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Nucleotide Sequence Analyses and Predicted Coding of Bunyavirus Genome RNA Species

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We performed 3′ RNA sequence analyses of [^{32}P]pCp-end-labeled La Crosse (LAC) virus, alternate LAC virus isolate L74, and snowshoe hare bunyavirus large (L), medium (M), and small (S) negative-stranded viral RNA species to determine the coding capabilities of these species. These analyses were confirmed by dideoxy primer extension studies in which we used a synthetic oligodeoxynucleotide primer complementary to the conserved 3′-terminal decanucleotide of the three viral RNA species (Clerx-van Haaster and Bishop, Virology **105**:564–574, 1980). The deduced sequences predicted translation of two S-RNA gene products that were read in overlapping reading frames. So far, only single contiguous open reading frames have been identified for the viral M- and L-RNA species. For the negative-stranded M-RNA species of all three viruses, the single reading frame developed from the first 3′-proximal UAC triplet. Likewise, for the L-RNA of the alternate LAC isolate, a single open reading frame developed from the first 3′-proximal UAC triplet. The corresponding L-RNA sequences of prototype LAC and snowshoe hare viruses initiated open reading frames; however, for both viral L-RNA species there was a preceding 3′-proximal UAC triplet in another reading frame that was followed shortly afterward by a termination codon. A comparison of the sequence data obtained for snowshoe hare virus, LAC virus, and the alternate LAC virus isolate showed that the identified nucleotide substitutions were sufficient to account for some of the fingerprint differences in the L-, M-, and S-RNA species of the three viruses. Unlike the distribution of the L- and M-RNA substitutions, significantly fewer nucleotide substitutions occurred after the initial UAC triplet of the S-RNA species than before this triplet, implying that the overlapping genes of the S RNA provided a constraint against evolution by point mutation. The comparative sequence analyses predicted amino acid differences among the corresponding L-, M-, and S-RNA gene products of snowshoe hare virus and the two LAC virus isolates.

The genomes of bunyaviruses (Bunyaviridae) consist of three RNA species, which are designated large (L), medium (M), and small (S) (1, 2). The viral S RNA codes for virion nucleocapsid polypeptide N (8), as well as a nonstructural polypeptide (18; F. Fuller and D. H. L. Bishop, J. Virol., in press). The viral M RNA codes for two virion glycoproteins, G1 and G2 (9), which elicit and interact with neutralizing antibodies (13) and are major determinants of virus virulence (16). The product encoded by the viral L RNA has not been determined yet, although a candidate is the large virion polypeptide (2).

To determine the coding properties of the three bunyavirus RNA species, we sequenced (by two different procedures) the 3′ termini of the three RNA species from three related viruses, snowshoe hare (SSH) virus, La Crosse (LAC) virus, and an alternate LAC virus isolate, designated L74. We undertook these studies not only to characterize the viral RNA sequences and to identify open reading frames in their complementary, putative mRNA sequences, but also to determine RNA and polypeptide sequence differences. The data obtained so far have suggested that the 3′-proximal sequences of the bunyavirus S-RNA species code for two gene products that are read in overlapping open reading frames, whereas the viral L- and M-RNA species code for gene products that are read in single open reading frames. Also, our data have indicated that the first UAC triplets on the L-RNA species of LAC and SSH viruses do not initiate contiguous open reading frame nucleotide sequences.

MATERIALS AND METHODS

Materials. Reverse transcriptase was kindly provided by J. W. Beard, St. Petersburg, Fla. The synthetic decadeoxynucleotide 5'-dAGTAGTGTAC was purchased from Collaborative Research, Inc., Waltham, Mass. 2',3'-Dideoxynucleoside triphosphates and deoxynucleoside triphosphates were obtained from P-L Biochemicals, Milwaukee, Wis. [α-^{32}P]dCTP (2,000 to 3,000 Ci/mmol) was from Amersham Corp., Arlington Heights, Ill. Cytidine 3',5'-bis[^{32}P]phosphate (1,000 to 3,000 Ci/mmol) was obtained from either Amersham Corp. or New England Nuclear Corp., Boston, Mass.

Viruses, virus growth, purification, and isolation of virus RNA. The origins of the prototype SSH and LAC viruses (7, 11) and the alternate LAC virus isolate L74 (obtained in 1974 from *Aedes triseriatus* collected in Albany, N.Y.) (14) have been described previously. The procedures used to grow and purify the viruses, extract the RNAs, and resolve and recover the individual L-, M-, and S-RNA species have also been described previously (5, 6).

3'-End-labeling of RNA, chemical and enzymatic digestion of end-labeled RNA, and resolution of the products by thin polyacrylamide gel electrophoresis. These procedures have been described previously (5).

