Mutational Analyses of the Nonconserved Sequences in the Bunyamwera Orthobunyavirus S Segment Untranslated Regions

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Bunyamwera virus (BUNV) is the prototype of both the genus Orthobunyavirus and the family Bunyaviridae. BUNV has a tripartite genome of negative-sense RNA composed of small (S), medium (M), and large (L) segments. Partially complementary untranslated regions (UTRs) flank the coding region of each segment. The terminal 11 nucleotides of these UTRs are conserved between the three segments and throughout the genus, while the internal regions are unique to each segment and largely nonconserved between different viruses. To investigate the functions of the UTR sequences, we constructed a series of BUNV S segment cDNA clones with deletions in the 3′ and/or 5′ UTR and then attempted to rescue these segments into recombinant viruses. We found that the genomic 5′ UTR was much more sensitive to mutation than the 3′ UTR and, in general, sequences proximal to the termini were more important than those flanking the coding region. Northern blot analyses of infected-cell RNA showed that the internal, nonconserved sequences of the S segment 3′ UTR play a role in the regulation of transcription and replication and the balance between these two processes. In contrast, deletions in the 5′ UTR caused attenuation of the recombinant virus but did not specifically affect levels of S segment RNAs or the encoded nucleocapsid protein. Thus, the internal regions of both UTRs are functional: most of the 5′ UTR is essential to viral growth, and, while nonessential, the internal 3′ UTR is important to the regulation of viral RNA synthesis.

The family Bunyaviridae comprises more than 350 viruses with worldwide distribution. They are divided into five genera: Hantavirus, Nairoivirus, Orthobunyavirus, Phlebovirus, and Tospovirus. With the exception of the hantaviruses, which infect rodents, bunyaviruses are arthropod borne and include several important human, livestock and plant pathogens, such as La Crosse orthobunyavirus, a leading cause of encephalitis in children in the Midwestern United States; Crimean-Congo hemorrhagic fever virus, a nairovirus found throughout Asia, the Middle East, and Africa that can lead to severe hemorrhagic fever in humans; and Rift Valley fever phlebovirus, which causes devastating epizootics among livestock in sub-Saharan Africa.

Bunyamwera virus (BUNV) is the prototype of the genus Orthobunyavirus and the family Bunyaviridae. BUNV has a tripartite, negative-sense RNA genome comprising small (S), medium (M), and large (L) segments. The three genome segments encode six proteins: the L segment codes for the viral RNA polymerase (L) protein; the M segment encodes a medium (M) and large (L) segments. The three genome segments encode six proteins: the L segment codes for the viral RNA polymerase (L) protein; the M segment encodes a nucleocapsid protein (N) as well as a small nonstructural protein called NSs. The N and NSs proteins are translated from the same mRNA species, using alternative start codons in overlapping reading frames. The N protein functions to encapsidate viral genomic and antigenic RNAs, forming ribonucleoproteins (RNPs) which are the templates for RNA synthesis by the L protein (13, 34). The NSs protein has been shown to play a role in the inhibition of cellular protein synthesis and to counteract the host immune response (6, 39).

Bracketing the open reading frame (ORF) of each segment are partially conserved and partially complementary 3′ and 5′ untranslated regions (UTRs). The UTRs of Bunyamwera virus are 85, 56, and 50 nucleotides in length at the 3′ end and 174, 100, and 108 nucleotides at the 5′ end of the S, M, and L segments, respectively. The first 11 nucleotides at each terminus are conserved between the three segments as well as throughout the Orthobunyavirus genus. The internal regions of the UTRs are unique to each segment, and, while conservation of these regions within serogroups extends for 4 nucleotides (S and L segments) or 3 nucleotides (M segment) beyond the terminal 11 nucleotides (11, 12), the bulk of each UTR consists of largely nonconserved sequence (Fig. 1).

