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5′-Terminal nucleotide sequence of Semliki Forest virus 18S defective interfering RNA is heterogeneous and different from the genomic 42S RNA

(defective interfering RNA/capped 5′ terminus/two-dimensional RNA fingerprinting)

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ABSTRACT **An 18S defective interfering (DI) RNA population was isolated from the cytoplasm of baby hamster kidney (BHK-21) cells infected with Semliki Forest virus from the 10th undiluted passage. The RNA was ≈2000 nucleotides long and contained a 5′-terminal cap with the structure ^{7}mGpppAp and a poly(A) tract. The DI RNA contained large T1 oligonucleotides derived from both the 42S RNA-specific region and the 3′ one-third of the genome common to 42S and 26S RNA. Several of the large oligonucleotides were present in nonequimolar ratios, suggesting that the RNA population is heterogeneous. As this population is approximately uniform in size, this suggests that the DI RNAs may be generated by internal deletions involving different regions of the genome. The 5′-terminal cap-containing RNase T1 oligonucleotide was isolated by two-dimensional gel electrophoresis from uniformly ^{32}P-labeled RNA and shown to be heterogeneous. Five T1 caps with the structure ^{7}mGpppA-U(A-U)$_n$C-A-U-G (n = 4–8) were identified. The two major T1 caps (n = 4 and 6) comprised about 75% of the total yield of T1 caps. The T1 caps were different from the genomic 42S RNA T1 cap (^{7}mGpppA-U-G), suggesting that the extreme 5′ end of the genome is not conserved in this defective interfering RNA.**

Defective interfering (DI) particles are generated in most viral systems when cultures are serially infected with undiluted inocula (1). These particles are deletion mutants, containing genomes that have retained the nucleic acid sequences necessary for replication and encapsidation and, in some cases, for translation (1–7). It appears that, for RNA viruses, at least two mechanisms exist for the generation of DI RNAs. The first is a mechanism by which an internal deletion(s) is generated [e.g., polio (3–5), vesicular stomatitis virus (7), Sindbis virus (8, 9), Semliki Forest virus (SFV; 2, 10, 11)] and, on continued passaging of the virus, the size of the deletion increases (8, 11) to the point where the RNA loses the nucleotide sequences necessary for replication or encapsidation. The second mechanism involves dissociation of the RNA polymerase from the template, followed by association with the newly synthesized strand and copying back on that strand. This mechanism, which results in inverted complementary sequences at the 3′ and 5′ ends of the RNA, has been elucidated in detail with vesicular stomatitis virus (7) and Sendai virus (6).

We have studied the structure and synthesis of DI RNAs produced in SFV-infected cells. In cells infected with standard virus, two species of RNAs are synthesized: the genomic 42S RNA and the subgenomic 26S RNA. The latter is identical to the 3′ one-third of the 42S RNA (2, 12) and serves as the mRNA for the structural proteins (13). DI RNAs of different size classes accumulate in cells on serial passaging of SFV at high multiplicity (11). Based on nucleic acid hybridization (8) and oligonucleotide fingerprinting (2, 9, 11) studies, it has been concluded that the DI RNAs of Sindbis virus and SFV have conserved the 5′ and 3′ ends of the 42S RNA and have large internal deletions that may vary in length. The size of the deletion increases with increasing passage number. Ultimately, a stable 18–20S RNA accumulates. This RNA, which contains only about 15% of the 42S RNA sequences, presumably represents the minimum-size RNA that is still able to replicate and to become encapsidated. A looping out of the template molecule and a polymerase jump mechanism appears to be the simplest explanation for the generation of Sindbis virus and SFV DI RNAs (2, 8–11).

In this report, we describe the isolation and characterization of an 18S DI RNA from SFV-infected baby hamster kidney (BHK21) cells. The results show that the nucleotide sequence at the 5′ terminus of the DI RNA is different from that of the 42S RNA and is heterogeneous. Furthermore, the DI RNA, although approximately uniform in size, appears to consist of a mixture of RNA species that contain different internal deletions.

