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Attenuation of Bunyavirus Replication by Rearrangement of Viral Coding and Noncoding Sequences

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Bunyamwera virus (BUN) is the prototype virus of the family *Bunyaviridae*. BUN has a tripartite negative-sense RNA genome comprising 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, while the internal regions are unique. The UTRs direct replication and transcription of viral RNA and are sufficient to allow encapsidation of viral RNA into ribonucleoprotein complexes. To investigate the segment-specific functions of the UTRs, we have used reverse genetics to recover a recombinant virus (called BUN MLM) in which the L segment open reading frame (ORF) is flanked by the M segment UTRs. Compared to wild-type virus, BUN MLM virus shows growth attenuation in cultured mammalian cells and a slower disease progression in mice, produces small plaques, expresses reduced levels of L mRNA and L (RNA polymerase) protein, synthesizes less L genomic and antigenomic RNA, and has an increased particle-to-PFU ratio. Our data suggest that the packaging of BUN RNAs is not segment specific. In addition, the phenotype of BUN MLM virus supports the finding that BUN UTRs differ in their regulation of RNA synthesis but suggests that the interplay between each segment UTR and its cognate ORF may contribute to that regulation. Since BUN MLM virus is attenuated due to an essentially irreversible mutation, the rearrangement of UTRs is a feasible strategy for vaccine design for the more pathogenic members of the *Bunyaviridae*.

The family *Bunyaviridae* comprises >300 mainly arthropod-borne viruses that share several fundamental characteristics: the virus particles are spherical, enveloped, and contain a tripartite RNA genome of negative polarity. Viral replication is carried out in the cytoplasm, while assembly and budding of virions occur at the Golgi apparatus. Several members of the family, including La Crosse, Hantaan, Rift Valley fever, and Crimean-Congo hemorrhagic fever viruses, cause encephalitis or hemorrhagic fever in humans; bunyaviruses are increasingly recognized as examples of emerging infections that pose a serious threat to human health (8).

The family is divided into five genera: *Hantavirus*, *Nairovirus*, *Orthobunyavirus*, *Phlebovirus*, and *Tospovirus*. Bunyamwera virus (BUN) is the prototype member of both the family *Bunyaviridae* and the genus *Orthobunyavirus*. The three genome segments (designated small, medium, and large, or S, M, and L), encode six proteins: the S segment encodes the nucleocapsid (N) protein and the small nonstructural protein, NSs; the M segment encodes a polyprotein which is cotranslationally cleaved to produce the two virion glycoproteins, Gn and Gc, and a second nonstructural protein called NSm; and the L segment encodes the viral RNA polymerase (the L protein). Genomic RNA serves as the template for transcription and replication to produce full-length, positive-sense RNAs called antigenomes, which in turn serve as templates for the production of progeny genomes. The L protein is responsible for all viral RNA synthetic events and recognizes as templates only genomes and antigenomes that are encapsidated by the N protein to form ribonucleoprotein complexes (reviewed in references 3, 4, 9, and 23).

Partially conserved and complementary untranslated regions (UTRs) flank the coding region of each RNA segment. The S, M, and L segments possess UTRs of 174, 100, and 108 nucleotides (nt), respectively, at the 5′ terminus and 85, 56, and 50 nt, respectively, at the 3′ terminus. The terminal 11 nt of each UTR are complementary and conserved between the three segments, as well as within the genus. The internal regions of the UTRs are unique to each segment and show significant lengths of complementarity. The termini anneal intramolecularly to form stable panhandle structures that are visible by electron microscopy (21). They direct viral replication (2, 7) and transcription (7), as well as encapsidation of genomic and antigenomic RNAs by the N protein (20). They are also thought to play a role in assembly of ribonucleoproteins into virions. In order to investigate further the functions of the UTRs, we have exploited our ability to recover infectious Bunyamwera viruses entirely from cDNAs (5, 17) to rescue a recombinant virus in which the L segment open reading frame (ORF) is flanked by the M segment UTRs. (Although constructs with the other five combinations of coding/noncoding sequences were made, so far, despite repeated attempts, none of these could be recovered into recombinant viruses.) Among the characteristics of the mutant virus, designated BUN MLM virus, are a small-plaque phenotype, growth attenuation in cultured cells, retarded disease progression in mice, a reduction in L mRNA and protein levels, reduced levels of genomic

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and antigenomic L segment RNA, and an increased particle-to-PFU ratio relative to wild-type (wt) BUN.

MATERIALS AND METHODS

Cells and viruses. BHK-21 (baby hamster kidney) cells were grown at 37°C in Glasgow modified minimal essential medium (MEM) supplemented with 2 mM glutamine and 10% heat-inactivated fetal bovine serum (FBS). BSR-T7/5 cells, which stably express T7 RNA polymerase (6), were kindly provided by K. K. Conzelmann (Max-von-Pettenkofer Institut, Munich, Germany) and were grown at 37°C in Glasgow MEM supplemented with 2 mM glutamine, 10% FBS, and 1 mg/ml G418. Vero-E6 cells were grown at 37°C in Dulbecco's MEM supplemented with 2 mM glutamine and 10% FBS. Bunyaviruses were plaque purified in Vero-E6 cells, and working stocks were grown in BHK-21 cells as described by Watret et al. (25).

