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Nucleotide Sequences That Affect Replicative and Transcriptional Efficiencies of Sendai Virus Deletion Mutants

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Structural features of the genomes of virus deletion mutants (DI virions) influence their replication efficiency. Among nonsegmented negative-strand RNA viruses, substitution of the genomic 3′ terminus by a complementary copy of the 5′ terminus (so-called "copy-back" sequence) could enhance replication either because the new 3′ end is a better promoter of RNA replication or because DI RNAs that possess this sequence are incapable of acting as templates for transcription. Here we provide evidence that both mechanisms operate in mixed infections with Sendai virus DI RNAs. RNAs incapable of transcription always outgrew RNA species that were transcribed. This was true even when the 3′-terminal sequence of the untranscribed RNA was identical to the genomic 3′ terminus, as in the case of an internally deleted DI genome (RNA Ra) rendered transcriptionally inert by point mutations of bases 47 and 51 at the 5′ end of the positive-strand leader RNA template. Nevertheless, Ra was outgrown by a copy-back DI RNA, indicating that the 3′ genomic end of Ra is a less efficient site for replication initiation than the copy-back sequence.

Deletion mutants arise spontaneously as by-products of the replication of many viruses (9). The survival of these mutants depends on the ability of their genomes to be recognized by the replicative machinery that serves the parental virus, their encapsidation by viral core proteins, and their packaging into virions. If a deletion mutant has a selective advantage over the parental virus, it will outgrow it and interfere with its replication. Hence, deletion mutants are commonly termed defective-interfering (DI) virions (9).

Interference can occur not only between a DI genome and its parent but also between DI genomes themselves. Among nonsegmented negative-strand RNA viruses, some observations of competition between vesicular stomatitis virus DI genomes have been reported, but these observations have not always been interpretable mechanistically (14–16). However, the relative rarity of vesicular stomatitis virus DI RNAs with internal deletions, compared to the abundance of DI RNAs of the copy-back type, possessing 3′ termini that are complementary copies of the genomic 5′ terminus, has suggested that the copy-back 3′ terminus confers a powerful selective advantage (16). The discovery of an abundance of internally deleted, easily propagated DI RNA species in the paramyxovirus Sendai virus (1, 17) not only called this premise into question, but presented the opportunity of putting it to experimental test.

MATERIALS AND METHODS

Virus passages. The chorioallantoic cavities of chicken embryos at day 11 of incubation were infected with individual DI virus strains or with mixtures of DI virus strains at various multiplicities, as described in Results, and incubated at 35°C for 72 h. When repeated passages were done, virus-containing allantoic fluids were diluted with phosphate-buffered saline (pH 7.4; divalent cation-free) to give the hemagglutinin (HA) titers used for inoculation.

Virion RNA. Virus particles were isolated from allantoic fluid by centrifugation through 30% sucrose for 2 h at 96,300 × *g* onto a cushion of 60% sucrose. The sucrose solutions were made up in 100 mM NaCl–0.1 mM EDTA–5 mM Tris hydrochloride (pH 7.4). The virus band was diluted to 30 ml with the same buffer lacking NaCl and pelleted by centrifugation at 158,000 × *g* for 45 min. The pellet was suspended in the salt-free buffer containing 1% sodium dodecyl sulfate and 200 μg of Proteinase K per ml, and the mixture was heated at 56°C for 15 min. RNA was then extracted twice with phenol-chloroform (1:1), ethanol precipitated, dissolved and reprecipitated twice from 0.4 M NaCl, washed with ethanol, dissolved in water at about 1 μg/μl, and stored at −20°C. Equal amounts (0.5 μg) of RNA species from each passage were labeled at their 3′ termini with cytidine 3′,5′-[5′-^{32}P]bis(phosphate) and T4 RNA ligase and analyzed by acid-urea-agarose gel electrophoresis (17).

RESULTS

Competition between copy-back and internally deleted DI RNAs. The nucleotide sequences of the Sendai virus DI RNA species used in this study are shown diagrammatically in Fig. 1. Virus strain 11 contains a single small copy-back RNA species, 11a (17, 18), and strain R contains a pair of large DI RNAs, internally deleted Ra and copy-back type Rb (17). All of the strain 7 DI RNAs are extensively deleted internally. They retain the transcriptional start signal of the viral *NP* gene, the transcription termination-poly(A) addition signal of the *L* gene, and adjacent fragments of these genes, and all are transcribed in vivo (8, 20).