MATERIALS AND METHODS

Cells and Virus. The origin and cultivation of the prototype strain of SFV in 1-liter roller bottles of BHK21 cells has been described (14).

Generation of an 18S DI RNA. One-liter roller bottles of BHK21 cells were infected with standard SFV at high multiplicity of infection (100 plaque-forming units per cell), and the virus was harvested 24 hr later. By using this virus undiluted as inoculum, new cultures were similarly infected. Serial passaging with undiluted virus inoculum was continued for up to 20 passages. The production of virus particles was monitored by infectivity and hemagglutination assays of the culture fluid. The synthesis of virus-specific RNAs was studied by analysis on 15–30% sucrose gradients of [^{3}H]uridine-labeled RNAs made in infected cells in the presence of actinomycin D at 1 μg/ml (15). An 18S DI RNA was detected by the fourth undiluted passage.

Labeling and Purification of DI RNA. The intracellular 18S DI RNA was labeled by using the virus from the 10th undiluted passage to infect several roller bottles of BHK21 cells. The virus was grown in the presence of actinomycin D (1 μg/ml) in Eagle's minimal essential medium supplemented with 0.2% bovine serum albumin and lacking inorganic phosphate. At 2.5 hr, 0.5 mCi/ml (1 Ci = 3.7×10^{10} becquerels) of carrier-free ^{32}P (Amersham, England) was added. Cytoplasmic extracts were prepared 10 hr later, and the virus-specific RNAs were purified on sucrose gradients as described (15). The DI RNA-containing

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Abbreviations: SFV, Semliki Forest virus; BHK21, baby hamster kidney cell line 21; DI, defective interfering; T1 cap, cap-containing RNase T1 oligonucleotide.

fractions were pooled, and the RNA was precipitated with ethanol and purified by oligo(dT)-cellulose chromatography (PL Biochemicals). The bound RNA (generally more than 90% of total RNA) was precipitated with ethanol twice and used for further analysis. The relative specific radioactivities of the monophosphates in the DI RNA, determined as described (16), were: CMP, 1.1; AMP, 0.9; GMP, 0.8; and UMP, 1.3.

Sequence Analyses of DI RNA. Two-dimensional gel electrophoresis to separate the RNase T1 oligonucleotides was carried out as described (15–17). The determination of the 5′-terminal structure and the sequence analysis of cap-containing RNase T1 oligonucleotide (the T1 caps) by using various enzymes and high-voltage paper electrophoresis have been described (15, 18).

RESULTS

Isolation of a SFV 18S DI RNA. When undiluted inocula of SFV were used to serially infect BHK21 cells, the infectivity titer decreased to ≈10% of its initial value by the fourth passage and to ≈0.1% of its initial value by the fifth passage. The virus from these passages interfered with the replication of standard virus in mixing experiments (data not shown). These results are characteristic of the production of DI particles and similar to those obtained by others (10, 11). Labeling of virus-specific RNAs of each passage with [^{3}H]uridine showed that, from the fourth passage on, a new RNA species was present that cosedimented with the 18S ribosomal RNA marker. The sedimentation profile of the intracellular RNAs from passage 11 (Fig. 1 *Lower*) shows that, at this passage, very little of the genomic 42S and the subgenomic 26S RNA is present. The 18S RNA, which was sensitive to digestion with ribonuclease A and was polyadenylated, was absent from cells infected with standard virus (Fig. 1 *Upper*). These results are similar to those reported by others (2, 8, 11), and we thus conclude that the 18S RNA represents a DI RNA.

