., 1995).
The non-coding regions of the BUN segments contain the promoter sequences that direct transcription and/or replication, and promoter strength in mammalian cells was determined as M > L > S (Barr et al., 2003; Kohl et al., 2004a). The first 11 nt at both the 3' and 5' ends, which are conserved between the three segments, are followed by 3 nt (M segment) or 4 nt (L and S segments) stretches that are conserved on a segment-specific basis throughout the genus Orthobunyavirus (Kohl et al., 2004a). Detailed analysis of the first 20 nt of the BUN S segment 3' and 5' ends showed that sequence, complementarity and structure all play a part in transcription; in addition, deletion mutagenesis showed that nt 20–33 were required for transcription (Kohl et al., 2004a). This is in accord with previous data that indicated that the S segment encapsidation signal, a potential stem–loop structure, is located within the first 32 nt of the 5' end (Osborne & Elliott, 2000).

Recently, a naturally occurring reassortant virus isolate causing haemorrhagic fever in East Africa was identified (Bowen et al., 2001). This virus was initially called Garissa, but subsequent analysis showed it to be identical to a previously isolated virus called Ngari (Gerrard et al., 2001). This virus was initially called Garissa, but subsequent analysis showed it to be identical to a previously isolated virus called Ngari (Gerrard et al., 2004). The L and S segments of this virus are derived from BUN, but its M segment sequence shows considerable divergence from that of BUN M and a closer relationship to a North American virus, Cache Valley virus (Gerrard et al., 2004).

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With regard to the non-coding regions, no obvious sequence similarity was found in the body (i.e. beyond the terminal 20 nt or so) of the Ngari/Garissa virus M segment and that of BUN; therefore, the authors suggested that, because the Ngari/Garissa virus M segment was efficiently transcribed and replicated by BUN L and N proteins and packaged into virions, either encapsidation and promoter sequence motifs were contained within the highly conserved terminal 20 nt or that considerable flexibility is allowed in these sequences (Gerrard et al., 2004). Given our S segment data (Kohl et al., 2004a) and a recent finding from our laboratory that deletion of nt 20–30 in the M segment 5' untranslated region results in the failure to rescue a virus from cDNA (A. C. Lowen & R. M. Elliott, unpublished observations), we decided to examine further the importance of the terminal 33 nt of the BUN M segment 3' and 5' ends.

Comparison of available sequences allowed identification of a few highly conserved nucleotides between nt 20 and 33 at the M segment 3' and 5' ends. Mutational analyses of minireplicons and RNA-folding predictions suggest that a stem–loop structure near or at the 5' end is an important functional feature of bunyavirus genomes. Packaging assays for the minireplicons were developed and, similar to results for a bunyavirus of the genus Phlebovirus, Uukuniemi virus (Flick et al., 2004), we found that the L segment-derived minireplicon was maintained most efficiently upon serial passage. We also show that viral coding sequences do not increase packaging efficiency of an S segment-derived minireplicon. These data provide several new insights into the packaging of BUN nucleocapsids.

METHODS

Sequence alignments. Sequences were aligned by using CLUSTAL_X (Thompson et al., 1997). GenBank accession numbers for bunyavirus sequences used in this study are: Germiston virus M segment, M21951; Cache Valley virus M segment, AF186243; Garissa virus M segment, AY959725; Maguari virus M segment, AY286443; Bunyamwera virus M segment, M11852; S segment, D00353; L segment, X14383.

Media, cells and viruses. BHK-21 and BSR-T7/5 cells (BHK-derived cells that express T7 RNA polymerase stably; Buchholz et al., 1999) were maintained in Glasgow minimal essential medium supplemented with 10% tryptose phosphate broth; 10% fetal calf serum and, for BSR-T7/5 cells only, 1 μg genetin ml−1. Cells were grown at 37°C. BUN working stocks were grown in BHK-21 cells at 33°C and titres were determined by plaque assays on BHK-21 cells as described previously (Bridge & Elliott, 1996; Watt et al., 1985).

