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Evolution of the 5′-End of Genomic RNA of Murine Coronaviruses during Passages *in Vitro*

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The 5′-ends of the genomic RNA and subgenomic mRNAs of murine coronavirus (MHV) have a stretch of approximately 70 nucleotides of leader sequences. The 3′-region of this leader sequence contains several repeats of a pentanucleotide (UCUAA), whose number varies among different MHV strains. It has been demonstrated that this UCUAA repeat plays crucial roles in the discontinuous transcription of MHV mRNAs. In the present study, we demonstrate that the number of UCUAA repeats in the leader sequence of MHV genome rapidly decreases during serial passages of viruses on susceptible cells. The downward evolution of the number of UCUAA repeats was not due to a higher growth rate of the viruses with fewer repeats, but seemed to be due to homologous interference between viruses with different numbers of UCUAA repeat. The ease with which these variant viruses arose suggests the high frequency of the occurrence of this deletion during RNA replication. This finding is in agreement with the proposed discontinuous and nonprocessive mode of coronavirus RNA synthesis. Analysis of the intracellular subgenomic mRNA species of viruses with different numbers of UCUAA repeats and of MHV recombinant viruses suggests that the number of this pentanucleotide repeat at the 3′-end of the leader sequence may regulate the synthesis of certain mRNA species, in agreement with the leader-primed transcription mechanism.

Mouse hepatitis virus (MHV) is a member of Coronaviridae, which have been associated with respiratory illnesses, gastroenteritis, and neurological diseases (*24*). The virus contains a single-stranded, positive-sense RNA genome of more than 6×10^6 Da. (*10, 23*). Several different MHV strains have been isolated from a variety of mouse strains in different geographical areas under diverse conditions. These MHV strains differ in their pathogenic potential, antigenic properties, and other biological properties. Oligonucleotide fingerprinting studies of the genomic RNAs of these MHV strains showed that their sequences are very heterogeneous (*8, 11*). Some of the heterogeneity of viral RNA probably resulted from single base mutations, which are frequently observed in RNA viruses (*3*). Another potential mechanism contributing to this genomic heterogeneity is RNA recombination, which has been demonstrated to occur at a very high frequency among coronaviruses (*12*). The relevancy of RNA recombination to the evolution of MHV has been suggested by the occurrence of recombination during coronavirus infection in mouse brain (*4*). We have proposed that the high frequency of coronavirus recombination is probably related to the observation that coronavirus RNA synthesis occurs by a mechanism of discontinuous and nonprocessive transcription, in which RNA synthesis frequently pauses at points of secondary structure (*1*). The RNA intermediates may dissociate from the template and subsequently reassociate with the template to continue transcription. This type of transcription may also account for the generation of defective-interfering (DI) RNA during high multiplicity passages of coronavirus (*13, 16*). Here we report another type of genomic heterogeneity and evolution of coronaviruses, which may also be the consequence of discontinuous and nonprocessive RNA synthesis.

The 5′-end of the genomic RNA of MHV contains a stretch of approximately 70 nucleotides of leader sequences (*5, 9, 22*), which are also present at the 5′-ends of every subgenomic mRNA (*5, 9, 22*). The leader sequence has been implicated in the priming for subgenomic mRNA synthesis of MHV (*15*). At the 3′-end of this leader sequence, there are several repeats of a pentanucleotide sequence, UCUAA (*21*), and the number of repeats of this sequence varies among different MHV strains (*14*). This repeat sequence has been shown to play a particularly crucial role in MHV subgenomic mRNA transcription (*14*). Our preliminary studies indicated that the uncloned MHV often contained a mixture of genomic RNA with different numbers of the UCUAA repeat at its 5′-ends (*21,* unpublished observations). This observation suggests that this repeat sequence may amplify or delete during passages. We therefore sought to determine the possible evolution of the 5′-end sequences of MHV genome during serial passages of the virus.