To test the premise that the copy-back 3′-terminal sequence is a better promoter of RNA replication than the genomic 3′ terminus (14, 16), we performed competition experiments between Sendai virus strain 7 (internally deleted DI RNAs) and copy-back strain 11. We first infected embryonated eggs with mixtures of various proportions of the two strains to obtain a seed in which both kinds of RNA were equally represented, a goal that was achieved at an input ratio of 10 parts of strain 7 to 1 part of strain 11 (Fig. 2, lanes 11:7). Then we passaged the mixture serially at an inoculum of 1 HA unit per embryonated egg and examined the RNA species in the yield from each passage. As shown in the right-hand lanes of Fig. 2, copy-back RNA 11a completely outgrew all of the internally deleted RNA 7 species by the seventh passage. Despite the slowness of this

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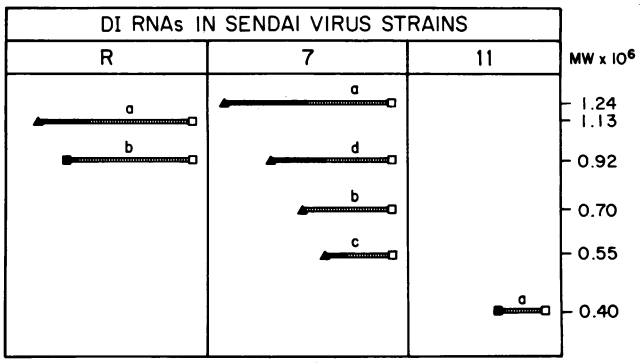

FIG. 1. Sequence compositions of the DI RNAs used in this work. All RNA species are depicted in the negative-strand sense, with 3′ termini at the left and lengths proportional to M_r (scale at right). Symbols: ▲, genome positive-strand leader RNA template; ■, antigenome negative-strand (copy-back) leader template; □, 5′-terminal sequence complementary to the antigenome leader template; filled bars, 3′-coterminal *NP* gene sequences; hatched bars, 5′-coterminal *L* gene sequences.

process, the result supports the conclusion that a copy-back RNA species has a competitive advantage over internally deleted DI RNAs, although the smaller size of RNA 11a might also have been responsible. We were able to address this alternative possibility in later experiments.

One HA unit of Sendai virus represents about 10^6 virus particles, yet the chorioallantoic membrane is lined by about 100-fold more cells (12, 13). Therefore, the input multiplicity used in these passages and in the work reported below

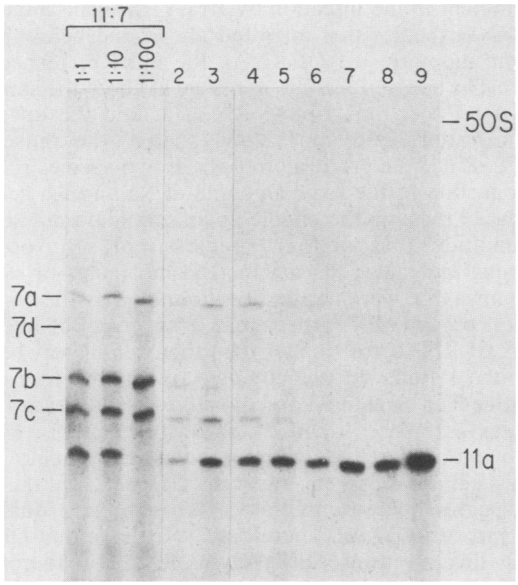

FIG. 2. Overgrowth of transcribed DI RNAs of Sendai virus strain 7 by the copy-back DI RNA 11a. Virions of the two strains were mixed in the proportions shown in lanes labeled 11:7 and inoculated into embryonated eggs. The yield containing equivalent amounts of the two types of DI RNAs (lane 1:10) was then passed repeatedly at an input of 1 HA unit per egg. All viral RNAs were labeled in vitro and analyzed by electrophoresis as described in Materials and Methods. The four internally deleted DI RNA species in strain 7, RNA 11a, and the electrophoretic position of intact (50S) viral genomes are indicated.

appears to be inadequate to ensure the simultaneous infection of cells by competing DI virions and standard helper virions. Yet, higher multiplicities were often unproductive due to DI particle interference (data not shown). The ability of DI RNAs to be replicated so efficiently at low input multiplicity is a special, unexplained feature of Sendai virus infection of the chorioallantoic membrane (13).