For several members of the Bunyaviridae, including BUNV (10, 40), La Crosse orthobunyavirus (4), Rift Valley fever phlebovirus (1, 19), Hantaan virus (14), Uukuniemi virus (17), and Crimean-Congo hemorrhagic fever virus (16) minireplicon systems based on reporter genes flanked by the UTR sequences have been developed, demonstrating that the UTRs alone are sufficient to allow transcription, replication, encapsidation, and packaging (15) of minigenome segments by viral proteins. Indeed, we have previously shown that a chloramphenicol acetyltransferase (CAT) reporter gene plus the terminal 33 nucleotides of each BUNV S segment UTR comprises a functional minireplicon (20).

In addition to being conserved, the terminal 11 nucleotides...
FIG. 1. Sequence alignment of the 3' (A) and 5' (B) S segment untranslated regions of nine members of the Orthobunyavirus genus. Red denotes identity in 80% of sequences; blue denotes conservation of a purine or pyrimidine in 80% of sequences. Abbreviations: INKV, Inkoo virus; JCV, Jamestown Canyon virus; LACV, La Crosse virus; TAHV, Tahyna virus; AKAV, Akabane virus; TINV, Tinaroo virus; MAGV, Maguari virus.
of each UTR are complementary, with the exception of a G-U pairing at position 9. Depending on the segment terminal complementarity extends for up to 25 nucleotides (12). These complementary regions are thought to base-pair intramolecularly to form stable panhandle structures in vivo, as evidenced by the observed circular confirmation of viral RNPs in electron micrographs (28, 30) and their ability to be chemically cross-linked (33). Furthermore, mutational analyses have shown that terminal complementarity up to nucleotide 14 at the 5′ and 3′ ends is essential for a minigene to form a functional template (2, 20). The same studies demonstrated that, in addition to complementarity, the sequences of the 3′- and 5′-terminal 14 nucleotides of the S segment are either essential for activity or important in the determination of promoter strength.

An investigation into the RNA binding properties of the BUNV N protein showed that the 5′-terminal 32 and 3′-terminal 33 nucleotides of the BUNV S segment bind selectively to the N protein and suggested a predicted stem-loop at the 5′ end as an encapsidation signal (29). Thus, studies performed to date on the BUNV UTRs have uncovered several functional sequences, all residing in the conserved terminal 20 to 30 nucleotides of either terminus.

Information is lacking on signals contained within the internal, nonconserved sequences of the UTRs. Bunyaviruses in general carry longer UTRs than other segmented, negative-strand RNA viruses such as influenza viruses (22) and Thogoto virus (23) and among the orthobunyaviruses in particular, members of the Bunyamwera serogroup have significantly longer S segment UTRs than viruses of the Simbu serogroup (Fig. 1). Thus, we postulated that the longer UTRs of other orthobunyaviruses may include nonessential sequences, and in this study, we sought to identify the minimal UTR sequence of the S segment sufficient for viral growth and to determine the function of the remaining sequences.

To that end, we attempted to rescue recombinant viruses carrying deletions in the 3′ and 5′ UTRs of the BUNV S segment. Our results confirm that the sequences closer to the termini are more important for viral growth. We found that the minimal 3′ UTR sequence required is the terminal 22 nucleotides and a BUNV S segment containing the 3′-terminal 29 nucleotides and the 5′-terminal 112 nucleotides is viable, while S segments with shorter UTRs could not be recovered. Through characterization of the recombinant viruses, we show that the nonconserved sequence of the S segment 3′ UTR functions in the regulation of transcription and replication and affects the balance between these two processes. A region of particular interest lies at nucleotides 25 to 28 of the 3′ UTR. In addition, the sequence context of the N start codon was found to be an important determinant of NSs expression levels. Our data suggest that the RNA sequence between the N stop codon and nucleotide 112 from the 5′ terminus may play a role in host protein shut-off. Finally, shortening of the S segment UTRs was found to attenuate growth of the recombinant viruses in cell culture.

