Short Communication

The S-genomic RNA of Hantaan virus contains only two trinucleotide repeats at its 5' end and the 3' end of S-specific mRNA is truncated and lacks poly-A tail

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Although previous studies of Bunyaviridae mRNA termination sites have been successful in mapping the termination position, the methods employed were limited to such that the position could only be determined within 4 or 5 nucleotides (Bouloy *et al.*, 1984). Furthermore, non-viral sequences such as poly-A tail, at the 3' end of mRNAs would not be detected by these mapping techniques. Because of these shortcomings, we attempted to clone and sequence intact 3' ends of Hantaan virus S genome-derived antigenomic RNAs using the crossover sequencing method. We report here the analysis of the 5' terminus of S genomic RNA and the 3' terminus of S-specific mRNAs using the crossover sequencing technique. We found that S genomic RNA contains only two trinucleotide repeats at its 5' end and the 3' ends of mRNAs are truncated and are not polyadenylated.

The hantavirus genus of family Bunyaviridae is comprised of several serologically distinct virus types; Hantaan, Seoul, Puumala, Dobrava/Belgrade, Prospect Hill, Thailand, Thottapalayam and Sin Nombre viruses (Chu *et al.*, 1994; Xiao *et al.*, 1994). Hantaan virus is the prototype of the genus hantavirus which is the etiologic agent of hemorrhagic fever with renal syndrome (HFRS). Hantaviruses are enveloped negative sense RNA viruses which contain three genomic segments; large (L), medium (M), and small (S). All three segments of many different strains of hantaviruses have been cloned and sequenced (Schmaljohn *et al.* 1986, 1987; Yoo and Kang, 1987; Giebel *et al.*, 1989; Parrington and Kang, 1990; Schmaljohn, 1990; Stohwasser *et al.*, 1990, 1991; Arikawa *et al.*, 1990; Antic *et al.*, 1991a, 1991b, 1992; Parrington *et al.*, 1991; Vapalahti *et al.*, 1991; Spiropoulou *et al.*, 1994). All the previous reports showed that there are three trinucleotide repeats at both the 3' and the 5' ends of all hantavirus genomic

segments (Schmaljohn, *et al.*, 1986, 1987, 1990; Yoo and Kang, 1987; Giebel *et al.*, 1989; Parrington and Kang, 1990; Schmaljohn, 1990; Stohwasser *et al.*, 1990, 1991; Arikawa *et al.*, 1990; Antic *et al.*, 1991a, 1991b, 1992; Parrington *et al.*, 1991; Vapalahti *et al.*, 1991; Spiropoulou *et al.*, 1994). Nucleotide sequence analysis of the L-segments revealed that L-segments contain approximately 6,500 nucleotides which has a single open reading frame to code for approximately 2,150 amino acids (Schmaljohn, 1990; Antic *et al.*, 1991b; Stohwasser *et al.*, 1991). The M segments of hantaviruses contain approximately 3,700 nucleotides which also has a single open reading frame which codes for approximately 1,140 amino acids (Yoo and Kang, 1987; Schmaljohn *et al.*, 1987; Giebel *et al.*, 1989; Arikawa *et al.*, 1990; Antic *et al.*, 1991; Parrington *et al.*, 1991; Vapalahti *et al.*, 1992; Spiropoulou *et al.*, 1994). The product of M segment is cleaved into G1 and G2 glycoproteins with approximate molecular masses of 68 and 55 kDa respectively. The S segments of hantaviruses contain approximately 1,700 nucleotides which code for nucleocapsid protein of approximately 430 amino acids (Schmaljohn *et al.*, 1987; Parrington *et al.*, 1991; Stohwasser *et al.*, 1990; Arikawa *et al.*, 1990; Vapalahti *et al.*, 1992; Spiropoulou *et al.*, 1994). We have recently reported that 5' ends of Hantaan virus mRNA contains host cell derived cap at the 5' end (Garcin *et al.*, 1995). This is consistent with previous studies that Bunyavirus, Phlebovirus and Uukuvirus genera which initiate mRNA transcription via a cellular cap-stealing mechanism, a process known to occur with influenza virus systems (Plotch *et al.*, 1981). The RNase T2 and S1 nuclease mapping techniques (Eshita *et al.*, 1985; Bouloy *et al.*, 1984; Raju *et al.*, 1987) revealed that the Bunyaviridae mRNAs are truncated at the 3' end. Unlike rhabdoviruses and myxoviruses, Bunyaviridae mRNAs believed to be not polyadenylated since the Bunyaviridae mRNAs do not bind to the oligo-dT columns.