Evolution of strain R DI RNAs. To learn whether the replicative advantage of a copy-back DI RNA is a general feature of Sendai virus DI RNAs, we performed repeated passages of Sendai virus strain R. This strain has the interesting property of possessing two large DI RNA species of different types (Fig. 1). RNA Ra, M_r 1.1×10^6, is internally deleted and retains the genomic 3′ terminus along with a portion of the adjacent *NP* gene (1, 17). RNA Rb, M_r 0.92×10^6, has a copy-back 3′ end (17). We surmised that a study of competition between these RNA species should clarify the ability of a copy-back RNA to outcompete an internally deleted RNA.

At an input multiplicity of 1 HA unit per egg, Rb became markedly predominant by the eighth passage (Fig. 3), although small amounts of Ra may have persisted through further passages (Fig. 4, lanes R_{11} and R_{12}). Genomic 50S RNA was barely visible in these yields, due to interference from the DI RNA species, but there was evidently sufficient helper virus present to maintain viability of the inocula. This experiment supported the conclusion that copy-back DI RNAs have a selective advantage over internally deleted species, although it left open the possibility that the slightly smaller size of Rb was the decisive factor. However, we had produced a virus stock consisting almost exclusively of a relatively large copy-back RNA species, enabling us to make a more conclusive test of the relative importance of RNA size and 3′ terminus structure in survival, as described next.

Rb outgrew strain 7 RNAs. Competition between the large copy-back RNA Rb and the internally deleted DI RNAs of strain 7, two of which are smaller than Rb (Fig. 1), was measured by infecting embryos with a constant amount of the yield from the 10th passage of strain R (Fig. 3, lane 10), containing Rb as its predominant DI RNA, mixed with

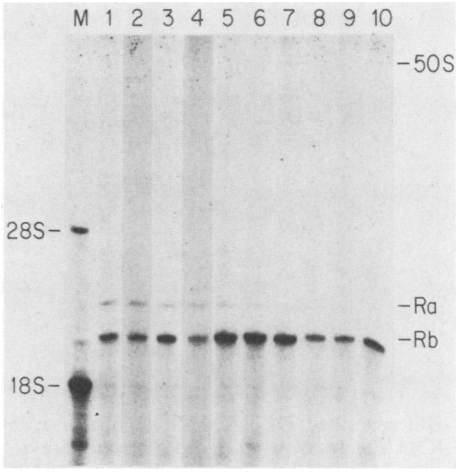

FIG. 3. DI RNA Rb outgrows its companion, Ra. Serial passages of Sendai virus strain R in embryonated eggs were performed at 1 HA unit per egg. RNA labeling and electrophoresis were done as in Fig. 2. Labeled are the two DI RNAs in strain R, the position of 50S viral genomic RNA, and the positions of 18S and 28S rRNA molecules in lane M.

various proportions of strain 7 (Fig. 4). As shown, Rb was a very effective competitor in a single passage, and only when the input of strain 7 was 100-fold or more in excess was there any evidence that its RNA species were replicated. Moreover, there were about equal amounts of Ra and the strain 7 DI RNAs in the yield from the infection with a 100-fold excess of strain 7. When that yield was passaged again, the major DI RNA that emerged was Rb [Fig. 4, lane $(1:100)_2$]. Thus, despite its large size, copy-back RNA Rb had a decisive selective advantage over all of the strain 7 DI RNAs, each of which is transcribed in infected cells (8). This demonstrates that internally deleted DI RNAs suffer from a replicative disadvantage, either because their genomic 3′ termini are relatively weak promoters of RNA synthesis or because part-time involvement in transcription decreases their overall replicative efficiency.

Unusual properties of RNA Ra. Comparison of the data in Fig. 3 and 4 reveals that DI RNA Ra was markedly more resistant to competition by its companion Rb than any of the strain 7 RNAs. This is puzzling, since Ra has the same internally deleted configuration as strain 7 RNAs. A possible explanation for this difference in behavior is that Ra is transcriptionally inactive owing to a mutation downstream from its 3′ terminus, since transcriptional activity may be a critical determinant of DI RNA replicative efficiency, as suggested above.

