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RNAs from two independently isolated defective interfering particles of Sindbis virus contain a cellular tRNA sequence at their 5′ ends

(cDNA cloning/alphavirus replication/$tRNA^{Asp}$)

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ABSTRACT **Defective interfering (DI) particles are deletion mutants that interfere specifically with the replication of homologous standard virus. We have determined the 5′-terminal nucleotide sequences of two DI RNA populations by the following methods: (*i*) cloning of the cDNA from one of the DI RNA populations and sequencing a representative clone, and (*ii*) using both DI RNA populations as templates for preparing primer-directed cDNA transcripts and sequencing these transcripts. The 5′ terminal sequences of the two DI RNA populations were not derived from standard Sindbis viral RNA but were almost identical to those of a cellular $tRNA^{Asp}$.**

Serial high multiplicity passaging of many viruses in cultured cells leads to the accumulation of defective interfering (DI) particles. These deletion mutants interfere specifically with the growth of standard virus and have been implicated as an important factor in moderating cytopathic infections (1, 2). The genomes of DI particles contain less sequence information than those of standard viruses, yet their selection and maintenance in the population depends on their having nucleotide sequences that are recognized for both replication and packaging. A comparison between sequences present in DI and standard nucleic acids should provide one means of identifying these recognition sites. We have been analyzing DI particles of Sindbis virus, an alphavirus, and describe here the surprising result that in two independently isolated DI RNAs the 5′ termini are almost identical to the sequence of a cellular $tRNA^{Asp}$.

METHODS

Generation of DI Particles and Purification of Viral RNAs. DI-2 particles were generated by 18 undiluted passages of Sindbis virus on chicken embryo fibroblasts (3). Before DI-3 particles were generated the standard virus (from an early passage) was cloned by three consecutive plaque purifications. The DI-3 virus was obtained after 16 passages of the cloned virus on chicken embryo fibroblasts.

Cloning and Sequence Analysis of cDNAs. cDNA clones were generated from DI-2 RNA by using standard procedures. The nucleotide sequence of a representative clone was determined by the chemical method of Maxam and Gilbert (4), using the modifications of Smith and Calvo (5). The details of these methods will be presented elsewhere.

Primer Extension of cDNAs. A 27-nucleotide *Hinf*I/*Hin*dIII primer fragment was prepared for analytical hybridizations by digestion of the cloned DNA with *Hinf*I, 3′-end-labeling using reverse transcriptase (James Beard, Life Sciences, St. Petersburg, FL), digestion with *Hin*dIII, and purification from an 8% polyacrylamide sequencing gel. The same fragment was prepared for large-scale hybridizations by digestion of the DNA with *Hin*dIII, removal of terminal phosphates with calf intestinal alkaline phosphatase, 5′-end-labeling using polynucleotide kinase, and digestion with *Hinf*I. Hybridizations were performed with a 5- to 10-fold molar excess of RNA in 80% (vol/vol) formamide/0.4 M NaCl/50 mM 1,4-piperazinediethanesulfonic acid (Pipes), pH 6.4/1 mM EDTA (6). The RNA and DNA were lyophilized together, dissolved at 0.2–1.0 μg/ml, denatured at 75°C for 10 min, and annealed at 30°C overnight. The hybrids were collected by precipitation with ethanol and the primer was extended in 50 mM Tris·HCl, pH 8.3/50 mM KCl/12 mM $MgCl_2$/10 mM dithiothreitol/the four dNTPs at 0.5 mM each/500 units of reverse transcriptase per ml. The extended products were resolved on 8% polyacrylamide sequencing gels and individual fragments were purified and sequenced as described above.

Blot Hybridization Analysis of Cellular RNA. Total cellular RNA was isolated from primary chicken embryo cells by extraction with phenol/chloroform and the 4–5S fraction was purified by sucrose velocity gradient sedimentation. Total and 4–5S RNA (10 μg each) were denatured in 1 M glyoxal/50% (vol/vol) dimethyl sulfoxide/10 mM sodium phosphate, pH 7, at 50°C for 1 hr and resolved on a 4% polyacrylamide/0.5% agarose composite gel (7). The gel was treated for transfer by successive 30-min incubations in 50 mM NaOH/250 mM sodium phosphate, pH 5.5/1 μg of ethidium bromide per ml and twice in 25 mM sodium phosphate, pH 5.5/1 mM EDTA. The RNA was electrophoretically transferred to activated paper at 10 V/cm for 3 hr in 25 mM sodium phosphate, pH 5.5/1 mM EDTA (8). By using these conditions of *in situ* nicking and electrophoresis, RNAs ranging in size between tRNA and standard 49S RNA were effectively transferred to the activated paper. The paper was prepared and activated essentially as described by Seed (9). The paper was incubated for 20 hr at 42°C in the buffer described by Alwine *et al.* (10). Hybridization was carried out at 42°C for 46 hr in the same buffer containing 10% dextran sulfate. The probe fragment was prepared from cloned DNA by *Hpa* II digestion, 3′ labeling with reverse transcriptase, gel purification of a 263-nucleotide fragment, *Hinf*I digestion, and gel purification of a 97-nucleotide fragment.

