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Nonviral heterogeneous sequences are present at the 5' ends of one species of snowshoe hare bunyavirus S complementary RNA

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ABSTRACT

Analyses of the 5' ends of snowshoe hare bunyavirus plus sense S RNA species (including mRNA) recovered from infected cells have revealed two types of termini. These include ends that are essentially exact copies of the 3' end of the viral S RNA and others that are similar, but additionally have 13-14 nucleotide extensions that are heterogeneous in sequence. The former probably represent replicative plus sense RNA species, the latter mRNA species that have host cell derived primer sequences.

INTRODUCTION

Snowshoe hare (SSH) virus is a member of the California encephalitis serogroup of arthropod transmitted bunyaviruses (Bunyavirus genus, family Bunyaviridae) (1). Members of the family have a genome consisting of 3 segments of negative sense, single stranded, RNA, designated on the basis of size differences as L (large), M (medium) and S (small) (2). The 3.3×10^5 dalton S RNA species of SSH virus codes for 2 proteins, a 26.8×10^3 dalton nucleoprotein, N, and a 10.5×10^3 dalton non-structural protein, NS_S (3-5).

A single size class of S viral complementary (plus sense) mRNA can be translated in vitro into N and NS_S (5). The two proteins are coded by overlapping reading frames in the mRNA (4,5). The first open reading frame codes for N protein and commences with an AUG equivalent to viral nucleotide residues 80-82, the second (NS_S) starts at residues 99-101 (4). It is not known whether there are discrete mRNA species for each gene product, or whether a single S mRNA species serves as translation template for both N and NS_S. In order to investigate the transcription strategy an oligodeoxyribonucleotide was synthesized (viral S RNA residues 121-149) and used on infected cell extracts containing SSH plus sense RNA species (including mRNA) to backcopy their 5' end(s). After poly dA tailing, cloning and sequencing, it was determined that 2 types of transcripts were made. The shorter of these transcripts was an extact copy of the viral RNA essentially up to the 3' end. The longer transcripts were similar, except that they contained additional, heterogeneous,

sequences extending 13-14 nucleotides beyond the 3' end of the viral RNA sequence.

Such non-viral mRNA extensions are similar to the cellular primers found on influenza mRNA species (6-9) and in vitro transcription products (10-12). These data suggest that bunyaviruses may, like influenza virus, use cellular primers to initiate mRNA synthesis in vivo.

MATERIALS AND METHODS

Oligodeoxyribonucleotide primer

For the specific backcopying of SSH plus sense RNA species (mRNA and putative plus sense replicative RNA species) a 29 nucleotide long oligodeoxyribonucleotide primer was synthesized (5' TATACCCTGCATCAGGATCA-AATCCATTT 3') by a solid phase phosphotriester method using a protected T residue (representing the 3' nucleoside of the primer) linked to a polystyrene resin (13, Vega Biochemicals, Tucson, Ariz., USA) and precursor protected dinucleotides (14-16). After uncoupling and deprotection, the oligonucleotide was purified by HPLC chromatography and phosphorylated at its 5' terminus by polynucleotide kinase.

Virus, cells and preparation of infected cell RNA

BHK-21 cells were grown to confluence in Eagle's medium containing 10% newborn calf serum and infected with SSH virus at a multiplicity of 5 to 10. The cells were incubated for 10 hr at 37°C in the presence of (^{3}H)adenosine to label RNA and extracted for total cytoplasmic RNA as described previously (5, 17).

In vitro translation of mRNA

Infected cell RNA was translated using rabbit reticulocyte lysates (18). The products were resolved by polyacrylamide gel electrophoresis (19).