Plasmids. The construction of pT7riboBUNS(+), pT7riboBUNM(+), and pT7riboBUNL(+) was described previously (5). Each of these plasmids encodes a full-length antigenomic BUN segment, with the bacteriophage T7 promoter immediately upstream and a hepatitis δ ribozyme, followed by the T7 terminator, immediately downstream. pT7riboBUNMLM(+) was made from pT7riboBUNM(+) and pT7riboBUNL(+) using PCR to incorporate restriction sites for the remote-cutting enzyme BbsI. The primers BbsI LORF+ (GGAGAAGACACATGGAGGACCA AGCTTATG) and BbsI LORF− (GGGGAAGACACTTCAGAAAAAGGTAA ATAG) were used to amplify the L ORF from pT7riboBUNL(+); the primers BbsI M3′UTR+ (GGGGAAGACACTGAAAATTAGACGG) and BbsI M5′UTR− (GGGGAAGACACCCATCTTGGAAATAAAG) were used to amplify the M segment 3′ UTR, the pT7ribo plasmid (13) backbone, and the M 5′ UTR from pT7riboBUNM(+) (BbsI sites are underlined). Both PCR products were then digested with BbsI (New England Biolabs), gel purified (using the QIAquick Gel Extraction kit from QIAGEN), and ligated with T4 DNA ligase (Invitrogen). The construct was verified by sequence analysis. PCR parameters are available from the authors on request.

Generation of the recombinant BUN MLM virus by reverse genetics. The three-plasmid rescue system used is detailed elsewhere (17). Briefly, subconfluent BSR-T7/5 cells (10^6 cells per 60-mm-diameter petri dish) were transfected with 0.8 μg pT7riboBUNM(+), 0.4 μg pT7riboBUNS(+), and 2 μg pT7riboBUNL(+) (for wt virus) or 0.5, 1, or 2 μg pT7riboBUN MLM(+) (for recombinant virus) using DAC-30 transfection reagent (Eurogentec). Transfection mixtures were prepared in polystyrene tubes as follows. Ten mocroliters of DAC-30 diluted in 350 μl Opti-MEM was added to 350 μl Opti-MEM containing the plasmid DNA, and the mixtures were incubated for 30 min at room temperature. The cells were washed once with Opti-MEM before the transfection mix was added and were incubated for 6 h, and then growth medium was added. Six days posttransfection, the supernatants were harvested, and Bunyamwera viruses therein were isolated by plaque formation on Vero-E6 cells as described previously (25).

Analysis of viral RNA. BHK-21 cells were infected with either wt BUN or BUN MLM virus at a multiplicity of infection (MOI) of 0.01 and incubated at 33°C for 5 days. Culture fluids were clarified by centrifugation at 3,000 × *g* for 5 min, and virus was precipitated by adding one-fifth volume of polyethylene glycol solution (40% [wt/vol] in 2.5 M NaCl). After 1 h on ice, virions were collected by centrifugation for 20 min at 20,800 × *g* in an Eppendorf 5417R microcentrifuge. RNA was extracted from the pellets using TRIzol reagent (Invitrogen) as directed by the manufacturer. The RNA extracted from virions released from 5 × 10^6 cells was resuspended in 30 μl diethyl pyrocarbonate-treated water. Twelve microliters RNA was then combined with 10 pmol of a forward primer specific to the 5′ UTR of the M segment (BUN M+: GGGGTACCCGTCTCATATA AGTAGTGTACTACCGAT) and heated at 65°C for 5 min. After 5 min on ice, 200 U Moloney murine leukemia virus reverse transcriptase (Promega), 10 μmol deoxynucleoside triphosphates, 40 U RNasin (Promega), and 4 μl of the provided buffer were added to a total volume of 20 μl, and the mixture was incubated at 42°C for 2.5 h. Two microliters of the cDNA product was used as the template in a PCR with primers BUN M+ and BUN L_{978}−, a reverse primer complementary to nucleotides 956 to 978 of the L segment (GCTCTAGACCT CTCTACCCCCCAAATAAAATGGATAGAAGG). The PCR product was isolated on an agarose gel and purified using the QIAquick Gel Extraction kit (QIAGEN), and its sequence was determined (GRI Genomics, Essex, United Kingdom).

Virus growth curves. BHK-21 cells in 35-mm-diameter petri dishes were infected at an MOI of 5 with either transfectant wt BUN or BUN MLM virus. One hour postinfection (p.i.), the inoculum was removed and the cells were washed three times with EDTA to remove unattached viruses. At the time points indicated, viruses in the cell supernatant were titrated by plaque assay on Vero-E6 cells.