Nuclease S1 Analysis. A 97-nucleotide primer fragment was prepared by *Hpa* II and *Hinf*I digestion of cloned DNA as described above, except for omission of the labeling step. The primer was hybridized to DI-2 RNA and extended with reverse

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Abbreviations: DI, defective interfering; Pipes, 1,4-piperazinediethanesulfonic acid.

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transcriptase as described above except that dATP, dGTP, and dTTP were used at 25 μM each and [α-^{32}P]dCTP was included at 5 μM. The sample was treated with alkali to hydrolyze the RNA. The cDNA and viral RNAs were then dissolved in hybridization buffer, denatured as described above, and annealed at 40°C for 16 hr. The samples were digested with nuclease S1 (6) and resolved on an 8% polyacrylamide sequencing gel.

RESULTS

The 5′-Terminal Sequences of cDNAs Derived from Sindbis DI RNAs. We have cloned a nearly full-length (≈2,300 nucleotides) cDNA copy of DI RNA generated from Sindbis virus in chicken embryo fibroblasts and determined its sequence. The details of this work will be published elsewhere, but the general pattern of deletions, duplication, and rearrangements of the standard RNA sequence resembled that found in DI RNAs derived from Semliki Forest virus (11, 12). A striking and unexpected finding, however, was that the 39 nucleotides at the end of the clone corresponding to the 5′-terminal region of the RNA were not present in either the standard RNA or in its complement (J. and E. Strauss, personal communication). One explanation for the presence of sequences at the 5′ end of the clone that are unrelated to Sindbis viral sequences is that they were generated during the cloning procedures. We were able to eliminate this possibility by determining the sequences of primer-extended cDNAs. Our results demonstrated that the sequences originated in the DI-2 RNA population. We used as primer a 27-nucleotide *Hin*fI/*Hin*dIII fragment complementary to nucleotides 102–129 of the standard viral RNA. For analytical reactions, the primer was labeled at the *Hin*fI site at the 3′ end of the cDNA strand. For preparative reactions, the primer was labeled at the *Hin*dIII site at the 5′ end of the cDNA strand.

As templates we used the DI RNA population (DI-2) from which the clone was derived, standard Sindbis virion RNA, and another population of DI RNAs (DI-3) independently derived by passaging on chicken embryo fibroblasts. The primer was hybridized to the appropriate RNA template and extended by reverse transcriptase, and the products were resolved on 8% polyacrylamide sequencing gels (Fig. 1). The major cDNA fragments were eluted from preparative gels and purified, and their sequences were determined (4). The deduced sequences of the RNA templates are compared in Fig. 2A.

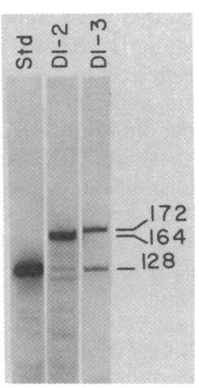

FIG. 1. Polyacrylamide gel electrophoresis of cDNA extension products. A 27-nucleotide primer was prepared from cloned DNA by *Hin*fI digestion, 3′ labeling, *Hin*dIII digestion, and gel purification. The primer was hybridized to standard Sindbis RNA, DI-2 RNA, and DI-3 RNA and extended with reverse transcriptase. After denaturation the cDNA products were resolved by electrophoresis on an 8% polyacrylamide sequencing gel (16 × 20 × 0.08 cm) at 10 W for 1 hr. The number of nucleotides in each fragment, as determined by sequence analysis, is indicated.

