

## The 5' Ends of Hantaan Virus (*Bunyaviridae*) RNAs Suggest a Prime-and-Realign Mechanism for the Initiation of RNA Synthesis

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**We examined the 5' ends of Hantaan virus (HTN) genomes and mRNAs to gain insight into the manner in which these chains were initiated. Like those of all members of the family *Bunyaviridae* described so far, the HTN mRNAs contained 5' terminal extensions that were heterogeneous in both length and sequence, presumably because HTN also "cap snatches" host mRNAs to initiate the viral mRNAs. Unexpectedly, however, almost all of the mRNAs contained a G residue at position -1, and a large fraction also lacked precisely one of the three UAG repeats at the termini. The genomes, on the other hand, commenced with a U residue at position +1, but only 5' monophosphates were found here, indicating that these chains may not have initiated with UTP at this position. Taken together, these unusual findings suggest a prime-and-realign mechanism of chain initiation in which mRNAs are initiated with a G-terminated host cell primer and genomes with GTP, not at the 3' end of the genome template but internally (opposite the template C at position +3), and after extension by one or a few nucleotides, the nascent chain realigns backwards by virtue of the terminal sequence repeats, before processive elongation takes place. For genome initiation, an endonuclease, perhaps that involved in cap snatching, is postulated to remove the 5' terminal extension of the genome, leaving the 5' pU at position +1.**

Hantaan virus (HTN), the prototype of the *Hantavirus* genus of the family *Bunyaviridae*, is the etiologic agent of Korean hemorrhagic fever, a severe form of hemorrhagic fever with renal syndrome. Other viruses of this genus, such as Four Corners virus (37) and Bayou virus (34), have also been recently recognized as significant pathogens responsible for a rare but frequently fatal respiratory illness. HTN, like other viruses in the family, has a three-segment negative-strand RNA genome (47). For HTN, each genome segment has one major open reading frame in the virus complementary or antigenomic sense. The small (S) segment encodes the nucleocapsid protein (48), the medium (M) segment encodes the envelope glycoproteins (49, 55), and the large segment encodes a polypeptide of approximately 240 kDa (1, 46) which is presumed to be the viral polymerase (reviewed in reference 9).

Primary transcription of bunyavirus negative-strand genome RNA to complementary mRNA is believed to occur by interaction of the virion-associated polymerase and the three genome templates (4, 43). Initiation of transcription is probably similar to that of influenza viruses, in which an endonuclease associated with the polymerase complex cleaves host cell mRNAs to generate capped fragments which act as primers, and the presence of a methylated 5' cap structure on the host mRNA is required for this cleavage to occur for both influenza viruses and bunyaviruses (24, 26, 40, 41). As in influenza virus mRNA, 5' terminal extensions of approximately 10 to 18

nucleotides that are heterogeneous in sequence and are not templated from genome RNA have been found on the mRNAs of viruses in the *Bunyavirus* (3, 5, 10, 21, 40), *Phlebovirus* (8, 18, 52), *Nairovirus* (20), and *Tospovirus* (25) genera of this family. Direct evidence for the presence of caps on these termini was obtained by using anti-cap antibodies to immunoselect the mRNAs (17, 53). In this report, we provide the first evidence that HTN mRNAs also contain 5' terminal nucleotide extensions.

The main impetus of this study, however, was the manner in which the synthesis of these genome and antigenome RNAs initiate. The 3' ends of the HTN negative-strand genome segments were determined by <sup>32</sup>Pcp labelling to be 3' OH AUC AUC AUC . . . (the spaces were introduced to highlight the terminal repeats) (48). As the ends of all *Bunyaviridae* genomes described to date are exactly complementary for 8 or 9 nucleotides (nt) (and mostly complementary for ca. 20 nt), the 3' ends of genomes and antigenomes, the presumptive promoters for genome replication, are very similar. Genome and antigenome synthesis is thus believed to initiate in the same manner with essentially the same sequence (24). In the case of La Crosse virus, for example, a member of the *Bunyavirus* genus whose genome ends are 3' OH UCA UCA . . . , the 5' ends of the genomes (templated from the 3' ends of the antigenomes; see Fig. 1A for a diagram of RNA synthesis) were found to be their exact complement, 5' AGU AGU . . . (38). This 5' end was triphosphorylated as well, indicating that genome synthesis had initiated with ATP opposite the presumptive 3' U of the antigenome template, i.e., at position +1 (Fig. 1A). For HNT, if genome and antigenome synthesis were to

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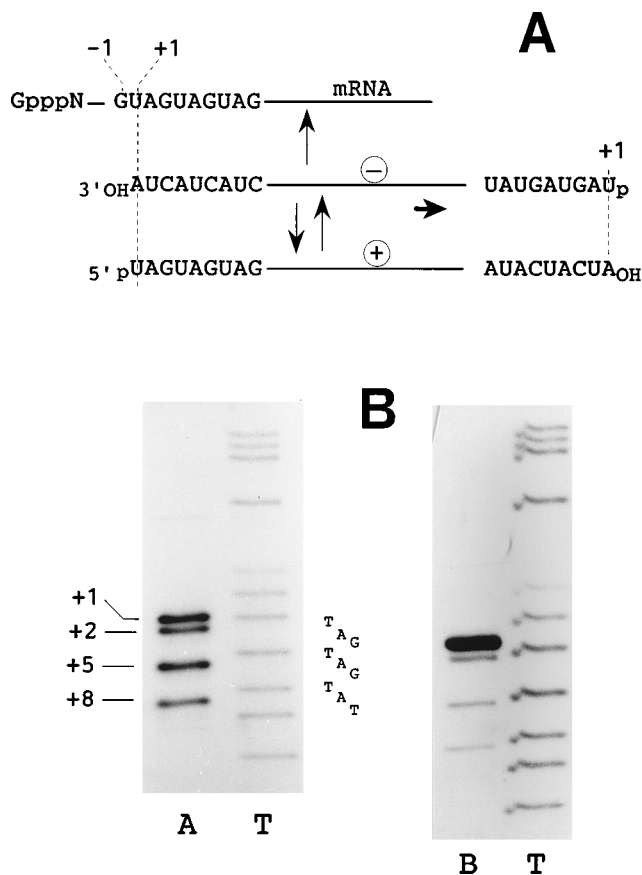


FIG. 1. The 5' end of HTN S genome RNA extends only to position +1. The various S segment RNAs are shown in panel A, and their origins are indicated by vertical arrows. Circled plus and minus signs refer to antigenomes and genomes, respectively, and the numbering system is shown above each end. A radiolabelled primer (horizontal arrow in panel A) was used to determine the position of the 5' end(s) of the S genome by its extension on two preparations of virion RNA (preparation 1 in lane A and preparation 2 in lane B, panel B). The ends of the extension products were determined by reference to a sequencing ladder of the cloned S segment generated with the same primer. Only the T lane of the ladder is shown, and the relevant sequence is written beside the left-hand T lane.

initiate opposite the 3' OH of each template, it would initiate with UTP. If so, this would be unique, as all viral RNA polymerases which initiate their chains described so far employ only ATP and GTP (1a).

