

# Complementarity, sequence and structural elements within the 3' and 5' non-coding regions of the Bunyamwera orthobunyavirus S segment determine promoter strength

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The genome of *Bunyamwera virus* (BUN; family *Bunyaviridae*) consists of three segments of negative-sense, single-stranded RNA that are called L (large), M (medium) and S (small), according to their size. The genomic RNAs are encapsidated by the viral nucleocapsid protein to form ribonucleoprotein complexes (RNPs). The terminal 3' and 5' non-coding sequences are complementary and interact to give a panhandle-like structure to the RNP. Located within these non-coding sequences are elements that control replication and transcription. The sequences of the terminal 11 nt are conserved among the genome segments and are followed by shorter, complementary nucleotide motifs that are conserved on a segment-specific basis. Here, a detailed analysis of the 3' and 5' non-coding regions of the BUN S segment is presented. By using a mini-replicon system, it was shown that a functional BUN S promoter requires complementarity, as well as defined sequences, within the terminal 15 nt of either end. It was also shown that the minimal requirement for transcription is localized within the terminal 32 nt of the S segment. A comparison of known strong BUN promoters led to the prediction of a structural element outside the terminal 15 nt; introduction of this motif into the BUN S sequence resulted in increased antigenome and mRNA levels and increased expression of S segment proteins, as shown by mini-replicon assays, as well as recovery of a recombinant virus.

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## INTRODUCTION

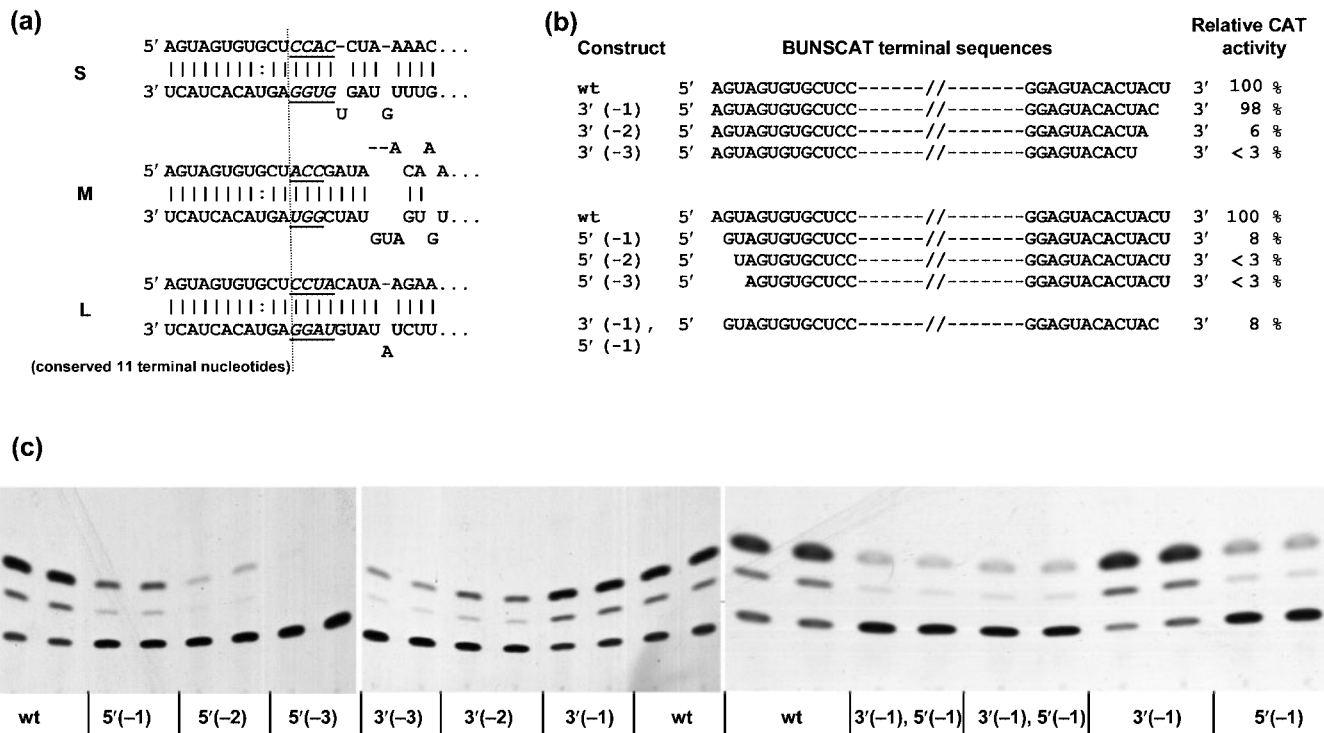
The family *Bunyaviridae* is divided into five genera: the arthropod-transmitted viruses in the genera *Orthobunyavirus*, *Nairovirus*, *Phlebovirus* and *Tospovirus* and the rodent-borne viruses in the genus *Hantavirus* (Elliott *et al.*, 2000). The family contains several important pathogens, such as *Rift Valley fever virus*, *La Crosse virus* and *Hantaan virus*, and bunyaviruses have become increasingly important as ecological changes modulate vector dynamics and humans become targets (Elliott, 1997). Bunyavirus replication takes place in the cytoplasm and newly synthesized virions are transported to the cell surface after budding through the Golgi apparatus. The viral genome consists of three segments of single-stranded, negative-sense RNA called, according to their size, L (large), M (medium) and S (small). All bunyaviruses encode four structural proteins, but there are differences in the pattern of sizes of genome

RNAs and viral proteins among the different genera and also whether non-structural proteins are encoded (reviewed by Elliott, 1996; Elliott *et al.*, 2000; Schmaljohn & Hooper, 2001).

*Bunyamwera virus* (BUN; genus *Orthobunyavirus*) has been used in our laboratory as the prototype virus for studying replication of this important family of viruses. The BUN L segment encodes an RNA-dependent RNA polymerase called the L protein, whilst the M segment encodes a precursor to the virion glycoproteins Gn and Gc, as well as a non-structural protein of unknown function (NSm). The S segment encodes two proteins in different, overlapping reading frames: the nucleocapsid protein, N, and a second non-structural protein, NSs, which are translated from the same mRNA. The N protein encapsidates viral genomes and antigenomes (complementary or replicative-intermediate RNA) to give RNA–N complexes termed ribonucleoproteins or RNPs (Elliott, 1996). Recent studies have shown that BUN NSs is an interferon antagonist and, in mammalian but not mosquito cells, NSs mediates host-protein shut-off (Kohl *et al.*, 2004; Weber *et al.*, 2001, 2002).