Pathogenicity studies. IFN-competent mice of inbred strain 129 and transgenic mice with targeted disruptions of the β subunit of the alpha/beta interferon (IFN-α/β) receptor ($IFNAR^{0/0}$, on a 129 background) were obtained from B&K Universal (United Kingdom) Ltd. Four- to 5-week-old mice were age and sex matched into groups of six mice per treatment. The mice were inoculated either intracerebrally with 1,000 PFU or intraperitoneally with 5,000 PFU of wt BUN or BUN MLM virus in phosphate-buffered saline containing 0.75% bovine serum albumin. The mice were then monitored for 14 days. All experiments were carried out under the authority of a United Kingdom Home Office animal license. Animals reaching defined clinical end points indicative of substantial encephalitis were euthanized and scored as having died that day. All animals were kept under specific-pathogen-free conditions with environmental enrichment and were supplied with food and water ad libitum.

Metabolic labeling of viral proteins. Vero-E6 cells in 35-mm-diameter petri dishes were infected at an MOI of 1 with either wt BUN or BUN MLM virus. Forty-two hours postinfection, the cells were labeled with 50 μCi per dish of [^{35}S]methionine for 2.5 h. Cell lysates were prepared and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), as described previously (25).

Northern blotting. Vero-E6 cells in 35-mm-diameter petri dishes were infected at an MOI of 0.5 with the different viruses. Forty hours postinfection, the cells were lysed and total cellular RNA was isolated using 1 ml TRIzol reagent (Invitrogen) per dish, as described by the manufacturer. RNA was quantified by spectrophotometry, and 10 μg cellular or 1 μg viral RNA (extracted from released virions as described above) was fractionated on a 1% agarose-2.2 M formaldehyde gel. The RNAs were transferred to a positively charged nylon membrane (Boehringer Mannheim) and hybridized with a mixture of three digoxigenin (DIG)-labeled RNA probes (120 ng each of probe, complementary to approximately the 5′ 1,000 bases of BUN L, M, and S genomic or antigenomic RNA segments) in 50% formamide buffer overnight at 68°C. The probes were synthesized and quantified using the DIG Northern Starter kit (Roche), as directed by the manufacturer. Washing at different stringencies and detection were carried out as described by Roche using reagents supplied in the DIG Northern Starter kit. Blots were exposed to X-OMAT S UV film (Kodak) for up to 2 min, and then the film was developed in a Canon film processor.

Determination of particle-to-PFU ratios. BHK-21 cells were infected with either wt BUN or BUN MLM virus at an MOI of 0.01 and incubated at 33°C for 5 days. Culture fluids were clarified by centrifugation at 2,000 × *g* for 5 min. The supernatant was layered onto a cushion of 96% glycerol (in 40 mM Tris-HCl, pH 7.0) and centrifuged at 121,600 × *g* for 2 h in a Sorvall AH629 rotor with 36-ml buckets. The band of virus at the interface of glycerol and medium was recovered, and 100 μl of the virus sample was titrated by plaque assay on Vero-E6 cells, as previously described (25). The remaining sample was further concentrated by centrifugation at 20,800 × *g* for 5 h in an Eppendorf 5417R microcentrifuge, and the pellet was resuspended in 5 μl phosphate-buffered saline. The virus suspension was mixed with 5 μl stain (phosphotungstic acid, pH 7.0) and 5 μl of latex beads of known concentration (Agar Scientific Ltd.). A 3-μl droplet was placed on a 200-mesh parlodion-coated grid and allowed to sit for 2 min. Excess mixture was drained from the grid with filter paper. The specimen was examined in a Jeol 100s electron microscope. Virus and latex were counted simultaneously, and the virus concentration was determined based on the known concentration of the latex beads. After correcting for the concentration step, particle-to-PFU ratios were calculated as particles per ml divided by PFU per ml.

RESULTS

Generation of a recombinant Bunyamwera virus. In order to investigate the segment-specific functions of the BUN UTRs (which are only partially conserved between segments), we designed a recombinant L segment in which the L ORF was flanked by the M segment UTRs. The recombinant antigenomic segment (called MLM) was constructed in pT7ribo (7) so that the bacteriophage T7 promoter was upstream of the 5′ UTR and the self-cleaving hepatitis δ ribozyme and T7 terminator were downstream of the 3′ UTR [analogous to pT7riboBUNL(+) described previously (5)]. When transfected into the T7 polymerase-expressing cell line BSR-T7/5, the pT7ribo plasmids allow both the production of full-length an-