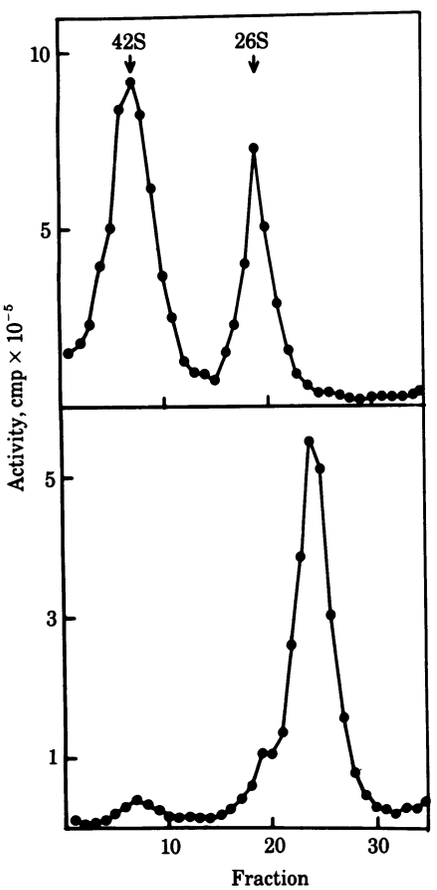

FIG. 1. Sucrose gradient sedimentation of SFV 18S DI RNA isolated from BHK21 cells. BHK21 cells were infected with either standard SFV (*Upper*) or the culture fluid from the 10th undiluted passage of SFV (*Lower*). Intracellular RNAs were labeled with [^{3}H]uridine (2 μCi/ml) in the presence of actinomycin D (1 μg/ml). After 6 hr, a cytoplasmic extract was prepared and the RNA was fractionated on a 15–30% sucrose gradient (15). Centrifugation was for 16 hr at 22,500 rpm and 23°C in a SW27.1 rotor. Fractions (0.5 ml) were collected, and their radioactivity was assayed. Sedimentation is from right to left.

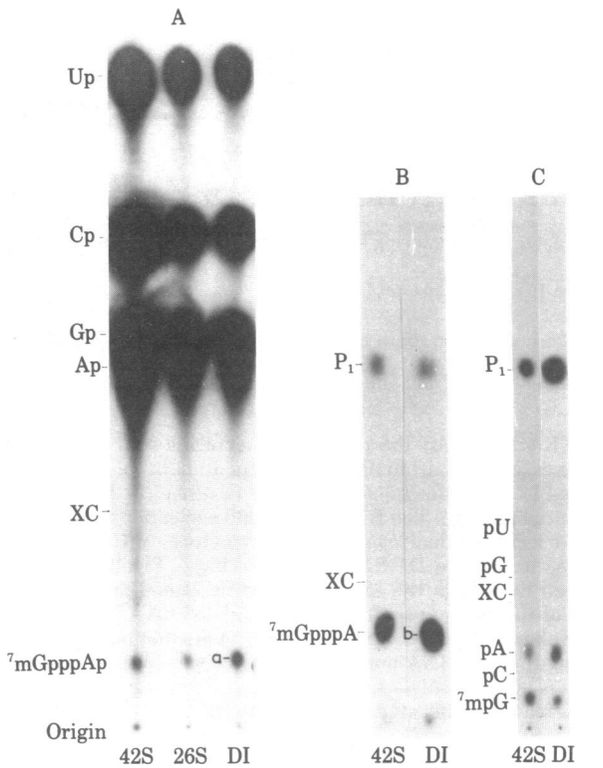

FIG. 2. Characterization of the 5′-terminal cap structure of SFV DI RNA. Uniformly ^{32}P-labeled 42S, 26S, and DI RNA were first digested with a mixture of RNases T1, T2, and A, and the products were analyzed by DEAE paper ionophoresis at pH 3.5 (*A* lanes). The tentative 5′ terminus of the DI RNA (spot a) and the corresponding spot from 42S RNA (^{7}mGpppAp) were eluted with triethylamine bicarbonate, and the eluates were lyophilized and treated with nuclease P1 (15). The products were analyzed by ionophoresis on 3MM paper (*B* lanes). Spot b and ^{7}mGpppA from the 42S RNA were eluted with water, and the eluates were lyophilized and digested with nucleotide pyrophosphatase (15). These products were analyzed on 3MM paper (*C* lanes). The radioactive spots were located by autoradiography using Kodak X-omat films and intensifying screens. Positions of ^{7}mpG and the 3′ and 5′ monophosphate markers are as indicated. XC = xylene cyanol.