Cloning DNA copies of the 5' ends of SSH S plus sense (including mRNA) species

Total cytoplasmic RNA extracted from SSH virus infected cells was resolved by centrifugation in gradients of sucrose and RNA recovered from individual fractions of the gradient and translated using rabbit reticulocyte lysates (see text). From a similar centrifugation, fractions of the gradient were pooled (see text), the RNA recovered and used as a template for cDNA synthesis using the oligodeoxyribonucleotide primer. Prior to cDNA synthesis approx. 10 µg RNA and 2 µg primer in 10 µl H_2O were denatured for 15 min at room temperature with 0.5 µl of 0.1M methylmercury hydroxide then 1 µl of 0.7 M 2-mercaptoethanol added. The sample was diluted into a reaction mixture (0.1 ml) containing (final concentrations) 70mM KCl, 50mM Tris-HCl, pH 8.0, 10mM $MgCl_2$, 0.5 mM each of dCTP, dTTP and dGTP, 0.5 mM (α-^{32}P) dATP (specific activity 500 mCi per mMole), 10 mM DTT plus 15 units of reverse transcriptase and incubated at 42°C for 2 hr. The reaction mixture was adjusted to 40% formamide, boiled for 5 min and resolved by 6%

polyacrylamide gel electrophoresis in 7M urea. Following autoradiography, bands from the gel were eluted in 0.4M NaCl, the cDNA recovered by alcohol precipitation and tailed with poly dA using terminal transferase (4). After phenol extraction and chromatography through a 1 ml column of Sephadex G50 (fine), the product DNA was converted into double-stranded DNA using a 5' phosphorylated oligo $(dT)_{12-18}$ primer, then treated with S1 nuclease, repaired with the "Klenow" fragment of DNA polymerase and blunt-end ligated into the Pvu II site of the E. coli plasmid pBr322 as described previously (4). Plasmid DNA was used to transform competent E. coli MC1061 cells (20, 21). Bacterial clones contained SSH cDNA inserts were identified by Grunstein-Hogness hybridization (22) using short copy cDNA prepared as described elsewhere (4).

Sequencing plasmids containing viral inserts

Sequencing was undertaken by the Maxam-Gilbert method (23, 24) using strand-separated Hinf I derived restriction fragments containing the total viral insert.

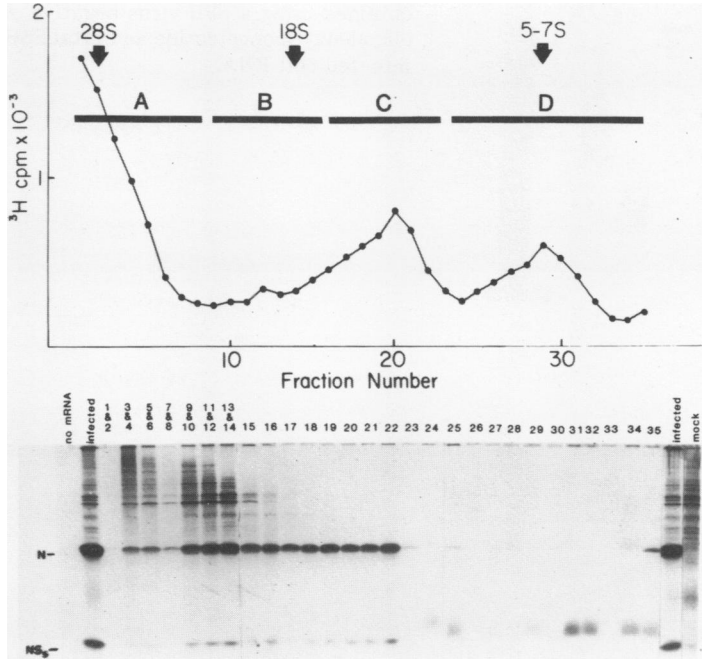

Fig. 1. Resolution of infected cell extracts in 15-30% gradients of sucrose containing 50mM Tris-HCl, pH 7.4, 0.1M NaCl, 1mM EDTA and 0.5% SDS (top panel) and in vitro translation of mock, or infected cell RNA, or RNA recovered from the gradient (bottom panel). Labelled RNA was centrifuged at 23,000 rpm for 23 hr at 23°C.

RESULTS

Preparation of cytoplasmic extracts containing SSH S mRNA species

To obtain SSH S mRNA free from most cellular RNA, (^{3}H)adenosine labeled infected cell RNA was resolved by centrifugation in gradients of sucrose (Fig. 1, top panel). The position of the viral S mRNA was determined by in vitro translation using rabbit reticulocyte lysates (Fig. 1, lower panel).