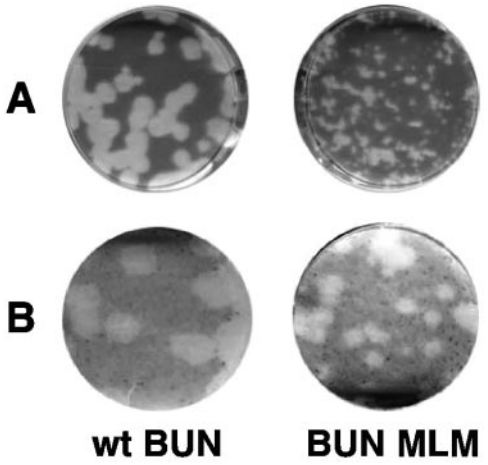

FIG. 1. Comparison of plaques produced by wt BUN (left) and the recombinant BUN MLM virus (right) in mammalian cells. Vero-E6 (A) or BHK-21 (B) cell monolayers were fixed with 4% formaldehyde at 4 days postinfection and stained with Giemsa's solution.

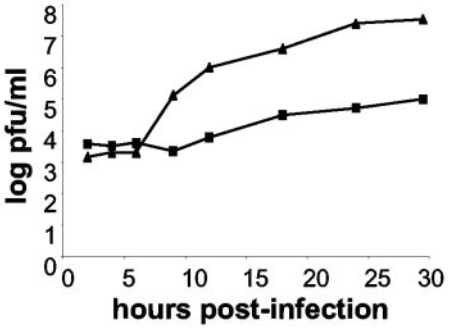

FIG. 2. Growth of wt BUN and BUN MLM viruses in cell culture. BHK-21 cells were infected with either wt BUN (triangles) or the recombinant BUN MLM virus (squares) at an MOI of 5. Virus in the cell supernatants was then titrated by plaque assay at the time points indicated.

tigenomic RNA segments and the expression of BUN proteins. Although during infection antigenomic RNAs are encapsidated by the N protein, in transfected cells the N protein is initially not present and the naked antigenome RNAs appear to function as mRNAs. Thus, the pT7ribo plasmids act as both the "support" and "transcription" plasmids traditionally used in negative-sense RNA virus rescue systems (5, 17).

Using various amounts of pT7riboBUNS(+), pT7riboBUNM(+), and pT7riboBUNMLM, rescue experiments were carried out as described in Materials and Methods. Combinations of 0.4 μg pT7riboBUNS(+), 0.8 μg pT7riboBUNM(+), and either 0.5, 1.0, or 2.0 μg pT7riboBUNMLM yielded virus. The titers of BUN MLM virus recovered from these rescue experiments were 6.5×10^2 PFU, 5×10^3 PFU, and 1.7×10^3 PFU, respectively, relative to 5×10^7 PFU of wt virus from the same experiment. The plaques generated by BUN MLM virus were smaller than those produced by wt virus in either Vero-E6 or BHK-21 cells (Fig. 1). Three plaques from the MLM rescue experiment were picked, and virus stocks were grown up, resulting in titers of 10^5 to 10^6 PFU/ml, approximately 100-fold lower than those of rescued wt BUN produced at the same time.

Analysis of viral RNAs by reverse transcription-PCR. To verify that the transfectant virus contained a recombinant L segment with M segment UTRs, viral RNAs were analyzed by reverse transcription-PCR. RNA extracted from isolated virions was reverse transcribed from a primer complementary to the 5′ UTR of the M antigenomic RNA. Subsequent PCR used the same M segment-specific forward primer with a reverse primer complementary to nucleotides 956 to 978 of the L segment ORF. A product of approximately 1 kb was thereby amplified. Nucleotide sequence analysis showed the product to be the entire M segment 5′ UTR fused to the L segment ORF (up to nucleotide 978), as expected (data not shown).

Growth of BUN MLM virus is attenuated in cultured cells. The single-step growth cycle of the mutant BUN MLM virus was investigated in BHK-21 cells and compared to that of the isogenic wt BUN generated by plasmid transfection in the same experiment. Infections were done at high MOI (5 PFU/cell), and virus in supernatants from infected cells was titrated by plaque assay at the time points indicated (Fig. 2). After 30 h, wild-type BUN yielded approximately 4×10^7 PFU/ml in BHK-21 cells, whereas BUN MLM virus grew less well, yielding 400-fold-lower titers (1×10^5 PFU/ml) (Fig. 2). In Vero-E6 cells, BUN MLM grew even more poorly, giving 1,000-fold lower yields than wt BUN (5×10^3 versus 5×10^6 PFU/ml; data not shown). Hence, this UTR rearrangement resulted in attenuation of virus replication in cultured mammalian cells.