5′ Terminus of the DI RNA Is Capped. We (15) and others (19) have shown that the 5′ termini of the 42S and 26S RNAs have the structure ^{7}mGpppAp. To study whether the DI RNA contains a similar cap structure, purified ^{32}P-labeled 42S, 26S, and DI RNAs were digested with a mixture of RNases T1, T2, and A, and the digestion products were analyzed by ionophoresis on DEAE-paper at pH 3.5. In addition to the four monophosphates, a spot comigrating with ^{7}mGpppAp derived from the 42S and 26S RNAs was seen (Fig. 2, spot a). No other tentative 5′ terminus could be identified, suggesting that most if not all of the DI RNA molecules are capped. This conclusion was also supported by quantitation of the radioactivity in the

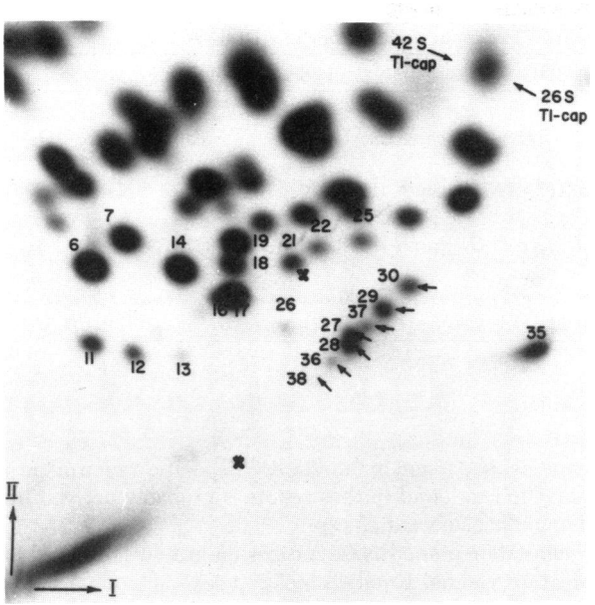

FIG. 3. Two-dimensional polyacrylamide gel analysis of T1 oligonucleotides from DI RNA. Purified DI RNA, labeled with ^{32}P was digested with RNase T1, and the products were analyzed on a two-dimensional gel as described (15–17). The positions of the oligonucleotides were located by autoradiography. The spots were eluted with water from the gel, and the eluates were analyzed for the presence of cap. Spots containing ^{7}mGpppAp are indicated by arrows. Positions of T1 caps from 42S and 26S RNAs are as indicated. Upper x = bromphenol blue; lower x = xylene cyanol.

cap relative to that in the monophosphates.

Spot a was analyzed by digesting the eluted material with penicillium nuclease P1 (see Fig. 2). This treatment released inorganic phosphate (P_i), whereas the bulk of the radioactivity (spot b) comigrated with ^{7}mGpppA derived from the 42S RNA. Spot b was then digested with nucleotide pyrophosphatase, which released P_i, pA, and p^{7}mG; i.e., the products obtained from the 42S RNA cap (see Fig. 2). Thus, the 5′ terminus of the DI RNA has the same structure as that of the 42S and 26S RNAs (^{7}mGpppAp). The specific radioactivity of the middle phosphate in the 5′-5′ linked triphosphate was much higher in the DI RNA cap than that of the corresponding phosphate in the 42S RNA cap, the ratio ^{7}mpG/P_i/pA being 0.9:1.9:1.0 for the 42S RNA cap (see also ref. 15) and 0.5:4.9:1.0 for the DI RNA cap.