Three different MHV strains, JHM, B1, and JHM-2c, were used for this study. JHM is a prototype of MHV. B1 is a recombinant virus between JHM and A59 strains of MHV, and contains mostly A59-derived se-

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quences, except for approximately 3 kb at the 5′-end, which is derived from JHM sequence (Fig. 3B) (*6*). JHM-2c is a JHM-derived small plaque mutant isolated from a persistently infected cell culture (*17*). All three viruses were plaque-purified before use. These viruses were used to infect DBT cells (*2*) at a multiplicity of infection (m.o.i.) of one. Progeny viruses were harvested from the media of infected cultures at the height of cytopathic changes, which occurred at approximately 14 hr postinfection (p.i.) for JHM and B1, and 24 hr p.i. for JHM-2c. The viruses were further serially passaged without dilution on DBT cells. The viruses collected at selected passage levels were used for biochemical characterization of the virion genomic RNAs. This was carried out by infecting DBT cells with viruses at an m.o.i. of 0.0002 to eliminate the DI particles, which were generated during serial undiluted virus passages (*16*) (data not shown). The virus samples collected from this low m.o.i. infection were propagated one more time without dilution, and a 10-fold-diluted virus stock was then used to infect a mass culture. The virus collected from this mass culture was purified and the virion genomic RNA was extracted (*16*). The genomic RNAs were used for the examination of the heterogeneity of the 5′-end sequences. This was carried out by primer extension studies using a specific primer (oligo 1,5′-AATGTCAGCACTATGACA-3′) complementary to nucleotides 123–140 from the 5′-end of the genomic RNA of JHM (*21*), thus representing sequences slightly downstream of the leader RNA. The 5′-end-labeled primer (*20*) was hybridized to the virion genomic RNA and extended with reverse transcriptase. Primer extension products were then analyzed by electrophoresis on 6% polyacrylamide gels containing 8 *M* urea. As shown in Fig. 1A, the original plaque-cloned virus of each strain yielded a single cDNA product, whereas viruses after several undiluted passages yielded an additional cDNA product, which appears to be smaller than the original cDNA by five nucleotides. The relative amount of the faster migrating cDNA band increased as passage levels of viruses increased. This result indicates that a small RNA species appeared during serial undiluted passages of these viruses, although the speed of appearance of this RNA species varied with different virus strains. These primer-extended products were eluted from the gels and sequence information was obtained by the Maxam–Gilbert method (*18*). This sequence represents the first 140 nucleotides at the 5′-ends of each genomic RNA species. Only the sequences around the UCUAA repeats are shown in Fig. 1B. The sequences of the original JHM and B1 were identical and both contained three UCUAA repeats. The faster migrating cDNA bands of both viruses at later passages had only two UCUAA repeats. In contrast, the original JHM-2c contained four UCUAA repeats but lacks nine nucleotides immediately following the UCUAA repeats. Similar to the cases of JHM and B1, the faster migrating cDNA band of JHM-2c contained one fewer UCUAA repeat. These results indicated that the number of UCUAA repeats in the leader sequence of MHV genome rapidly evolved from a high number to a lower number during serial undiluted passages. We have not passaged JHM-2c further; thus, we do not know whether a virus with only two UCUAA repeats will eventually appear from JHM-2c after additional passages. Since JHM-2c (four repeats) was originally derived from JHM (three repeats) during persistent infection (*17*), we have also performed serial passages of JHM under conditions of persistent infection in an attempt to isolate viruses with increased repeat number. However, such viruses have not been detected by primer extension studies as described above. Thus, the exact growth conditions favoring viruses with a higher repeat number is not yet clear.

The finding that viruses with a lower number of UCUAA repeats rapidly became the dominant virus population suggests that these viruses may have a growth advantage over those corresponding viruses with a higher number of repeats under the conditions of serial undiluted passages. We therefore compared the growth properties of viruses with different number of the UCUAA repeats. We isolated several plaque-cloned viruses with two, three, and four UCUAA repeats, respectively. This was achieved by isolating individual plaques derived from JHM strain at passage 11, JHM-2c strain at passage 15, and B1 strain at passage 5, when the viruses with higher and lower numbers of the pentanucleotide repeat were roughly equivalent in amount (see Fig. 1A). The individual plaque isolates were then screened for the number of UCUAA repeats by primer extension studies as described above. The pairs of the same virus strains with different number of repeats were then examined for the kinetics of virus growth and the amount of viral RNA synthesis. No difference in either of these two parameters between any two pairs of viruses was found (data not shown). This result indicates that the accumulation of viruses with fewer UCUAA repeats was not due to a higher growth rate of these viruses. We then examined whether the accumulation of low-repeat virus population was due to the possible interference between the two viruses during serial passages. For this purpose, DBT cells were coinfected with plaque-cloned JHM virus with two UCUAA repeats (JHM(2)) and JHM virus with three repeats (JHM(3)) at an m.o.i. of 1 each. The virus harvested was propagated without dilution for an additional four times to mimic the conditions of virus passage used in this study. The viruses harvested at