Arenaviruses are another family of segmented negative-strand RNA viruses in which a similar situation occurs. For Tacaribe virus (11), the genome 3' ends are OHGC GU . . . , whereas the 5' ends are pppG CG CA . . . . When genomes and antigenomes are annealed intermolecularly or their complementary ends are annealed intramolecularly, there is a single-base overhang at each 5' end which can be specifically removed with RNase T<sub>1</sub> in a high salt concentration and which appears to be pppG (13, 42). Despite the 3' terminal G of the template, the available evidence suggests that the GTP which started the chain did so opposite the penultimate C (position +2; Fig. 1A contains a description of the numbering system) (14). This pppG is thought to end up as a 5' overhang via a slippage mechanism by being extended to a pppGpC dinucleotide (opposite positions +2 and +3), the pppGpC is proposed to realign upstream on the template by two positions such that its cytidine is opposite the template 3' terminal G, and the 5' pppG overhang is locked in place when the replicase resumes

RNA synthesis processively (14). We were thus particularly interested in locating the positions of the 5' ends of the HTN genomes and antigenomes. We report here that the 5' end is in fact a U at position +1 but that only a 5' monophosphate could be found at this position. The manner in which this 5' end could have arisen is discussed.

## MATERIALS AND METHODS

**Virus and RNA.** RNA was isolated from purified viruses (48) by phenol extraction (preparations 1, 2, and 3A) or with guanidine-CsCl density gradients (7) (preparation 3B).

**cDNA synthesis, PCR, and cloning of the 5' ends of the HTN S genome.** A lightly <sup>32</sup>P-labelled primer (5' CCTCATTTGATTGCTCCTTG 3'), complementary to positions 47 to 28 on the S genome, was extended on virion RNA with reverse transcriptase (RTase). Reaction products were separated on a sequencing gel, and after autoradiography, the portion of the gel corresponding to positions +1 to +8 and the corresponding cDNAs were eluted in 500 mM NH<sub>4</sub>Ac-10 mM MgAc-0.1% sodium dodecyl sulfate-1 mM EDTA overnight at 4°C and then ethanol precipitated with 10 μg of glycogen. Purified primer extension products were cloned by rapid amplification of cDNA ends (RACE) (12). An *EcoRI-PstI-Sall* adaptor, 5' CGAATTCTGCAGTCGAC, that contains a 5' PO<sub>4</sub> and whose 3' end is blocked with ddATP added via terminal transferase (Boehringer) was ligated to the 3' end of the cDNA with T<sub>4</sub> RNA ligase (Biolabs). A primer complementary to the adaptor and a nested primer, 5' GGAATTCGATTGCTCCTTGATTG, complementary to positions 39 to 24 on the S genome and also containing an *EcoRI* site at its 5' end, were then used to amplify the cDNA by PCR. Twenty-five cycles were carried out as follows: 95°C for 10 s, 50°C for 20 s, and 72°C for 10 s. PCR products were checked on a nondenaturing 10% acrylamide gel, phenol extracted, digested with *EcoRI*, cloned into pGEM3, and sequenced.

**Nature of the 5' ends of the S genome. (i) Anchored PCR.** An unmodified adaptor (5' and 3' OH ends), a 17-mer corresponding to the T3 promoter, was ligated with T<sub>4</sub> RNA ligase to differently treated HTN RNAs (Fig. 2). The ligation products were reverse transcribed with a primer complementary to positions 47 to 28 on the S genome and then subjected to PCR with the above-described T3 primer and a nested primer complementary to positions 24 to 39 on the S genome as described above. PCR products were analyzed with a further-nested 5'-<sup>32</sup>P-labelled primer, 5' GATTGTCTTTTTAGGGAG, complementary to positions 27 to 11 on the S genome, in a primer extension reaction with T<sub>7</sub> DNA polymerase (Sequenase) followed by electrophoresis in a 12% sequencing gel.

**(ii) Immunoselection with anti-cap antibodies.** Capped (positive control) RNA was made in vitro with T<sub>7</sub> RNA polymerase and a plasmid (pGEM3HS-17) containing S sequences from positions +1 to +39 in the presence of 250 μM <sup>7m</sup>GpppG. Equal amounts of in vitro-capped T<sub>7</sub> RNAs and HTN RNAs (estimated by primer extension reactions) were reverse transcribed with 5' <sup>32</sup>P-GATTGCTCCTTGATTG 3' (complementary to positions 39 to 24 on the S genome) by using RNase H-negative murine RTase (Gibco, BRL). Polyclonal rabbit anti-cap serum (36) or control serum (anti-Sendai virus C protein) was adsorbed to protein A-Sepharose as previously described (13). The immunoselected capped RNA-cDNA duplexes were analyzed on 10% sequencing gels.

**(iii) Capping, reverse transcription, and RNase treatment.** Equal amounts of pppG-initiated transcripts (made in vitro with T<sub>7</sub> RNA polymerase and pGEM3HS-17) and HTN S RNAs (estimated by primer extension reactions) were capped with the vaccinia virus guanylyl and methyl transferases (BRL) as described by Moss (35) with 100 μCi of [α-<sup>32</sup>P]GTP in the presence of 100 μM S-adenosyl methionine in 100-μl reaction mixtures. After phenol extraction, samples were combined with 20 pmol of an unlabelled primer (complementary to positions 39 to 24 on the S genome) and ethanol precipitated. Primer extensions were performed with RNase H-negative murine RTase. A 200-μl volume of RNase digestion buffer containing 375 mM NaCl, 25 mM Tris (pH 7.4), 2.5 mM EDTA, 20 μg of RNase A per ml, and 5 U of RNase T<sub>1</sub> per ml was added, and the mixture was incubated for 45 min at 25°C. A 28-μl volume of 1% sodium dodecyl sulfate and 40 μg of proteinase K per ml was added, and the mixture was incubated for 30 min at 37°C. Samples were phenol extracted, ethanol precipitated, and analyzed on a 10% acrylamide sequencing gel.