With the standard RNA as template the expected 128-nucleotide extension product was obtained (Fig. 1). The RNA sequence deduced from the cDNA sequence was identical to that previously obtained for the 5′ end of standard Sindbis RNA (13). In contrast, the major cDNA extension products obtained when DI-2 and DI-3 RNA were used as templates were longer than the standard product (Fig. 1). Sequence analysis of these fragments indicated that the two DI RNA templates contained the same 67 nonstandard nucleotides, including the 39 found in the clone, joined to standard sequences at slightly different locations (Fig. 2). Primer extension using the DI RNAs as templates also resulted in products that migrated with the standard product (Fig. 1). These fragments were identical in sequence to the standard product and may have been generated from low levels of standard RNA present in the DI RNA preparations.

Under the conditions we used for synthesis, the standard RNA was accurately copied to the extreme 5′ end, thus in this case the enzyme transcribed through the hairpin structure predicted to occur at the terminus (13). The extension products obtained from the DI RNAs were discrete sizes, suggesting the reverse transcriptase also copied to the end of these templates. The sequence derived from the DI extension products is consistent with the partial sequence information obtained by direct 5′ end sequence analysis of DI-2 RNA (13), providing evidence that it is the 5′ end of the DI RNAs.

Sequences at the 5′ End of Two DI RNAs of Sindbis Virus Are Homologous to Those of a Cellular tRNA. The lack of homology between the 5′-terminal sequences of DI RNAs and sequences in Sindbis viral RNAs raised the possibility that these sequences were derived from the host cell. To search for these sequences in uninfected cells we prepared from the cloned DNA a 97-nucleotide *Hpa* II/*Hin*fI fragment labeled in the cDNA strand at the 3′ *Hpa* II site. This fragment contains 26 nucleotides from the non-Sindbis sequence plus 71 nucleotides complementary to Sindbis RNA (Fig. 2*B*). Total cytoplasmic chicken cell RNA was denatured with glyoxal, resolved on a polyacrylamide/agarose composite gel, electrophoretically transferred to activated paper, and hybridized to the labeled fragment. As seen in Fig. 3, hybridization was observed to cellular RNA migrating at the position of tRNA.

We examined the homology between the cellular and the DI sequences by the extent to which the RNAs could protect a cDNA probe from S1 nuclease digestion. The radioactive probe was prepared by hybridizing the 97-nucleotide *Hpa* II/*Hin*fI fragment to DI-2 RNA and extending this primer by using reverse transcriptase in the presence of [α-^{32}P]dCTP. The major product was 138 nucleotides long, but radioactivity had been incorporated only in the 3′-terminal 41 nucleotides copied from non-Sindbis sequences (Fig. 2*B*). This cDNA was hybridized to standard, DI-2, DI-3, and chicken cell RNAs. After hybridization the samples were digested with S1 nuclease and the protected DNA fragments were resolved on an 8% polyacrylamide sequencing gel (Fig. 4). None of the labeled cDNA was protected by standard Sindbis RNA even though this RNA would be expected to hybridize to the unlabeled part of the probe (Fig. 4). DI-2 RNA, which served as a template for the synthesis of the cDNA, protected the full-length probe. Sequence analysis of the primer-extended product of DI-3 RNA had shown that it contains the same nonstandard sequence plus eight more nucleotides of standard sequence than does DI-2 RNA (Fig. 2). These eight nucleotides would not be base paired when DI-3 RNA was hybridized to the probe and so should be sensitive to nuclease S1 digestion. The cDNA probe would then be cleaved opposite the nick in DI-3 RNA, giving rise to the 67-nucleotide protected fragment complementary to the nonstandard se-

A

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STD  RNA   1   ..TTGGCGGC GTAGTACACA CT                                  22

DI-2 RNA   1   .ATATAGTGG TGAGTATCCC CGCCTGTCAC GCGGGAGACC GGGGTTCGGT    50
DI-3 RNA   1   .......TGG TGAGTATCCC CGCCTGTCAC GCGGGAGACC GGGGTTCGGT    50


STD  RNA  23                ATTG AATCAAACAG CCGACCA                     43

DI-2 RNA  51   TCCCCGACGG GGAGCCA         AACAG CCGACCA                   79
DI-3 RNA  51   TCCCCGACGG GGAGCCATTG AATCAAACAG CCGACCA                   87
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B