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**Fig. 1.** Effect of terminal deletions on BUNSCAT RNA. (a) Sequences of the 5' and 3' termini of the L, M and S segments. Segment-specific complementary bases are italicized and underlined. (b) Sequences of the terminal deletion mutants of BUNSCAT and summary of CAT activity results. (c) CV-1 cells transiently expressing BUN L and S segment proteins were transfected in duplicate with equimolar amounts of BUNSCAT RNAs containing wt or deleted terminal sequences, as indicated. Cells were harvested at 20 h post-transfection, cell extracts were prepared and equivalent amounts of protein were assayed for CAT activity [the results from two independent clones of the BUNSCAT 3'(-1), 5'(-1) construct are shown].

A feature of bunyavirus genome RNAs is the conservation of the 3'- and 5'-terminal sequences, from which genus-specific consensus sequences can be derived (Elliott *et al.*, 2000). For the genus *Orthobunyavirus*, the consensus extends to 11 nt that are complementary apart from a conserved U-G pairing at position 9 (Fig. 1a). These terminal 11 nt are followed by 3 (M segment) or 4 (S and L segments) nt that are complementary and conserved on a segment-specific basis (Fig. 1a). Beyond these terminal 14 or 15 nt, variable lengths of complementarity are found, which are generally unique to an individual virus and segment (Elliott *et al.*, 1991). The non-coding regions (NCRs) vary among different segments and different viruses; for the BUN S segment, the 3' NCR (genome sense) is 85 nt and the 5' NCR is 174 nt (Elliott, 1989). Signals within these NCRs are poorly understood.

Bunyavirus RNPs are observed to be circular when examined by electron microscopy (Obijeski *et al.*, 1976; Pettersson & von Bonsdorff, 1975; Raju & Kolakofsky, 1989) and RNase-resistance and chemical cross-linking experiments (Pardigon *et al.*, 1982; Raju & Kolakofsky, 1989) suggest that the terminal sequences are indeed base-paired. The panhandle structure seems to be a recurring

theme among negative-stranded segmented RNA viruses; it has also been described for orthomyxoviruses, such as influenza viruses and *Thogoto virus* (Hsu *et al.*, 1987; Leahy *et al.*, 1997; Martín-Benito *et al.*, 2001), and arenaviruses, such as *Lymphocytic choriomeningitis virus* (Perez & de la Torre, 2003). In a recent study on BUN, it was suggested that cooperation between the 3'- and 5'-terminal sequences is necessary for efficient transcription and translation (Barr & Wertz, 2004). However, the sequences in the reporter RNAs used in the experiments described in that paper contained several errors, compared with the authentic BUN NCRs (see Fig. 4).

We have previously described mini-genome systems that have been used to study BUN transcription (Dunn *et al.*, 1995; Weber *et al.*, 2001). By using these assays, we show here that small deletions at the termini are tolerated and demonstrate the existence of a structural element within the panhandle that defines promoter strength. We were able to define the minimum sequence requirements from the 3' and 5' ends of the S segment that are necessary for reporter-gene transcription and, by using chimeric M/L/S mini-genomes, to determine the basic sequence and complementarity requirements for transcription.

## METHODS

**Cells and viruses.** BHK-21 and BSR-T7/5 cells (which stably express T7 RNA polymerase; Buchholz *et al.*, 1999) were maintained in Glasgow's minimal essential medium supplemented with 10% tryptose phosphate broth and 10% fetal calf serum (FCS). Geneticin was added to a final concentration of 1 mg ml<sup>-1</sup> for the maintenance of BSR-T7/5 cells. CV-1 cells were grown in Dulbecco's minimal essential medium containing 10% FCS. All cells were grown at 37 °C. Working stocks of BUN were grown in BHK-21 cells at 33 °C and titres were determined by plaque assays on BHK-21 cells, as described previously (Bridgen & Elliott, 1996; Watret *et al.*, 1985). A recombinant vaccinia virus expressing T7 RNA polymerase, vTF7-3 (Fuerst *et al.*, 1986), was grown and titrated in CV-1 cells.

**Plasmids.** Plasmids pTM1-BUNN, pTM1-BUNL and pTM1-BUNNSs, expressing bunyavirus proteins, and the reporter RNAs pBUNSCAT, pT7riboBUNLRen(-), pT7riboBUNMRen(-), pT7riboBUNSRen(-), pT7riboBUNSRen(-)mut16 and pTM1-FF-Luc have been described previously (Dunn *et al.*, 1995; Kohl *et al.*, 2003, 2004; Weber *et al.*, 2001). Transcripts generated from pT7ribo plasmids mimic bunyavirus RNAs and can be encapsidated, transcribed and replicated after transfection into cells expressing the BUN N and L proteins (Dunn *et al.*, 1995).

Plasmids pT7riboBUNS(+), pT7riboBUNM(+) and pT7riboBUNL(+) used in virus rescue have been described by Bridgen & Elliott (1996). Plasmids pT7riboBUNSRen(-)mut946 and pT7riboBUNS(+)-mut946, carrying the C946A point mutation used in mini-replicon assays and virus rescue, were generated by using a QuikChange mutagenesis kit according to the manufacturer's instructions (Stratagene). All constructs were verified by DNA sequencing. Details of cloning strategies and oligonucleotide sequences can be obtained from the authors on request.

**Transfection, mini-replicon reconstitution and reporter-gene assays in BSR-T7/5 cells.** Approximately 5 × 10<sup>5</sup> cells in 35 mm Petri dishes were transfected with 1 µg pTM1-BUNL, 0.5 µg pTM1-BUNN and 0.1 µg pTM1-FF-Luc, together with 0.5 µg pT7riboBUNLRen(-), pT7riboBUNMRen(-) or pT7riboBUNSRen(-) DNA, by using 5 µg DAC-30 (Eurogentec). Transfection efficiencies were normalized by measuring luciferase expression from co-transfected pTM1-FF-Luc. Luciferase activities were determined by using a Dual-Luciferase Assay kit (Promega); cells were lysed in a total volume of 200 µl lysis buffer at 24 h post-transfection and luciferase activity was measured in 1 µl cell extract.

**Infection, metabolic labelling and Western blotting.** BHK-21 cells (1 × 10<sup>6</sup>) grown in 35 mm Petri dishes were infected at an m.o.i. of 1 with wild-type (wt) BUN or BUN S(C946A) virus. Cells were labelled with 50 µCi [<sup>35</sup>S]methionine as indicated. Extracts were prepared by using RIPA buffer containing Complete protease inhibitor mix (Roche) and analysed by SDS-PAGE. Quantification was performed by using a Molecular Dynamics phosphorimager and ImageQuant software. For Western blotting, cell extracts were separated by SDS-PAGE and blotted on to Hybond-C pure membrane, followed by incubation with appropriate antibodies, as described previously (Kohl *et al.*, 2004).