**cDNA synthesis, PCR, and cloning of the 5' ends of HTN S and M mRNAs.** Twenty micrograms of total infected cell RNA (7) was reverse transcribed with either 5' TGGATCTTGTTCCTTCCC (complementary to positions 279 to 262 of the S genome) or 5' GCAAGACAGGTCGTAACAGG (complementary to positions 439 to 429 of the M genome). The RACE procedure of Frohman et al. (12) was carried out, except that the cDNAs were tailed with CTP rather than ATP, and the products were amplified with 5' ATCCGAGGATCCGTCGAC ATCGAAAGGGGGGGGGG (for the C tails) and nested primers 5' gacgtgacGCAATCCTATCTGCC (complementary to positions 243 to 229 of the S genome; sequences in lowercase are nonviral and were added to facilitate cloning) and 5' gacgagctCAGTGGACACTGTCTC (complementary to positions 344 to 329 of the M genome). PCR products were purified on agarose gels, digested with restriction enzymes, cloned in pBluescript, and sequenced.

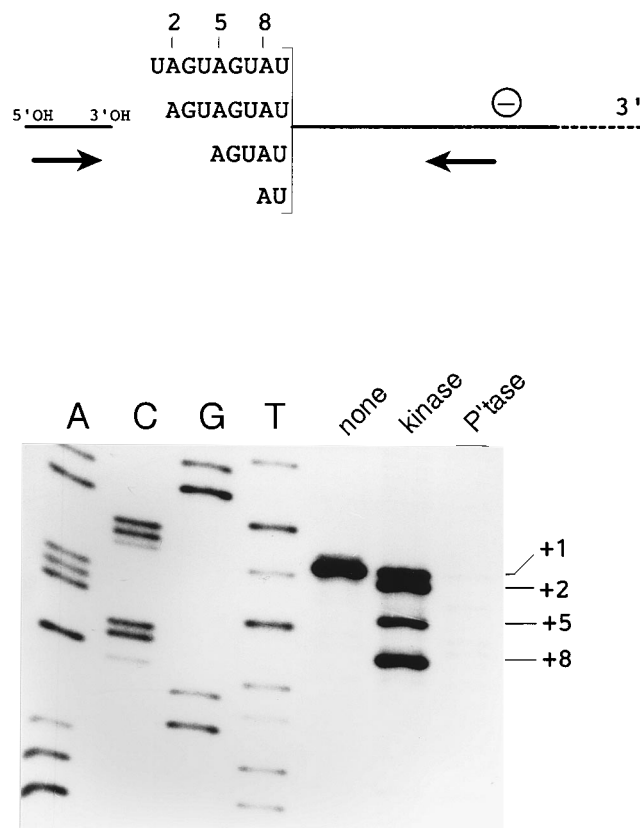


FIG. 2. Determination of the nature of the 5' ends by enzyme treatment and RACE. The experiment is outlined at the top, where the various 5' ends of the S genome determined as shown in Fig. 1 are shown oriented towards the left side. The RACE adaptor, with its 5' and 3' OH ends indicated, is shown on the left, and the primers used for amplification after ligation of the adaptor are shown as horizontal arrows. The RNA of virion preparation 1 was either not treated (lane none), incubated with PK and ATP (lane kinase), or incubated with calf intestinal alkaline phosphatase (lane P'tase) (see Materials and Methods). The various samples were then incubated with the adaptor and T4 RNA ligase and amplified by PCR, and the right-hand (radiolabelled) primer was extended back on the amplified products and also used to generate a sequencing ladder from a cloned S segment (lanes A, C, G, and T). The positions of the various 5' ends shown were determined after taking into account the length of the adaptor.

## RESULTS AND DISCUSSION

**The 5' end of the HTN S genome RNA is the exact complement of its 3' end.** To avoid the possibility that the amplification and cloning involved in RACE procedures might bias the result, we examined the 5' ends of the antigenomes and genomes by primer extension directly on these RNAs. Preparations of intracellular bunyavirus RNAs enriched for antigenomes can be obtained from the 1.31-g/ml fraction of CsCl density gradients, but for HTN, only very small amounts of antigenomes are present and they are also significantly contaminated with mRNAs (data not shown), so these were not examined directly (see below). Genomes, in contrast, can be obtained in relatively pure from virions. An oligonucleotide complementary to positions 47 to 28 was therefore extended on the S genome segment with RTase, and the same primer was used to generate a sequencing ladder from a plasmid containing the 5' end of the S genome, i.e., one which began with 5' UAG UAG UAU . . . (Materials and Methods). The results of this analysis with two preparations of HTN virion RNA (Fig. 1B) show that the primer extended to positions +1, +2, +5, and +8 but that the relative proportions of these ends

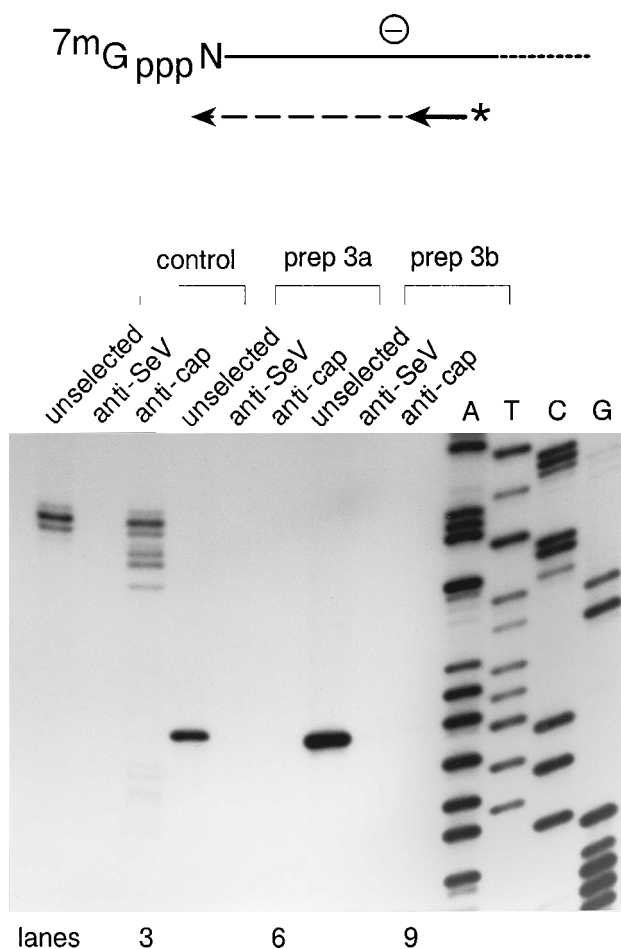


FIG. 3. Inability to demonstrate 5' cap groups on the S genome RNA. A radiolabelled primer (solid arrow marked with an asterisk; outlined at the top) was extended with RNase H-negative RTase on a capped T7 RNA polymerase transcript of the S genome (made in vitro; the 5' end is at position -16) as a positive control and on a third batch of virion RNA prepared either by phenol extraction (preparation [prep] 3A) or without phenol by using guanidine and CsCl density gradients for deproteinization (preparation 3B). Thirty percent of each reaction was incubated with protein A-Sepharose beads coated with an unrelated control antibody (to the Sendai virus C protein [anti-SeV, lanes 2, 5, and 8]), and an equal amount (30%) of the reaction was incubated with beads coated with anti-cap antibodies (lanes 3, 6, and 9). The bound material was recovered by phenol extraction and separated on a 10% sequencing gel, alongside a sequencing ladder of the S genome plasmid generated with the same primer. Fifteen percent of each reaction was not selected with antibodies (unselected, lanes 1, 4, and 7).