Materials and Methods

Cells and viruses. BHK-21 cells were grown at 37°C in Glasgow modified minimal essential medium supplemented with 2 mM glutamine and 10% heat-inactivated fetal bovine serum. BSR-T7/5 cells, which stably express T7 RNA polymerase (7), were kindly provided by K. K. Conzelmann (Max-von-Pettenkofer Institute, Munich, Germany) and were grown at 37°C in Glasgow modified minimal essential medium supplemented with 2 mM glutamine, 10% fetal bovine serum, and 1 mg/ml G418. Vero-E6 cells were grown at 37°C in Dulbecco’s modified Eagle’s medium supplemented with 2 mM glutamine and 10% fetal bovine serum. Bunyaviruses were purified by plaque formation on BHK-21 cells, and working stocks were grown in BHK-21 cells as described previously (35).

Plasmids. The construction of pT7ribobUNL(S)+, pT7ribobUNL(S)+, and pT7ribobUNL(S)+ has been described previously (5). Each of these plasmids encodes a full-length, antigenic-sense BUNV segment, with the bacteriophage T7 promoter immediately upstream and a hepatitis δ ribozyme, followed by the T7 terminator, immediately downstream. Deletions in the S segment UTRs were introduced by a PCR procedure using primer pairs in outward orientation flanking each region to be deleted (35) and pT7ribobUNL(S)+ as the template. Single nucleotide substitutions were introduced into pT7ribobUNL(S)+ by PCR using specific primers carrying the appropriate point mutation. Primer sequences and PCR parameters are available from the authors on request. All constructs were verified by sequence analysis.

Generation of recombinant viruses from cDNA. Recombinant viruses were produced using the recently described three-plasmid system (26). Briefly, subconfluent BSR-T7/5 cells (106 cells per 60-mm-diameter petri dish) were transfected with 1 μg each pT7ribobUNL(S)+, pT7ribobUNL(S)+, and pT7ribobUNL(S)+ (for wild-type virus), or the appropriate mutant pT7ribobUNL(S)+ construct, using Fugene 6 transfection reagent (Roche), according to the manufacturer’s protocol. Four days posttransfection, supernatants were harvested, and Bunyamwera viruses therein were isolated by plaque formation on BHK-21 cells as described previously (38). Recovered viruses were confirmed to carry the correct mutations by reverse transcription-PCR followed by sequence analysis of virion-derived S segment RNA. Two independent isolates of each virus were analyzed and found to have consistent phenotypes (data not shown).

Virus growth curves. BHK-21 cells in 35-mm-diameter petri dishes were infected at a multiplicity of infection of 1 with either transfected wild-type BUNV or recombinant viruses. One hour postinfection, the inoculum was removed and cells were washed three times with phosphate-buffered saline to remove unattached viruses. At the time points indicated, viruses in the supernatant fluid were titrated by plaque assay on Vero-E6 cells.

Metabolic labeling of viral proteins. BHK-21 cells in 35-mm-diameter petri dishes were infected at a multiplicity of infection of 1 with either wild-type BUNV or recombinant viruses. Twenty-four hours postinfection, cells were labeled with 50 μCi per dish of [35S]methionine for 2.5 h. Cell lysates were prepared and analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) as described previously (36).

Western blotting. BHK-21 cells in 35-mm-diameter petri dishes were infected at a multiplicity of infection of 1 with either wild-type BUNV or recombinant viruses. Twenty-four hour postinfection cell lysates were prepared by scraping into 200 μl protein dissociation solution containing 0.05 M EDTA, pH 8.0, 0.5% (wt/vol) SDS; 10% (wt/vol) Ficoll; and 0.12% (wt/vol) bromophenol blue. Equal amounts of cell extract were separated by SDS-18% PAGE and transferred to Hybond-C Extra membrane (Amersham), followed by overnight incubation in saturation buffer (phosphate-buffered saline containing 10% dry milk and 0.1% Tween 20). The membrane was incubated for 2 h in saturation buffer containing either an anti-NSs antibody (T. J. Hart et al., unpublished data) at a dilution of 1:500, an anti-N antibody at a dilution of 1:3,000, followed by incubation with an alkaline phosphatase-labeled anti-rabbit antibody (Cell Signaling Technology) diluted 1:1,000 in saturation buffer. After three washes in phosphate-buffered saline-0.1% Tween 20, the membrane was rinsed in phosphate-buffered saline, and detection was performed using SuperSignal WestPico chemiluminescent substrate (Pierce).