**Recovery of recombinant BUN from cDNA.** Recombinant BUN was recovered from cDNAs by using recent modifications (A. C. Lowen, C. Noonan, A. McLees & R. M. Elliott, unpublished results) to our original protocol (Bridgen & Elliott, 1996). In brief, BSR-T7/5 cells were transfected with 1 µg each of pT7riboBUNM(+), pT7riboBUNL(+) and either pT7riboBUNS(+) for recovery of wt virus or pT7riboBUNS(+)-mut946 for recovery of BUN S(C946A) virus by using 5 µg DAC-30. Support plasmids pTM1-BUNN,

pTM1-BUNL and pTM1-BUNM were co-transfected (1 µg each). After 1 week or when cytopathic effects were visible, supernatants were plated onto BHK cells and several plaques were isolated and grown up. The presence of the C946A mutation was confirmed by RACE (rapid amplification of cDNA ends) analysis of the viral S RNA segment terminal sequences. One plaque isolate of each virus was chosen for further characterization.

**Purification of RNA transcripts by using spin columns.** RNAs were transcribed *in vitro* from *Bbs*I-linearized plasmids (Dunn *et al.*, 1995) and purified on RNeasy mini spin columns (Qiagen) as described by the manufacturer. The eluted RNA transcripts were either used immediately or aliquotted and stored at -70 °C. The concentration of the RNA transcripts was determined by measuring A<sub>260</sub>.

**Transfection of CV-1 cells and CAT assay.** Procedures for transfection of plasmid DNA and *in vitro*-transcribed RNA were as described previously (Dunn *et al.*, 1995). In brief, subconfluent monolayers of CV-1 cells in 35 mm Petri dishes were infected with vTF7-3 at an m.o.i. of 10 for 1 h at 37 °C. After washing, cells were transfected with plasmid DNAs that expressed BUN proteins, followed 2.5 h later by *in vitro*-transcribed RNA. Cells were harvested 16 h later and chloramphenicol acetyltransferase (CAT) enzyme activity was determined as described previously (Gorman *et al.*, 1982). For quantitative purposes, extracts were diluted as described previously (Dunn *et al.*, 1995) and chromatography plates were analysed by using a Molecular Dynamics phosphorimager and ImageQuant software.

**Northern blots.** BHK-21 cells in 35 mm Petri dishes were infected with BUN at an m.o.i. of 1. At 18 h post-infection, total cellular RNA was extracted by using TRIzol reagent (Invitrogen). RNA was quantified by spectrophotometry and 10 µg was combined with 5.5 µl formaldehyde, 15 µl deionized formamide and 1.5 µl 10 × MOPS buffer in a total volume of 30 µl. Samples were heated at 55 °C for 15 min and then cooled on ice. Aliquots of 10 × loading buffer (3 µl; 40 mg bromophenol blue ml<sup>-1</sup>, 40 mg xylene cyanol ml<sup>-1</sup>, 2.5 µg Ficoll 400 ml<sup>-1</sup>) were added and samples were loaded on a 1.5% agarose/2.2% formaldehyde gel. Electrophoresis was performed at 75 V for 5 h in MOPS buffer. RNAs were transferred by capillary blotting overnight in 20 × SSC on to a positively charged nylon membrane (Nytran). After UV cross-linking, 150 ng digoxigenin (DIG)-labelled RNA probe complementary to either the S genome or antigenome segment was hybridized to the membrane in 4 ml 50% formamide buffer overnight at 68 °C. Synthesis and quantification of probes using a DIG Northern starter kit (Roche), washing of the membrane and subsequent detection were as directed by the manufacturer. Blots were exposed to X-OMAT UV film (Kodak) for up to 2 min.

**RNA folding.** Predicted RNA structures at 28 and 37 °C (temperatures at which the virus replicates in mosquito and mammalian cells, respectively) were determined by using Mfold (Zuker *et al.*, 1991); a minimum of 25 nt from the 5' and 3' termini (unless otherwise indicated) was analysed.

## RESULTS

### Effect of terminal deletions of the S segment 3' and 5' NCRs on mini-replicon activity

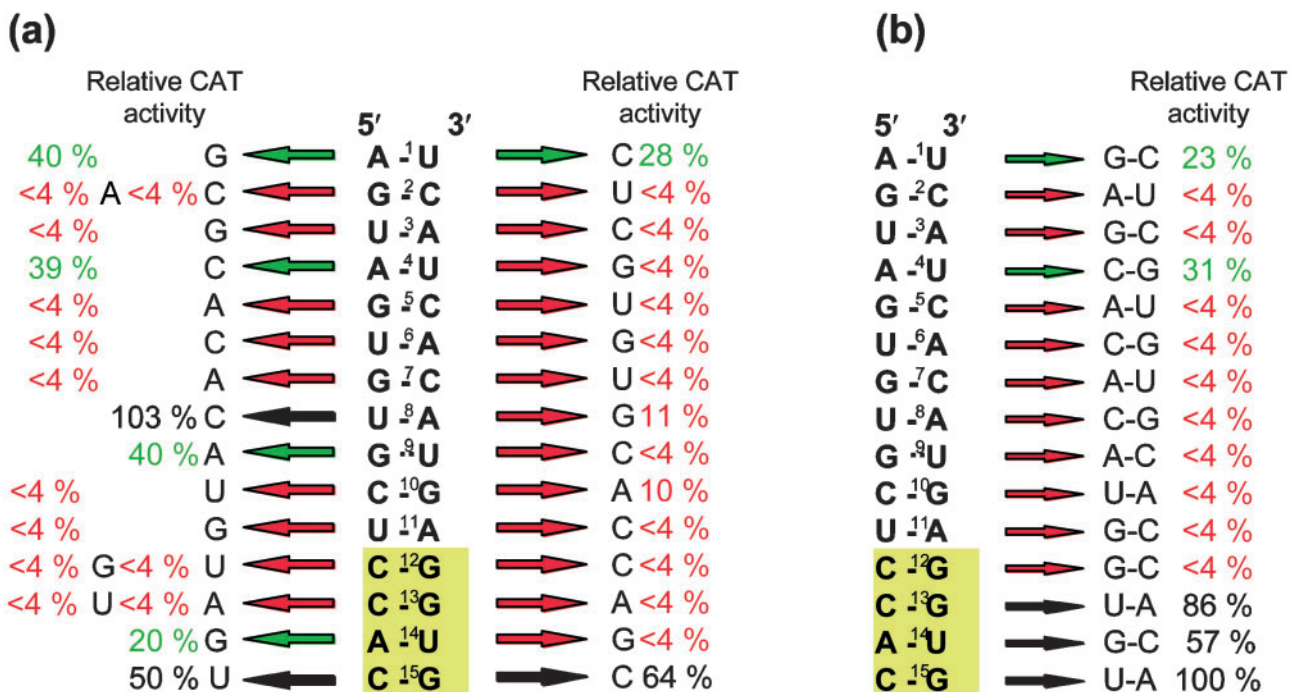
We reported previously that a BUNSCAT RNA transcript [comprising the negative-sense CAT reporter gene flanked by the 5' and 3' NCRs of the BUN S RNA segment] lacking 5 nt at the 3' terminus was inactive in the reporter assay (Dunn *et al.*, 1995). Here, we investigated the effects