varied in the two preparations; they were roughly equally abundant in preparation 1, but position +1 clearly predominated in preparation 2. In a third preparation, moreover, the band at position +1 was the only one visible (Fig. 3). The reason for the variability of ends among different preparations is unclear but appears to be variable degradation with a specific nuclease. The primer extension products from preparation 1 were also cloned by a RACE procedure (Materials and Methods). Fifty-nine clones were sequenced, and remarkably, only three sequences were found. The majority, 40, started with U at position +1, 11 started with A at position +2, 8 started with A at position +5, and all contained the predicted sequence to at least position +35. No clones were found that started at +8. The predominant 5' end of this segment is thus what is expected to be the exact complement of the 3' end of the antigenome, i.e., an RNA that starts with U<sup>+1</sup>. Five prime ends

that begin at position  $-1$ , and which characterize arenavirus genomes in particular, are clearly absent.

**The HTN S genome RNA contains a 5' monophosphate.** RACE, which facilitated determination of the 5' ends of the HTN RNA, was also used to examine the nature of these ends. The amplification procedure, carried out as described above, used an oligonucleotide adaptor whose 5' end was monophosphorylated so that it could be ligated to the 3' OH end of the extended primer, and its 3' end was blocked with dda so that it could not ligate to itself. We examined the nature of the 5' ends of the HTN RNA by using as an adaptor an oligonucleotide that was unmodified at either end (5' OH and 3' OH), so that it could be ligated only to RNAs that contain a 5' monophosphate. The positions of the 5' ends were then determined by (nested) primer extension on the PCR-amplified products, taking into account the additional sequence of the adaptor. When the RNA of preparation 1 was thus examined, only the  $U^{+1}$  RNA was found to contain 5' monophosphate ends by these criteria. Pretreatment of the RNA with calf intestinal phosphatase presumably eliminated its ability to be ligated to the unmodified adaptor (lane P'tase in Fig. 2), as expected. On the other hand, pretreatment of the RNA with polynucleotide kinase (PK) and ATP led to the reappearance of bands equivalent to positions  $+2$ ,  $+5$ , and  $+8$  (lane kinase in Fig. 2), and each of these bands was present in amounts roughly equivalent to that of the  $U^{+1}$  band, i.e., similar to their distribution when the RNA was examined directly by primer extension (Fig. 1B). No change in the pattern of bands occurred when ATP was omitted during the PK pretreatment (data not shown). Since the relative amount of the  $U^{+1}$  band did not increase on PK treatment, this RNA appears to contain relatively few 5' hydroxyl groups. The 5' ends of the  $A^{+2}$ ,  $A^{+5}$ , and  $A^{+8}$  RNAs, on the other hand, appear to contain predominantly 5' hydroxyl groups (they are unlikely to contain di- or triphosphates or caps [see below]).

**Inability to detect 5' di- or triphosphates or cap groups on HTN S genome RNAs.** The above-described results, of course, do not exclude the possibility that the 5' ends of the HTN RNAs contain other groups, in particular, di- or triphosphates or cap groups, i.e., groups that would indicate that RNA synthesis had initiated at position  $+1$ . The 5' monophosphate groups found on at least some of the  $U^{+1}$  RNAs could have arisen by breakdown of these other groups. We first examined the HTN RNAs for the presence of a cap group with the aid of anti-cap antibodies (36). As a positive control, we used a capped T7 transcript (made in vitro) that contains the S genome sequence but starts at position  $-16$ . A radiolabelled primer specific for the S genome was then extended on both RNAs with RNase H-negative RTase, and samples of the resulting RNA-cDNA hybrids were selected with anti-cap antibodies, or an unrelated antibody, bound to protein A-Sepharose beads. This method has previously been shown to be relatively sensitive in detecting capped RNAs, such as rare arenavirus mRNAs (13). As shown in Fig. 3, ca. half of the T7 transcript could be selected with the anti-cap antibody, whereas undetectable amounts of this RNA were selected with the unrelated control serum. Since the extent of capping of the T7 transcript is unknown but is unlikely to be complete, a sizeable fraction of the capped RNA can thus be recovered by this method. None of the HTN S genome RNA, in contrast, could be selectively recovered by the anti-cap antibodies (Fig. 3).

The possible presence of di- or triphosphorylated 5' ends on HTN RNA was investigated by determining whether this RNA could be modified in vitro by the vaccinia virus capping enzyme. This enzyme normally modifies the 5' ends of RNAs

which start with pppA, but it is known to be able to cap directly the 5' diphosphate ends of homopolymers of all four nucleotides (30). Triphosphate ends of primary transcripts are processed by first being converted to diphosphates by the enzyme. However, the enzyme's ability to remove a phosphate from pppU... ends has not been determined (35a). As this information is critical for our approach, we first tried to synthesize an RNA with the precise 5' sequence of the S segment with either T3, T7, or SP6 RNA polymerase, i.e., one which began with 5'-pppUACUACU... Despite numerous attempts, however, we were unable to force these viral RNA polymerases to start on  $U^{+1}$ ; all of the transcripts we obtained started on  $A^{+2}$ . Uncapped RNAs which naturally start with pppU are apparently extremely rare, and we were able to learn of only one example, that of the transcript of insertion element IS1 of *Escherichia coli* (29). RNA was therefore transcribed from a plasmid containing this element with *E. coli* RNA polymerase in vitro. The position of its 5' end was determined by extending a specific primer on this RNA and comparing the products to a sequencing ladder (as for Fig. 2). This end was found to correspond to the sequence 5'-UUUUUGAGGU..., as expected (29). The 5' ends of these transcripts could be capped by the vaccinia virus enzyme in vitro (data not shown). This enzyme can thus be used to determine whether an RNA begins with 5' pppU...