Northern blotting. Northern blotting was performed essentially as described previously (25). Briefly, BHK-21 cells in 35-mm-diameter petri dishes were infected at a multiplicity of infection of 1 with either wild-type BUNV or recombinant viruses. Twenty-four hours postinfection RNA was extracted using TRizol reagent (Invitrogen). Five micrograms RNA in sample buffer was electrophoresed through a 1.5% agarose–2.2 M formaldehyde gel and then transferred to a positively charged nylon membrane (Roche). After hybridization with digoxigenin-labeled RNA probes complementary to the 5′ most 1 kb of the M and S antigenome RNAs or the M and S genome RNAs (150 ng of each probe), detection was carried out using a DIG Northern starter kit (Roche). The Chemi Genius 2 bioimaging system (Syngene) was used for quantification of digoxigenin-labeled RNA.

Nucleotide sequence accession numbers. Sequences were aligned using Clustal W. The accession numbers of the S segment sequences used are as follows: Aino virus (M22011), Akabane virus (AB000851), BUNV S (NC_001927), Inko virus
RESULTS

Rescue of recombinant Bunyamwera viruses. We designed 20 pT7riboBUNS(+1) constructs in which an internal portion of either the 3' or 5' or both genomic UTRs was deleted (Fig. 2, upper part). As shown in Fig. 1, the deleted regions correspond to largely nonconserved sequences. The recombinant plasmids were then cotransfected into BSR-T7/5 cells with pT7ribo plasmids encoding wild-type M and L segments, and viruses were recovered from 11 of the transfections, as indicated in Fig. 2. All negative results were confirmed by carrying out at least one additional rescue transfection (where each attempt yielded...
greater than $10^6$ PFU/ml wild-type virus from a parallel transfection). For each recombinant virus, two independent isolates were obtained by plaque purification on BHK-21 cells. After amplification in BHK-21 cells, the resulting virus stocks were used for further analysis. Both isolates of each virus were found to share identical phenotypes, and thus data from only a single isolate of each are shown. The S segment RNA of each recovered virus was analyzed by reverse transcription-PCR and sequencing to confirm the presence of the expected deletions.

**Growth characteristics of S segment UTR deletion mutants in cell culture.** The plaque phenotypes of the rescued viruses were examined in Vero-E6 cells and all of the recombinant viruses produced smaller plaques than wild-type BUNV (Fig. 2, lower part). Similarly, recombinant viruses also formed smaller plaques in BHK cells (data not shown). In general, the observed reductions in plaque size correlated with the size of S segment deletion for each virus, with the exception of the S22/174 virus, which produced larger plaques than either of the S24/174 or S28/174 viruses. In addition, viruses containing the 5’ UTR deletion were found to produce especially small plaques. Since plaque size is related to growth rate, the growth curves of the mutant viruses were investigated in BHK-21 cells and compared to that of isogenic wild-type BUNV also generated from plasmid transfection. Infections were done at a multiplicity of 1 PFU/cell and virus in supernatants from infected cells was titrated by plaque assay on Vero-E6 cells at the time points indicated (Fig. 3).

At 30 h postinfection wild-type virus yielded $8 \times 10^7$ PFU/ml. Several of the viruses with 3’ UTR deletions (S62/174, S33/174, S29/174, and S22/174) produced a similar titer after 30 h but grew more slowly than wild-type BUNV. Consistent with their relative plaque sizes, the S22/174 virus grew to a higher titer than the S24/174 and S28/174 viruses, which showed a 10-fold reduction in yield relative to the wild-type virus. The 5’ UTR deletion mutant S85/112 grew more slowly than wild-type BUNV and to a 30-fold lower titer. The three viruses carrying double deletions exhibited the greatest attenuation; the virus with the shortest S segment (S29/112) grew to a 1,000-fold-lower titer than wild-type BUNV.