Plasmids. Plasmids pTM1-BUNN, pTM1-BUNS, pTM1-BUNL and pTM1-BUNLm express BUN proteins under control of the T7 RNA polymerase Φ10 promoter and encephalomyocarditis virus internal ribosome entry site sequences, and have been described previously (Kohl et al., 2003; Weber et al., 2001). Plasmid pTM1-FF-Luc contains the firefly luciferase gene and was used as an internal control (Weber et al., 2001); pT7riboBUNLREN(−), pT7riboBUNMREN(−) and pT7riboBUNSREN(−) contain the Renilla luciferase gene cloned in antisense orientation between the respective L, M or S segment 5' and 3' non-coding regions under control of a T7 promoter (Weber et al., 2001). The original pT7riboBUNMREN(−) plasmid was found to have a C to T point mutation at position 37 from the antigenomic 3' end compared with the published BUN M segment sequence. This mutation was corrected and both constructs were found to have identical minireplicon activity. For all experiments described here, the corrected pT7riboBUNMREN(−) was used. Mutations in the 5' non-coding regions (pFMut series) or 3' non-coding regions (pTMut series) were produced by PCR-based methodologies, either quick change for point mutations or excise for deletions (Shi & Elliott, 2002; Shi et al., 2004); details of the oligonucleotides and PCR conditions are available from the authors on request. To produce minireplicons containing the firefly luciferase gene, the firefly luciferase ORF was amplified by PCR using KOD polymerase (generating blunt ends; Novagen) and 5'-phosphorylated primers FFlucRe (5'-TTTACCAGCGGCTATCTTTCGCCCTTCC-3') and FFlucFfd (5'-ATGAGAGACGCCCCAAAACATAGG-3'). The purified PCR product was cloned in antisense orientation into the SmlI site of pT7ribo-based plasmids containing the S, M or L segment genome-sense non-coding regions (Dunn et al., 1995) to give pT7riboBUNS(−)FFLuc, pT7riboBUNM(−)FFLuc and pT7riboBUNL(−)FFLuc. For the construction of pT7riboBUNS124RENS747 and pT7riboBUNS185RENS686, the first three ATG codons (including those for N and NSs) in the S segment were first mutated to AGG by PCR. pT7riboBUNS(−) was amplified by using primers containing the appropriate nucleotide substitutions in two separate PCRs, yielding the construct pT7riboSAATG(−). pT7riboSAATG(−) was then used as the template in two further PCRs, using primers complementary to either nt 124–104 (BUNS124−) and nt 747–752 (BUNS747+) of the BUNS segment, or nt 185–104 (BUNS185−) and nt 686–709 (BUNS686+) of the BUN L segment. The purified PCR products from these two reactions were combined and added to an insert corresponding to the Renilla luciferase gene, which had been excised from pT7riboBUNMREN(−) by using NruI and Stul restriction enzymes, and ligation reactions were performed. Clones with the Renilla luciferase sequence in the negative-sense orientation were selected. All
constructs were confirmed by DNA sequencing: automated DNA sequencing was carried out by the University of Dundee (UK) sequencing service.

Transfection, minireplicon reconstitution and reporter-gene assays. For minireplicon experiments, approximately 5 x 10^6 BSR-T7/5 cells grown in 35 mm diameter dishes were transfected with 0.25 µg pT1M1-BUNL, 0.1 µg pT1M1-BUNN, 0.1 µg pT1M1-FF-Luc and 0.3 µg p77ribos-based minireplicon plasmid DNA, using 2.5 µg DAC-30 (Eurogentec) as transfectant (Kohl et al., 2004a). Transfection efficiencies were normalized by co-transfection of pT1M1-FF-Luc in order to calculate induction levels of Renilla luciferase in minireplicon-reconstitution assays. As negative control, a non-functional L clone (pT1M1-BUNLmut; Kohl et al., 2003) was co-transfected instead of pT1M1-BUNL. Luciferase activities were determined by using Dual-Luciferase assay kits (Promega); cells were lysed in 200 µl lysis buffer at 24 h post-transfection and luciferase activities were measured in 10 µl cell extract.