Both HTN RNA and, as a positive control, the T7 transcript of the S genome (which starts with pppG at position  $-16$ ) were incubated with [ $\alpha$ - $^{32}$ P]GTP, S-adenosylmethionine, and capping enzyme to radiolabel their 5' ends. We also combined these two RNAs in a single reaction to determine whether the presence of one RNA is inhibitory to the capping of the other. The presence or absence of a radiolabelled cap group on the RNAs was then determined by extending a nonradioactive primer on these templates with RNase H-negative RTase and treating the resulting RNA-cDNA hybrids with RNase in a high salt concentration to trim the single-stranded RNA tail (see outline in Fig. 4 and Materials and Methods). To provide a size marker for the expected cap-labelled RNA fragment and an estimation of the amount of each S genome RNA present in the capping reactions, a radiolabelled primer was also extended on an equal amount of each uncapped RNA and then treated identically (lanes uncapped in Fig. 4). The results of this experiment show that under conditions in which a significant amount of the control RNA could be capped (i.e., roughly equivalent to the amount of radiolabelled primer which could be extended on this RNA), none of the HTN RNA could be shown to contain a cap group. This inability of the HTN RNA to be capped did not appear to be due to contaminating inhibitors, as its presence did not interfere with the capping of the control RNA in the mixed reaction (HTN + control in Fig. 4). We were thus unable to show the presence of 5' di- or triphosphates on the HTN RNA by this test.

**The 5' ends of HTN mRNAs.** The sequences present at the 5' ends of the HTN mRNAs were determined by a RACE protocol in which a specific primer is extended back to the 5' end of the viral positive-strand RNA present in infected cells. The 3' OH end of the resulting cDNA was then tailed with C residues, and the oligo(C) stretch served as an anchor for a primer extending towards the 3' end of the mRNA. The amplified products were then cloned and sequenced, and the results obtained with the more abundant S and M genome mRNAs were very similar (Fig. 5). Twenty of the 22 S RNAs (Fig. 5A) and 12 of the 13 M RNAs (Fig. 5B) sequenced contained 8 to 17 additional nt, i.e., those upstream of position  $+1$ . These include the mRNAs which lack a TAG repeat (see below). Thirty of the 32 nontemplated sequences were differ-

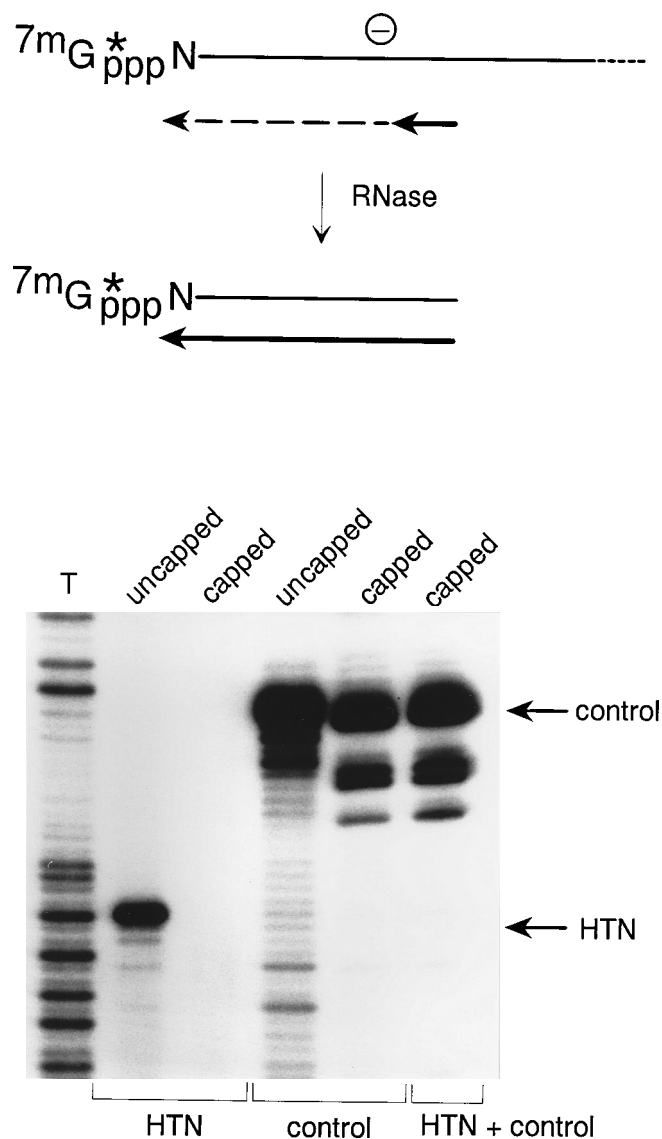


FIG. 4. Inability to detect 5' (p)ppN ends on HTN S genome RNA. HTN S genome RNA (preparation 3A), a pppG-initiated T7 RNA polymerase transcript made in vitro (as a positive control), and a mixture of the two RNAs were incubated in a capping reaction including [ $\alpha$ - $^{32}$ P]GTP. A nonradioactive primer was then extended on the treated RNAs with RNase H-negative RTase, and the resulting RNA-DNA hybrids were treated with RNases A and T<sub>1</sub> in a high salt concentration to trim the single-stranded RNA tails as outlined above and separated on a 10% sequencing gel. To provide a size marker for the cap-labelled RNA fragment and an estimation of the amount of each S genome RNA present in the capping reaction, a radiolabelled primer was also extended on an equal amount of these RNAs, and these samples (lanes uncapped) were then treated identically to those described above.

ent, but a single (and different) sequence was represented twice in each of the S and M RNAs. The remaining 3 of the 35 S and M positive-strand RNAs, which appear to start directly with a U at position +1, are most likely antigenomes.

These results were not unexpected, as nontemplated nucleotides heterogeneous in both length and sequence (and similar in size distribution) are found on mRNAs of the *Bunyavirus*, *Phlebovirus*, *Tospovirus*, and *Nairovirus* genera. These additional sequences are believed to result from a "cap-snatching" mechanism for the initiation of mRNA synthesis that is similar to that described for influenza virus but occurs in the cytoplasm