Reduced virulence of BUN MLM virus in mice. Groups of six 4- to 5-week-old wild-type (strain 129) or interferon-receptor knockout (strain 129-IFNAR$^{0/0}$) mice were inoculated with wt BUN or BUN MLM virus, either intracerebrally with 1,000 PFU or intraperitoneally with 5,000 PFU per mouse, and monitored for 14 days (Fig. 3). Wild-type mice inoculated intracerebrally with wt BUN died by 4 days p.i., while wt mice inoculated intracerebrally with BUN MLM virus survived to at least day 7 p.i. All but one mouse in the latter group eventually died by day 14 (Fig. 3A). Of the mice inoculated intraperitoneally with wt BUN, all but one died by 10 days postinfection. By contrast, all wt mice inoculated intraperitoneally with BUN MLM virus survived.

Intracerebral inoculation of IFNAR$^{0/0}$ mice was lethal in all cases; however, while mice inoculated with wt BUN died between 1 and 3 days postinfection, mice inoculated with BUN MLM virus succumbed only between days 5 and 7 p.i. (Fig. 3B). All mice inoculated intraperitoneally with wt BUN died within 3 days, whereas all mice inoculated by this route with BUN MLM virus survived. Thus, wt BUN was virulent following both intraperitoneal and intracerebral inoculation and in both 129 and IFNAR$^{0/0}$ mice. As expected, this infection was more rapidly virulent following intracerebral than intraperitoneal inoculation and in IFNAR$^{0/0}$ mice than in 129 mice. In contrast, BUN MLM virus was virulent only following intracerebral inoculation. As with wt virus, this was more rapid in the IFNAR$^{0/0}$ than in the 129 mice. These results indicate that, in vivo, BUN MLM virus has a viable phenotype but is highly attenuated relative to wt virus.

Decreased synthesis of polymerase protein. To analyze the proteins synthesized by the recombinant virus, Vero-E6 cells were infected at an MOI of 1 with either wt BUN or BUN

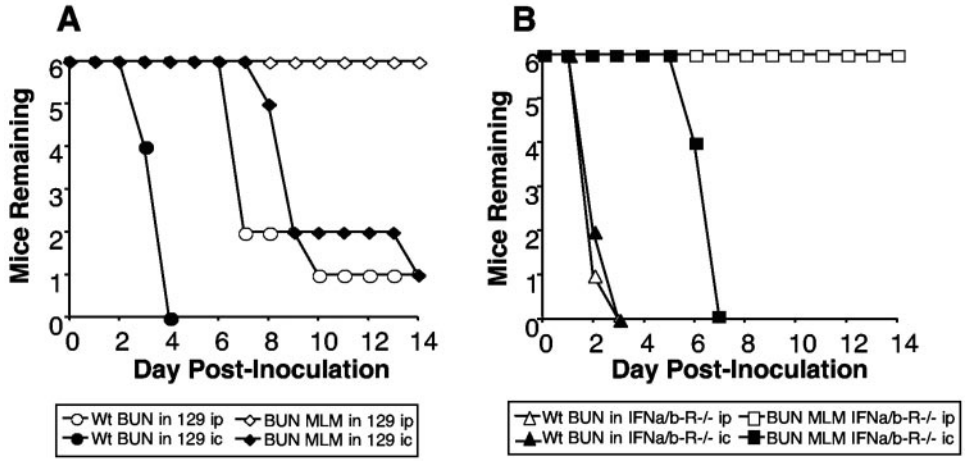

FIG. 3. Comparison of wt BUN and BUN MLM virus infections in mice. Either wt (A) or IFN α/β receptor knockout (IFNAR$^{0/0}$ [IFNa/b-R−/−]) mice (B) were infected intracerebrally (ic) or intraperitoneally (ip) with wt BUN or BUN MLM virus and monitored for 14 days. Wild-type BUN infection was lethal to all but one mouse by day 10 postinfection when injected intraperitoneally, whereas all mice infected intraperitoneally with BUN MLM virus survived. When injected intracerebrally, BUN MLM virus induced a slower disease progression than wt virus.

MLM virus and labeled with [^{35}S]methionine at 42 h postinfection, and cell extracts were analyzed by SDS-PAGE followed by autoradiography (Fig. 4). No impairment in the ability of the virus to decrease host cell protein synthesis was observed (Fig. 4, compare lane 1, mock infected, to lanes 2 and 3). All the viral proteins were expressed at similar levels in each infection, except the polymerase (L) protein. The L protein, though detectable, was present in much smaller quantities in cells infected with BUN MLM virus than in those infected with wt BUN.