Therefore, we examined cells infected by strain R for poly(A)-containing transcripts of the two DI RNAs in the inoculum (Fig. 5, lane R). These cells produced abundant mRNAs specified by the nondefective helper virus, as well as a very large amount of a putative polytranscript (band 2, Fig. 5) and small amounts of a larger putative polytranscript (band 1) that were also seen in cells infected by standard virus and by copy-back strain 11.

The electrophoretic mobility of band 1 (Fig. 5) is also consistent with the possibility that it represents the mRNA

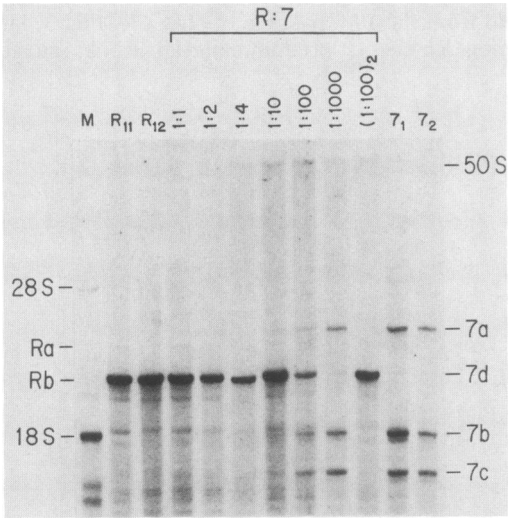

FIG. 4. Untranscribed DI RNA Rb competes with the transcribed DI RNA species of strain 7. RNA species are designated as in Fig. 3. Lanes: M, rRNA markers; R_{11}, R_{12}, 7_1, 7_2, RNA species in separate passages of the two original DI virus strains; R:7, single-passage yields of mixtures of the two DI strains inoculated in the proportions indicated at a total of 1 HA unit per egg. Lane $(1:100)_2$ contains the yield from a second passage of the yield from lane (1:100), which contained equivalent amounts of the two types of DI RNAs.

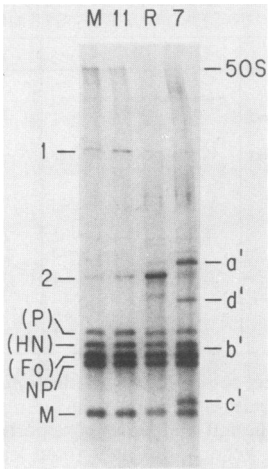

FIG. 5. Lack of transcription from RNA Ra. At 48 h after infection, chicken embryo lung cells were labeled with $^{32}P_i$ in the presence of actinomycin D for 4 h. RNA was extracted, passed through oligo(dT)-cellulose, denatured with formamide, and analyzed by electrophoresis in an acid-urea-agarose gel (8). Lanes: M, standard, nondefective Sendai virus; 11, copy-back DI virus strain 11; R, the original strain R, containing internally deleted DI RNA Ra and copy-back RNA Rb; 7, DI strain 7. The positions of the M and NP mRNAs and the putative positions of the remaining viral mRNAs specified by the helper virus are shown. Bands a′ through d′ are transcripts of the four strain 7 DI RNAs. Bands 1 and 2, present in all lanes, are thought to represent polytranscripts (7).

for the L protein, but band 2 does not correspond to any mRNA specified by the helper virus or any of the DI genomes. We have no idea why this band should have been so prominent in the infection by strain R. Small amounts of other RNA species that migrated above and below band 2 have the mobilities of DI RNAs Ra and Rb, respectively (Fig. 5). The upper, Ra band, migrated at about the same rate as RNA a′, the transcript of RNA 7a, and the lower, Rb band, migrated slightly more slowly than d′, the transcript of RNA 7d. Since the proportion of these two species reflected the proportion of the large amounts of Ra and Rb genomes that passed through the oligo(dT) column (data not shown), we conclude that neither species represent poly(A)-containing molecules but are instead molecules of strain R DI genomes that were not totally eliminated by the oligo(dT) selection procedure. Furthermore, transcripts of internally deleted DI RNAs are smaller than their templates, because their poly(A) tails do not compensate for the loss of 112 nucleotides of negative- and positive-strand leader RNA templates at their 5′ and 3′ ends, respectively (8, 18). Consequently, the RNA species with the mobility of Ra genomes cannot represent transcripts. Band 2 and the minor RNA species below it migrated too rapidly to qualify as transcripts of RNA Ra. Therefore, we believe that DI RNA Ra is as inactive transcriptionally as RNA Rb. In contrast, transcriptionally active strain 7 genomes yielded transcripts in amounts comparable to the yields of the helper virus mRNAs (Fig. 5, lane 7), whereas cells infected with the transcriptionally inert copy-back genome of strain 11 expressed only the helper virus mRNAs (Fig. 5, lane 11).