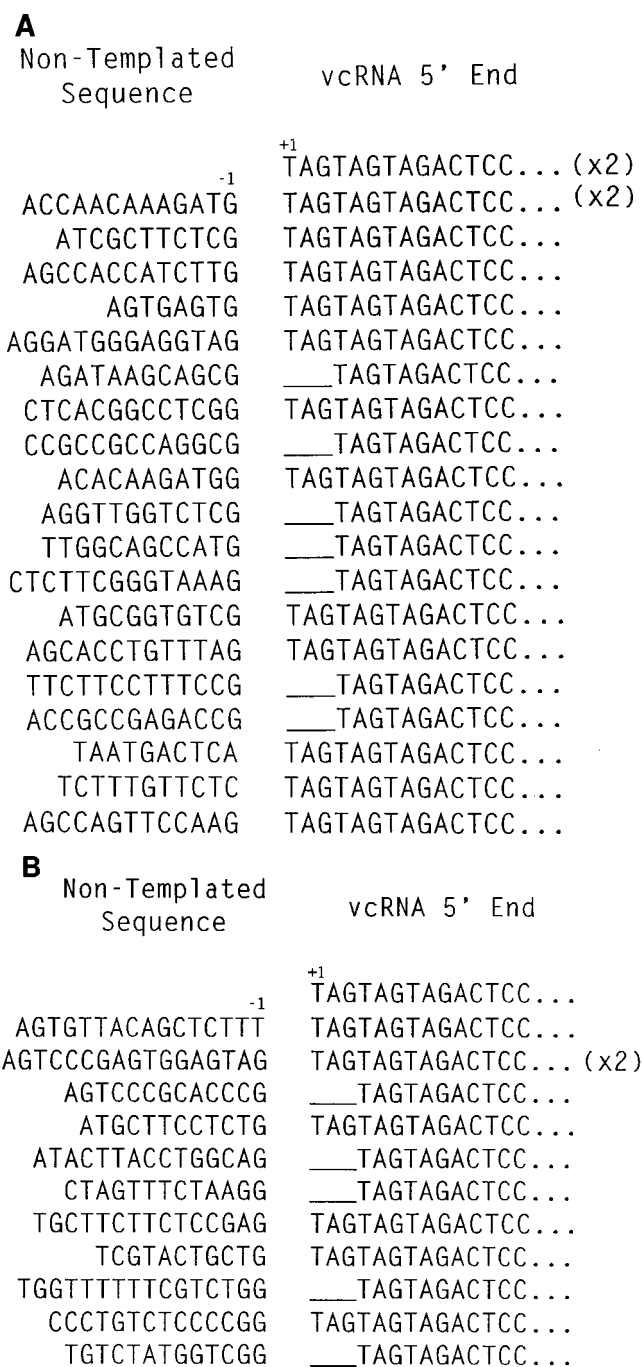


FIG. 5. The 5' ends of the HTN S mRNAs. The number of clones with the same sequence is shown in parentheses. Sequences missing from the expected virally templated sequence are underlined. The sequences of the S and M positive-strand RNAs are shown in panels A and B, respectively.

(44). During cap snatching, the viral polymerase is believed to bind to the capped 5' end of host cell mRNAs, to cleave them (in this case) at ca. positions 7 to 18 from the cap, and to use the capped fragment as a primer for mRNA synthesis. The general heterogeneity of the 5' ends of the HTN mRNAs presumably reflects the large numbers of host mRNAs which serve as a source of the capped primers. There are, however, two features of these sequences which stand out: (i) there is a very strong preference for the base at position -1 (29 of 32

mRNAs contain a G at this position), and (ii) 12 (38%) of 32 mRNAs appear to lack precisely 3 nt at the start of the templated sequence, i.e., they lack one of the three UAG repeats.

The preference for certain bases at positions  $-1$  to  $-3$  of mRNAs initiated by cap snatching has long been noted, and there is also the curious similarity of this preferred sequence at the 3' end of the host primer and the 5' end of the antigenome (20). For influenza viruses whose antigenome begins with 5' AGCA . . . , there is a slight preference for CA or GCA at the 3' end of the primer (2, 27, 50). For phleboviruses whose antigenome begins with 5' AC AC, 6 of 11 Uukuniemi virus sequences analyzed also contained AC at positions  $-2$  and  $-1$  (52). For the *Bunyavirus* genus, whose antigenomes begin with 5' AGU AGU . . . , 9 of 19 Germiston virus mRNA primers contained GU at positions  $-2$  and  $-1$  (5), and 10 of 21 Bunyamwera virus mRNA primers contained AGU at positions  $-3$  to  $-1$  (21). However, the strongest preference reported to date is that for the *Nairovirus* genus, whose antigenomes are expected to begin with 5' UC UC . . . and, like those of the hantaviruses, the 3' ends of whose genomes contain an A rather than a U residue. Of 24 Dugbe virus mRNA primers analyzed, 23 contained a C at position  $-1$ , 14 contained UC at positions  $-2$  and  $-1$ , and 11 contained CUC at positions  $-3$  to  $-1$  (20). Since in virtually all of these cases the mRNAs analyzed were formed in vivo and the particular host mRNAs whose capped ends were snatched as primers are unknown, it is possible that the preferred sequence at the 3' end of the primer simply represents the preference of each viral endonuclease to cut after each preferred sequence, such that in each case a specific subset of host mRNAs would be used. More recently, however, the primers on 13 Germiston bunyavirus mRNAs formed in vitro were analyzed and 12 were found to be derived from rabbit  $\alpha$ - and  $\beta$ -globin mRNAs (53). This too was not unexpected, as bunyavirus mRNA synthesis in vitro, in most cases, is dependent on concurrent translation (reviewed in reference 23), and these Germiston virus mRNAs were made in the presence of a rabbit reticulocyte lysate. Most importantly, in 4 of these 12 cases, a U residue was found interposed between the globin mRNA sequence and that templated from the 3' end of the genome, and in a 5th case, GU was found to be interposed. The preferred U at position  $-1$  in this case could not have been derived from the host mRNA.

Jin and Elliott (20) have pointed out that these apparently nontemplated U and GU residues, as well as the C, UC, and CUC residues at the 3' ends of *Nairovirus* mRNA primers, could be explained by a slippage mechanism similar to that proposed to account for the apparently nontemplated pppG present at the 5' ends of arenavirus genomes. This mechanism, as it applies to the initiation of HTN mRNAs, is depicted in Fig. 6A. (i) There is a strong preference for the viral endonuclease to cut the host mRNA after a G; (ii) the 3' G of the resulting capped fragment aligns opposite the C<sup>+3</sup> of the template; (iii) the primer is elongated for a few nucleotides, e.g., until G<sup>+6</sup> (capped primer-GUAG<sub>OH</sub>), as shown in Fig. 6A, but elongation by only a single nucleotide is sufficient and perhaps preferable; and (iv) before further elongation proceeds, the nascent chain realigns on the template such that the original 3' G of the primer is at position  $-1$  and the following UAG is opposite positions  $+1$  to  $+3$ , thus creating the nearly ubiquitous G at position  $-1$ . mRNAs that lack precisely the first UAG repeat could result when the original G of the capped primer first aligns opposite the template C<sup>+6</sup> and, after elongation by a few nucleotides, realigns on the template such that this G is opposite position  $+3$  rather than position  $-1$  (Fig. 6). This is equivalent to placing this G at position  $-1$  and leaving a 3-nt gap, as in Fig. 5. The curious similarity of the sequences