Decreased synthesis of L segment RNA. RNAs synthesized by BUN MLM virus were analyzed by Northern blotting of RNA isolated from infected Vero-E6 cells. The cells were infected at an MOI of 0.5 with either wt BUN or BUN MLM virus and lysed at 42 h postinfection, and the total cellular RNA was extracted. Duplicate samples were subjected to agarose gel electrophoresis and transferred onto nylon membranes. One blot was probed with DIG-labeled RNAs complementary to each of the BUN genome segments (Fig. 5A); the

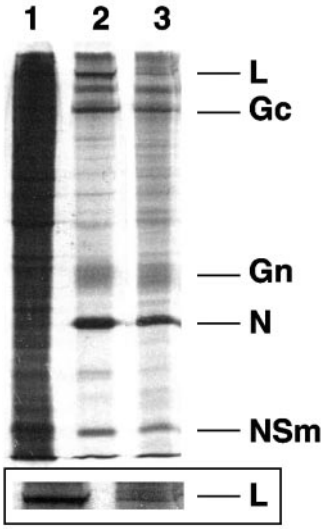

FIG. 4. Protein profiles of wt BUN and BUN MLM virus-infected cells: reduced expression of the L protein by BUN MLM. Vero-E6 cells were mock infected (lane 1) or infected with wt BUN (lane 2) or BUN MLM (lane 3) and labeled with [^{35}S]methionine for 2.5 h at 42 h postinfection. Cell lysates were separated by SDS-PAGE, and labeled proteins were visualized by autoradiography. Positions of bunyavirus proteins are indicated. The box shows an enlargement of the portion of the gel containing the L protein band in lanes 2 and 3.

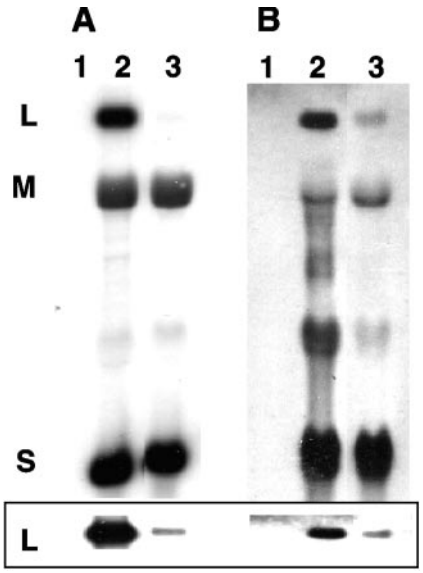

FIG. 5. Northern blot analysis of viral RNA in infected cells. Vero-E6 cells were mock infected (lane 1) or infected with wt BUN (lane 2) or BUN MLM (lane 3). Total cellular RNA was extracted 42 h postinfection, subjected to Northern blotting, and probed with DIG-labeled RNAs complementary to (A) BUN genomic mRNA or (B) BUN antigenomic RNA and mRNA. Positions of viral RNAs are indicated. The box presents longer exposures of the blots to show the L segment RNAs.

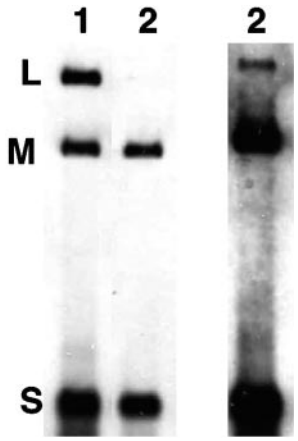

FIG. 6. Northern blot analysis of RNA extracted from isolated virions. Cells were infected with wt BUN (lane 1) or BUN MLM-10 (lane 2). Virus particles released into the cell supernatant were concentrated, and viral RNA was extracted. L segments packaged into BUN MLM virions were not detectable with a 1-min exposure (left) but were detected with a 5-min overexposure (right). Viral RNAs are identified on the left.

other blot was probed with DIG-labeled RNAs complementary to the BUN antigenome segments and mRNAs (Fig. 5B). Antigenomic RNA and mRNAs were not resolved on this gel, and the identified bands therefore represent the sum of both positive-sense species of viral RNA. Synthesis of all L segment RNA species by BUN MLM virus was decreased relative to the wt. The effect was greatest in the genomic RNAs, which were detectable in BUN MLM-infected cells by overexposure of the blot, but was still significant in the positive-sense RNAs. Thus, the M segment transcription and replication promoters (which are contained in the UTRs) are unable to direct the synthesis of L segment RNA to wt levels.

BUN MLM virions incorporate less L segment RNA than wt BUN. To examine the RNA actually packaged by BUN MLM virus, Northern blotting was carried out on RNA extracted from isolated virions, using probes complementary to genomic RNA (Fig. 6). The results show that significantly less L segment RNA was packaged into BUN MLM virions than into wt virus. While it is possible that packaging signals were disrupted in the recombinant L segment, thereby inhibiting packaging, such an effect cannot be distinguished from the decreased availability of L genome segments in the viral infection (Fig. 5A).