**S segment RNA levels in recombinant-virus-infected cells.** To examine the synthesis of S segment genomic, antigenomic, and mRNAs by the recombinant viruses, total cellular RNA extracted from infected BHK-21 cells at 24 h postinfection was analyzed by Northern blotting. A single time point was chosen for convenience rather than optimizing for each virus such that the absolute amounts of viral RNA detected might vary due to
the different growth characteristics of each virus. In addition to S segment RNAs, M segment RNAs were detected and their signal was used to act as an internal control.

Figure 4A shows the blot hybridized with a probe to detect both antigenome and mRNAs; these two species were not resolved for S segments containing the 5’ UTR deletion due to the shortened length of the antigenome RNAs. The signal for the M segment represents a mixture of both positive-sense RNAs, as these species were also not resolved because of the relatively small length difference between full-length antigenome RNA and mRNA. Deletion of nonconserved regions of the S segment 3’ UTR did affect RNA synthesis activities from this promoter. Specifically, steady-state levels of S segment antigenome RNAs (normalized to the corresponding M segment) were decreased in the S33/174, S29/174, and S28/174 viruses relative to wild-type BUNV, while the S62/174, S24/174, and S22/174 viruses showed increases in S antigenome levels. Accumulation of S segment mRNA in infected cells, on the other hand, was similar between the wild-type and S62/174 viruses, moderately decreased with S24/174 and S22/174 (1.3-fold), and drastically decreased with S33/174, S29/174, and S28/174 (5- to 10-fold relative to the wild-type virus). Interestingly, the ratio of S segment mRNAs to antigenome RNAs was found to be altered in cells infected with viruses containing the 3’ UTR deletions. In wild-type-infected cells, S mRNA was 1.4-fold more abundant than antigenome RNA, while in cells infected with the 3’ UTR mutants, antigenome levels were up to fivefold greater than the corresponding mRNA levels (Fig. 4B).

In contrast to the positive-sense species, the abundance of S genome segments was not found to be decreased in any of the recombinant-virus-infected cells relative to the corresponding M segments (Fig. 4C and D). Most deletion mutants expressed amounts of S genome RNA similar to that of the wild-type virus, with the exceptions of the S22/174, S24/174, S85/112, and S29/112 viruses, which produced relatively more.

**Protein synthesis in recombinant-virus-infected cells.** To determine whether deletion of nonconserved UTR sequences altered the expression of viral proteins, we first used in vivo labeling of proteins in infected cells. In an analysis of samples
labeled for 2 h at 24 h postinfection (Fig. 5A), we found that levels of N protein were reduced in S29/174, S28/174, and S29/112 virus-infected cells, while the levels of other viral proteins and N protein from the remaining recombinant viruses appeared similar to those in wild-type BUNV-infected cells.

We also observed that the shut-off of host protein synthesis normally seen in a BUNV virus infection was impaired in cells infected with viruses carrying the 5′ UTR deletion (Fig. 5A, lanes 9 to 12), which show little shut-off. Thus, some other factor or process contributing to the inhibition of host translation must be disrupted in these viruses.

DISCUSSION

In this study, we used reverse genetics to investigate the functions in the viral life cycle of nonconserved sequences in the BUNV untranslated regions. We focused on the S segment, which has the longest UTRs of the three viral RNAs, and for which it has previously been shown that the terminal 33 nucleotides are required for efficient transcription, replication, and encapsidation in the context of a minireplicon (20). Sequences internal to these 33 nucleotides at either end are not conserved between the S, M, and L segments, and, with the exception of a single nucleotide C+A-rich region and a 19-nucleotide G+U-rich region found in the 5′ UTR (9), are largely nonconserved between viruses of the Orthobunyavirus genus. Thus, we predicted that these sequences would be nonessential for viral growth and that it would be possible, therefore, to recover recombinant viruses with large deletions in the internal portions of the S segment UTRs.