The induced syntheses of N and NS_S identified the location of the S mRNA species (Fig. 1, lower panel). Based on the incorporation of amino acid precursors and the distribution of radioactivity, it was determined that the peak of S mRNA activity was resident in fractions #16-22. From a similar gradient, gradient fractions were pooled (A-D, see Fig. 1). Although SSH S mRNA is slightly smaller (25) than the viral S RNA species (negative sense RNA) it cannot be resolved from viral S RNA by gradient centrifugation (unpublished data). It was probable therefore that the gradient fractions containing S mRNA also contained both viral plus and negative

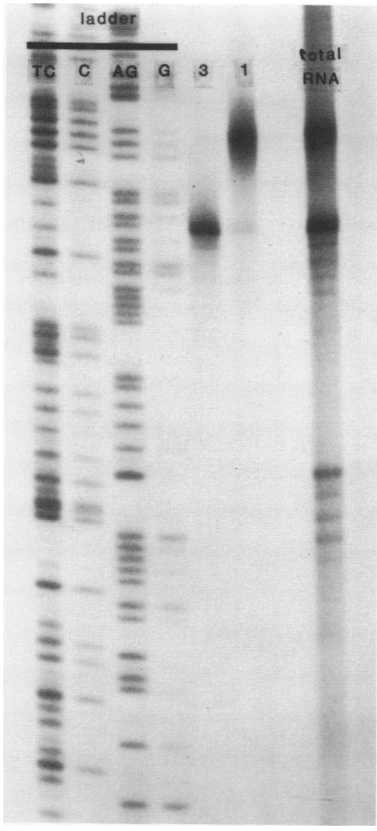

Fig. 2 Gel electrophoresis of cDNA transcripts obtained using a SSH virus negative sense oligodeoxyribonucleotide and total SSH virus infected cell RNA.

sense RNA. This was confirmed, in part, by the observation that self-annealing the pooled RNA fraction C completely abolished its ability to be translated in vitro.

cDNA transcripts made from SSH plus sense RNA

Total SSH virus infected cell RNA was used with a SSH S viral sense oligodeoxyribonucleotide primer to make cDNA (see Materials and Methods). As shown in Fig. 2, two size classes of large transcripts were made (designated 1 and 3) plus some smaller species. By reference to a sequencing gel of a known size restriction fragment of Punta Toro DNA, it was determined that transcripts 1 and 3 had average sizes of approximately 160-170 and 140-150 nucleotides, respectively. The smaller species had sizes of approximately 115-125 nucleotides.

In order to determine which size classes of RNA directed the syntheses of the various cDNA species, SSH virus infected total cytoplasmic RNA was first resolved on a gradient of sucrose (Fig. 1). A similar quantity (10%) of each of the pooled RNA fractions A-D (Fig. 1) was then used with the oligonucleotide primer to template cDNA synthesis. As shown in Fig. 3, fraction C RNA (and to lesser extents fractions A and B) made the cDNA species 1 and 3. The smaller cDNA transcripts came from RNA fraction D (i.e., RNA that was not translated into N or NS_S). The remainder of the pool C RNA was used to synthesize and, after 6% polyacrylamide gel electrophoresis, recover cDNA transcripts 1 and 3. As shown in the lanes labelled 1 and 3 (Fig. 2), the eluted cDNA species represented the two size classes of cDNA. Each cDNA species was tailed with poly dA, backcopied with the "Klenow" fragment of DNA polymerase using oligo dT as primer and cloned into the Pvu II site of pBr322.

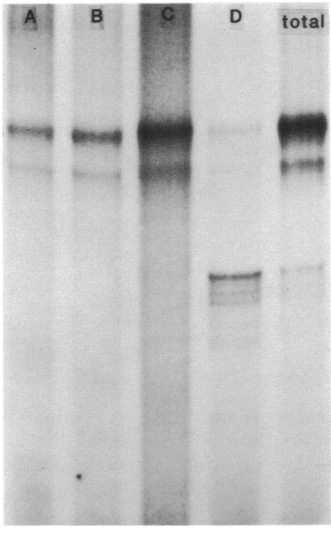

Fig. 3 cDNA synthesis by gradient resolved SSH virus infected cell RNA. Gradient purified RNA preparations (pools A-D, Fig. 1) and total RNA were used with an oligonucleotide primer to template cDNA synthesis. The products were resolved by 6% polyacrylamide-7M urea gel electrophoresis.