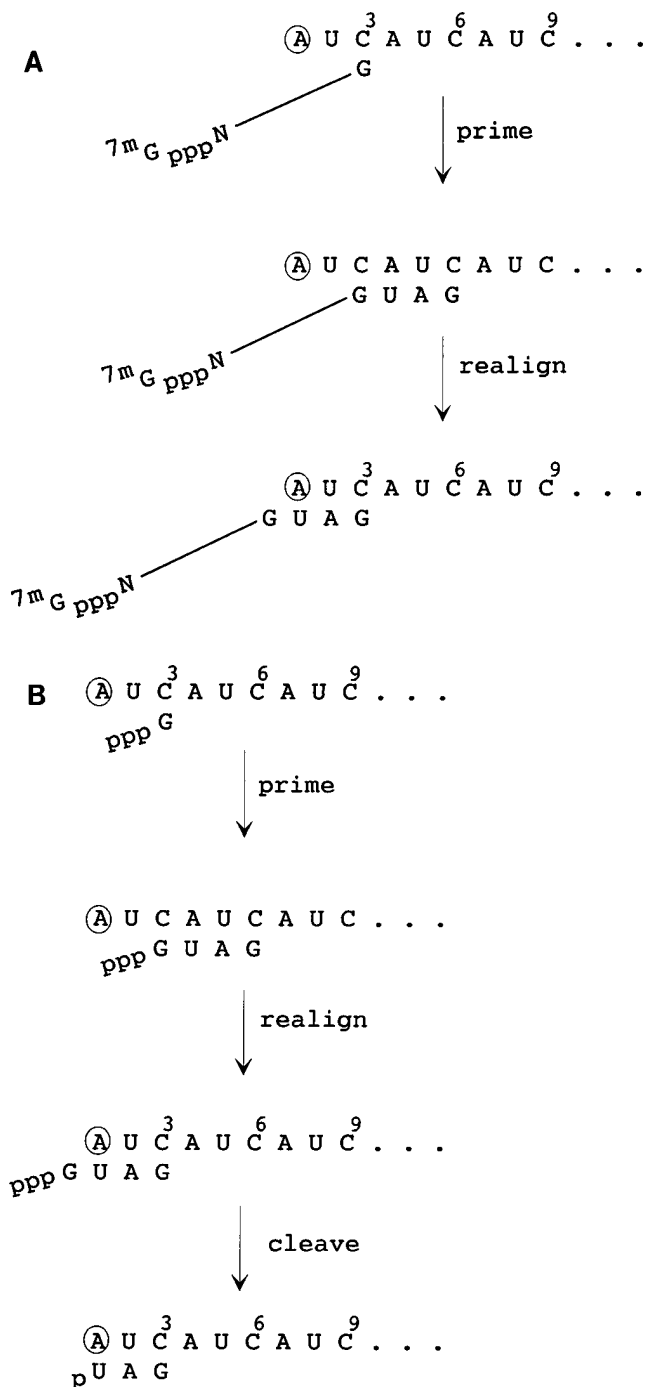


FIG. 6. Prime-and-realign model for initiation of HTN mRNA and genome synthesis. (A) mRNA initiation. The 3' end of the template is shown as the top strand with the 3' terminal A circled; the numbers above the sequence refer to nucleotide positions. The capped host cell primer, ending in a G residue (shown as the bottom strand) first aligns with the template C<sup>3</sup> (top of diagram) and primes mRNA synthesis for a few nucleotides (middle of diagram). The priming of 3 nt (UAG) is shown, but addition of 1 nt would be sufficient. Before further elongation, the slightly extended primer realigns upstream on the template such that the original 3' G of the host primer is placed at position  $-1$  (bottom of diagram). If the 3' terminal G of the host primer were first to align opposite C<sup>6</sup> of the template and were after addition of a few nucleotides, to realign upstream so that the original 3' G of the primer were opposite C<sup>3</sup>, the result would be deletion of one of the terminal triplet repeats. (B) Genome initiation. The proposed mechanism is very similar to that of mRNA initiation, except that the genome chain is initiated with GTP and a cleavage step occurs after realignment to remove the initiating triphosphate, leaving pU at position  $+1$ .

at the 3' end of the host primer and the 5' end of the antigenome, as well as the precise deletion of a single trinucleotide repeat in a large fraction of the HTN mRNAs, can thus be explained by this prime-and-realign mechanism, which may be a general feature of mRNA initiation in the family *Bunyaviridae*.

**A prime-and-realign model for initiation of genome chains.** All of the viral RNA polymerases described to date which initiate their chains with triphosphates do so with either ATP or GTP. This preference for purines for chain initiation occurs, remarkably, even when the 3' end of the viral genome is itself a purine and RNA would be expected to start with a pyrimidine triphosphate at position +1, i.e., opposite the 3' terminal purine. Besides the Tacaribe arenavirus genomes mentioned earlier, those of bacteriophage  $\phi$ B are germane to this study, as they also end in adenosine. The 3' ends of the linear  $\phi$ B genomes and antigenomes are  ${}_{\text{OH}}\text{ACCC} \dots$ , whereas their 5' ends are pppGGG  $\dots$ , and when they are aligned, there is a single A at each 3' end for which there is no partner at the 5' end, i.e., an overhang. Despite the 3' A, however, RNA synthesis begins with GTP opposite the penultimate cytosine (at position +2). The overhang arrangement of genome ends is maintained because the 3' adenosine is presumably added in a nontemplated manner by the viral replicase, e.g., in the act of terminating RNA synthesis (54). Thus, even for arenavirus and bacteriophage RNA genomes which contain purines at their 3' ends, RNA synthesis is not initiated by a pyrimidine triphosphate opposite these ends. Rather, unusual mechanisms which initiate with GTP are employed, and the resulting overhang arrangements of ends are apparently maintained by compensatory additions-deletions during chain termination. The reason for this preference for purines in initiation is unclear, but it appears to be a general property of RNA polymerases. Eucaryotic RNA polymerase III also appears to initiate its chains only with ATP or GTP (15). RNA polymerase II, on the other hand, initiates its RNAs with CTP or UTP ca. one-third of the time, judging by the distribution of 2-*O*-methylated nucleotides 5' to the cap within heterogeneous nuclear RNA (45). Most *E. coli* RNA polymerase transcripts are also initiated with ATP or GTP. However, when position +1 of *E. coli* promoters is changed to C or U, the start site becomes less precise but a significant fraction of the chains do start with a pyrimidine triphosphate (28). Some RNA polymerases can thus start with CTP or UTP, but even here there is a preference for ATP or GTP.