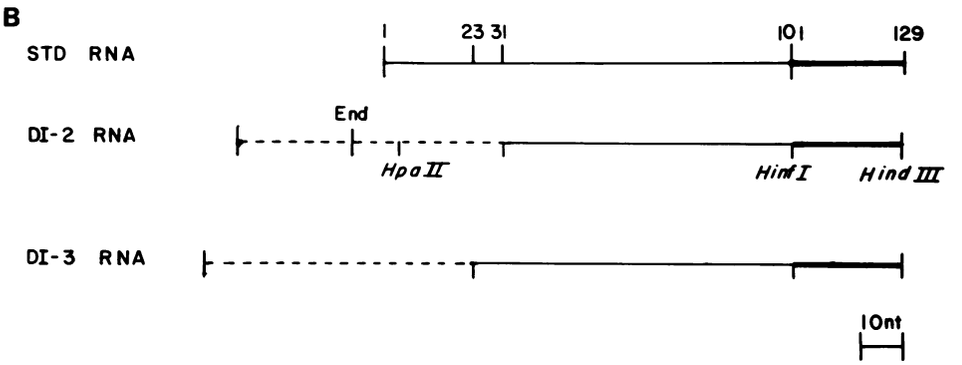

FIG. 2. Nucleotide sequence of cDNA extension products. The 5′-labeled *Hind*III/*Hinf*I primer fragment from cloned DNA was hybridized to standard Sindbis RNA, DI-2 RNA, and DI-3 RNA. The cDNA was extended with reverse transcriptase, the major products were eluted from denaturing polyacrylamide gels, and their sequences were determined by the method of Maxam and Gilbert (4). (*A*) The deduced sequences of the 5′ ends of the RNAs are presented as DNA (T instead of U) to indicate that it was the cDNA that was actually analyzed. Dots represent nucleotides not determined. To align the sequences with DI-3 RNA, gaps were introduced at nucleotide 22 in the standard RNA and nucleotide 67 in the DI-2 RNA. (*B*) The 5′ ends of the RNAs are represented schematically. The solid lines indicate regions of standard sequence and the broken lines indicate regions of nonstandard sequence. The primer is indicated by the dark solid line. The DI-3 cDNA sequence was not determined in the region corresponding to nucleotides 43–101 of the standard. The positions of several restriction enzyme recognition sites and the end of the clone are marked on the DI-2 RNA. nt, Nucleotides.

quence (Fig. 4). The small RNAs isolated from chicken cells also protected the same size fragment as did the DI-3 RNA (Fig. 4). Thus, the entire non-Sindbis sequence present at the 5′ end

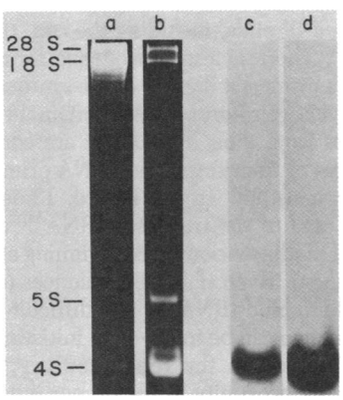

FIG. 3. Polyacrylamide gel electrophoresis of chicken cellular RNAs. Total cellular RNA was isolated from chicken embryos and a 4–5S fraction was purified by sucrose velocity gradient sedimentation. Samples were denatured for 1 hr at 50°C in 1 M glyoxal/50% (vol/vol) dimethyl sulfoxide/10 mM sodium phosphate, pH 7. The gel (12 × 16 × 0.15 cm) contained 4% acrylamide, 0.5% agarose, and 10 mM sodium phosphate at pH 7, and electrophoresis was at 10 V/cm for 1.75 hr. The resolved samples were denatured, stained, and electrophoretically transferred to activated paper. The samples on the paper were hybridized to a 3′-labeled 97-nucleotide *Hpa* II/*Hinf*I fragment from cloned DNA, washed, and exposed to x-ray film. Lanes a and c, 10 μg of total cellular RNA; lanes b and d, 10 μg of 4–5S RNA. Lanes a and b show ethidium bromide-stained gel before transfer; lanes c and d, autoradiogram of paper after hybridization and washing.