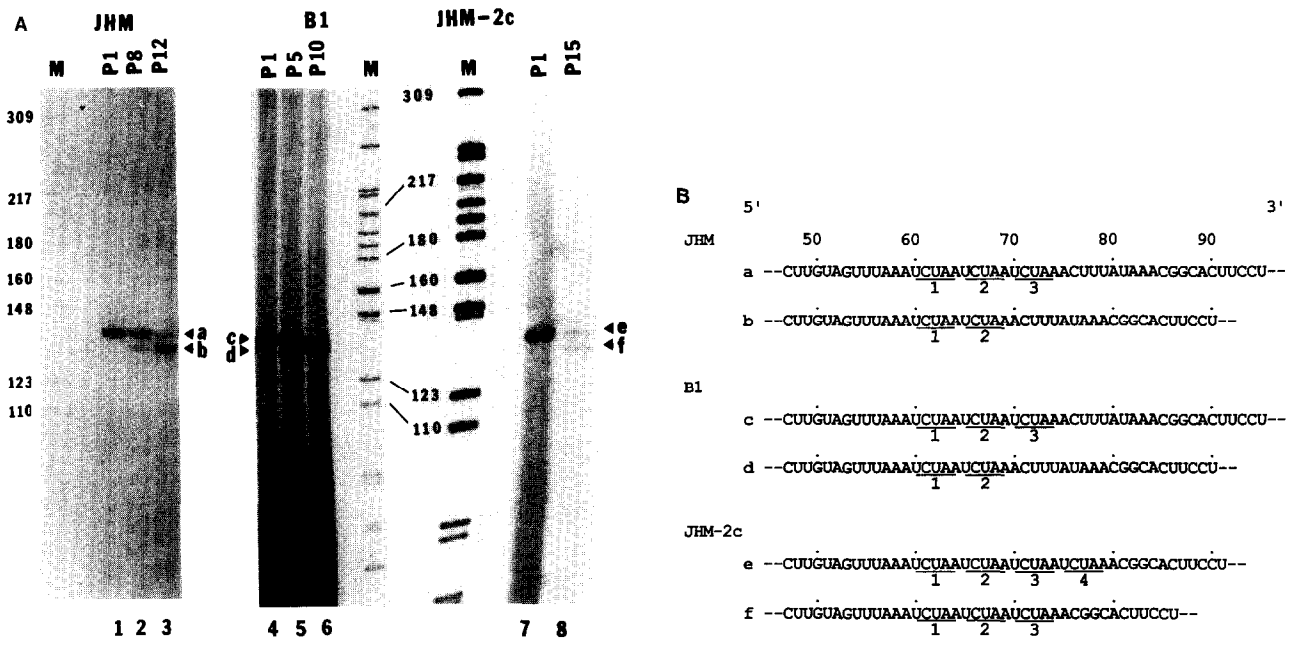

FIG. 1. Primer extension analysis of the 5'-end of genomic RNA of MHVs obtained after serial passages. (A) A synthetic oligodeoxyribonucleotide, oligo 1, was 5'-end-labeled with [γ-^{32}P]ATP by polynucleotide kinase (*20*). Genomic RNA of MHV was incubated in 8 μl of distilled water containing 10 m*M* methyl mercury. After 10 min of incubation at room temperature, RNA was further incubated in 50 μl of buffer containing 60 units of RNasin (Promega Biotech), 10 m*M* $MgCl_2$, 100 m*M* KCl, 50 m*M* Tris–HCl, pH 8.3, at 42°, 10 m*M* DTT, 1.25 m*M* each of dATP, dCTP, dGTP, and TTP, 28 m*M* β-mercaptoethanol, 5'-end labeled oligo 1, and 20 units of AMV reverse transcriptase (Life Science) at 42° for 1 hr. Reaction products were extracted with phenol/chloroform, precipitated with ethanol, and analyzed by electrophoresis on 6% polyacrylamide gels containing 8.3 *M* urea. Lanes 1, 2, and 3 represent primer-extension products of JHM genomic RNA isolated from passages 1, 8, and 12, respectively. Lanes 4, 5, and 6 represent primer-extension products of B1 genomic RNA isolated from passages 1, 5, and 10, respectively. Lanes 7 and 8 represent primer-extension products of JHM-2c genomic RNA isolated from passages 1 and 15, respectively. M represents marker DNAs. (B) The RNA sequences obtained from (A) are compared. Only the sequences at the leader junction region are shown. Numbers from 50 to 90 denote the nucleotide number from the 5'-end of genomic RNA (*21*). The five-nucleotide repeats are underlined and numbered. Sequences of a to f correspond to the cDNA bands of a to f in (A).