Fig. 4 Sequence analyses of the ends of 2 cDNA clones representing SSH virus plus sense S RNA species. The adjacent numbers represent the nucleotide residues of the viral complementary strand (4). The specificities of the 4 chemical reactions are indicated above the respective lanes. The 4 left lanes came from clone 1-3, the 4 right lanes from clone 1-2.

Sequence analyses of cloned DNA made from SSH plus sense RNA

The nucleotide sequence of the first 150 residues of cloned SSH S DNA, representing the 3' end of the viral RNA, lacks a Hinf I site (4). Since the plasmid Pvu II site is located in a Hinf fragment of some 344 nucleotides, all the clones obtained from SSH plus sense RNA species yielded Hinf I restriction fragments greater than 490 nucleotides in length. For each clone analyzed these end-labelled fragments were recovered, strand separated, and sequenced by the Maxam-Gilbert method. Two representative sequences obtained from cDNA transcript 1 are shown in Fig. 4. By comparison with the previously determined sequence of cloned SSH S DNA (4), it was determined that their sequences were exact copies of the viral 3' end up to the penultimate nucleotide of the S RNA, thereafter the sequences diverged prior to the poly T sequence (i.e., the complement of the poly dA residues added to the cDNA transcripts).

The sequences obtained from 3 clones derived from cDNA transcript 1 are shown in Fig. 5. All 3 have 14 additional nucleotides beyond the penultimate residue of the viral RNA. The clone 1-3 lacks 2 nucleotides in the primer region of the sequence. This was presumably due to the lack of a dinucleotide addition during the synthesis of that primer molecule. Since dinucleotides were also missing from clone

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                                                                    1
Clone 1-6   TTTTTTTTTT  GGTCTCTTGCTGT                    AGTAGTGTAC  TCCACTCGAA  TACTTTGAAA
Clone 1-2   TTTTTTTTTT  GATCTTGGCGGCTG                     GTAGTGTAC  TCCACTCGAA  TACTTTGAAA
Clone 1-3   TTTTTTTTTT  GAGGTGACGGTTCC                     GTAGTGTAC  TCCACTCGAA  TACTTTGAAA

Clone 3-1   TTTTTTTTTT                                      TAGTGTAC  TCCACTCGAA  TACTTTGAAA
Clone 3-2   TTTTTTTTTT                                      TAGTGTAC  TCCACTCGAA  TACTTTGAAA

                                                                                      N
            31
Clone 1-6   ATACTTTGTT  GCTAATTGTT  TTTACCTAAG  GGATTGACTT  GGAAGTGTGA  TGTCCGATTT
Clone 1-2   ATACTTTGTT  GCTAATTGTT  TTTACCTAAG  GGATTGACTT  GGAAGTGTGA  TGTCCGATTT
Clone 1-3   ATACTTTGTT  GCTAATTGTT  TTTACCTAAG  GGATTGACTT  GGAAGTGTGA  TGTCCGATTT

Clone 3-1   ATACTTTGTT  GCTAATTGTT  TTTACCTAAG  GGATTGACTT  GGAAGTGTGA  TGTCCGATTT
Clone 3-2   ATACTTTGTT  GCTAATTGTT  TTTACCTAAG  GGATTGACTT  GGAAGTGTGA  TGTCCGATTT

                  NS
                    S
            91
Clone 1-6   GGTGTTTTAT  GATGTCGCAT  CAACAGGTGC  AAATGGATTT  GATCCTGATG  CAGGGTATA
Clone 1-2   GGTGTTTTAT  GATGTCGCAT  CAACAGGTGC  AAATGGATTT  GATCCTGATG  CAGGGTATA
Clone 1-3   GGTGTTTTAT  GATGTCGCAT  CAACAGGTGC  AAATGGATTT  GATCCTGATG  CAG  TATA