We searched for mutations that might explain the inability of Ra to be transcribed by sequencing across the leader template-*NP* gene junction (Fig. 6). We found only two base changes, at positions 47 and 51 from the 3′ end of the negative strand (3, 19, 21). Position 51 lies just before the

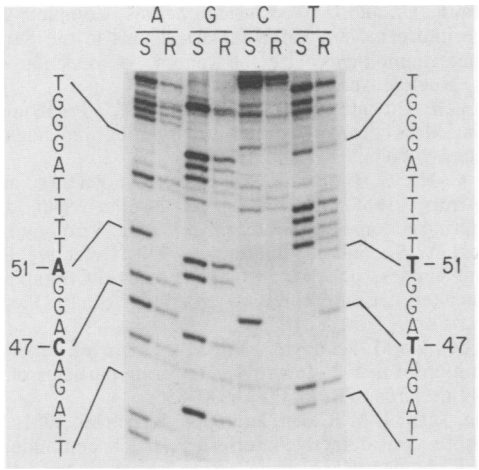

FIG. 6. Point mutations in DI RNA Ra. Nondefective 50S RNA (S) and gel-purified (17) DI RNA Ra (R) were sequenced by the dideoxy chain termination method using as a primer a synthetic oligonucleotide complementary to bases 13 to 24 from the 3′ end of the virus genome. The numbers given are relative to the 3′ genomic terminus.

group of four A residues at the junction with the *NP* mRNA start sequence (Fig. 7), and the change introduces a fifth contiguous A residue. The change at residue 47 also introduces an A residue in a position close enough to the leader-*NP* junction to qualify as a likely participant in *cis*-acting signals that regulate viral RNA transcription. These alterations evidently account for the failure of RNA Ra to be transcribed, since its 3′-terminal sequence was otherwise identical to the viral genome from base 1 to 25 (17) and from base 26 to more than 150 bases within the *NP* gene (this work; data not shown).

DISCUSSION

A clear finding of our competition studies with Sendai virus DI RNAs is that the copy-back type has a marked replicative advantage over the internally deleted type, which is independent of the size of the copy-back RNA molecule. This advantage could depend on superior promoter activity of the copy-back 3′ terminus, the transcriptional inertness of copy-back RNAs, or combined effects of both factors. The last possibility is supported by the finding that internally deleted RNA species Ra, possessing the genomic 3′ terminus, but rendered transcriptionally inert by point mutations in a sequence important for control of transcription, was only slowly outgrown by copy-back RNA Rb. The fact that Ra was eventually outgrown by Rb shows the superiority of the copy-back 3′ terminus as a promoter; the slow rate of this process compared to the rapid outgrowth by Rb of transcriptionally active strain 7 DI RNAs demonstrates the replicative advantage conferred by transcriptional inactivity. Nevertheless, we must remember that internally deleted, transcriptionally active DI RNAs are capable of surviving and of interfering with replication of the parental genome in virus strains that are free of copy-back DI RNAs (17; unpublished data). Likewise, influenza virus copy-back DI RNAs have not been encountered, but transcriptionally active, internally deleted influenza virus DI RNAs interfere strongly with the parental virus (2).