The DI RNA Population Is Heterogeneous. It has been shown (2) that the DI RNA of SFV contains T1 oligonucleotides from both the 42S RNA-specific region of the genome and the 3′ region, which is common to both 42S and 26S RNAs. To see whether our 18S RNA had a similar structure, ^{32}P-labeled RNA was digested with RNase T1, and the resulting oligonucleotides were fractionated by two-dimensional polyacrylamide gel electrophoresis (Fig. 3). As expected, the complexity of the fingerprint was much lower than that of the 42S and 26S RNAs (see ref. 15). In contrast to the 42S and 26S RNA fingerprints, the DI RNA fingerprint showed unequal intensities of spots representing large (longer than nine bases) oligonucleotides. Both strong spots (e.g., nos. 6, 7, 14, 16, 17, 18, and 19) and fainter spots (e.g., nos. 11, 12, 13, 22, 25, and 26) are evident. To study the origin of the oligonucleotides, they, as well as a large number of spots (e.g., nos. 6, 7, 14, 16, 17, 18, and 19) and fainter spots from the gels, the eluates were digested with RNase A, and the products were analyzed by ionophoresis of DEAE paper. By comparing the digestion products, the majority of the spots (about 75%—nos. 6, 7, 11, 12, 13, 14, 17, 18, 19, 27, and 30)

Table 1. Molar yields of T1 oligonucleotides from SFV RNA

Spot, no.	RNase A composition	Length, no. of nucleotides	Origin*	Molar† yield
6	A_2U,AU,$(AC)_2$,C_3,U,G	14	42S	1.0
11	A_3C,A_2U,A_2C,$(AU)_2$,C_4,AG	20	42S	0.1
12	$(A_2C)_2$,AU,AC,C_3,U,G	19	42S	0.1
14	$(AU)_2$,C_5,U,A_2G	13	42S	0.8
18	A_4U,C_4,U_2,G	12	42S	0.3
19	A_3U,AC,C_2,U_2,G	11	42S	0.6
21	A_2U,AU,$(AC)_2$,U_2,G	12	26S	0.2
22	$(AU)_2$,C_4,U_2,G	11	26S	0.1
25	$(AU)_2$,AC,C_2,U_2,G	11	26S	0.1
30	A_2U,$(AU)_2$,C_2,U_3,G	15	42S	0.1
35	$(AU)_3$,U_{10},G	17	26S	0.2

* 42S = 42S RNA-specific region (5′ two-thirds of the genome); 26S = 3′ one-third of the genome (common to 42S and 26S RNA).
† Relative to oligonucleotide 6, which is the most abundant one.

were identified as being derived from the 42S-specific portion of the genome and spots 21, 22, 25, and 35 were identified as being derived from the common 3′ one-third of the genome. In addition to the above oligonucleotides, spot 26 and a cluster of diagonally located spots (nos. 28, 29, 36, 37, and 38) were not found in the 42S (nor the 26S) RNA fingerprint. These oligonucleotides are thus DI RNA specific (see below).

The identification of both strong and weak spots as virus-specific oligonucleotides suggested heterogeneity of the RNA. To estimate the molar representation of the oligonucleotides, the spots were cut from the gel and the radioactivity was quantitated (Table 1). As can be seen, there was a large variation in the molar yields of the various oligonucleotides, strongly suggesting heterogeneity of the RNA. Secondary digestion of the oligonucleotides with RNase A suggested that the major spots (nos. 6, 7, 14,