We were in fact able to rescue a virus in which the 85-nucleotide S segment 3′ UTR was shortened to just the 22 terminal nucleotides. In contrast, we found that the 174-nucleotide 5′ UTR was more sensitive to mutation: the minimum 5′ UTR sequence for which we could recover a virus consisted of the terminal 112 nucleotides. These 112 nucleotides contain the conserved C+A- and G+U-rich regions. The length of the 5′ UTR, independent of its sequence, does not appear to be the limiting factor, as S85/46 and S85/67, which contain a 113-nucleotide 5′ UTR, were not viable. By combining deletions in the two UTRs we constructed three double-deletion mutants and identified S29/112 as the minimal S segment recoverable in our system. It is noteworthy that this recombinant segment has UTRs of similar lengths to the S segment UTRs.
of members of the Simbu serogroup (33 to 35 nucleotides at the 3' end and 104 to 123 nucleotides at the 5' end; see Fig. 1).

Despite the fact that they are nonessential, nonconserved sequences in the BUNV S 3' UTR were found to affect the balance of S segment RNAs present in infected cells. Deletion of sequences in the 3' UTR internal to the conserved panhandle region specifically decreased levels of S segment mRNAs while having either a lesser negative effect or, in some cases, a positive effect on the amount of antigenome RNAs. As a result, the ratio of S mRNA to S antigenome RNA, which for the wild-type virus is 1.4 at 24 h postinfection, was inverted for the 3' UTR deletion mutants. Similar results have been reported from analyses of the vesicular stomatitis virus leader region: here, the first 15 nucleotides were found to be sufficient to support a minimal level of replication but not transcription, and sequences upstream of the leader had a greater influence on levels of transcription than replication (24, 41). In influenza virus, mutation of nonconserved nucleotides in the 3' and 5' UTRs of the NA segment was found to decrease levels of both NA antigenome RNA and mRNA (42), whereas alterations to conserved nucleotides in the double-stranded region of the NA genomic promoter lead to a preferential reduction in mRNA levels, the cause of which was identified as a defect in polyadenylation (18).

Several stages contribute to the overall process of viral mRNA synthesis, and impairment at any stage would lead to a decrease in mRNA levels. Thus, the mRNA phenotypes of the S segment 3' UTR deletion mutants could be explained by the following. (i) Reduced binding of the L protein or host factors involved in viral RNA synthesis. Polymerase binding is likely the same for transcription and replication, due to the fact that both processes initiate at the 3' end of the genome. However, it is possible that a host factor(s) is involved in one or both modes of RNA synthesis and that nucleotides 23 to 85 of the S segment play a role in their recruitment. Indeed, a putative transcription factor from the insect vector of tomato spotted wilt virus, a tospovirus, was shown to bind both the viral polymerase and viral RNA and to improve the efficiency of replication, but not transcription, in vitro (8). (ii) Impaired cap-snatching activity. The cleavage of cellular mRNAs 12 to 18 nucleotides from their capped 5' ends (cap snatching) and initiation from the resulting host-derived primer are events unique to transcription; thus, disrupting these events would lead to a preferential reduction in mRNA synthesis. Although little is known about the mechanism of BUNV cap snatching, the analogous endonuclease activity of the influenza virus RNA polymerase is dependent on the binding of UTR sequences (22). (iii) Impaired transcription elongation. It has been shown that active translation is required for transcription elongation, but not initiation, in La Crosse orthobunyavirus and Germiston orthobunyavirus-based in vitro assays (3, 36). Thus, if the internal sequences of the 3' UTR promote ribosomal binding or scanning of nascent mRNA molecules, premature termination of S segment transcription would be expected in viruses with 3' UTR deletions, and would account for decreased levels of S segment mRNAs. (iv) Inefficient transcription termination. Termination of transcription has been shown to occur 100 to 110 nucleotides upstream of the 5' end of the BUNV S genomic template (29), whereas antigenome RNAs are full-length copies of genome RNAs; therefore, if transcription termination was perturbed, full-length mRNAs would likely result (and would appear to be antigenomes on a Northern blot). Although termination occurs at the 5' end of the genome, the 3' end of the template and factors recruited during initiation are thought to be involved in transcription termination by the cellular RNA polymerase II (31, 32), and a similar interplay could take place during bunyavirus transcription. (v) One possibility we cannot discount is that reduced accumulation of mRNA is due to its decreased stability rather than decreased synthesis.