of limited deletion of the conserved terminal residues at both the 3' and 5' ends of BUNSCAT RNA. Mutants lacking 1, 2 or 3 nt from either end, or lacking both the 3'- and 5'-terminal nucleotides, were generated by PCR-directed mutagenesis (Fig. 1b). Equimolar amounts of BUNSCAT RNAs were transfected into CV-1 cells that were infected with vTF7-3 and expressed the L and S proteins. As shown in Fig. 1(c), deletion of 1 nt from the 3' end of the RNA had little effect on CAT activity, compared with wt BUNSCAT RNA. However, deletion of 2 or 3 nt led to a strong reduction in activity, representing only 6 or <3% of wt BUNSCAT activity, respectively. Deletions from the 5' end were tolerated less well than 3'-end deletions. The construct missing the 5'-terminal nucleotide gave a CAT signal that was only 8% of that of wt BUNSCAT, whilst deletion of 2 or 3 nt resulted in <3% CAT activity, compared with wt. Similarly, removal of both the 5'- and 3'-terminal residues led to a dramatic loss of CAT activity (Fig. 1c), comparable with that caused by removal of just the 5'-terminal nucleotide. As the exact complementarity between the ends was restored in this construct, this demonstrated that the reduced CAT activity obtained by deleting 1 nt from the 5' end was not due to a 3' overhang, but was indeed an effect of the 5' deletion.

### Mutagenesis of the terminal regions of the S-segment 3' and 5' NCRs

It has previously been shown that a C→G mutation at position 12 of the BUN S genome segment strongly reduces CAT activity in the reporter assay (Dunn *et al.*, 1995). That a single point mutation could exert such an effect on the functionality of the reporter RNA suggested that conservation of the sequence and/or the structure of the BUN segment NCRs might be of great importance.

A series of single point mutations was made in pBUNSCAT (using a PCR-based mutagenesis strategy) in the 3' and 5' NCRs at each of the 15 nt at either end. *In vitro*-transcribed RNA was transfected into vTF7-3-infected CV-1 cells expressing BUN L and S proteins and CAT activities were compared with that of wt BUNSCAT. The results are summarized in Fig. 2(a). It can be seen that most single point mutations in either the 3' or 5' terminus resulted in a marked reduction in CAT activity (all <11%, with most <4% of wt activity), except for mutations at positions 1, 4, 8, 9 and 15 at the 5' end and positions 1 and 15 at the 3' end, where the mutant RNAs maintained significant CAT activity (>40% that of wt BUNSCAT RNA).

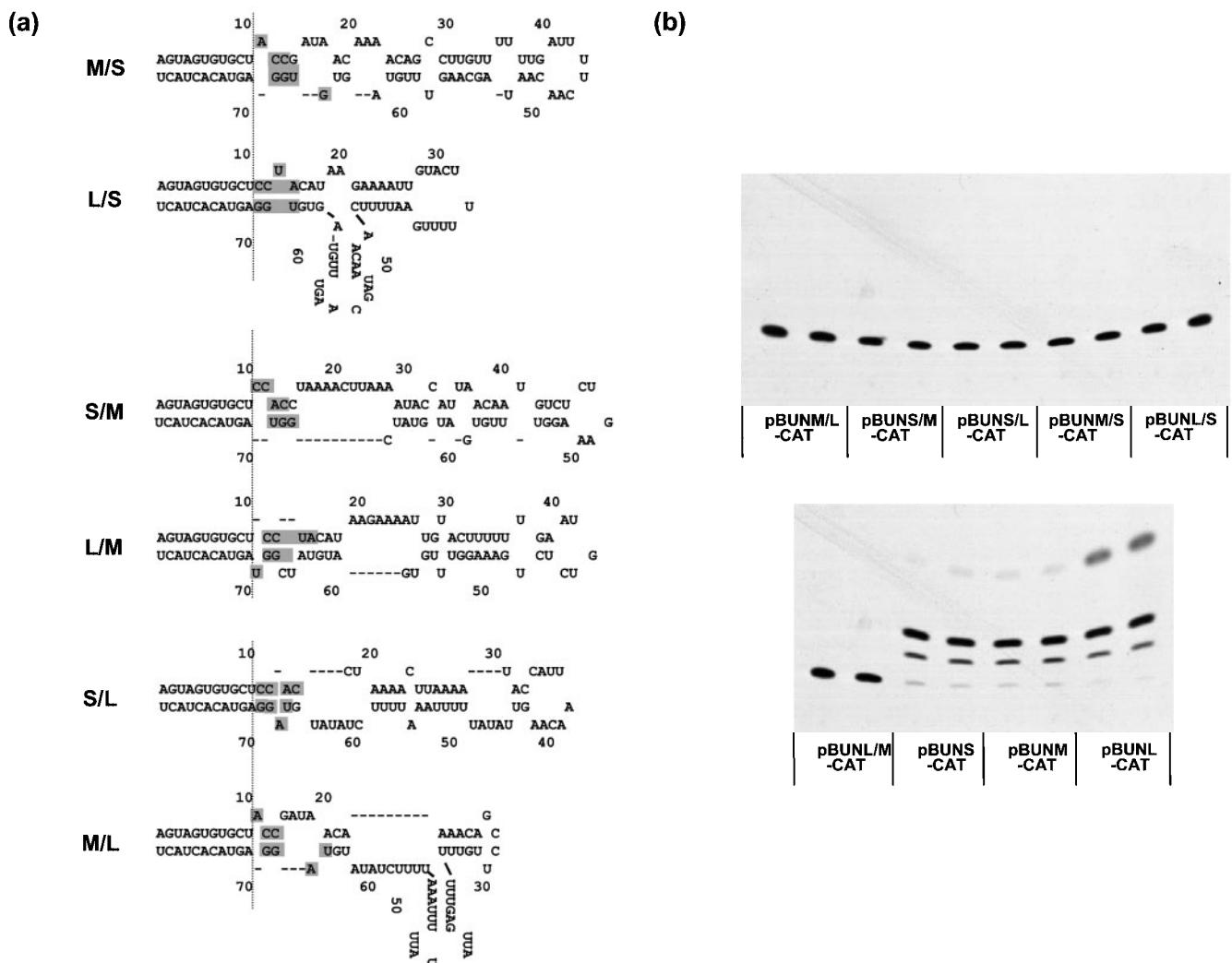


**Fig. 2.** Mutagenesis of the terminal sequences in BUNSCAT RNA. (a) Effect of single point mutations in the BUN S-segment 5' and 3' termini. CV-1 cells transiently expressing BUN L- and S-segment proteins were transfected with equimolar amounts of mutated BUNSCAT RNAs. Equivalent amounts of protein were assayed for CAT activity. The effect of each mutation is expressed as a percentage of the activity of wt BUNSCAT RNA. Red, <20% CAT activity; green, CAT activity between 20 and 49%; black, CAT activity >50% of wt. Nucleotides 12–15, boxed in yellow, represent those nucleotides that are conserved among orthobunyavirus S segments. (b) Effect of mutations in BUNSCAT RNA that restore complementarity. CAT activity is expressed relative to that of wt BUNSCAT RNA.