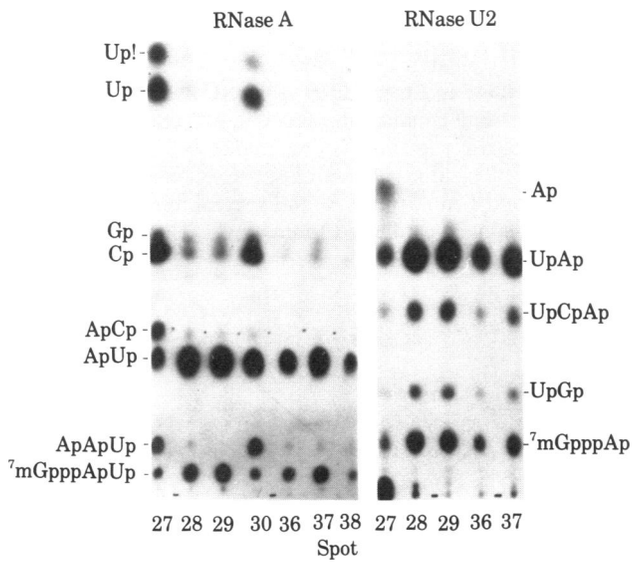

FIG. 4. Sequence analysis of T1-containing oligonucleotides from DI RNA. Spots were eluted from the gel (see Fig. 3) with water, and the eluates were lyophilized. The oligonucleotides were digested with either RNase A or RNase U2, and the products were analyzed by ionophoresis on DEAE paper at pH 3.5 (RNase A) or pH 1.9 (U2). The identity of the digestion products was confirmed by redigesting them with RNase T2 followed by analysis of the digests on 3MM paper. (In this run the positions of Cp and Gp are reversed as compared with those in Fig. 2.)

Table 2. RNase A and U2 digestion products of cap-containing T1 oligonucleotides from SFV DI RNA

	Spot 29	Spot 37	Spot 28	Spot 36	Spot 38
RNase A product	cap AU,$(AU)_5$,C,G	cap AU,$(AU)_6$,C,G,	cap AU,$(AU)_7$,C,G	cap AU,$(AU)_8$,C,G,	cap AU,$(AU)_9$,C,G,
RNase U2 product	cap A,$(UA)_4$,UCA,UG	cap A, $(UA)_4$,UCA,UG	cap A, $(UA)_5$,UCA,UG	cap A, $(UA)_6$,UCA,UG	Not done
Sequence deduced	cap A-U$(A-U)_4$C-A-U-G	cap A-U$(A-U)_5$C-A-U-G	cap A-U$(A-U)_6$C-A-U-G	cap A-U$(A-U)_7$C-A-U-G	cap A-U$(A-U)_8$C-A-U-G
Length in nucleotides*	14	16	18	20	22
Molar yield†	1.00	0.36	0.79	0.17	0.02

* Excluding the ^{7}mG in the cap.
† Relative to the radioactivity in cap AU (RNase A) and cap A (RNase U2).

16, 17, 18, 19, and 35) are homogeneous and do not contain mixtures of two or more oligonucleotides having different sequences but identical charges and sizes.

The 5′-Terminal Nucleotide Sequence Is Heterogeneous. We have recently shown that the sequences of the 42S and 26S RNA 5′-terminal T1 oligonucleotides (the T1 cap) are ^{7}mGpppAUG and ^{7}mGpppAUUG, respectively (15). If the DI RNA is indeed generated by an internal deletion of the 42S RNA but having preserved 5′ and 3′ ends, one would expect the 5′ end to have the same structure as the 42S RNA. To study this, spots were eluted from the gel, digested with RNase T1, T2, and A and the digestion products were analyzed on DEAE paper. This procedure will identify the spot that contains the cap structure (ref. 15). No cap was found in the region to which the 42S and 26S RNA T1 caps are known to migrate (see Fig. 3). Instead, the cap was present in several spots (see Fig. 3, arrows) that migrated more slowly in both dimensions than the cap A-U-G and -A-U-U-G oligonucleotides. These T1 caps are DI RNA-specific, as they are not seen in the fingerprints of 42S and 26S RNA. The DI-specific spot 26 did not contain a cap.