RNA sequence analyses by the dideoxy incorporation technique. We used the dideoxy incorporation technique of Sanger et al. (15), as described by Zimmern and Kaesberg (19), with minor modifications. Each complete reaction mixture (5 μl) contained 50 mM Tris-hydrochloride (pH 8.3), 40 mM KCl, 8 mM $MgCl_2$, 10 mM dithiothreitol, 100 μM dATP, 100 μM dGTP (or dITP), 100 μM dTTP, 40 μM [α-^{32}P]CTP, 10 pmol of the decanucleotide primer, 0.1 μg of purified viral L, M, or S RNA, and 4 U of reverse transcriptase. Reaction mixtures containing additional dideoxynucleotide precursors contained either 15 μM dideoxyadenosine triphosphate (ddATP), 15 μM ddGTP, 15 μM ddITP, 15 μM ddTTP, or 5 μM ddCTP. The reaction mixtures were incubated for 30 min at 37°C; then 1 μl of a solution containing all four unlabeled deoxynucleoside triphosphates at a concentration of 0.5 mM was added, and the reaction mixtures were incubated for an additional 15 min. Before loading onto thin (0.4-mm) 12, 8, or 6% polyacrylamide gels (10), 4 μl of an 8 M urea-dye mixture was added (5), and the preparation was heated at 100°C for 1 min.

RESULTS

Analyses of the 3'-terminal L-, M-, and S-RNA sequences of prototype SSH virus, prototype LAC virus, and alternate LAC virus isolate L74. Limited analyses of the 3'-terminal sequences of [^{32}P]pCp-end-labeled L-, M-, and S-RNA species from prototype SSH virus and the serologically closely related prototype LAC virus have been described previously (5, 12). In both SSH and LAC viruses a conserved 3'-terminal sequence of 11 nucleotides ($_{HO}$UCAUCACAUGA) was identified at the ends of all three viral RNA species; this sequence was followed by a region of limited sequence identity (5). In the analyses reported here, these preliminary studies were extended by additional analyses of [^{32}P]pCp-end-labeled L-, M-, and S-RNA species from SSH and LAC viruses and the alternate LAC virus isolate L74, which was recovered from *A. triseriatus* mosquitoes (14). The results were confirmed and further extended by the dideoxy sequence analysis technique of Sanger et al., using a synthetic decadeoxynucleotide primer (5'-dAGTAGTGTAC) that was complementary to 10 of the 11 nucleotides at the 3' termini of the three viral RNA species of each virus. Figure 1 shows representative dideoxy sequence gels of SSH virus L-, M-, and S-RNA species. The results obtained with LAC, SSH, and L74 viruses are shown in Fig. 2 through 4. The LAC virus, L-, M-, and S-RNA data were used as references, and sequencing results for SSH and L74 virus RNA species are shown. (Identical nucleotides in corresponding sequences are represented by solid circles, and missing nucleotides are represented by spaces in the sequences.)

So far, analyses of SSH bunyavirus mRNA species have identified single mRNA species for each of the three viral RNA segments of this virus (3). From studies of the viral RNA sequences that are protected from RNase digestion by mRNA annealing, it has been concluded that the SSH virus mRNA species are initiated at the 3' termini of the negative-stranded viral RNA species (3, 5). For this reason we analyzed the SSH, LAC, and L74 virus 3'-terminal sequences for possible initiation sites of translation on the complementary, putative mRNA species (i.e., mRNA AUG codons, represented by underlined UAC triplets in the viral RNA sequences [Fig. 2 through 4]). We recognized that these correlations might not necessarily identify all of the viral gene products encoded by these RNA sequences (e.g., if mRNA splicing occurred); however, these analyses allowed us to determine whether there was more than one extensive open reading frame on the complementary RNA sequences of each RNA species and, possibly, more than one encoded gene product. Figures 2 through 4 also show the deduced amino acid sequences representing putative mRNA initiation sites (viral UAC triplets), the mRNA termination triplets (i.e., antiopal ACU, antiochre AUU, and antiamber AUC codons, indicated by the superscripts I, II, and III, respectively, representing the reading frames in which they occur), and the intervening encoded amino acids. The data obtained for the three viruses are discussed below in relation to each size class of viral RNA.