The present study of HTN has also failed to provide evidence that these viral genomes start with UTP. The only 5' ends which could be demonstrated are 5' monophosphates, and we see two explanations for their origin. They could have arisen by the action of a pyrophosphatase which cannot remove the  $\alpha$ - $\text{PO}_4$  (e.g., the acid pyrophosphatase from tobacco [51]) on ends which were originally triphosphorylated, although a similar enzyme has not been described for animal cells. It is unlikely that the 5' pU arose from the action of the more common phosphatases, as these would preferentially remove the  $\alpha$ - $\text{PO}_4$ , and we were unable to demonstrate 5' OH ends by using PK (Fig. 2). The second and more intriguing possibility is that the 5' pU ends arose by endonuclease action on a longer chain. All *Bunyaviridae* polymerases are thought to contain a cap-dependent endonuclease (39), which in this case appears to cleave host cell mRNAs after G residues, leaving a 5'  $\text{PO}_4$  downstream. We also found evidence in this study that during the initiation of HTN mRNA synthesis, the 3' G of the capped host RNA fragment primes mRNA synthesis not at the precise 3' end of the template but internally and then realigns upstream on the template. A similar prime-and-realign mech-

anism has been suggested to account for how arenavirus genomes acquire a 5' pppG at position -1. One possible explanation for the 5' pU at position +1 is that HTN genomes also initiate with GTP at an internal position, presumably opposite the template  $\text{C}^{+3}$  as suggested for the initiation of its own mRNAs (Fig. 6B). Further, the genome chain would again be extended for only a few nucleotides before it realigned upstream and the polymerase could continue processively, such that the initiating G would be at position -1. The proposed difference between the arenavirus and HTN mechanisms is that the endonuclease activity of the HTN polymerase would then cleave the unpaired pppG at -1, leaving the  $\text{pU}^{+1}$  as the new 5' end. Thus, in contrast to mRNA initiation, the same polymerase-associated endonuclease would cleave the nascent chain in the same way (3' to an unpaired G), except that this cleavage would occur after rather than during initiation, and the requirement for a cap group would somehow be relieved. Although it is highly speculative, this or a similar mechanism is required to account for how the 5' ends of the HTN genomes are generated if this viral polymerase is not to be the first which initiates its chains with a pyrimidine triphosphate.

There is much recent evidence that bacterial and eucaryotic RNA polymerases are capable of extraordinary internal flexibility (6a). When RNA polymerase ternary complexes (with template and nascent RNAs) are stalled or arrested, their catalytic sites can be displaced upstream along the nascent RNA chain by as much as 17 nt without disrupting the ternary complex (21a, 37a). When in this altered state, they can be induced to act as an endonuclease (by GreA or GreB in *E. coli* and TFIIIS in *Saccharomyces cerevisiae*) by adding water across an internal phosphodiester bond to generate a 3' OH end upstream and a 5'  $\text{PO}_4$  downstream, similar to their action during pyrophosphorolysis (the reverse reaction to nucleotide addition) (44a). This endonuclease activity is thought to be an intrinsic property of all RNA polymerases, and although described so far only during the elongation phase of RNA synthesis, it may well have evolved into the endonuclease which cleaves host cell mRNAs in those animal and plant viral polymerases which cap snatch to initiate their mRNAs (influenza viruses, bunyaviruses, arenaviruses, and tenuiviruses [17a, 42a], i.e., all of the segmented negative-sense or ambisense RNA viruses). The propensity of RNA polymerases to slip back on the template during initiation while retaining the nascent chain, thus causing repetitions at the 5' end of the nascent RNA, may also be a more general property of these enzymes. *E. coli* RNA polymerase is known to do this spontaneously on certain promoters, and this phenomenon has been called primer shifting (3a, 49a). Interestingly, primer shifting is also simulated by GreA or GreB, which suggests that the endonuclease and slippage activities may be linked for the bacterial enzyme (10a).

**Maintenance of genome integrity.** Prime-and-realign (or slip-back or jump-back) mechanisms which initiate viral genome synthesis require terminal sequence repetitions, and all five *Bunyaviridae* genera contain such di- or trinucleotide repeats at their ends. There is some evidence that pseudotemplated RNA synthesis (19) occurs to a greater or lesser extent during the initiation of mRNA synthesis in all members of the family *Bunyaviridae*, but this mechanism seems to apply only to the *Hantavirus* (and possibly the *Nairovirus*) genus for genome synthesis. A similar mechanism for genome initiation has also been proposed for DNA viruses with linear genomes and primer-dependent polymerases, in which priming occurs from a nucleotide linked to the precursor of a terminal protein (pTP). The most recent example of this is adenovirus (type 5), whose genome ends in 3'  ${}_{\text{OH}}\text{GTA GTA} \dots$  and DNA synthesis is

initiated with pTP-dC. By using an *in vitro* system, King and van der Vliet (22) have found that the primer is first elongated to pTP-CAT, but opposite the second 3' GTA at positions 4 to 6, and then jumps back to realign opposite positions 1 to 3 before elongation can proceed. The adenovirus polymerase is known to change its affinity for dCTP and its sensitivity to inhibitors of DNA synthesis when in the initiation and elongation modes, and these researchers have suggested that the proposed jumping back is coupled to a conformational change in the polymerase's active site. Very similar sliding-back mechanisms have been proposed for the initiation of bacteriophage  $\phi$ 29 (31) and PRD1 (6) DNA synthesis, and this may be a general mechanism for protein-primed DNA synthesis.

There is one other feature of this mechanism that requires comment, namely, its ability to repair damaged genome ends by restoring small terminal deletions and mutations. The slipping-back mechanism was first suggested to explain the regeneration of intact adenovirus termini from plasmids lacking 2 nt at either end (16), and a similar repair of the missing 2 nt has been shown to occur *in vitro* (22). The regeneration of damaged ends by using pseudotemplated synthesis and terminal sequence repetitions would, of course, also apply to RNA viruses and may be important in maintaining virus infectivity when these ends undergo limited damage. Genomes which lack a few nucleotides at the 3' end can be repaired by simply extending this end on an intact complementary 5' end. Genomes which lack a few nucleotides at the 5' end, such as those noted in the present study for HTN or for arenaviruses (32, 33), however, require a different mechanism for repair, as conventional RNA-DNA synthesis takes place only in the 5'-to-3' direction. The net result of the prime-and-realign mechanism is that the 5' end of the RNA or DNA has, in fact, grown in the 3'-to-5' direction.

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