Increased particle-to-PFU ratio of BUN MLM virus. Since a bunyavirus particle must contain at least one of each genomic RNA to be infectious, we predicted that BUN MLM, a virion population with relatively few L segment RNAs, would contain an increased proportion of noninfectious virus particles. To test this prediction, particle-to-PFU ratios of BUN MLM and wt BUN viruses were determined by carrying out electron microscopic particle counts and titrations of the same virus samples. Three independent virus preparations were analyzed and produced particle-to-PFU values for wt BUN of 2.6, 5.6, and 7.2. In contrast, BUN MLM virus was found to have particle-to-PFU ratios of 295, 1,033, and 1,267, that is, 100-fold higher than the wt. This result not only confirms our prediction based on virion RNA levels but also corresponds closely to the observed 100- to 1,000-fold growth attenuation of BUN MLM virus in cell culture. Inhibition of virus growth may be largely accounted for by the failure of BUN MLM virus to incorporate its L segment into virions and thereby produce infectious viruses.

DISCUSSION

The successful recovery of BUN MLM virus shows that two segments with the same UTRs can be accommodated in a single virion. Thus, the failure so far to rescue viruses containing rearranged genome segments of the form SLS, MSM, LSL, LML, or SMS cannot be attributed to packaging constraints. The attenuated phenotype and reduced protein and RNA levels of BUN MLM virus show that the rearrangement of UTRs relative to ORFs does have drastic effects on the viral life cycle. Hence, it seems likely that the combination of M UTRs with the L coding sequence constituted a set of *cis*-acting sequences most similar to that of a wt segment, whereas in the other five recombinant segments such sequences were disrupted to an extent that compromised the ability to recover infectious virus.

Minigenome-based studies of BUN virus RNA synthesis have localized the signals directing transcription and replication to the UTRs (7). Similar studies have also established that the levels of synthesis driven from the S, M, and L promoters are not equal; rather, reporter gene activity (which reflects a combination of transcription and replication of the minigenome) varies with the UTRs used in the minigenome so that the proportions are M > L > S (15). This was confirmed by Barr et al. (2), who directly labeled S, M, and L genome analogues in the minigenome system and defined the relative replicative abilities of the BUN UTRs also as M > L > S. These results support the hypothesis that the unique regions of the BUN UTRs allow differential regulation of gene expression.

Levels of viral RNAs in LaCrosse virus (genus *Orthobunyavirus*)-infected BHK cells were characterized by Rossier et al. (22). Although the three genomic RNA species were present in similar amounts, there was more S mRNA than M mRNA and more M mRNA than L mRNA (the numbers of mRNA copies per cell were estimated to be 20,000 S, 5,000 M, and 500 L). Thus, it appears that some factor besides UTR promoter strength plays a role in the regulation of viral transcription within infected cells; however, it is noteworthy that in both assays, transcription from the M segment was greater than transcription from the L segment.

Our characterization of BUN MLM virus has shown that replacing the L segment UTRs with M segment UTRs grossly disrupts regulation of L gene expression. We found that synthesis of all species of L segment RNA and expression of the L protein were reduced in BUN MLM virus relative to wt BUN. Although at first glance this result appears not to agree with previous data on the ranking of BUN promoter strengths, the two are not necessarily contradictory. Both the minireplicon system (15), which measures reporter gene activity, and the methods of Barr et al. (2), which examine RNA levels directly, are based on BUN genome analogues in which a common nonviral sequence (the *Renilla* luciferase gene) is bracketed by BUN S, M, or L UTRs. By contrast, in an authentic viral genome, the different promoters drive expression of very dif-

ferent coding sequences, the most obvious difference being their lengths (<1 kb for S, 4.5 kb for M, and 6.8 kb for the L segment). In addition, the two results were obtained in contrasting cellular environments: the minigenomes are expressed in healthy cells, whereas the authentic genomes are expressed in virus-infected cells suffering host cell shutoff. Thus, while the BUN M UTRs may comprise a strong promoter, they do not do so in the context of the L coding sequence during a viral infection. Potential reasons for this discrepancy include the following: (i) there may be *cis*-acting regulatory elements within the BUN coding regions that function only in proximity to the correct promoter, (ii) a host cell factor(s) present in uninfected cells may act in *trans* to affect BUN promoter strength, and (iii) the L segment is six times longer than *Renilla* minigenomes and, whether through secondary-structure formation or the efficiency of elongation by the L protein, template length could affect apparent promoter strength.

Little is known about the mechanism by which members of the *Bunyaviridae* package their genomes into virus particles. At least one of each genomic segment must be present for a virion to be infectious. Two basic models, representing opposite extremes, explain how this might be accomplished for segmented genome viruses. The first model describes a specific mechanism in which each genome segment is targeted individually for assembly, allowing the efficient production of virions containing complete and unduplicated genomes. This model is exemplified by the selective and sequential packaging of the three double-stranded RNA segments of bacteriophage phi 6 (18). The second describes a random mechanism in which all viral segments compete indiscriminately for a common niche, so that infectious viral particles arise only as dictated by chance. In such a system, the packaging of more than the required number of segments increases the probability of infectious particles arising (10).