L-RNA analyses. When the LAC virus L-RNA sequences were analyzed for possible coding capabilities, we found that the first UAC triplet

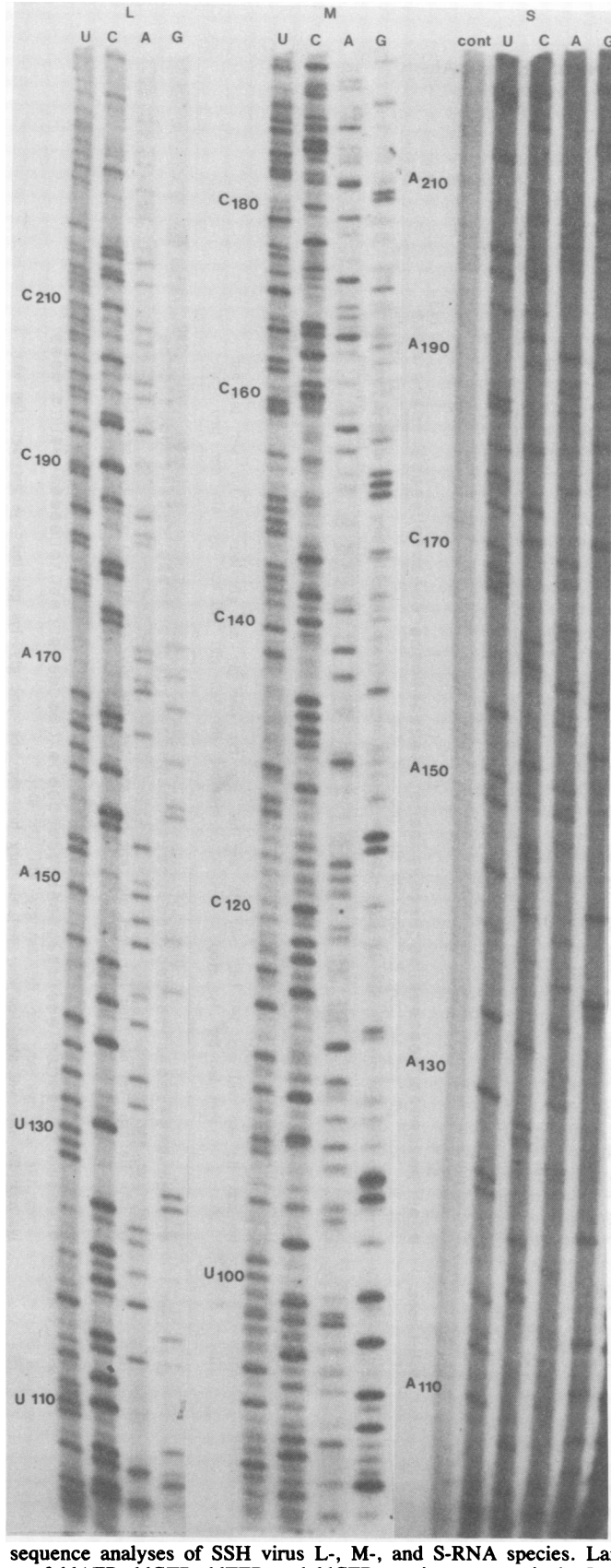

FIG. 1. Dideoxy sequence analyses of SSH virus L-, M-, and S-RNA species. Lanes U, C, A, and G contained the products of ddATP, ddGTP, ddTTP, and ddCTP reactions, respectively. In the lane marked cont, no chain-terminating nucleotide was used.