To determine the nucleotide sequence of these "aberrant" spots, they were eluted from the gel, and the eluates were digested with RNase A or U2 (Fig. 4 and Table 2). After RNase A digestion, the eluates from spots 28, 29, 36, 37, and 38 all yielded the same major products—cap AU, AU, C, and G and, in addition, submolar amounts of A-A-U, A-C and U+U!. These products probably represent contaminants from spots 27 and 30. Spots 27 and 30 yielded relativelv less cap A-U and A-U; instead, they contained 1 mol of A-A-U and several moles of C and U. In addition, spot 27 contained 1 mol of AC. These two oligonucleotides were present in the 42S RNA-specific region of the genome. The cap found in these spots is probably derived from contaminating adjacent T1 caps.

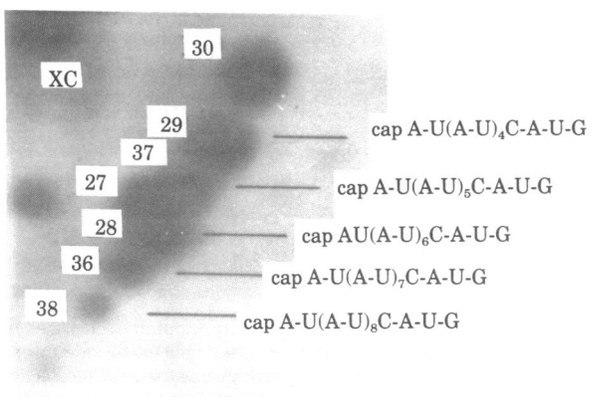

FIG. 5. Sequence of 5′ terminal T1 oligonucleotides from DI RNA. Region of the two-dimensional gel containing the T1 caps. Nucleotide sequences for each oligonucleotide were deduced from the RNase A and U2 digestion products shown in Fig. 4. XC = xylene cyanol.

Quantitation of the RNase A digestion products showed that the T1 caps yielded 1 mol each of cap A-U, C, and G, but different amounts (5–9 residues) of A-U. After RNase U2 digestion, all spots yielded 1 mol each of cap A, (U,C)A and U-G, and variable amounts of U-A. As no A-C or free U were found in the oligonucleotides after RNase A digestion, the C must be located between two A-U residues as A-U-C-A-U. Quantitation of the RNase digestion products suggested that spots 29 and 37 both contained four U-A residues and that spots 28, 36, and 37 contained one U-A residue less per oligonucleotide than expected based on the quantitation of the RNase A digestion products. Exact determination of the number of A-U or U-A residues was hampered by the unequal labeling of the RNA and the higher than average specific radioactivity of the middle phosphate in the cap structure. The combined information from the RNase A and U2 digestions, as well as that from nuclease P1 digestions (data not shown) suggested that the cap-containing oligonucleotides have the sequence cap A-U[$(A-U)_n$,C]A-U-G. The location of the C could not be determined with the enzymes used. However, the most likely position would seem to be between the two A-U residues located closest to the 5′ end (see below), giving the tentative sequences cap A-U$(A-U)_{4-8}$C-A-U-G (Fig. 5). The total molar yields (see Table 2) of the T1 caps was 0.3–0.4 relative to oligonucleotide 6 (the most abundant of the large ones). The two most abundant T1 caps (spots 28 and 29) represented about 75% of the total yield of T1 caps.

DISCUSSION

Our results suggest that SFV 18S DI RNA is composed of a heterogeneous population of RNA species and that these species have heterogeneous 5′-terminal nucleotide sequences with the peculiar structure ^{7}mGpppA-U$(A-U)_n$C-A-U-G (n = 4–8).