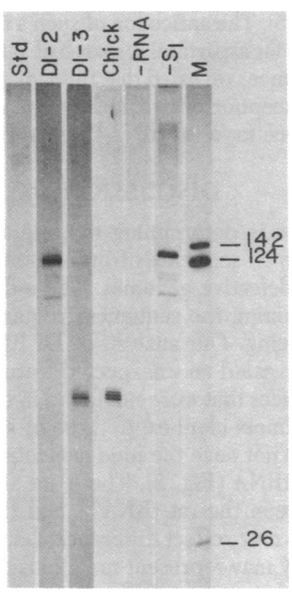

FIG. 4. S1 nuclease analysis of viral and cellular RNAs. A cDNA probe was generated by hybridizing a 97-nucleotide *Hpa* II/*Hinf*I fragment from the cloned DNA to DI-2 RNA and extending with reverse transcriptase in the presence of [α-^{32}P]dCTP. The probe was hybridized to approximately equimolar amounts of the RNAs in 80% (vol/vol) formamide/0.4 M NaCl/50 mM Pipes, pH 6.4/1 mM EDTA at 40°C for 16 hr. The samples were digested with S1 nuclease at 40°C for 90 min and resolved on an 8% polyacrylamide sequencing gel (30 × 40 × 0.04 cm) by electrophoresis at 30 W for 2 hr. The number of nucleotides in the marker restriction fragments (lane M) is indicated. Controls in which RNA or S1 nuclease were omitted are also shown.

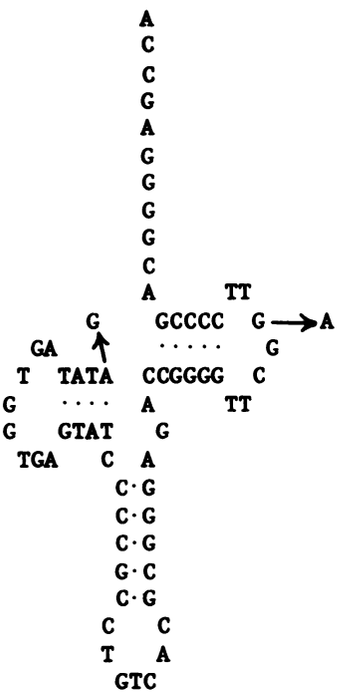

FIG. 5. Cellular $tRNA^{Asp}$ sequence at the 5′ end of DI-2 and DI-3 RNAs. The sequence is presented as DNA and is folded into the cloverleaf structure typical of tRNAs to indicate the sequence homology. The two nucleotide differences between this sequence and the rat $tRNA^{Asp}$ gene sequence (14) are indicated.

of DI-2 and DI-3 RNAs was also present in chicken RNA, the size of tRNA. We were able to arrange this sequence to fold into a cloverleaf structure typical of a tRNA, but missing the 5′ terminal stem (Fig. 5). The anticodon of such a tRNA (GTC) is one of the anticodons for aspartate. A search of published tRNA and tRNA gene sequences revealed that the sequence is actually the same, with the exception of two nucleotides, as nucleotides 10–75 of the sequence for a rat $tRNA^{Asp}$ gene (14).

DISCUSSION

A major objective in determining the sequences of DI RNAs is to establish which sequences from standard viral RNAs are retained by the defective genomes. These data should be valuable for determining the sequences required for viral replication and packaging. Our analysis of DI RNAs of Sindbis virus, however, revealed an unexpected feature: the 5′ termini contained sequences that were not found in the standard virion RNA but were almost identical to those of a cellular $tRNA^{Asp}$. The DI RNAs do not have the nine nucleotides forming the 5′ terminus of the tRNA (Fig. 5). There are also two nucleotide differences between the rat $tRNA^{Asp}$ and the DI 5′-terminal sequences; these may reflect differences between chicken and rat tRNAs or they may represent mutations that have occurred after the tRNA became attached to the viral sequences. Mutations in RNA genomes occur at a much higher frequency than those in DNA sequences, which are subject to repair mechanisms (15).

tRNA-like structures, as measured by their ability to be aminoacylated, have been described previously in RNA viruses. In several plant viruses, the 3′ ends of the RNA, although not identical to tRNAs, have amino acid accepting activity (16). In the two DI RNAs from Sindbis virus the 3′ end of the cellular $tRNA^{Asp}$ was covalently attached to viral sequences and was unavailable for charging. In this regard, RNAs from two animal picornaviruses, mengovirus and encephalomyocarditis virus, were found to be charged with specific amino acids *in vitro* (17, 18). In both instances the aminoacylation occurred at internal sites after degradation of the RNA.