In the case of vesicular stomatitis virus, the copy-back 3′ terminus had been thought to be a better promoter of viral RNA replication than the genomic 3′ terminus, in keeping with the preferential synthesis of genomes compared with antigenomes during virus replication (16). However, we are aware of only one test of this point by DI RNA competition (15). Several transcriptionally inactive DI RNAs with copy-back 3′ termini independently overgrew DI-LT RNA, which is internally deleted and transcriptionally active (4, 11, 15). But DI-LT RNA is difficult to propagate and maintain on its own, so its outgrowth by other DI RNAs might simply reflect its replicative infirmity. A recently described collection of several internally deleted vesicular stomatitis virus DI RNAs (10) might contain species with more replicative vigor than DI-LT, but none of these has been tested in competition studies. Our results with DI RNAs of a paramyxovirus suggest that the preferential replication of the genomes of nonsegmented negative-strand viruses is not solely a consequence of superior promoter activity of the antigenomic 3′ terminus; the transcriptional inertness of antigenomes also makes a contribution.

It may seem fortuitous that Ra should have acquired point mutations that enabled us to distinguish so conveniently the advantage of transcriptional inactivity in DI survival. However, Ra probably could not have survived in our stocks in competition with its copy-back companion Rb if it had not lost its ability to be transcribed, in view of the rapidity with which untranscribed DI RNA species outgrew transcribed DI RNAs in our competition studies with the internally deleted RNA species in strain 7.

Ra is not the only Sendai virus deletion mutant that has revealed informative changes in a sequence that regulates RNA transcription. We reported earlier that RNA 7a contains the entire 3′ end of the Sendai virus genome up to the third U residue in the 5-base oligo(U) sequence that constitutes the initiation site for viral mRNA polyadenylation (5, 19). This alteration eliminated termination and polyadenylation at the end of the *NP* gene. Thus, in the case of RNA 7a, we obtained evidence for the importance of downstream sequences in the mRNA termination step. Now, in the case of RNA Ra, we have gained a glimpse of the sequence requirements for positive-strand leader termination and *NP* mRNA initiation in a region just upstream of the leader-*NP* junction. It is not possible to deduce whether both mutations in Ra affect leader RNA readthrough into the *NP* gene, but we suspect that they do. The four A residues at positions 52 to 55 of the leader template resemble the purine triplets that separate transcription stop and start signals in the virus genome (6, 19; Fig. 7). Therefore, these four A residues may be functionally equivalent to those intergenic triplets, segregating the leader terminus from the *NP* mRNA start site. Likewise, the region from base 40 through base 51, including the two mutated sites in Ra, may represent a signal for leader

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                  40         50         60         70
Ra:      3'...UACUUCAAUCUAUCCA AAAA UCCCAGUUUCAUAGGUG...
Genome:     ...UACUUCAAUCUGUCCU AAAA UCCCAGUUUCAUAGGUG...
                * *  ***  *  *
Consensus:     ...UNAUUCU-UUUU GAA  ...
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FIG. 7. Mutations in DI RNA Ra in the context of the leader RNA template-*NP* gene junction. Sequences are presented in the negative-strand sense, and numbering is relative to the 3′ terminus of the genome. The mutations at bases 47 and 51 are emphasized, as are the consensus bases in the *NP* mRNA start signal (6). The sequence of four A residues adjacent to the *NP* mRNA start signal is separated by spaces. Also shown is the consensus sequence at the end of each gene, aligned for maximum similarity with the leader template. Identical bases are indicated by asterisks. Following the consensus sequence is the GAA intergenic sequence (6). N represents a variable nucleotide.

RNA termination, being functionally equivalent to the mRNA termination-poly(A) addition signals at the end of each virus gene.

Although bases 40 through 51 of the leader template are not closely similar to the 11-base mRNA termination-poly(A) addition signals, careful examination suggests relationships, as indicated in Fig. 7. If the G residue at position 47 of the leader template is deleted, 7 of the remaining 10 bases are identical in the two sequences. The probability of this much similarity occurring by chance is 7.6×10^{-3}, supporting the idea that the two sequences have an evolutionary relationship and that the leader template dodecamer is involved in termination of leader RNA synthesis. It appears that insertion of the G residue at position 47 and the substitution of two C residues at positions 49 and 50 have inactivated the $(U)_5$ poly(A) addition signal, assuring that the leader RNA is not polyadenylated. The substitution of an A for a U at position 43 may contribute to this effect, since position 43 of the leader template would correspond to position 2 of the consensus 3'-AUUC that precedes each $(U)_5$ and participates in mRNA termination-polyadenylation (5; Fig. 7).

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