the end of five passages were used for primer-extension study using the 5'-end-labeled oligo 1 as the primer (Fig. 2). It is evident that, in the coinfected cells, although the amount of virus with two UCUAA repeats was almost equal at passages 1 and 5, the yield of the virus with three repeats was significantly lower after five passages. In contrast, the yields of both viruses remain constant through five passages in single infections (Fig. 2). This result suggests that the virus with two repeats could interfere with the replication of the virus with three repeats. It is significant that under the conditions of virus passages used here, no DI particles were detected (data not shown). Thus, the accumulation of the viruses with lower repeat numbers during serial passages was not due to resistance to DI particles.

This finding that various strains of MHVs rapidly evolved into viruses with fewer UCUAA repeats suggests that the generation of these RNA species occurred very frequently. It has been proposed that MHV RNA replication occurs by a discontinuous and nonprocessive mechanism, i.e., RNA synthesis is frequently interrupted at sites of secondary structures on template RNA, releasing the incomplete RNA products which subsequently rejoin the template to resume synthesis (*1*). It is significant that the UCUAA repeat sequences are located within a probable hairpin loop (*21*). If the rejoining of incomplete RNA products containing at least one UCUAA sequence occurs imprecisely at UCUAA repeat site on the template RNA during RNA replication, a genomic RNA with a different number of UCUAA repeats will be generated. The precise mechanism of the dominance of the viruses with fewer UCUAA repeats is not clear, although our data indicate that virus interference is at least one of the mechanisms.

The data obtained above thus suggest that the number of the UCUAA repeats has significant effects on the biological properties of the virus. Since we have previously demonstrated that the pentanucleotide sequence plays a crucial role in the leader-primed transcription of coronavirus subgenomic mRNAs (*14*), it is possible that the number of UCUAA repeats at the 3'-end of leader sequence may affect the binding of leader

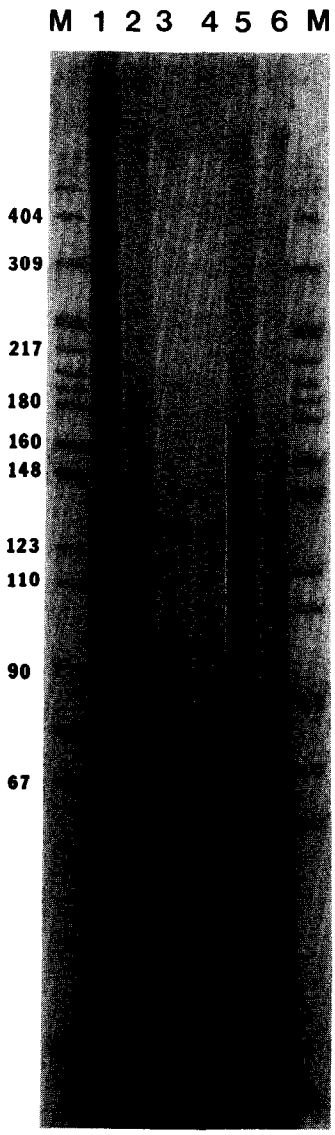

FIG. 2. Primer extension analysis of the 5′-end of JHM genomic RNA obtained from passages 1 and 5. The 5′-end-labeled oligo 1 was hybridized to JHM genomic RNA and primer-extended as described in Fig. 1A. Lane 1, passage 1 of JHM(3). Lane 2, passage 5 of JHM(3). Lane 3, passage 1 of JHM(2). Lane 4, passage 5 of JHM(2). Lane 5, passage 1 of coinfection with JHM(2) and JHM(3). Lane 6, passage 5 of coinfection with JHM(2) and JHM(3). Marker DNAs were run in parallel.