Northern blotting. To prepare minireplicon RNAs for Northern blot analysis, approximately 1 x 10^6 BSR-T7/5 cells grown in 60 mm diameter dishes were transfected with 1 µg each of pT1M1-BUNL and pT1M1-BUNN and 2 µg p77ribosBUNMREN(−) by using 10 µg DAC-30. At 24 h post-transfection, total cellular RNA was extracted by using TRIzol LS reagent (Invitrogen). RNA was quantified by spectrophotometry and 20 µg was combined with 5-5 µl formaldehyde, 15 µl deionized formamide and 1-5 µl 10 x MOPS buffer [0.4 M 3(N-morpholino)propanesulfonic acid, 0.1 M sodium acetate, 0.01 M EDTA, pH 7.0] in a total volume of 30 µl. Samples were heated at 65 °C for 15 min, cooled on ice and loaded on a 1-5 % agarose, 2-2 M formaldehyde gel. Samples were run at 75 V for 5 h in 1 x MOPS buffer. After transfer to a positively charged nylon membrane (Roche) and UV cross-linking, 150 ng of a DIG-labelled probe complementary to either the positive or negative sense of the full-length Renilla luciferase gene was hybridized at 68 °C. Probes were synthesized and quantified by using a DIG Northern starter kit (Roche) as directed by the manufacturer. Washes and detection were carried out as described by the manufacturer, using the reagents supplied in the DIG Northern starter kit. Blots were exposed to X-OMAT UV film (Kodak).

Packaging of minireplicon RNA into virions. Approximately 5 x 10^6 BSR-T7/5 cells grown in 35 mm diameter dishes were transfected with 0.25 µg pT1M1-BUNL, 0.1 µg pT1M1-BUNN, 0.1 µg pT1M1-FF-Luc and 0.3 µg p77ribosBUNMREN(−) by using 2.5 µg DAC-30. For the negative control, pT1M1-BUNLmut instead of pT1M1-BUNL was used. At 24 h post-transfection, cells were infected with BUN at an m.o.i. of 2 (1 for competition experiments; see below). The BUN virus used was a high-titre stock grown under conditions to minimize defective-particle production (Watret et al., 1985). Twenty-four hours later (when all cells showed cytopathic effects), 500 µl infectious supernatant from transfected cells was used to infect approximately 1 x 10^6 BHK cells grown in 35 mm diameter dishes. This was repeated for the indicated number of passages. Renilla luciferase activity in these infected BHK cells was determined after 24 h further incubation. For competition experiments, 0.15 or 0.3 µg (as indicated) of each Renilla or firefly luciferase-encoding minireplicon DNA was transfected with 0.25 µg pT1M1-BUNL and 0.1 µg pT1M1-BUNN by using 1-5 µl DAC-30. Passage of supernatants from transfected/infected cells was as described above, and both Renilla and firefly luciferase activities were determined by using a Dual-Luciferase assay kit (Promega).

RNA folding. RNA structures at 28 or 37 °C were predicted by using MFOLD (Zuker et al., 1991) with 33 nt from the 5’ or 3’ terminus of the BUN M segment (unless otherwise indicated).

RESULTS

Identification and mutation of conserved nucleotides in the BUN M segment 3’ and 5’ non-coding regions

Previous work indicated that the regions encompassing nt 20–33 from either the 3’ or 5’ end of the BUN S segment were important for transcription and replication (Kohl et al., 2004a). To identify conserved nucleotides in these regions, we first compared the BUN M, L and S termini with each other, and then compared BUN M sequences with those of other Bunyamwera serogroup viruses. We assumed that the most essential bases or motifs would be conserved within the various sequences and should thus be readily identifiable, as other sequence elements involved in replication/transcription, such as the 11 terminal bases at the 3’ and 5’ ends, are also highly conserved (Kohl et al., 2004a).

As shown in Fig. 1(a), an AAxA motif was identified in the 5’ end when L, M and S segments were aligned: AACA in the M and AAAA in the L and S segments. Aligning the BUN M 5’ terminus with the 5’ ends of other Bunyamwera serogroup virus M segments (Fig. 1b) revealed that, in addition to the AACA motif, nt A(21) was conserved. This nucleotide was also conserved when Bunyamwera serogroup sequences were aligned with the M segments of orthobunyaviruses from the California serogroup (not shown). Based on these comparisons, we created constructs (pFMut-series plasmids) with A(21) at the 5’ end deleted or substituted (Fig. 1c; pFMut2, pFMut2A). The highly conserved AACA motif (nt 23–26 from the 5’ end) was mutated to GGUG in pFMut4 (Fig. 1c). As putative positive controls, we created a mutant with position 30 at the 5’ end changed from U to G (pFMut1, Fig. 1c), as this nucleotide did not seem to be conserved regardless of the sequences aligned, and a deletion mutant covering nt 18–22 from the 5’ end, spanning conserved and non-conserved sequence (pFMut3).