Clone 3-1   GGTGTTTTAT  GATGTCGCAT  CAACAGGTGC  AAATGGATTT  GATCCTGATG  CAGGGTATA
Clone 3-2   GGTGTTTTAT  GATGTCGCAT  CAACAGGTGC  AAATGGATTT  GATCCTGATG  C    TATA

                                                           PRIMER
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Fig. 5 The sequences of 5 cDNA clones transcribed from SSH virus plus sense RNA species. The deduced sequences (shown as the viral complementary strands) obtained from 3 clones recovered from cDNA transcript 1 (clones 1-6, 1-2, 1-3) and 2 from cDNA transcript 3 (clones 3-1, 3-2) are given (see Fig. 3). The primer location (residues 121-149) and the first ATG representing the initiation of the N protein open reading frame, and ATGATG sequence representing the initiation of the NS_S open reading frame, are underlined (4,5).

3-2, primer variations probably contributed to the heterogeneity seen in the cDNA transcripts (Fig. 2). Otherwise the sequences were faithful replicas of the viral S RNA sequence up to the penultimate or, for clone 1-6, the terminal residue (4). Also shown in Fig. 5 are the sequences obtained from 2 clones derived from cDNA transcript 3. Their sequences were found to be faithful copies up to residue 3 of the viral S RNA sequence without additional nucleotides before the homopolymeric sequence. Both sets of clones contained the ATG triplets representing the start of the N and NS_S open reading frames.

It was concluded from these results that cDNA transcript 1 contained sequences in addition to the viral S RNA sequence. By contrast, cDNA transcript 3 contained only viral S RNA sequences.

DISCUSSION

Sequence analyses of the 5' ends of SSH plus sense S RNA species were undertaken in order to determine their transcription strategies. The protocol employed a 29 nucleotide long oligonucleotide primer that was identical in sequence to an internal coding region of the negative sense viral S RNA (i.e., within the N and NS_S genes). This long sequence was selected in order to specifically transcribe viral plus sense RNA sequences. Due to the difficulties in obtaining bunyavirus mRNA preparations with 3' polyA sequences (17, and unpublished data), we elected to obtain S size class RNA by separating infected cell extracts on gradients of sucrose. The RNA preparations therefore contained both S mRNA and viral RNA and presumably other plus sense species. Using this material, 2 types of cDNA transcripts were identified. One of these corresponded in length and sequence to that expected of a copy back to the 3' end of an exact complementary copy of the viral RNA. The other by size and sequence had an additional 14 nucleotides beyond the penultimate residue of the viral RNA. These 14 nucleotides varied from clone to clone, indicating that the 5' ends of this type of plus sense RNA are heterogenous in sequence. Although direct sequence analyses of SSH mRNA species have not been undertaken, similar heterogenous sequences on the 5' ends of RNA have been identified for influenza mRNA species recovered from infected cell extracts (6-9). Such sequences derive from host cell mRNA species that are utilized by the virus to initiate mRNA synthesis. The analogous results obtained for cDNA transcript 1 suggest that they may represent SSH S mRNA sequences with 5' host cell derived primers. The heterogeneous sequences do not correspond to any known S RNA sequence (4), and it is unlikely that they represent replicative intermediates.

Whether the clones derived from cDNA transcript 3 represent alternate S mRNA species is not known. A more plausible interpretation is that they represent

replicative plus sense RNA species. The viral or alternate origins of the shorter (115-125 nucleotide) transcripts derived from RNA species that are smaller than the S mRNA species have not been investigated. Cloning and sequencing will be needed to establish their identities. Some studies have been reported that indicate that bunyaviruses can productively infect enucleated cells (25), other reports indicate that the viruses are inhibited by enucleation (26). However, it has been shown that bunyavirus mRNA synthesis is not curtailed in cells treated with actinomycin D, or a-amanitin, although virus yields are reduced (27). This is in contrast to the results obtained for influenza virus where either drug (28, 29), or enucleation (30), inhibits intracellular transcription and the development of a productive infection. Conceivably, bunyaviruses may cannibalize preformed cytoplasmic mRNA species.

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