RNA during subgenomic mRNA transcription, and consequently alter the amount and species of mRNAs synthesized. We therefore examined the intracellular virus-specific mRNA of various viruses with different numbers of pentanucleotide repeats. As shown in Fig. 3A, all the virus strains examined synthesized seven well-characterized MHV-specific mRNA species (*7*) although the amounts of several mRNAs were variable; for instance, mRNA 4 of B1 virus with three repeats (B1(3)) and that of JHM(3) were relatively lower in quantity. In addition, JHM(2) synthesized an extra RNA species, 2a, while B1(3) synthesized an extra RNA, 3a. Only a trace amount of RNA 3a and RNA 2a was synthesized in cells infected with B1 virus with two UCUAA repeats (B1(2)) and with JHM(3), respectively. An additional minor RNA species, 1a, was also noted in B1(3) and JHM(3). We have examined several different clones for each virus strain. The intracellular RNA patterns of different clones were identical (data not shown). Thus, these RNA patterns were characteristic of the genotype of each virus. In contrast, JHM-2c with three or four UCUAA repeats does not synthesize any extra RNA species (*17*) (data not shown). Since MHV mRNAs have a nested-set structure, and mRNA synthesis involves the binding of the 3′-end of a free leader RNA to the template RNA at the intergenic start sites (*15, 21*), the synthesis of any novel RNA species suggests the presence of a new sequence complementarity between the 3′-end pentanucleotide repeats of free leader RNA and an internal site of the template RNA. It should be noted that B1 virus is a recombinant virus whose genome consists of the leader RNA of JHM and most of the coding sequence from A59 (*6*), and that the 3′-end of A59 and JHM leader sequences are identical, except that A59 has two UCUAA repeats while JHM has three (*14*). Thus, we propose that the interaction between the two UCUAA repeats with JHM sequence in gene B region (see Fig. 3B) generated RNA 2a. In contrast, the interaction between the three UCUAA repeats and A59 sequences in gene C region generated RNA 3a. The minor RNA 1a may be the result of interaction between three repeats and the JHM sequence in gene A, or alternatively it could represent a DI RNA. To support these interpretations, we have examined intracellular virus-specific RNAs of additional recombinant viruses (*4, 12*) (Fig. 3A), the structure of which is illustrated in Fig. 3B. In agreement with the interpretation above, the presence of mRNA species 2a correlated with a two-repeat leader and JHM gene B sequence, whereas RNA 3a could be detected only from viruses with a three-repeat leader region and A59 gene C sequence (Figs. 3A and 3B). RNA 1a appears to be the result of combination of three repeats and the JHM sequence. In addition, the amounts of mRNAs 4 and 5 were quite variable among different viruses. Whether this variation is also correlated with any genetic sequence of virus is not known at this time.

The data obtained here provide additional support for the leader-primed transcription mechanism of MHV subgenomic mRNAs, in which the 3′-end of a free leader RNA binds to the complementary sequence at the intergenic sites of the template RNA and then serves as the primer for mRNA transcription (*21*). The finding that the presence or absence of novel RNA spe-