Comparisons of the 33 nt at the 3’ terminus of BUN M again revealed conserved bases between nt 20 and 33 (Fig. 1a). The U triplet (positions 25–27 in BUN M) was conserved between the BUN M, L and S segments, as were A(30), A(31) and U(23). As shown in Fig. 1(b), U triplet from the California serogroup (not shown). Based on these comparisons, we created constructs (pFMut-series plasmids) with A(21) at the 5’ end deleted or substituted (Fig. 1c; pFMut2, pFMut2A). The highly conserved AACA motif (nt 23–26 from the 5’ end) was mutated to GGUG in pFMut4 (Fig. 1c). As putative positive controls, we created a mutant with position 30 at the 5’ end changed from U to G (pFMut1, Fig. 1c), as this nucleotide did not seem to be conserved regardless of the sequences aligned, and a deletion mutant covering nt 18–22 from the 5’ end, spanning conserved and non-conserved sequence (pFMut3).
Transcriptional activities of 5’ and 3’ M segment-derived minireplicon mutants

Minireplicon-expressing plasmids were co-transfected into BSR-T7/5 cells along with BUN L and N protein-expressing plasmids, incubated for 24 h and then Renilla luciferase activities were determined. As shown in Fig. 2(a), minireplicons carrying mutations or deletions at the 3’ end (pTMut series) showed luciferase activity similar to that of the original pT7riboBUNMREN(+) positive control. Similarly, most mutations at the 5’ end of the M segment-derived minireplicons did not show significant effects on luciferase activity compared with wild type (Fig. 2a). However, substitution of the highly conserved AACA motif at the 5’ M terminus (FMut4) resulted in four- to fivefold reduction in minireplicon activity compared with the wild-type control, pT7riboBUNMREN(+). No Renilla luciferase activity was measured in the negative controls.

It has been shown previously that reporter activity of minireplicons is the result of both transcription and replication (Barr et al., 2003; Flick et al., 2002; Kohl et al., 2004a). To confirm the effects seen on reporter-gene activity, we analysed minireplicon-derived negative- and positive-sense RNAs in transfected cells by Northern blotting. In the case of the M segment-derived minireplicon, antigenomic and mRNAs were indistinguishable in size (Barr et al., 2003). The levels of antigenomic transcripts were similar for all minireplicons except for pFMut4, which was detected at reduced levels (Fig. 3a). Two forms of negative-sense transcripts were detected: a slower-migrating form, corresponding to the initial T7 polymerase transcript containing the ribozyme sequence at the 3’ end, and a faster-migrating form, corresponding to the BUN minireplicon. This band contains both the initially processed RNA after ribozyme cleavage, as well as progeny minireplicon RNA produced by BUN-specific replication. Similar levels of the initial transcript were detected for all minireplicons, indicating

![Fig. 1. Sequence comparisons to identify highly conserved nucleotides in the non-coding regions of the BUN M segment. (a) Comparison of the 5’- or 3’-terminal 33 nt of BUN L, M and S genomic RNAs. Conserved nucleotides are indicated by asterisks; numbers correspond to the M sequence. (b) Comparison of the 5’ or 3’ genomic terminal nucleotides of the M segments of various Bunyamwera serogroup viruses. Conserved nucleotides are indicated by asterisks; numbers correspond to the BUN M sequence; conserved nucleotides of particular interest are indicated by arrows. Arrowheads indicate position of nucleotides within the shown sequence. Abbreviations: Gar, Garissa virus; CV, Cache Valley virus; Mag, Maguari virus; Ger, Germiston virus; Bun, Bunyamwera virus. (c) Mutations introduced between nt 20 and 33 in either the 5’ or 3’ end of the M segment-derived minireplicon. Represented are the genomic RNA sequences as transcribed from the pFMut- or pTMut-series plasmids. Original sequence with conserved nucleotides (marked with asterisks) as indicated. Substitutions are marked in blue, deletions in red and short stuffer sequence in green.](image-url)
equivalent efficiencies of T7 polymerase-directed transcription. However, the amount of actual minireplicon RNA was reduced in the case of pFMut4 compared with the other minireplicons (Fig. 3b), suggesting that this minireplicon is replicated less efficiently than other RNAs described in this study.