Determination of particle-to-PFU ratios is a classical way to assess the efficiency of viral genome packaging. Here, we report wt BUN particle-to-PFU ratios of 2.6, 5.6, and 7.2. These relatively small values imply some degree of specificity in packaging. The particle-to-PFU ratio for BUN MLM virus, in contrast, is 100-fold higher, which indicates that particles which do not have the S-M-L particle constellation can form; thus, packaging cannot be absolutely specific.

A specific mechanism would likely rely on a packaging signal that is unique to each segment. The bunyavirus UTRs are known to be sufficient to allow packaging of a reporter gene into virus-like particles (5, 11; unpublished observations). Work using Uukuniemi phlebovirus minigenomes was reported in which packaging of an L segment-based minigenome was maintained for seven passages, whereas the reporter gene flanked by M or S segment UTRs was maintained only up to three passages (11). Thus, it appears that for Uukuniemi virus, the L segment UTRs allow more efficient packaging than the M or S segment UTRs, though this information does not answer the question of specificity. In the case of influenza A virus, Bancroft and Parslow (1) found that minigenomes consisting of a reporter gene flanked by the viral UTRs did not compete specifically for packaging. However, Fujii et al. and Watanabe et al. (12, 24) used minigenomes containing varying lengths of the neuraminidase (NA) (12) or hemagglutinin (24) coding sequences in addition to the corresponding UTRs to show not only that packaging is much more efficient when a portion of the viral coding sequence is included in the minigenome, but also that the presence of the coding sequence allows the specific recognition of the segment for packaging.

The recovery of BUN MLM virus provides some insight into the degree of specificity involved in bunyavirus assembly. The rescue of BUN MLM shows that a segment with noncognate UTRs can be packaged. Thus, if there is a segment-specific packaging signal that would preclude incorporation of two like segments, it is not contained within the UTRs, and either it is not disrupted in the MLM chimeric segment or a weak signal is re-formed by placing the L coding sequence within the M UTRs. The viability of BUN MLM virus does not, however, exclude the possibility that specific packaging signals exist only in the open reading frames, as seen with influenza A virus. Indeed, Muster et al. (19) were able to rescue a recombinant segment consisting of the influenza A virus NA ORF flanked by the influenza B virus NS UTRs into the influenza A/WSN/33 virus background. While this result showed that the influenza A virus polymerase recognizes the influenza B virus promoter sequences, the fact that the chimeric segment was packaged is probably due to the presence of packaging signals in the NA ORF (12).

Evidence from particle-to-PFU ratios and Northern blots of virion RNA suggest that S and M wt segments were packaged preferentially over the MLM recombinant segment, perhaps due to a disrupted signal in the MLM RNA. This conclusion must be regarded with caution, however, because in a BUN MLM virus infection, effects on packaging are obscured by effects on replication: that is, the paucity of L segments present in viral particles and the high particle-to-PFU ratio of BUN MLM virus can be explained by the reduced availability for packaging of the L segment in BUN MLM virus-infected cells.

Our results show that BUN MLM virus grows to 100- to 1,000-fold-lower titers than wt virus in cell culture. This level of attenuation corresponds well to the increase in the particle-to-PFU ratio of BUN MLM (100-fold), suggesting that it is the inability of the virus to produce infective progeny virions that is the cause of its attenuation. It is also likely, however, that the deregulation of L gene expression plays a role. Previous studies using minigenomes of both BUN and Rift Valley fever viruses (7, 16) have shown that a specific L/N ratio is critical for optimal replication of the minigenome. In addition, a BUN virus carrying a promoter up mutation in the 3′ UTR of its S genome segment was attenuated in cell culture (14). It is also noteworthy that gene rearrangement in the nonsegmented vesicular stomatitis virus, carried out by Wertz et al. (26), led to effects comparable to those of UTR rearrangement described here for BUN virus. Translocation of the nucleoprotein gene to successive positions downstream in the vesicular stomatitis virus genome resulted in stepwise decreases in N protein expression and led to attenuation of the recombinant viruses in cell culture and in mice (26). In a like manner, the decreased level of polymerase expressed by BUN MLM virus probably contributes to its attenuation.

Pathogenicity studies showed BUN MLM virus to be viable in vivo but to be less virulent than wt BUN. Mice inoculated intraperitoneally with BUN MLM virus all survived the treatment (compared to a 92% mortality rate with wt BUN), while those inoculated intracerebrally with BUN MLM virus lived

longer than those inoculated intracerebrally with wt BUN. Disease caused by both viruses progressed more quickly in the IFNAR$^{0/0}$ mice; however, death still came later with BUN MLM virus infection, suggesting that the attenuation of BUN MLM virus is not linked to the host immune response but rather is intrinsic to the virus.

The data presented in this paper provide insight into the segment-specific functions of bunyavirus UTRs. In addition, we describe an attenuated bunyavirus carrying an essentially irreversible mutation and thereby demonstrate that UTR rearrangement is a possible strategy for vaccine design for the more pathogenic members of the *Bunyaviridae*.

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