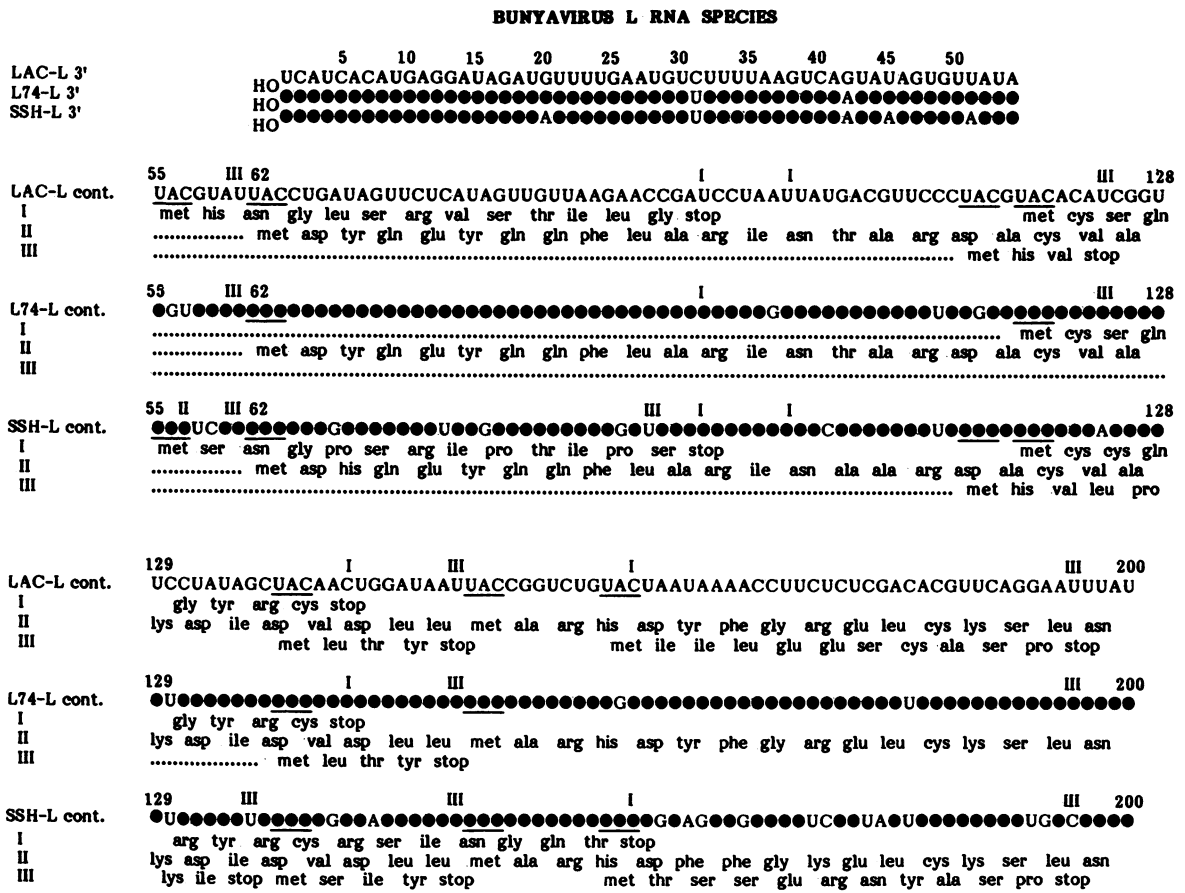

FIG. 2. Deduced prototype LAC virus, prototype SSH virus, and alternate LAC virus L74 L-RNA 3′-terminal sequences and predicted L-RNA-encoded gene products. This figure shows the L-RNA sequences that were obtained from both [^{32}P]pCp-end-labeled L-RNA analyses and dideoxynucleotide DNA product chain termination analyses with a 3′-terminal complementary primer (see text). The nucleotides in the SSH and L74 viral L-RNA sequences that were identical to those in the LAC virus L-RNA sequences are indicated by solid circles. UAC triplets (possible AUG translation initiation codons on the complementary RNA) are underlined. After the putative translation initiation sites, the amino acids that were coded in the same reading frame (reading frame I, II, or III) are shown under the corresponding triplets. The positions of the translation stop signals (antiopal ACU, antiochre AUU, and antiamber AUC) are indicated by the superscripts I, II, and III, corresponding to the reading frames in which they occur.