L and S segments is necessary to allow RNA synthesis and that complementarity of the NCRs extending beyond the conserved 11 terminal residues is required. However, the sequences and structures presented in that paper (see Fig. 1A of Barr & Wertz, 2004) do not represent authentic BUN sequences (these are compared directly in Fig. 4); thus, we made constructs containing the correct sequences to verify these findings. A series of chimeric mini-replicons containing the antisense CAT ORF flanked by heterologous combinations of NCRs were constructed; the sequences of the terminal 40 nt of these constructs are shown in Fig. 5(a). When transfected into vTF7-3-infected cells

expressing BUN L- and S-segment proteins, no CAT activity was detected with any chimeric construct (Fig. 5b), whereas constructs with homologous NCRs gave different activities of CAT, depending on the origin of the NCR, as reported previously (Barr *et al.*, 2003; Kohl *et al.*, 2004). Comparison of the predicted base-pairing between homologous segment termini (Fig. 1a) and the heterologous termini (Fig. 5a) shows disruption of predicted complementarity beyond the conserved terminal 11 nt. A consistent feature was disruption of the segment-specific base-pairing over nt 12–14 or 15; these residues are highlighted in Fig. 5(a). To investigate whether increasing



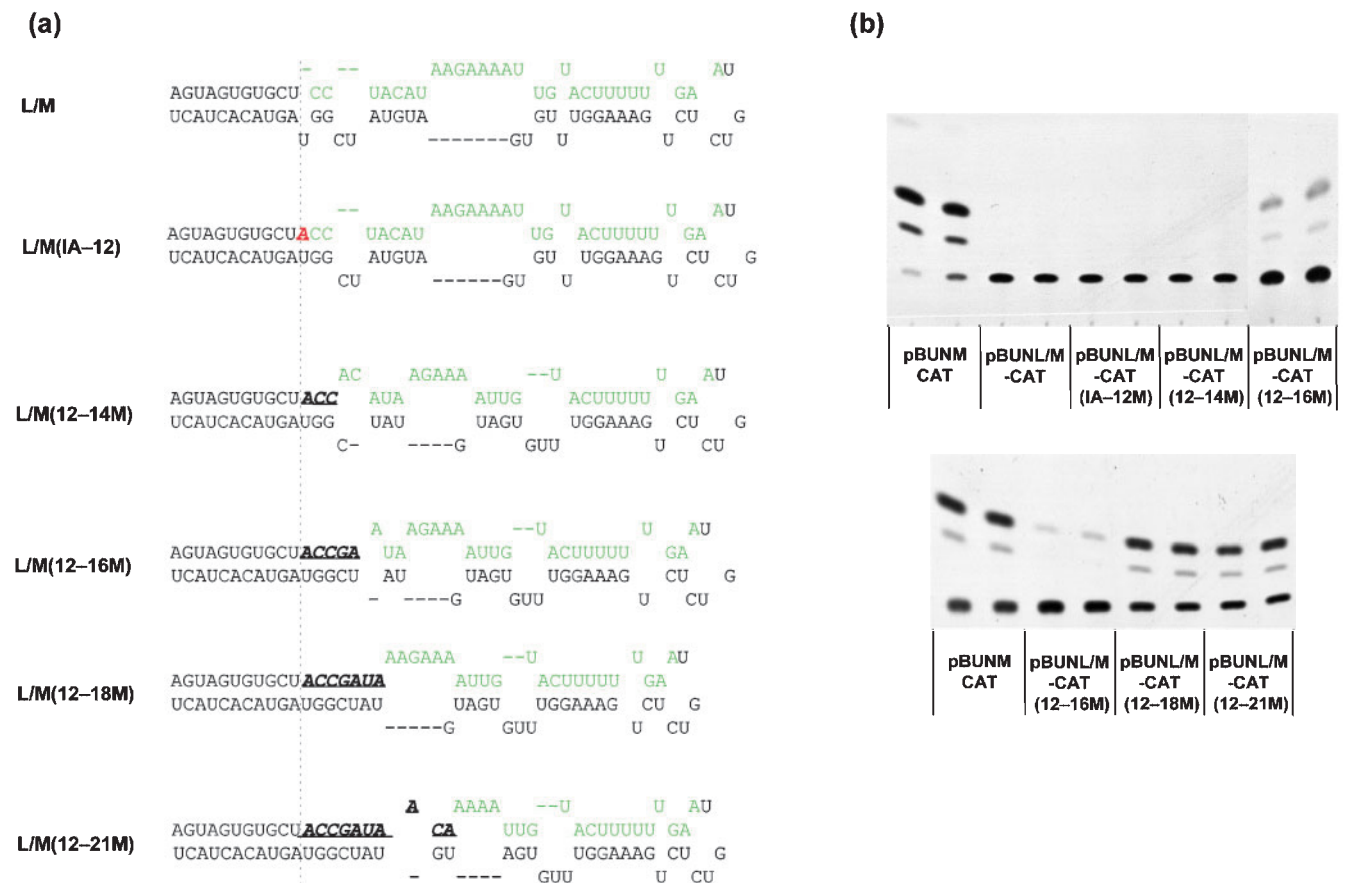
**Fig. 5.** Reporter RNAs containing heterologous NCR sequences. (a) Possible base-paired structures between the 5' and 3' termini of chimeric RNA transcripts. The terminal 40 nt of the 5' and 3' termini were paired together in a 5'→3' manner in the genomic sense. The dotted line marks the boundary of the terminal 11 nt conserved in all genomic segments. Shaded regions highlight nucleotides that are conserved in a segment-specific basis in bunyavirus RNA segments. (b) CAT activity of chimeric RNAs. Run-off RNA transcripts from *Bbs*I-linearized templates from each of the chimeric CAT constructs and the three homologous BUN-segment CAT constructs were transfected in duplicate into CV-1 cells transiently expressing BUN viral proteins. CAT activity in cell extracts was determined at 20 h post-transfection.

complementarity across this region resulted in a functional template, mutagenesis was performed on the pBUNL/M-CAT construct, so that the 5' NCR (L segment-derived) was mutated to resemble the sequence of the M-segment NCR (the pBUNL/M-CAT construct was chosen as the L- and M-segment NCRs are similar in length). A series of mutants was constructed by PCR-directed mutagenesis and is shown in Fig. 6(a). Run-off transcripts from each of the *Bbs*I-linearized templates were transfected into vTF7-3-infected CV-1 cells expressing BUN L- and S-segment proteins. CAT activities for each mutant are shown in Fig. 6(b). Weak CAT activity was detected when nt 12–16 of the L NCR were mutated to those of the M NCR (6% of wt level). When the mutated region was extended to nt 12–18 and 12–21 of the M 5' NCR, as in mutants pBUNL/M-CAT(12–18M) and pBUNL/M-CAT(12–21M), CAT activity was found to increase dramatically (71 and

65%, respectively, of wt pBUNMCAT activity). This indicated that base-pairing is required not only between the terminal 11 nt, but also, at least for the M segment, between nt 12 and 18 of the 5' and 3' ends. This was in agreement with the results shown in Fig. 2(b) for double mutations that restored complementarity at nt 13–15 in the S-segment-derived BUNSCAT RNA.