The large T1 oligonucleotides identified in the DI RNA fingerprint were present in nonequimolar ratios. The composition of the RNase A digest showed that the oligonucleotides were homogeneous and not composed of a mixture of oligonucleotides having identical sizes and charges but different nucleotide sequences. The presence of oligonucleotides in nonequimolar ratios could mean (*i*) that the DI RNA was contaminated by 42S RNA-specific sequences, (*ii*) that some of the oligonucleotides were present in more than one copy per DI RNA molecule, or (*iii*) that the DI RNA population was heterogeneous. The first possibility seems excluded, as only a few of the 42S RNA-specific spots were found in the DI RNA fingerprint and random contamination would be expected to yield all 42S-specific T1 oligonucleotides. The RNA was both size and oligo(dT)-cellulose selected to minimize contamination by 42S RNA-specific se-

quences. The second possibility cannot be excluded at present. Even if some of the oligonucleotides were duplicated in the DI RNA, this would not be sufficient to explain the nonequimolar yields. Therefore, we interpret the results to mean that the RNA population is heterogeneous. The finding of heterogeneity in the 18S DI RNA is surprising in light of results reported by Kennedy (2), who used a fingerprinting technique similar to ours to show that several large oligonucleotides were present in equimolar ratios in a small SFV DI RNA (DIssl, M_r 0.8×10^6) and in the 42S RNA and found no evidence of heterogeneity (11). Although the reason for this difference in results remains unclear, the fact that a slightly different protocol was used to generate the DI particles could have resulted in the production of a more homogeneous DI-particle population.

All of the DI RNA molecules appeared to contain a 5′-terminal cap similar to the one found in the 42S and 26S RNAs. Contrary to the expected cap A-U-G, as found in the 42S RNA, the DI RNA had a different and heterogeneous 5′-terminal sequence. This was unexpected, and contradicts the proposal that the DI RNAs of SFV and Sindbis virus have conserved the extreme 5′ and 3′ sequences from the 42S RNA (2, 8, 9). The series of cap-containing oligonucleotides found on the two-dimensional gel suggests that the 5′ terminus is synthesized by a mechanism that is different from that of the 42S RNA and allows addition of a variable number of A-U residues. The C residue found in the T1 caps could not be located exactly; the results from the RNase A and U2 digestions were consistent with its being located between any of the A-U residues. It has tentatively been placed between the two A-U residues located closest to the 3′ end, because one possible way by which a variable number of A-U residues could be synthesized is by the RNA polymerase initiating on the template at different positions of an uninterrupted A-U stretch. This would result in a variable number of A-U residues next to the cap. The RNase digestion results show that the 3′ end of the T1 cap is always A-U-G. Whether this A-U-G in fact corresponds to the authentic 42S RNA 5′ end (cap A-U-G) remains to be determined. The DI RNAs possess all the characteristics of an eukaryotic mRNA: a 5′-terminal cap, an A-U-G close to the cap, and a poly(A) tract. So far we have not been able to show either *in vitro* or *in vivo* that these RNAs direct the synthesis of any polypeptide(s) (unpublished data). It is, however, possible that the DI RNAs could direct the synthesis of polypeptides, perhaps only short oligopeptides, that have not been detected by $NaDodSO_4$ gel analysis.

The mechanism for the synthesis of our DI RNA, and especially the 5′ termini, is so far unknown. Our data do not contradict the model proposed by Stark and Kennedy (11), Guild and Stollar (8), and Dohner *et al.* (9), which postulates that the DI RNAs have internal deletions with some conserved sequences at the 5′ and 3′ ends of the genome. Our results raise some doubts as to whether the extreme 5′-terminal sequence indeed is conserved unmodified. The heterogeneity of the RNA suggests that the internal deletion may involve different regions of the RNA. The conserved sequences must in any event contain signals for RNA replication and encapsidation derived from the 42S RNA-specific region, because the 26S RNA is neither replicated nor encapsidated. Thus, by studying several different clones of stable DI RNAs and by identifying the conserved regions, it should be possible to characterize these regulatory sequences in detail.

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