**Packaging of minireplicons into virions is affected by deletions in the 3′ and 5′ non-coding regions**

The non-coding regions at the 3′ and 5′ ends of bunyavirus genomes or antigensomes interact to form a panhandle in the RNP, and these non-coding sequences also contain promoter elements that regulate replication levels for each segment (Barr et al., 2003; Flick et al., 2002; Kohl et al., 2004a). Here, we show that the BUN M segment non-coding regions also contain signals that direct efficient packaging. BSR-T7/5 cells expressing firefly luciferase and BUN L and N proteins, as well minireplicon RNAs, were superinfected with BUN virus as described in Methods and, at 24 h post-infection, infectious supernatants from transfected BSR-T7/5 cells as described in Methods. Renilla luciferase activities obtained from infected BHK-21 cells 24 h after infection are expressed in arbitrary light units.

**Fig. 2.** Transcription, replication and packaging of minireplicons. (a) Minireplicon activity. BSR-T7/5 cells were transfected with support plasmids expressing BUN L and N proteins as well as pT7riboBUNMREN (–) (positive control, +) or mutant minireplicon plasmids as indicated. In the negative control (–), a non-functional L protein-expressing plasmid was co-transfected. *Renilla* luciferase activities were normalized to an internal standard and are expressed as arbitrary light units. NT, Not transfected. (b) Packaging of minireplicon RNAs into BUN virions. BHK-21 cells were infected with infectious supernatants from transfected BSR-T7/5 cells as described in Methods. *Renilla* luciferase activities obtained from infected BHK-21 cells 24 h after infection are expressed in arbitrary light units.

**Fig. 3.** Northern blot analysis of minireplicon transcripts. RNAs were hybridized to probes that detect positive-sense (both antigenome (cRNA) and mRNA) transcripts in (a) or negative-sense transcripts in (b). In (b), the positions of unprocessed initial T7 polymerase-directed transcript and minireplicon RNAs are indicated.
were detected with pT7riboBUNMREN(−)-derived nucleocapsids (positive control, +), and similar or only slightly decreased activities were detected following transduction of nucleocapsids derived from pTMut3, pFMut2 or pFMut2A (Fig. 2b). Thus, mutation of U(25) at the 3′ end or A(21) at the 5′ end did not seem to affect transcription and had only minimal effects on packaging. Deletion of larger sequence blocks at the 3′ and 5′ ends (pFMut3, pTMut2E and pTMut5E) resulted in decreased packaging efficiency (approx. three-, 10- and 20-fold, respectively), even though these deletions did not affect minireplicon activities. Substitution of the highly conserved AACA motif at the 5′ genomic end (pFMut4) resulted in only low transduced minireplicon activity (reduced 30-fold compared with the positive control), in addition to the observed strong decrease in minireplicon activity. Unexpectedly, RNA transcribed from plasmid pFMut1, in which a non-conserved nucleotide was substituted (U to G at position 30 from the 5′ end), reproducibly showed a threefold decrease in packaging efficiency. No minireplicons were packaged or transduced from the negative controls (see Methods). Note that, in these experiments, minireplicon activities were assayed on the original transfected BSR-T7/5 cells in parallel, to verify transfection efficiencies (data not shown).

A structural model for 5′ genome termini

Previous work on encapsidation of the BUN S segment had shown that an encapsidation signal lies within the first 32 nt of the 5′ genome end, and it was suggested that a stem–loop acts as a recognition signal for N (Osborne & Elliott, 2000). To extrapolate from there to our current work, we analysed various RNA structures by using MFOLD (Zuker et al., 1991). Thirty-three nucleotides of the L, M and S genomic and antigenomic termini, as well as the relevant sequences from the pFMut-series (genome-sense) or pTMut-series (antigenome-sense) transcripts, were analysed. Representative models (S genome, M genome/antigenome, FMut2, TMut5E, FMut4) are shown in Fig. 4. Genomic and antigenomic RNAs are characterized by stem–loops of variable lengths that are predicted at the 5′ ends, with the stem beginning generally after the first or, in the case of the S segment, the second nucleotide. The most energetically favoured structures at 37 °C are shown; identical or very similar structures, maintaining the stem–loop at the 5′ end, were also predicted at 28 or 33 °C, the temperatures that we normally use to propagate the virus in insect or mammalian cells. All RNAs from the pFMut series conform to that pattern (only FMut2 RNA is shown), with the exception of the transcript from pFMut4, where the stem–loop structure is predicted to be shifted 7 nt away from the 5′ end. As the structure ultimately depends on the RNA sequence, the conserved AACA motif might be important in keeping the stem–loop in the correct position relative to the 5′ end.