### A motif outside the terminal 11 nt determines the efficiency of RNA synthesis

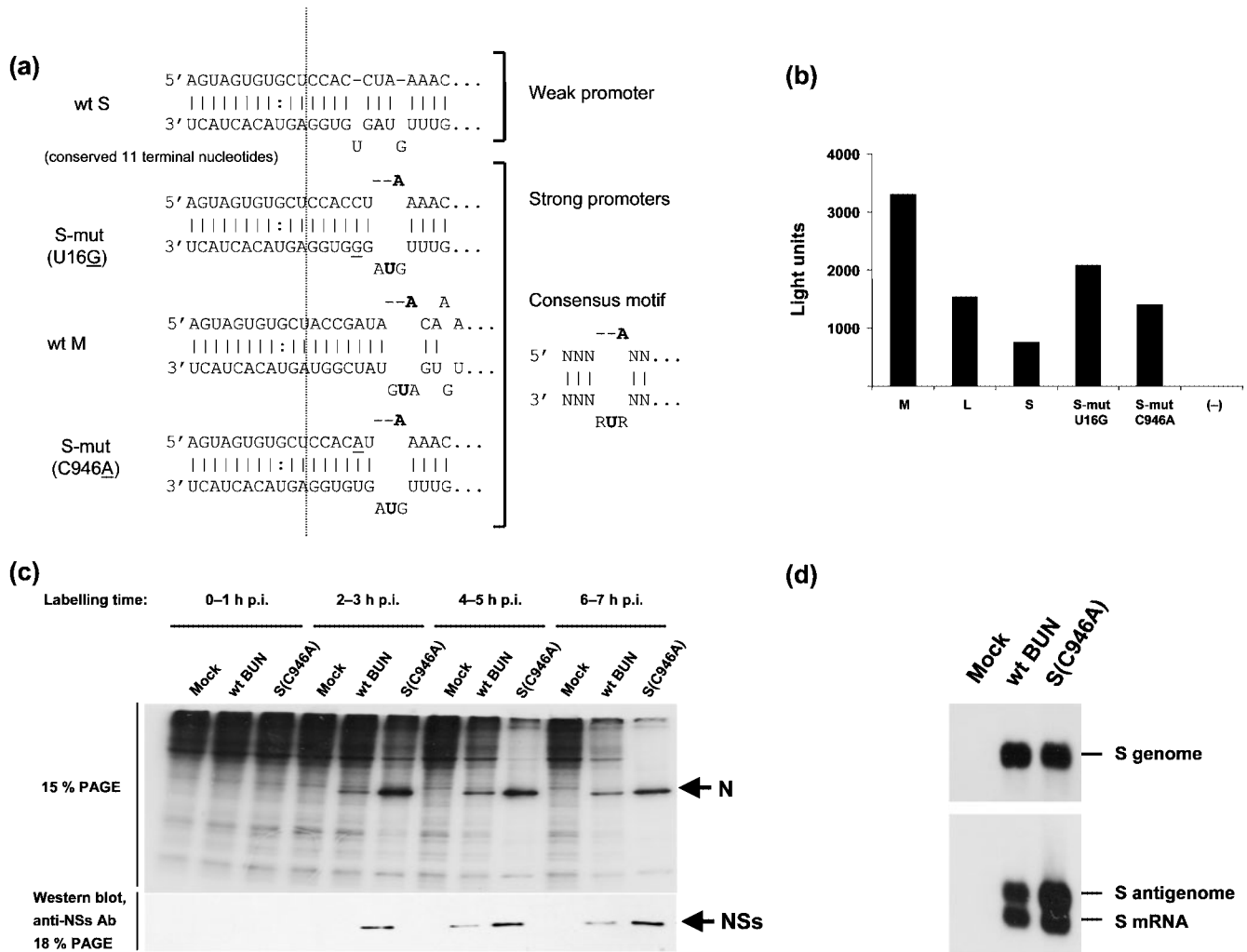
We previously described a recombinant BUN, S-mut(U16G), that does not express NSs (Bridgen *et al.*, 2001). This virus was found to overexpress N protein in mammalian cells due to a U→G mutation at nt 16 at the 3' end of the viral genome (Kohl *et al.*, 2003), which apparently transformed the naturally weak S-segment



**Fig. 6.** Mutation of the L-segment 5' NCR in the BUNL/M-CAT chimera to increase the possible base-pairing between the 5' and 3' termini. (a) RNA secondary structure predicted by Mfold. The conserved terminal 11 nt and the M-segment NCR sequences are shown in black and L-segment NCR sequences are shown in green. The insertion mutation in L/M(IA-12) is shown in red (bold, italic and underlined) and other mutations are shown in black (bold, italic and underlined). (b) Reporter-gene expression from pBUNL/M-CAT BUN L-segment 5' NCR mutants. CV-1 cells transiently expressing BUN viral proteins were transfected in duplicate with equal quantities of RNA transcripts from the BUNL/M-CAT mutants as indicated; pBUNMCAT and pBUNL/M-CAT were included as controls. Cells were harvested at 20 h post-transfection and CAT activity was assayed in cell extracts containing equal amounts of protein.

promoter into a stronger one. To determine whether a naturally strong BUN promoter, i.e. from the M segment, had sequence elements in common with the structure of S-mut(U16G), we compared their predicted base-pairing by using Mfold. As shown in Fig. 7(a), a consensus motif seemed to be present shortly beyond the conserved terminal 11 nt, involving unpaired residues flanked by base-paired nucleotides. To test experimentally whether this motif could indeed enhance RNA synthesis and gene expression,

we made a C→A mutation at nt 946 of the BUN S segment (at the 5' end) that gave a structure predicted to be similar to that of S-mut(U16G) (Fig. 7a). This mutation was first introduced into pT7riboBUNSRen(-) to give pT7riboBUNSRen(-)mut946. By using our *Renilla* luciferase-based reporter system (Kohl *et al.*, 2004), we compared activity against the wt L-, M- and S-segment promoters, as well as against S-mut(U16G). As shown in Fig. 7(b), *Renilla* luciferase activities obtained for the wt