The amount of N protein present in cells infected with each recombinant virus followed the same pattern as S mRNA levels, indicating that effects on N protein synthesis were likely the direct result of effects on transcription. This was not the case for the NSs protein, which exhibited a markedly different pattern of expression from the N protein. The N and NSs proteins are translated from the same mRNA species: the NSs start codon is 17 nucleotides downstream of the N start codon in a +1 reading frame. Thus, in order to initiate translation of the NSs protein, a ribosome must first scan through the N start codon in a process which has been well characterized for several cellular and viral transcripts, termed leaky scanning (21, 37). By this mechanism, translation of the downstream open reading frame is dependent on the efficiency of recognition of the first AUG by a scanning ribosome, an event which is dictated by the sequence context of that AUG. The optimal sequence context is GCC(A/G)CCAUUGG, where the −3 and +4 positions (the A of the AUG codon is designated +1) are the most important to determining the efficiency of initiation (21, 37).

The wild-type BUNV S segment contains a U residue at the −3 position relative to the N start codon, putting this AUG in a suboptimal context. By engineering deletions immediately upstream of the N open reading frame, we altered the nucleotide at the −3 position in the recombinant S segments. Consequently, NSs expression levels were affected: each mutant virus with a purine residue at the −3 position relative to the N start codon exhibited reduced levels of NSs protein, while those with pyrimidine residues at the same position accumulated amounts of NSs protein similar to that of wild-type BUNV. Thus, we have inadvertently determined that the sequence context of the N start codon has a profound influence on NSs expression. We did not find that reduced NSs levels correlated with a defect in the shut-off of host protein synthesis despite the fact that NSs has been shown to be necessary for shut-off (6). We expect the reason for this is that NSs fulfills a catalytic requirement leading to the inhibition of host protein synthesis and is therefore required in small quantities.

We found that recombinant viruses possessing S segments with 3' UTRs of 33, 29, or 28 nucleotides showed reductions in S segment antigenomic RNA, mRNA, and N protein. In contrast, viruses with shorter 3' UTRs of 22 or 24 nucleotides exhibited increases in S segment RNA and N proteins levels, indicating that nucleotides 23 to 28 may contain a negative regulatory element. Experiments to test this hypothesis are under way.

Deletion of nucleotides 788 to 849, the 62 nucleotides adjacent to the N open reading frame stop codon in the 5' UTR, was found to cause general attenuation of the resulting recombinant virus (S85/112), but did not specifically affect levels of S
segment RNA or protein levels. All four viruses containing the 5' UTR deletion grew more slowly and to lower titers than wild-type BUNV (or their corresponding 3' UTR deletion mutants), with the 5' UTR deletion alone causing a 30-fold reduction in yield for virus S85/112. Quantification of total S segment RNA normalized to the corresponding M segment RNA for this virus indicated that both positive- and negative-sense S RNA levels were moderately increased relative to that detected by Western blot, was decreased in S85/112 virus-specific protein production, although the mechanism by which this might occur is not apparent.

In summary, we have shown that nonconserved sequences in the BUNV S 3' UTR are nonessential to viral growth but contain regulatory signals contributing to the balance of transcription and replication activities. The BUNV S 5' UTR, by contrast, is more sensitive to mutation. In addition, nucleotides 788 to 849 of the 5' UTR contribute to the efficiency of viral growth, not through an effect on RNA or protein synthesis from the S segment, but perhaps through a possible role in the shut-off of host protein synthesis. Finally, deletion of nonconserved elements of the S segment UTRs was found to attenuate BUNV growth in cell culture, an observation which may be relevant to vaccine design for pathogenic members of the Bunyaviridae family.

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