We also analysed the predicted structures of antigenomic RNAs derived from pTMut-derived transcripts, as even large deletions in the 3′ end did not affect minireplicon activities (as measured by Renilla luciferase). As shown in Fig. 4, none of the deletions affected formation of a stem–loop structure at the 5′ end, although for TMut2E and TMut3 RNAs, the stem begins with the first, rather than the second, 5′ nucleotide (not shown).

Packaging of BUN L, M and S segment-derived minireplicons

Previous work on packaging of minireplicon RNAs of Uukuniemi virus (genus Phlebovirus) into virions, as measured by passage of reporter activity, showed that S segment-based replicons were lost rapidly; M segment-derived RNAs were maintained better, whereas L segment-derived minireplicons were maintained for several passages (Flick et al., 2004). However, competition between segments, i.e. whether one segment has an advantage for incorporation over another, and distinction between random or selective packaging remain important issues that have yet to be addressed. We reproduced an experiment similar to the one

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**Fig. 4.** Structure predictions of BUN genomes and antigenomes using MFOLD (Zuker et al., 1991). The most energetically favoured structures predicted at 37 °C are shown. These or very similar structures are also obtained at 28 or 33 °C. Structures are based on 33 nt, except for TMut5E (28 nt). FMut-based genomic structures and TMut-based antigenomic structures are indicated. Arrows mark beginning of stem–loop structures from the 5′ end. Red dots indicate GC pairs and blue dots indicate AU and GU pairs.
described for *Uukuniemi virus*. BSR-T7/5 cells were transfected with L and N expression plasmids as well as M-, L- and S-derived minireplicons and, subsequently, superinfected with BUN virus as described in Methods. Reporter-gene activities were determined 24 h post-transfection to evaluate transfection (T) efficiency. Supernatants were passaged (P) on BHK-21 cells for four successive passages and *Renilla* luciferase activities were measured. As reported previously (Kohl et al., 2004a), the M segment-based minireplicon had the most active transcriptional promoter (Fig. 5, T). Similar to *Uukuniemi virus*, we observed a sharp drop in S segment-derived minireplicon activity after a single passage and subsequent disappearance of the signal. Passaged M segment-derived *Renilla* luciferase activity decreased more slowly and was just measurable at passage 3. The L segment-derived minireplicon activity appeared more stable with passage, however, as luciferase activity at passage 3 was greater than that seen with the M segment-derived minireplicon, although the *Renilla* luciferase activity was significantly lower in the original transfection.}

In order to evaluate the effects of competition between segments – both native BUN segments and minireplicons – we constructed firefly luciferase-encoding minireplicons whose activities could be distinguished from those of *Renilla* luciferase-encoding replicons (see Methods). BSR-T7/5 cells were transfected with expression plasmids for BUN L and N proteins, as well as combinations of firefly or *Renilla* luciferase-encoding minireplicons (firefly/*Renilla*: M/M, L/L, S/S, M/L, M/S, L/M, L/S, S/M, S/L). As a negative control, we co-transfected the M/M combination (which would give high activities) with an inactive L mutant (Lmut)-expressing plasmid. As shown in Fig. 6, *Renilla* luciferase activities were as expected in the order M > L > S, and the level of activity for a particular minireplicon was not affected by the co-transfected partner minireplicon. Firefly luciferase activities were generally lower and, similar to minireplicons encoding the CAT gene (Kohl et al., 2004a), the L segment-derived minireplicon was the most active and the S segment-based minireplicon the least active. There was also variation in the levels of firefly luciferase activity for some combinations of minireplicons e.g. M/L and M/S gave higher levels than the M/M combination, although the reasons for this are unclear.