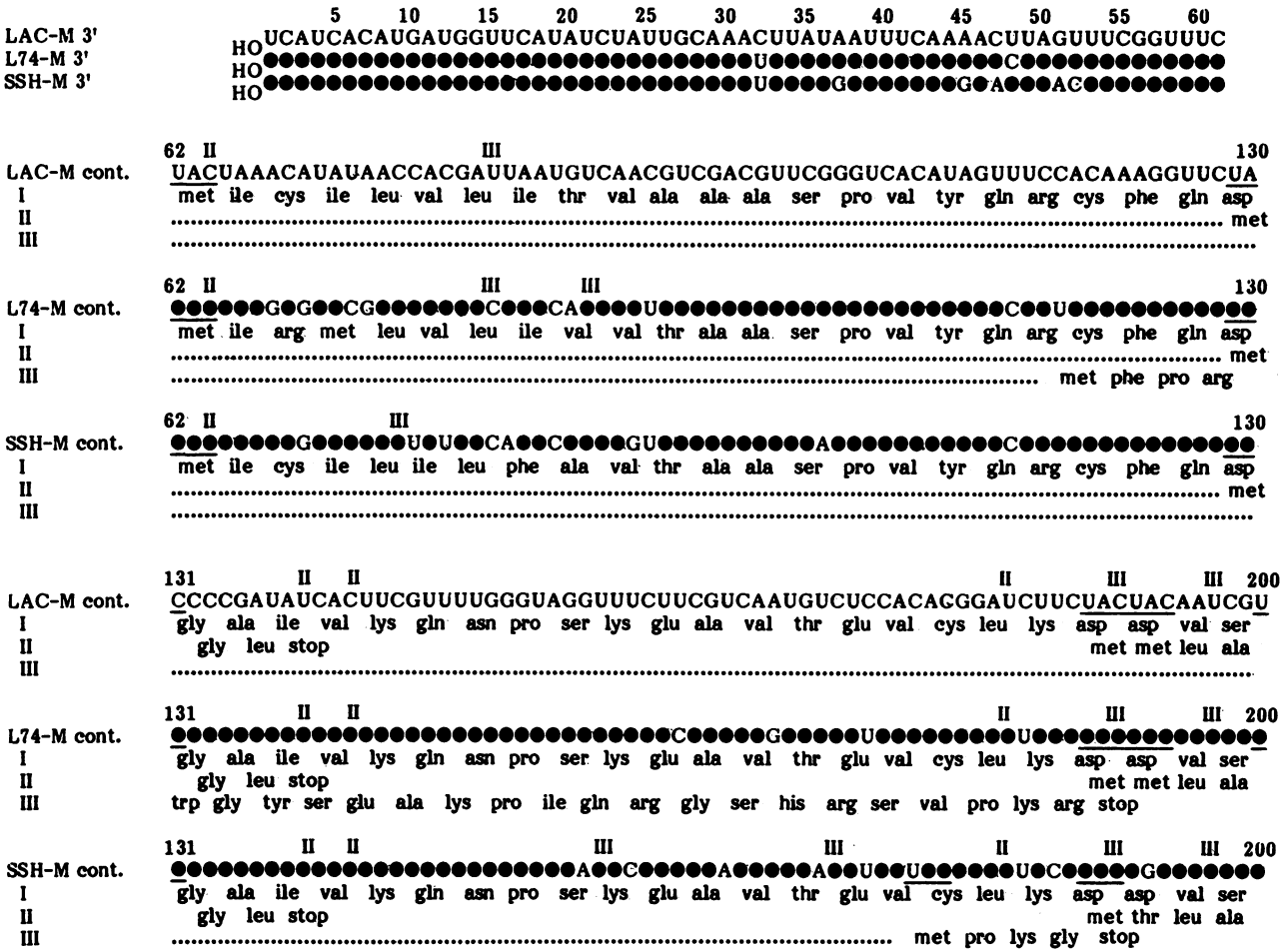

FIG. 3. Deduced prototype LAC virus, prototype SSH virus, and alternate LAC virus L74 M-RNA 3'-terminal sequences and predicted M-RNA-encoded gene products. For details, see text and the legend to Fig. 2.