In order to clone and sequence the 3' ends of Hantaan virus antigenomic RNAs, we first isolated the plus sense S-specific RNAs away from the cellular RNA background. The S segment-derived mRNAs were selected using an S segment specific cDNA clone bound to nitrocellulose filter. This method allowed purification to the point of homogeneity of the S specific plus-sense RNAs which contains both mRNAs and antigenomic RNAs. We then carried out an intramolecular RNA ligation, which allows

Figure 1. 5'-3' junction sequences of the S segment-specific plus-sense antigenomic RNA. The S segment-specific plus-sense antigenomic RNAs were extracted from Hantaan virus-infected cells and purified by a guanidinium thiocyanate-based lysis followed by acid phenol extraction and CsCl gradient centrifugation (Chomzynski and Sacchi, 1987; Raju and Kolakofsky, 1987). Hybridization selection of antigenomic RNA was carried out by the technique described previously (Parnes *et al.*, 1981) using a full length cDNA clone of the Hantaan virus S genomic segment. Hybrid selected antigenomic RNAs were treated with 20 units of tobacco acid pyrophosphatase (TAP; Promega) to remove the 5' cap, phenol chloroform extracted, and ethanol precipitated with 0.3 M sodium acetate and 20 mg of glycogen (Mandl *et al.*, 1991). The dried RNA pellet was resuspended in 200 μ l of RNA ligation buffer (Promega), added 100 units of T4 RNA ligase (Pharmacia LKB) and the reaction was carried out at 4°C for 18 h. The circularized RNAs were used as templates for cDNA synthesis by the reverse

transcription (RT)-PCR (Frohman *et al.*, 1988) using Superscript reverse transcriptase (Gibco-BRL) and three sets of primers representing S genomic RNA (Schmaljohn *et al.*, 1987). The reaction products were electrophoresed in agarose gels and DNA bands of the expected size were eluted by binding to glass beads (GeneClean, Bio101). Following restriction enzyme digestion, DNA fragments were ligated into pBlueScript (Stratagene Corp) and sequenced using dideoxynucleotide chain termination method (Sequenase, USB). Sequences represent the viral complementary RNA. Numbers on the top represent the position of the nucleotide in S genomic RNA.

a 5' end primer to be extended across the 5'-3' junction (Mandl *et al.*, 1991) of the circular RNA. Because Hantaan virus mRNAs contain host cell derived caps at the 5' end (Garcin *et al.*, 1995) which blocked the 5' phosphate group, it was necessary to remove the cap before we can make 3'-5' ligation. We have used tobacco acid pyrophosphatase (TAP) to ensure the 5' ends were available for ligation. Following RNA ligase treatment cDNA was synthesized and products having both 5' and 3' end sequences were amplified by PCR. Seven clones were obtained that possessed sequence from both 5' and 3' (Figure 1). Two of the clones have 3' end sequence up to the last base position 1,696 of the S genomic segment, and thus likely represent the full length antigenome (Figure 1, clone #1 and #2). The other five clones possess 3' end sequence up to position 1,470 (Figure 1, clones #3 to #7). After base 1,470, the sequence UC appears (which is a repeat of bases 1,469 and 1,470) followed by an A residue. Furthermore, clone #3 has a single base deletion at the position 1,465 while clone #4 has a single U residue inserted at position 1,466. These data show that the 3' terminus of

S-specific mRNAs are truncated and lack poly-A tail.

Analyses of the 3' ends of the antigenome of S segment revealed the presence of both a full length and truncated species of RNAs (Figure 1). The full length RNA representing the antigenome of S segment contains only two UAG trinucleotide repeats which is different from all previous reports (Schmaljohn *et al.*, 1986, 1987; Yoo and Kang, 1987; Giebel *et al.*, 1989; Parrington and Kang, 1990; Schmaljohn, 1990; Stohwasser *et al.*, 1990, 1991; Arikawa *et al.*, 1990; Antic *et al.*, 1991a, 1991b, 1992; Parrington *et al.*, 1991; Vapalahti *et al.*, 1991; Spiropoulou *et al.*, 1994). All the previously published data showed that there are three trinucleotide repeats at the 5' end of S genomic RNA. We and others (Schmaljohn *et al.*, 1986; Stohwasser *et al.*, 1990; Arikawa *et al.*, 1990; Vapalahti *et al.*, 1991; Spiropoulou *et al.*, 1994) made this mistake because every laboratory has used primers, for cloning, representing the 3' end of hantavirus genomic RNAs which contains three trinucleotide repeats. We discovered this mistake unexpectedly when we sequenced the 3' termini of plus-sense antigenomic RNAs isolated from

Hantaan virus infected cells. The presence of the two trinucleotide repeats at the 5' end have been confirmed by another laboratory (Chizhikov and Nichol, personal communication). Our result suggests that the 5' ends of all hantavirus genomic RNAs contain only two trinucleotide repeats like other Bunyaviruses.

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