We then looked at luciferase activities of the various minireplicon combinations after passage of supernatants onto BHK cells. The amounts of cell extract used to determine firefly luciferase activity were adjusted to give activities similar to the corresponding *Renilla* luciferase activities. As shown in Fig. 7, luciferase activity produced by packaged L

![Fig. 5. Transcription and packaging of BUN minireplicons. BSR-T7/5 cells were transfected with support plasmids expressing BUN L and N proteins, as well as 0-3 μg *Renilla* luciferase-based minireplicons pT7riboBUNSREN(-) (filled bars), pT7riboBUNMREN(-) (empty bars) or pT7riboBUNLREN(-) (hatched bars). Luciferase activity was measured after 24 h (T). For packaging experiments, BHK-21 cells were infected with infectious supernatants from transfected/infected BSR-T7/5 cells (P1) or infected BHK-21 cells (P2-4) as described in Methods. *Renilla* luciferase activities are expressed in arbitrary light units.](http://vir.sgmjournals.org)

![Fig. 6. Transcription of firefly and *Renilla* luciferase-encoding minireplicons. BSR-T7/5 cells were transfected with support plasmids expressing BUN L and N proteins, as well as combinations of 0-15 μg *Renilla* luciferase-encoding minireplicons pT7riboBUNLREN(-), pT7riboBUNMREN(-) or pT7riboBUNSREN(-) with firefly luciferase-encoding minireplicons pT7riboBUNLFFLuc(-), pT7riboBUNMFFLuc(-) or pT7riboBUNSFFLuc(-), as indicated. The combinations are indicated as firefly luciferase/*Renilla* luciferase-encoding constructs (M/M, L/L etc.). For the negative control, M/M(-), a non-functional L protein-expressing plasmid was co-transfected with the N expression plasmid and the M minireplicon combination. Luciferase activities are expressed as arbitrary light units. Activities were determined in a dual luciferase assay. Upper panel, *Renilla* luciferase activities; lower panel, firefly luciferase activities.](http://vir.sgmjournals.org)
segment-derived minireplicons was maintained through repeated passages and was easily detectable at passage 4, regardless of the co-transfected partner. M segment-based minireplicons decreased slowly over four passages and were undetectable by passage 5. S segment-derived minireplicons lost activity more quickly after passage, showing a dramatic decrease between passages 1 and 2.

These results show that, independent of the co-transfected competing minireplicon, all segments display individual characteristics when packaged and passaged by co-infecting BUN virions.

Coding sequences do not contribute to packaging of the S segment

The poor packaging of S segment-based minireplicons raises the question of why these genomes are eliminated so quickly. Work on another segmented-genome negative-strand RNA virus, influenza virus, indicated that coding sequences, in addition to non-coding sequences, can contribute to efficient packaging (Fujii et al., 2003, 2005; Watanabe et al., 2003). To investigate this possibility, we constructed S segment-derived minireplicons in which the Renilla gene was flanked by portions of coding sequence (Fig. 8a). Both the N and NSs translation-initiation codons were eliminated by site-directed mutagenesis so that the authentic Renilla luciferase AUG codon was used. These minireplicons were co-transfected with L and N expression plasmids, as well as pT7riboBUNSFFLuc(−) as competitor. Transfected cells were infected with BUN and supernatants were passaged twice on BHK cells. As shown in Fig. 8(b), Renilla and firefly luciferase activities were lost rapidly during passage. This confirms our previous findings and suggests that the terminal coding regions do not play a role in packaging of BUN S segments.

DISCUSSION

The non-coding regions at the ends of the bunyavirus genome segments are presumed to contain signals for encapsidation by the viral N protein to form RNPs, signals
regulating transcription and replication of the genome and signals for packaging the RNP s into virions. Previous studies on BUN, the prototype of the family Bunyaviridae, have concentrated mainly on the importance of the extreme terminal 20 nt at the genome 3’ and 5’ ends in these events (Barr & Wertz, 2004, 2005; Barr et al., 2003; Dunn et al., 1995; Kohl et al., 2004a; Osborne & Elliott, 2000). Here, we report analyses of the importance of nucleotides internal in the genome segments, between residues 20 and 33. We identified several nucleotides in this region that are highly conserved by comparison of the respective BUN M, L and S sequences, as well as comparing M segments of various Bunyamwera serogroup viruses (Fig. 1), and proceeded to mutational analysis.

Substitutions and deletions at the 3’ end, as shown Fig. 1(c), had no effect on minireplicon activity in comparison to a wild-type M segment-derived minireplicon. A possible explanation for this observation was offered by structural predictions of the corresponding antigenomic RNAs, in that the mutation did not affect the location of a potential stem–loop structure (a structure suggested as being necessary for efficient encapsidation of the BUN S segment; Osborne & Elliott, 2000) at the 5’ end, thus keeping it functional for transcription.