All of our sequence analysis and hybridization data were obtained by using cDNA copies of DI RNA generated by reverse transcriptase; therefore it was necessary to establish that the tRNA was not added to the DI RNAs during *in vitro* reverse transcription. There are several lines of evidence that make this possibility very unlikely. First, DI-2 RNA protected from nuclease S1 digestion the full length of the cDNA probe containing both tRNA and standard virus sequences (Fig. 4). This result is consistent with the tRNA sequences being covalently attached to viral sequences in the DI-2 RNA. Second, after electrophoresis of the DI RNA preparation under denaturing conditions, the only material that contained detectable $tRNA^{Asp}$ sequences migrated with the DI RNAs (data not shown). This result demonstrated that $tRNA^{Asp}$ molecules were not present in detectable amounts in the DI RNA preparations. Finally, the sequence of the first 11 nucleotides of DI-2 RNA have been determined directly after decapping of the RNA (13), and the data are consistent with the results we obtained from determining the sequence of the primer-extended product.

There are two ways in which we can envision the covalent linkage between $tRNA^{Asp}$ and viral RNA sequences being formed: there could have been a copy-choice event during synthesis of the negative strand such that the replicase switched templates from DI RNA to $tRNA^{Asp}$, or the tRNA could have served as a primer for the transcription of plus strand DI RNA. Our suggestion that $tRNA^{Asp}$ may serve as a primer for DI RNA synthesis implies that it might also serve a similar role in standard viral RNA synthesis. In the latter case, however, the tRNA primer would be removed before the RNA was encapsidated.

There is precedence for the utilization of cellular RNA sequences in the synthesis of viral RNA. Influenza virus mRNA synthesis is initiated with primers derived from the 5′ ends of cellular mRNAs (19). The recognition of the cellular RNAs does not involve base pairing with the viral RNA templates, and influenza virus mRNAs found in infected cells contain 10–15 nucleotides at their 5′ ends, including the cap, that are nonviral sequences (19). There is also precedence for a cellular tRNA functioning as a primer; it does so in the synthesis of retrovirus cDNA (20). Avian retrovirus RNAs contain 18–19 bases 100–180 nucleotides from their 5′ end that are complementary to the 3′ sequences of their respective tRNA primers and include the C-C-A terminus (20). In this regard, 13 out of 14 nucleotides at the 5′ end of the truncated $tRNA^{Asp}$ are homologous to the standard Sindbis virion RNA beginning at nucleotide 892 (unpublished data). Even if these sequences (on the negative strand) are able to bind $tRNA^{Asp}$, it is difficult to imagine how a site at this location can be involved in initiation of plus strand RNA synthesis unless in the replication complex this site is brought into close proximity to the proper initiation site. Furthermore, we have not detected sequences in the virion RNA that could provide a binding site for the 3′ terminus of the tRNA.

Although there is no evidence to support a role for $tRNA^{Asp}$ in Sindbis RNA replication there is reason to believe that the incorporation of the tRNA into the DI RNAs was not a random event. It has been reported that the predominant $tRNA^{Asp}$ in uninfected chicken cells constitutes only about 5% of the total tRNA (21), making it improbable that the same tRNA would be selected at random. Furthermore, the two populations of DI RNAs that have the identical tRNA sequences at their 5′ termini were independent isolates. The standard virus was plaque-purified three times between the generation of DI-2 and DI-3 and no DI RNAs could be detected in the early passages of

this virus. The identity of the tRNA sequence in the two DI RNAs includes the deletion of the nine 5′-terminal nucleotides of $tRNA^{Asp}$. We do not know if the presence of $tRNA^{Asp}$ or any other tRNA will be a common feature of the 5′ termini of DI RNAs of alphaviruses. The 5′ termini of DI RNAs from Semliki Forest virus are heterogeneous and different from those of the standard virus (22).

The presence of a cellular tRNA at the 5′ end of replicating DI RNAs complicates the identification of the important recognition signals for synthesis of Sindbis virus positive strand RNA. The complement of the tRNA at the 3′ end of the negative strand DI RNA is presumably the initiation site for synthesis of DI progeny positive strand. The recognition site for the polymerase, however, need not be at the extreme 3′ end of the template and may involve an internal region of conserved standard sequence as is true for the replicase of Qβ bacteriophage (23). The DI RNAs containing the $tRNA^{Asp}$ are replicated at the expense of standard RNA and interact with the viral structural proteins to be released as particles. They do represent species that have a selective advantage during multiple cycles of passaging, indicating that the tRNA does not interfere with and may enhance the ability of these molecules to be replicated.

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