**Fig. 7.** Generation of a stronger S-segment promoter. (a) Predicted RNA structures of the 5' and 3' termini of S, M and mutant S and M segments. Point mutations to create S-mut(C946A) are underlined. Nucleotides conserved within the conserved strong promoter structure are indicated in bold. The dotted line indicates the conserved terminal 11 nt. (b) Mini-replicon activity. BSR-T7/5 cells were transfected with BUN L and N expression plasmids, as well with pT7riboBUN constructs M, L, S, S-mut16 and S-mut946, as described in Methods. *Renilla* luciferase activities were measured at 24 h post-transfection and standardized against an internal reporter. (c) Protein expression. BHK-21 cells were infected with wt BUN or BUN S(C946A), or mock-infected, labelled with [<sup>35</sup>S]methionine at the indicated times post-infection and analysed by 15% SDS-PAGE. Replicate samples were analysed by 18% SDS-PAGE, blotted onto a membrane and probed with anti-NSs antibody. (d) RNA analysis. Total cellular RNA was extracted from mock-infected BHK-21 cells or cells infected with wt BUN or BUN S(C946A). RNAs were fractionated by denaturing agarose gel electrophoresis, followed by blotting onto a membrane. Duplicate membranes were probed with strand-specific probes to detect genome RNA or antigenome and mRNA, as indicated.

mini-genomes gave the gradient M>L>S that has been observed previously (Barr *et al.*, 2003; Kohl *et al.*, 2004). The activity of pT7riboBUNSRen(-)mut946 was found to be double that of the wt S mini-replicon, indicating that this particular mutation can indeed enhance mini-replicon activity.

To analyse the effect of this mutation on virus replication and gene expression, we rescued a recombinant virus carrying the C946A mutation, BUN S(C946A), by reverse genetics. We then compared viral protein synthesis in BHK cells infected with wt BUN and BUN S(C946A) by [<sup>35</sup>S]methionine pulse-labelling and Western blotting at early times post-infection. As shown in Fig. 7(c), in cells infected with BUN S(C946A), expression of S-segment proteins (N and NSs) was detected earlier than in cells infected with wt BUN. N levels expressed by BUN S(C946A) were found to be approximately four to five times higher than those of wt BUN. NSs levels were also higher in recombinant infected cells, and host-protein synthesis shut-off was obvious earlier after infection than in wt BUN-infected cells. Total infected-cell RNA was analysed by Northern blotting using probes to detect negative-sense RNA (genomes) or positive-sense RNA (antigenomes and mRNAs); as seen in Fig. 7(d), the amount of S genome RNA was similar in wt BUN- and BUN S(C946A)-infected cells, but there was a strong increase in the amount of S antigenome and S mRNA in mutant-infected cells. BUN S(C946A) gave similar-sized plaques to those obtained with wt BUN, although they gave approximately 10-fold lower titres compared with wt BUN after 48 h. This is similar to other BUNs that overexpress the N protein (Kohl *et al.*, 2003).

## DISCUSSION

In this study, we analysed the role of sequence and structural elements within the BUN S, M and L segment 3' and 5' NCRs. We mainly used a mini-replicon containing the CAT gene as a reporter; CAT activity is the end result of encapsidation, transcription and replication of the mini-replicon, as well as translation of the transcribed mRNA. The available evidence indicated that measuring reporter-gene activity was a good method for examining promoter strength, as there was excellent correlation between reporter activity and directly labelled RNA levels in our mini-replicon system (Barr *et al.*, 2003). In addition, the production of virus carrying the C946A mutation in the S segment gave increased levels of positive-sense RNA, as would be predicted from the mini-replicon experiment (Fig. 7). Of course, it is possible that individual mutations may have had effects that we could not measure by reporter activity, but to perform direct RNA analyses on all the mutated templates described here is beyond the scope of the present paper. It is also possible that some mutations could have affected translation efficiency of the reporter mRNA but, interestingly, we found essentially no difference in CAT activity between S or L segment-derived reporters in

which the CAT ORF was placed in an optimal translation context, compared with when the reporter was downstream of the authentic bunyavirus sequence (E. F. Dunn & R. M. Elliott, unpublished data). Hence, we consider the reporter assay to be a valid and convenient approach to investigate bunyavirus promoter sequences.

We found that deletion of 1–3 nt from the 3' end of the S segment still allowed transcription (although this was very weak if more than 1 nt was removed), similar to what has previously been described for *Rift Valley fever virus* (Prehaud *et al.*, 1997). This suggested that signals for polymerase recognition and interaction were still functional, although impaired. As deletion of nucleotides from the 5' end was tolerated much less well, the data presented here would lend support to a model of bunyavirus transcription similar to that described for influenza virus (Li *et al.*, 1998), in which the 5' terminus is involved in recognition by the L polymerase and the 3' end is involved in transcription initiation.

We also carried out mutagenic analysis of the first 15 nt of the BUN S 5' and 3' ends. As most of the changes made to these sequences of either end dramatically reduced CAT activity, we investigated whether restoring complementarity would restore CAT activity; our data showed that base-pairing was important, as well sequence specificity within the termini. The importance of complementarity was also shown by the use of S/L/M–CAT chimeric RNAs that were inactive in our assay. CAT activity was only detected when at least 14–16 nt was complementary between the 3' and 5' termini; further increasing the complementarity further increased the CAT signal. However, as the different segments have different complementary sequences after the conserved terminal 11 nt, complementarity *per se* is the important feature of this region. This is in agreement with a previously published report (Barr & Wertz, 2004) although, as mentioned above, the templates reported in that paper apparently contained base substitutions compared with the authentic BUN genome segments (Fig. 4). These results indicated that complementarity that extends past the extreme conserved terminal 11 nt is critical, possibly for forming the correct secondary structure at the termini, to allow transcription and replication.

Mini-genomes containing flanking sequences of either 13 or 20 nt of the S-segment 5' and 3' termini resulted in insignificant CAT activity, but a signal close to wt activity was obtained by flanking the reporter with 32 nt of the S-segment 5' NCR and 33 of the S-segment 3' NCR. This suggested that the minimal requirement of nucleotides for the S-segment NCR is more than 20 and less than 32. Any encapsidation signal has to be localized within these 32 nt. Finally, comparison of known 'strong' promoters allowed us to identify a structural motif within the terminal 20 nt of the M and S(U16G) RNAs. Whilst structural prediction has limitations, we successfully created a mutant S segment carrying this structure (C946A) that showed increased

reporter-gene expression. When introduced into a viable virus, the mutant overexpressed the N and NSs proteins and produced more antigenomes and mRNAs in infected cells. This suggested that structure, rather than sequence, defined this element, as in the case of the U16G mutation described previously (Kohl *et al.*, 2003).