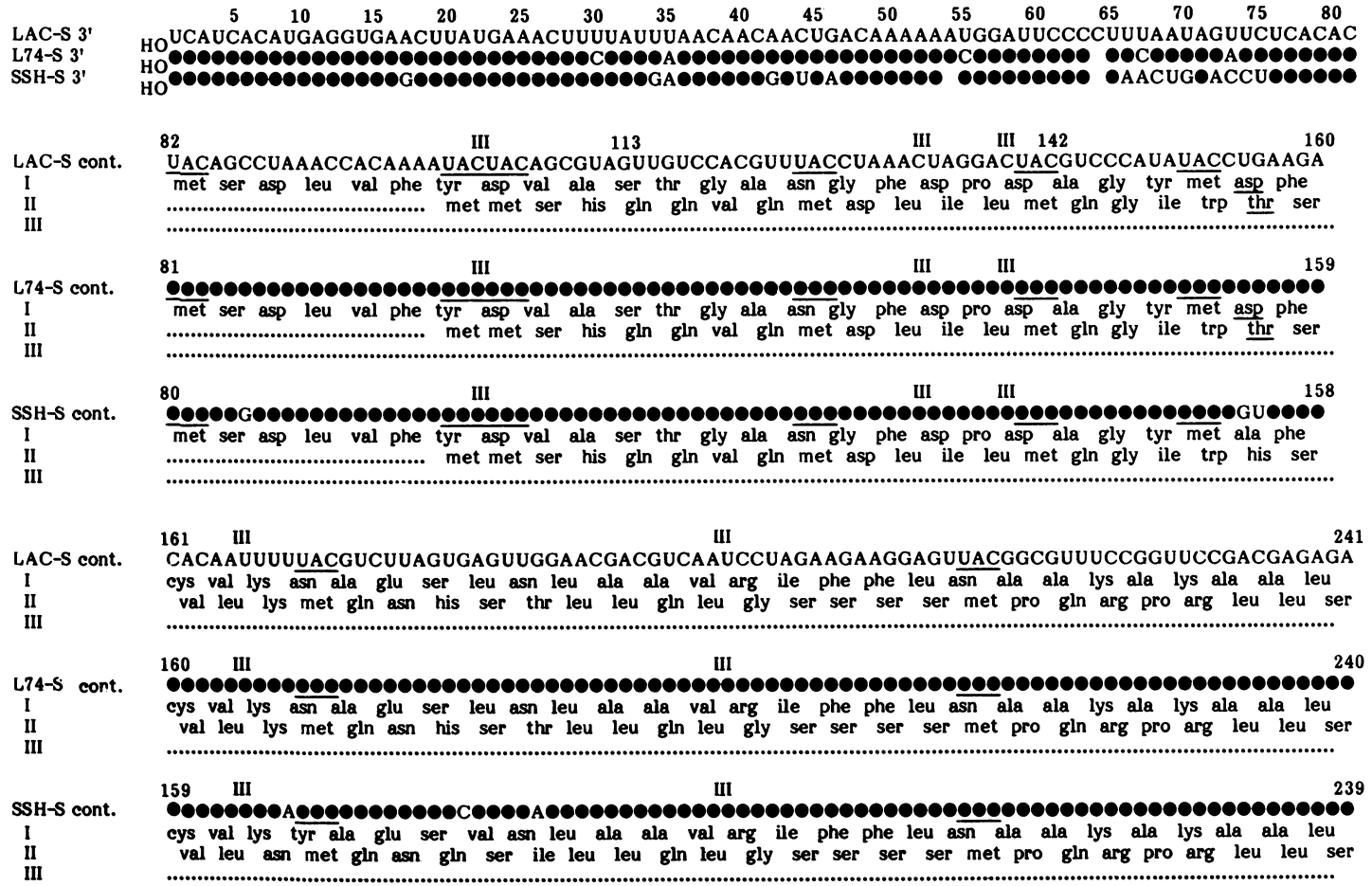

FIG. 4. Deduced prototype LAC virus, prototype SSH virus, and alternate LAC virus L74 S-RNA 3'-terminal sequences and predicted S-RNA-encoded gene products. For details, see the text and the legend to Fig. 2. Gaps in the SSH and L74 viral RNA sequences represent missing nucleotides compared with the prototype LAC virus S-RNA sequence.

(equivalent to an AUG on the complementary plus strand) was present at residues 55 through 57 from the 3′ end. By arbitrary assignment, this triplet was designated reading frame I; reading frames II and III started at residues 56 and 57, respectively. As Fig. 2 shows, the first reading frame of LAC virus L-RNA predicted an amino acid sequence that terminated in an antiamber (AUC) codon at nucleotide residues 94 through 96 (indicated by a superscript I). By contrast, a UAC triplet at residues 62 through 64 (reading frame II) initiated an open reading frame sequence through the 200 nucleotides that have been characterized. All other UAC triplets in reading frames I and III initiated triplet reading sequences that terminated in antiamber (AUC), antiopal (ACU), or antiochre (AUU) codons.

When the SSH virus L-RNA sequences were compared with those of LAC virus L RNA, we found that there were UAC triplets in the SSH virus L-RNA genome at positions equivalent to those identified in LAC virus L RNA. Also, as in the LAC virus L-RNA sequences, all of the UAC triplets representing reading frames I and III initiated triplet reading sequences that led to termination codons.

In contrast, analyses of the L74 virus L-RNA 3′ sequence showed that it did not have a UAC triplet at residues 55 through 57. However, a UAC triplet was located at residues 62 through 64 (reading frame II for LAC and SSH viral L-RNA species). As in the LAC and SSH viral L-RNA sequences, this UAC triplet initiated an open reading frame. All of the other UAC triplets in reading frames I and III initiated triplet reading sequences that led to termination codons (superscripts I and III).

By comparing the 200-nucleotide sequence of LAC virus L RNA, we identified 10 nucleotide substitutions in the L74 virus L-RNA sequence and 31 nucleotide changes in SSH virus L RNA. Five of the L74 virus substitutions were shared with SSH virus. Four of the L74 virus substitutions and seven of the SSH virus substitutions occurred before residues 62 through 64 (i.e., where the open reading frame commenced). Thus, we found neither significantly more nor fewer nucleotide substitutions per length of RNA before the putative mRNA translation initiation site than after this site.

For the product of reading frame II, the six base substitutions in the L74 virus genome were all third-position changes that did not dictate amino acid changes. In the SSH virus L RNA, only 6 of the 24 nucleotide changes caused amino acid changes.

In summary, the L-RNA sequence analyses of LAC, SSH, and L74 viruses identified a single open reading frame for the complementary plus strand. This reading frame commenced at genome nucleotide residue 62. If this sequence coded for a virus gene product, then the analyses of both alternate LAC virus isolate L74 and the serologically related SSH virus indicated that, although some SSH virus amino acid changes were predicted, most of the base substitutions in these viral L-RNA sequences did not cause amino acid changes.