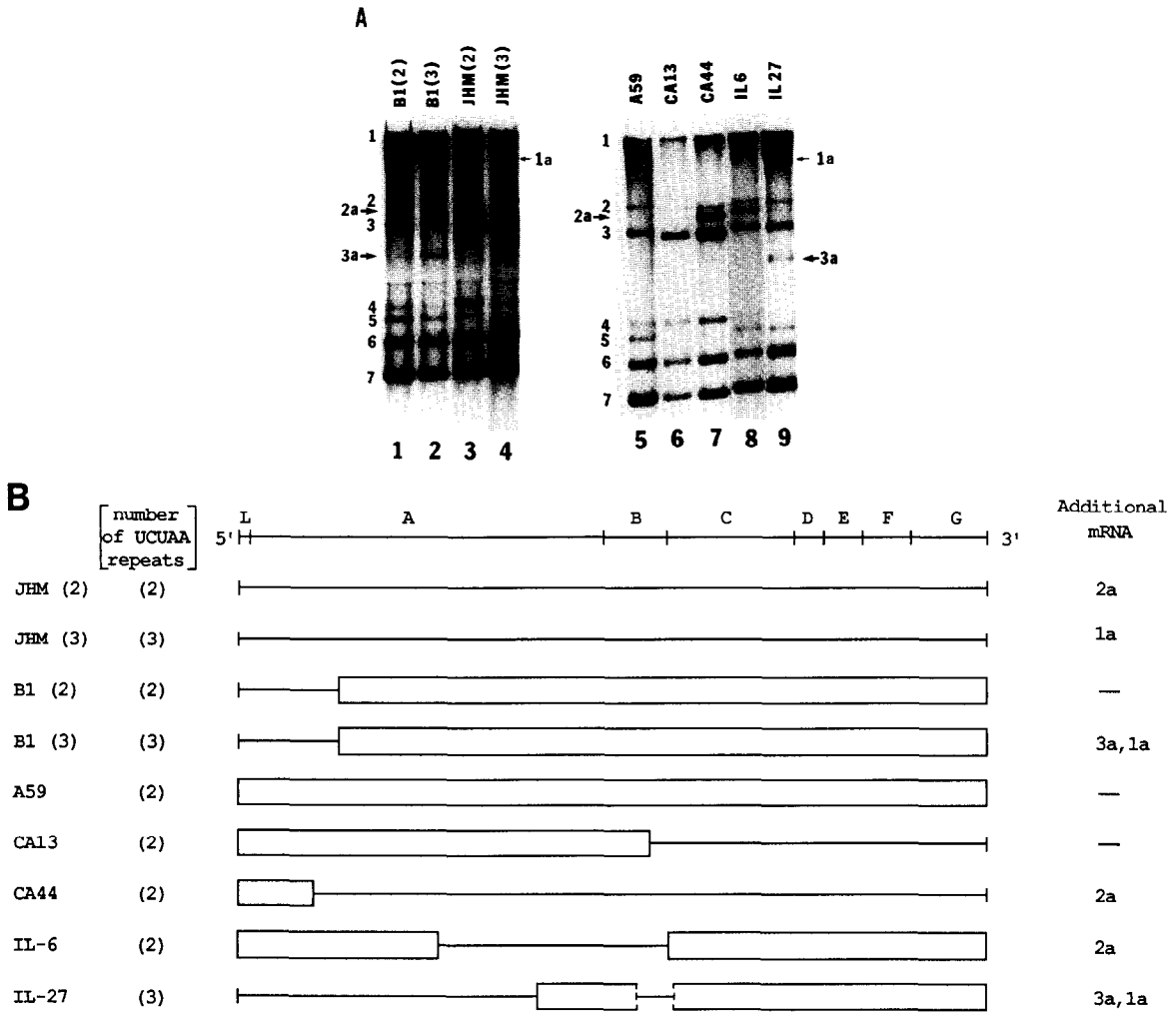

FIG. 3. Intracellular virus-specific mRNA species of different MHVs. (A) ^{32}P-labeled RNA from MHV-infected cells was electrophoresed in 1% agarose gels after denaturation with glyoxal (*18*). Numbers 1–7 at the left sides of each gel represent the MHV-specific mRNA species. Additional mRNAs, 1a, 2a and 3a, are indicated by arrows. Isolation and characterization of each recombinant MHV were described previously (*4, 6, 12*). (B) Schematic drawing of the genetic structure of the MHV recombinants. L represents the leader region. A through G are the seven genes described (*7*). The solid line designates JHM-derived sequences and the boxed areas represent A59-derived sequences. Presence of RNA 1a, RNA 2a, and RNA 3a is also indicated.

cies in some MHV strains correlated with either two or three UCUAA repeats and either A59 or JHM internal genomic sequences gives an additional support for the importance of this repeat sequence in leader-primed transcription (*14*). It is tempting to speculate that the amount of mRNAs 4 and 5 synthesized is also regulated by the interaction between the repeat sequence and the intergenic regions. The exact sequence requirement for MHV mRNA initiation may be learned from sequences of the initiation sites of RNAs 2a and 3a. Such studies are currently in progress.

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