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## REFERENCES

- Barr, J. N. & Wertz, G. W. (2004). Bunyamwera bunyavirus RNA synthesis requires cooperation of 3'- and 5'-terminal sequences. *J Virol* **78**, 1129–1138.
- Barr, J. N., Elliott, R. M., Dunn, E. F. & Wertz, G. W. (2003). Segment-specific terminal sequences of Bunyamwera bunyavirus regulate genome replication. *Virology* **311**, 326–338.
- Bridgen, A. & Elliott, R. M. (1996). Rescue of a segmented negative-strand RNA virus entirely from cloned complementary DNAs. *Proc Natl Acad Sci U S A* **93**, 15400–15404.
- Bridgen, A., Weber, F., Fazakerley, J. K. & Elliott, R. M. (2001). Bunyamwera bunyavirus nonstructural protein NSs is a nonessential gene product that contributes to viral pathogenesis. *Proc Natl Acad Sci U S A* **98**, 664–669.
- Buchholz, U. J., Finke, S. & Conzelmann, K.-K. (1999). Generation of bovine respiratory syncytial virus (BRSV) from cDNA: BRSV NS2 is not essential for virus replication in tissue culture, and the human RSV leader region acts as a functional BRSV genome promoter. *J Virol* **73**, 251–259.
- Dunn, E. F., Pritlove, D. C., Jin, H. & Elliott, R. M. (1995). Transcription of a recombinant bunyavirus RNA template by transiently expressed bunyavirus proteins. *Virology* **211**, 133–143.
- Elliott, R. M. (1989). Nucleotide sequence analysis of the small (S) RNA segment of Bunyamwera virus, the prototype of the family Bunyaviridae. *J Gen Virol* **70**, 1281–1285.
- Elliott, R. M. (1996). The *Bunyaviridae*: concluding remarks and future prospects. In *The Bunyaviridae*, pp. 295–332. Edited by R. M. Elliott. New York: Plenum.
- Elliott, R. M. (1997). Emerging viruses: the *Bunyaviridae*. *Mol Med* **3**, 572–577.
- Elliott, R. M., Schmaljohn, C. S. & Collett, M. S. (1991). *Bunyaviridae* genome structure and gene expression. *Curr Top Microbiol Immunol* **169**, 91–141.
- Elliott, R. M., Bouloy, M., Calisher, C. H., Goldbach, R., Moyer, J. T., Nichol, S. T., Petterson, R., Plyusnin, A. & Schmaljohn, C. S. (2000). *Bunyaviridae*. In *Virus Taxonomy: Seventh Report of the International Committee on Taxonomy of Viruses*, pp. 599–621. Edited by M. H. V. van Regenmortel, C. M. Fauquet, D. H. L. Bishop, E. B. Carstens, M. K. Estes, S. M. Lemon, J. Maniloff, M. A. Mayo, D. J. McGeoch, C. R. Pringle & R. B. Wickner. San Diego: Academic Press.
- Fuerst, T. R., Niles, E. G., Studier, F. W. & Moss, B. (1986). Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. *Proc Natl Acad Sci U S A* **83**, 8122–8126.
- Gorman, C. M., Moffat, L. F. & Howard, B. H. (1982). Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol Cell Biol* **2**, 1044–1051.
- Hsu, M.-T., Parvin, J. D., Gupta, S., Krystal, M. & Palese, P. (1987). Genomic RNAs of influenza viruses are held in a circular conformation in virions and in infected cells by a terminal panhandle. *Proc Natl Acad Sci U S A* **84**, 8140–8144.
- Kohl, A., Bridgen, A., Dunn, E., Barr, J. N. & Elliott, R. M. (2003). Effects of a point mutation in the 3' end of the S genome segment of naturally occurring and engineered Bunyamwera viruses. *J Gen Virol* **84**, 789–793.
- Kohl, A., Hart, T. J., Noonan, C., Royall, E., Roberts, L. O. & Elliott, R. M. (2004). A Bunyamwera virus minireplicon system in mosquito cells. *J Virol* **78**, 5679–5685.
- Leahy, M. B., Dessens, J. T. & Nuttall, P. A. (1997). Striking conformational similarities between the transcription promoters of Thogoto and influenza A viruses: evidence for intrastrand base pairing in the 5' promoter arm. *J Virol* **71**, 8352–8356.
- Li, M.-L., Ramirez, B. C. & Krug, R. M. (1998). RNA-dependent activation of primer RNA production by influenza virus polymerase: different regions of the same protein subunit constitute the two required RNA-binding sites. *EMBO J* **17**, 5844–5852.
- Martín-Benito, J., Area, E., Ortega, J., Llorca, O., Valpuesta, J. M., Carrascosa, J. L. & Ortín, J. (2001). Three-dimensional reconstruction of a recombinant influenza virus ribonucleoprotein particle. *EMBO Rep* **2**, 313–317.
- Obijeski, J. F., Bishop, D. H. L., Palmer, E. L. & Murphy, F. A. (1976). Segmented genome and nucleocapsid of La Crosse virus. *J Virol* **20**, 664–675.
- Pardigon, N., Vialat, P., Girard, M. & Bouloy, M. (1982). Panhandles and hairpin structures at the termini of Germiston virus RNAs (Bunyavirus). *Virology* **122**, 191–197.
- Perez, M. & de la Torre, J. C. (2003). Characterization of the genomic promoter of the prototypic arenavirus lymphocytic choriomeningitis virus. *J Virol* **77**, 1184–1194.
- Pettersson, R. F. & von Bonsdorff, C.-H. (1975). Ribonucleoproteins of Uukuniemi virus are circular. *J Virol* **15**, 386–392.
- Prehaud, C., Lopez, N., Blok, M. J., Oby, V. & Bouloy, M. (1997). Analysis of the 3' terminal sequence recognized by the Rift Valley fever virus transcription complex in its ambisense S segment. *Virology* **227**, 189–197.
- Raju, R. & Kolakofsky, D. (1989). The ends of La Crosse virus genome and antigenome RNAs within nucleocapsids are base paired. *J Virol* **63**, 122–128.
- Schmaljohn, C. S. & Hooper, J. W. (2001). *Bunyaviridae*: the viruses and their replication. In *Fields Virology*, 4th edn, pp. 1581–1602. Edited by D. M. Knipe & P. M. Howley. Philadelphia, PA: Lippincott Williams & Wilkins.
- Watret, G. E., Pringle, C. R. & Elliott, R. M. (1985). Synthesis of bunyavirus-specific proteins in a continuous cell line (XTC-2) derived from *Xenopus laevis*. *J Gen Virol* **66**, 473–482.
- Weber, F., Dunn, E. F., Bridgen, A. & Elliott, R. M. (2001). The Bunyamwera virus nonstructural protein NSs inhibits viral RNA synthesis in a minireplicon system. *Virology* **281**, 67–74.
- Weber, F., Bridgen, A., Fazakerley, J. K., Streitenfeld, H., Kessler, N., Randall, R. E. & Elliott, R. M. (2002). Bunyamwera bunyavirus nonstructural protein NSs counteracts the induction of alpha/beta interferon. *J Virol* **76**, 7949–7955.
- Zuker, M., Jaeger, J. A. & Turner, D. H. (1991). A comparison of optimal and suboptimal RNA secondary structures predicted by free energy minimization with structures determined by phylogenetic comparison. *Nucleic Acids Res* **19**, 2707–2714.