M-RNA analyses. Single open reading frames (designated I) were identified for the M-RNA sequences of LAC and SSH viruses and the alternate LAC virus isolate (Fig. 3). As in the L RNA, in each M RNA this reading frame commenced at nucleotide residue 62. In the two other reading frames (reading frame II, starting at residue 63, and reading frame III, starting at residue 64), each of the possible mRNA translation initiation codons (UAC triplets in the genome RNA) was followed a few triplets later by a termination codon (indicated by superscripts II and III). We also determined that nucleotide residues 201 through 203 were ACU (i.e., an antiopal termination codon in the second reading frame) (data not shown). By comparison with the LAC virus M-RNA sequence data, we found that the alternate LAC virus isolate had two nucleotide substitutions before residue 62 and 14 nucleotide substitutions between residues 62 and 200. For SSH virus, we identified six base substitutions in the sequence before residue 62 and 19 substitutions after residue 62. Eight of these nucleotide substitutions were shared by L74 and SSH viruses. For both SSH virus and the alternate LAC virus isolate, the only amino acid substitutions that were caused by these base changes were all localized within 11 amino acids of the amino terminus of the predicted gene product. All other nucleotide changes did not cause amino acid changes (Fig. 3).

It has been shown that the SSH and LAC viral M-RNA species code for two viral glycoproteins, G1 and G2 (9). Although the gene order of G1 and G2 is not known, it is likely that these proteins are derived from a common polypeptide precursor, as suggested recently by analyses of Uukuniemi (17) and Qalyub (4) viruses (members of the *Uukuvirus* and *Nairovirus* genera of the Bunyaviridae, respectively) (1). If this is true, then based on analyses of other viral glycoprotein precursors, we could expect an amino-terminal hydrophobic signal sequence on the nascent precursor glycoprotein. Such a hydrophobic sequence was evident in the predicted terminal 18-amino acid sequence of the M-RNA gene products shown in Fig. 3, although whether it functioned as a signal sequence is not known.

S-RNA analyses. As described previously (5), the UAC triplet on LAC virus S RNA occurs at residues 82 through 84, whereas in SSH virus

this triplet occurs at residues 80 through 82. In the alternate LAC virus isolate the comparable UAC triplet was present at residues 81 through 83. By comparison with the SSH virus sequence, the LAC virus S-RNA sequence had both an extra adenine residue (LAC virus residues 49 through 54) and an extra cytosine residue (residues 61 through 64). The alternate LAC virus strain had the extra cytosine residue, but not the extra adenine residue (Fig. 4). When the S-RNA sequences of the three viruses were compared, we found that there was considerably more sequence conservation after the UAC triplet (i.e., between SSH virus residues 80 and 239) than before this triplet. Thus, of 23 differences, 6 were located after residue 80, and 17 were located before residue 80. In the alternate LAC virus isolate, compared with the prototype LAC virus, there were only five differences, all of which were located before residue 81. The only common nucleotide substitution in the SSH and L74 viral sequences was an adenine residue at position 35.

When the S-RNA sequences that followed the first UAC triplet were analyzed for their coding potentials, we observed that all four of the termination codons were in reading frame III; i.e., there were two open reading frames, one which commenced with the UAC triplet at residue 82 (LAC virus reading frame I) and another which started with a UAC triplet at residues 101 through 103 (LAC virus reading frame II). Four of the nucleotide substitutions in SSH virus S RNA predicted amino acid changes in both reading frame I and reading frame II.

There is a potential region of secondary structure between residues 113 and 142 on the LAC viral S RNA (and the corresponding alternate LAC and SSH viral S-RNA sequences). This region contains 11 base pairs and directly precedes a reading frame II UAC triplet and (nine residues later) a reading frame I UAC triplet. Whether this hairpin structure functions in the selection of ribosome translation initiation sites is not known; the answer to this question will involve determining the NH_2-terminal amino acids of the S-RNA-encoded gene products and the mRNA ribosome binding sites.