Mutations at the 5’ end of the negative-sense minireplicon had different effects. Substitution or deletion of A(21) had no effect, although it was conserved in different M segments. Substitution of the conserved AACA motif in the M 5’ non-coding region resulted in strongly reduced minireplicon activity. pFMut4 antigenomes and genomes were detected at reduced levels compared with other RNAs. One can speculate that the pFMut4 mutation, for which the RNA-folding algorithm predicts a shift of the 5’ stem–loop structure inwards on the RNA, resulted in diminished transcription and replication rates, possibly by affecting encapsidation. However, it is also possible that instability of genomic pFMut4 RNAs resulted in a decrease in RNA levels. From our structural predictions, it seems that location of the stem–loop at the 5’ end of the genome is important for efficient replication and transcription. pFMut4-derived
transcripts, with the stem–loop shifted 7 nt towards the 3’ end of the RNA, can act as templates for, albeit inefficient, transcription. Deletion of non-conserved bases at the 5’ end (pFMut3) had no effect on replication or transcription and did not affect the predicted location of the stem–loop.

The overall efficiency in terms of transcription and replication by minireplicons carrying large deletions at the 3’ and 5’ ends raises the question of why the viral genome segments carry sequences that apparently have no direct role in RNA synthesis, at least in mammalian cells. The answer to this question might be provided by the packaging experiments presented here. Packaging of minireplicons into virions has been previously shown for other bunyaviruses, such as La Crosse virus, Uukuniemi virus, Crimean–Congo hemorrhagic fever virus and Hantaan virus (Blakqori et al., 2003; Flick et al., 2003a, b, 2004). In the case of Uukuniemi phlebovirus (which uses an ambisense strategy for its S segment), it was shown that L segment-derived minireplicons were packaged preferentially to those based on M and S segments, with S segment minireplicons being lost particularly quickly (Flick et al., 2004), although specific signals have not yet been described. Our results confirm those for all other bunyaviruses studied so far, in that the non-coding regions are sufficient to package minireplicon-derived RNA into progeny virions. Further, our data with BUN M-, L- and S-based minireplicons are in agreement with those described for Uukuniemi phlebovirus, in that L segment-based minireplicons were generally better maintained on passage than M segment-based minireplicons, whilst S segment-derived minireplicons were lost rapidly. This raised the question of how other viral segments influence packaging. Competition experiments with minireplicons carrying different luciferase reporter genes showed that these characteristics are indeed segment-specific and are not affected by the presence of other segments. This is of importance during virion assembly, as copies of all three segments must be packaged to produce an infectious particle. Indeed, it has recently been shown that rearrangement of non-coding and coding sequences, where a recombinant BUN virus with the L ORF flanked by M segment non-coding regions was created, resulted in an attenuated virus expressing less L protein and producing fewer infectious particles (Lowen et al., 2005). These results also show that transduced minireplicon activities do not just reflect different promoter activities, begging the important question of how and why differential packaging of the three segments is mediated in infected cells. If similar mechanisms and efficiencies occurred for packaging of all three segments, we suggest that this would probably result in the most efficiently replicated segment eliminating the other segments and resulting in a high proportion of non-infectious particles. Based on available data, it is not possible to say at what level discrimination between segments occurs, but it could be at the level of recognition of RNA sequences by a viral protein(s) involved in packaging. In the case of *Influenza A virus*, it has been shown for the haemagglutinin-, neuraminidase- and NS-encoding segments that both non-coding and portions of the adjacent coding regions (particularly at the 3’ end) are necessary for efficient packaging of genome RNA (Fujii et al., 2003, 2005; Watanabe et al., 2003). Our results suggest that requirements for packaging are different for BUN RNAs, and we show that packaging of the S segment could not be improved by adding portions of the coding region (Fig. 8).

As shown in Fig. 2(b), deletions in either the 5’ or 3’ M segment non-coding regions strongly affected packaging efficiency, whilst not affecting transcription/replication. This offers a possible explanation as to why apparently excess sequence is maintained within the non-coding regions: segments carrying deletions might well be replicated and transcribed, but inefficient packaging would inevitably lead to loss of the segment. Given the considerable sequence variation in the L, M and S segment non-coding regions, the differences between the three segments in terms of packaging may be due to regulatory elements within these regions. In conclusion, our results show that sequence elements between nt 20 and 33 from the 3’ and 5’ ends influence packaging of the genome and that these elements are conserved throughout the Bunyamwera serogroup.

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**REFERENCES**