DISCUSSION

We undertook 3′ analyses of the L-, M-, and S-RNA species of prototype LAC virus, an alternate LAC virus isolate, and prototype SSH virus to identify and make gene products predictions from the open reading frames in the complementary mRNA species. Previous analyses of SSH virus have shown that there are single complementary mRNA species which are essentially equivalent in length to the viral M- and L-RNA species (3). For SSH viral S-RNA species a single mRNA has been identified, and this mRNA is smaller (3.8×10^5 daltons) than the virion S RNA (4.5×10^5 daltons). From ^{32}P-labeled viral RNA oligonucleotide protection analyses, it has been deduced that for each viral RNA the principal mRNA species are initiated at the 3′ termini of the viral RNA species (3, 5). Thus, in this study we analyzed the sequence data obtained for SSH virus, LAC virus, and the alternate LAC virus isolate on the premise that the viral complementary 3′ RNA sequences represent mRNA sequences. However, direct mRNA analyses were not undertaken due to the low amounts of virus-induced mRNA that can be recovered from infected cells (3), so that these predictions have to be considered tentative until mRNA or gene product analyses have been undertaken. Notwithstanding these restrictions, our analyses of the three viruses identified single open reading frames for the L- and M-RNA species and two overlapping open reading frames for the S-RNA sequences. Interestingly, in the LAC and SSH viral L-RNA species, the first 3′ UAC triplet on the viral RNA (residues 55 through 57) does not initiate an extensive open reading frame on the complementary RNA, although the second UAC triplet (residues 62 through 64) does. This latter UAC triplet constitutes the first UAC triplet in the L74 viral L-RNA sequence due to nucleotide substitutions in its RNA at residues 56 and 57.

Compared with the prototype LAC viral RNA species, the nucleotide substitutions present in the SSH and L74 viral L- and M-RNA species appear to be evenly spread over the sequences and are evidently sufficient to cause some of the oligonucleotide fingerprint differences that have been demonstrated in the L- and M-RNA species of these viruses (6, 14). For the S-RNA species of either virus there are more nucleotide substitutions in the first 80 residues than in the following 160 residues. Again, these substitutions predict oligonucleotide fingerprint differences, as demonstrated previously (6, 14). Overall, for the genome RNA sequences that have been analyzed, the L74 virus nucleotide substitutions occur at a frequency of 1 in 20, and the SSH virus changes occur at a frequency of 1 in 8.

The L- and S-RNA substitutions of the alternate LAC virus isolate predict no changes in the 46 encoded amino acids of the L-RNA sequence that has been analyzed so far or in the 53 (reading frame I) or 47 (reading frame II) encoded amino acids of the S RNA. For the M RNA of virus isolate L74, the four predicted amino acid substitutions are located near the amino terminus of the 46-amino acid sequence so far deduced for the encoded gene product. Thus, we concluded that, for the sequences

analyzed, the majority of the nucleotides substitutions identified in the L-, M-, and S-RNA species of the alternate LAC virus isolate do not code for amino acid changes. By comparison with the prototype LAC virus data, amino acid substitutions are predicted in the gene products of the L-, M-, and S-RNA species of SSH virus. However, the majority of the SSH virus nucleotide substitutions also do not predict amino acid changes.

The frequencies of occurrence of antiopal, antiochre, and antiamber codons for the LAC virus RNA sequences are essentially equivalent, both for the total RNA sequences that have been obtained (13:10:10, respectively) and for the putative coding regions of the genome (8:5:6, respectively). Similar conclusions have been drawn from the data for the SSH virus and the alternate LAC virus isolate.

The fact that one-half of the L74 and SSH viral nucleotide substitutions in the L- and M-RNA species of these viruses are shared is in contrast to the S-RNA data, which indicate many fewer shared nucleotide substitutions. Clearly, more S-RNA sequence data are needed to determine whether this difference is real. If this difference is indeed real, then the larger number of common L- and M-RNA nucleotide substitutions in L74 and SSH viruses suggests that these RNA species may have come from a common ancestor more recently (due to RNA segment reassortment) than the S-RNA species of these viruses.

Although there are evidently fewer nucleotide substitutions in the coding regions of the S-RNA species of L74 and SSH viruses compared with the S-RNA species of the LAC virus, in SSH virus there is a region of considerable nucleotide substitution (SSH virus residues 64 through 73). It is reasonable to postulate that the paucity of S-RNA substitutions in the S-RNA coding region is related to the existence of genes in two overlapping reading frames, whereby evolution by point mutation is restricted since two genes are affected simultaneously. This agrees with the difficulties which we have experienced in obtaining any temperature-sensitive mutants representing S-RNA defects (2). The two S-RNA products can be translated either from two initiation sites on the same mRNA or from separate mRNA species produced by splicing. Preliminary in vitro translation studies have indicated that both N and NS proteins are translated from the same size class of S mRNA, so that we cannot distinguish between these two possibilities.

The hypermutated region of the SSH virus S-RNA sequence that precedes the open reading frames may be explained if this region of the genome has no function (i.e., no coding, structural, or morphogenetic function). Further analyses of the S-RNA species of these and other bunyaviruses are planned to investigate these questions.

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