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A new series of RNAs associated with the genome of spleen focus forming virus (SFFV) and poly(A)-containing RNA from SFFV-infected cells

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ABSTRACT

A series of low molecular weight RNAs (4.5 to 5.5S) as well as other 4 to 7S RNAs were dissociated from genomic RNA of spleen focus forming virus (SFFV) by heating. On two dimensional polyacrylamide gel electrophoresis, this series of RNAs gave a series of more than thirty spots. RNase T1 fingerprints of these spots were identical except for differences in 3'-terminal oligonucleotides, which were mainly due to different numbers of uridylic acid residues, larger RNA-molecules containing poly(U) sequences at their 3'-termini. This series of RNAs is also associated with poly(A)-containing nuclear and cytoplasmic RNAs from SFFV-infected cells.

INTRODUCTION

Virions of retrovirus contain low molecular weight 4 to 7S RNAs as well as high molecular weight genomic RNA (1-9). Most of these low molecular weight RNAs are released into solution by disruption of the virions, but some are associated with genomic RNA in a 70S complex, and are released by denaturing the complex (6-9). Most of these low molecular weight RNAs are tRNAs, 5S RNAs, 5.5S RNA, 7S RNA, etc. of host origin (7,10,11). One of the 4S RNAs associated with the genome has been identified as the primer for initiation of genomic RNA-dependent DNA synthesis by reverse transcriptase *in vitro* : $tRNA^{Trp}$ for Rous sarcoma virus (11-14) and $tRNA^{Pro}$ for murine leukemia virus (15,16). However, the functions of the other small molecular weight RNAs in the virions are still unknown.

During studies on identification of the primer for DNA synthesis of Moloney murine leukemia virus (Mo-MuLV), Peters *et al.* found a series of RNAs in the 70S-associated 4S RNA fraction. This series of RNAs is composed of five components that are

structurally identical except for differences in the number of uridylate residues at one end (15). In the present work we found that not only this series of RNAs, but also much longer molecules, are associated with genomic RNA of spleen focus forming virus (SFFV) and poly(A)-containing nuclear and cytoplasmic RNAs from SFFV-infected cells.

MATERIALS AND METHODS

Materials. RNases T1 and T2 were obtained from Sankyo Co. Ltd. Pancreatic RNase was purchased from Sigma Chemical Co. Poly(U)-Sepharose and poly(A)-Sepharose were products of Pharmacia Fine Chemicals. Acrylamide and N,N'-methylene-bisacrylamide were obtained from Eastman Organic Chemicals. Cellulose acetate (Separax) was purchased from Fuji Film Co.

Preparation of ^{32}P-labeled RNA. T3-K-1 cells (K-1 cells)(17), a subclone of differentiation inducible Friend leukemia cell line T3-C1-2(18,18'), were used in these experiments. These cells release a large excess of SFFV over helper lymphoid leukemia virus (LLV)(17). Before labeling, cells were incubated in phosphate-free Dulbecco's modified Eagle's medium supplemented with 10% dialyzed fetal calf serum (5×10^5 cells/ml) for 16 hours. Then carrier free $^{32}PO_4^{3-}$ was added at a final concentration of 0.5 mCi/ml and the cells were labeled for 4 or 24 hours.

Extraction, fractionation and stepwise heat denaturation of viral RNA from K-1 cells labeled for 24 hours were performed as described previously (15,19). On a sucrose gradient of K-1 viral RNA, genomic RNA of SFFV(~60S), amounting to almost 80% of the total genomic RNA, gave a big peak, while helper LLV(~70S) appeared as a small shoulder.

K-1 cells that had been labeled for 4 or 24 hours were washed twice with TSE buffer (0.02M Tris-HCl, pH 7.5; 0.1M NaCl; 0.001M EDTA), and nuclear RNA was extracted from the purified nuclei (21) by the hot phenol-SDS method (20). The cytoplasmic fraction obtained during isolation of the nuclei was centrifuged at 10,000 g for 10 min, and the resulting supernatant was adjusted to 0.1M NaCl and 0.5% SDS, and extracted with phenol-chloroform mixture (1:1,v/v). The phenol layer was re-extracted with the same volume of 0.1M Tris-HCl(pH 9.0), 0.1M NaCl, 1mM

EDTA, and 0.5% SDS(22). The two upper layers were then combined and extracted with chloroform-isoamyl alcohol (24:1,v/v). The aqueous phase was adjusted to 0.3M NaCl and cytoplasmic RNA was precipitated from it with 2 volumes of ethanol.

Poly(A)-containing RNA was purified by affinity chromatography on poly(U)-Sepharose as follows. Nuclear or cytoplasmic RNA was dissolved in 0.4 ml of NETS buffer (0.1M NaCl; 0.01M Tris-HCl, pH 7.5; 0.01M EDTA; 0.2% SDS) and applied to a column (0.5×4cm) of poly(U)-Sepharose equilibrated with NETS buffer at room temperature. The column was washed with 7ml of NETS buffer and then poly(A)-containing RNA was eluted with formamide buffer (90% formamide; 0.01M Tris-HCl, pH 7.5; 0.01M EDTA) and precipitated from the eluate with 2 volumes of ethanol. Affinity chromatography of poly(U)-containing RNA on poly(A)-Sepharose was performed in the same manner.

Two dimensional polyacrylamide gel electrophoresis. Cellular or viral low molecular weight RNAs were separated by two dimensional polyacrylamide gel (2-D gel) electrophoresis. The procedures used for application of the sample, electrophoresis, quantitation of each RNA spot, and elution of material from the gel were essentially as described previously (15,19,23), except that gel electrophoresis in the first dimension was carried out at high voltage and low buffer concentration (24), since this gave better resolution in the region of tRNA.

Fingerprint analysis. Fingerprints of RNase T1 and pancreatic RNase digestion products were prepared as described elsewhere (15,25).

RESULTS

Analysis of virion-associated low molecular weight RNA. Low molecular weight RNAs of ^{32}P-labeled SFFV virions were analyzed by 2-D gel electrophoresis. Figure 1a shows the pattern of free 4S RNA. The autoradiographic pattern is much simpler than that of cytoplasmic tRNA (Fig. 1c). Fingerprint analysis of each spot showed that free 4S RNAs of SFFV and Mo-MuLV (15) were qualitatively and quantitatively very similar (data not shown). Figure 1b shows the 2-D gel pattern of the low molecular weight RNAs associated with the genome. This autoradiogram was also similar

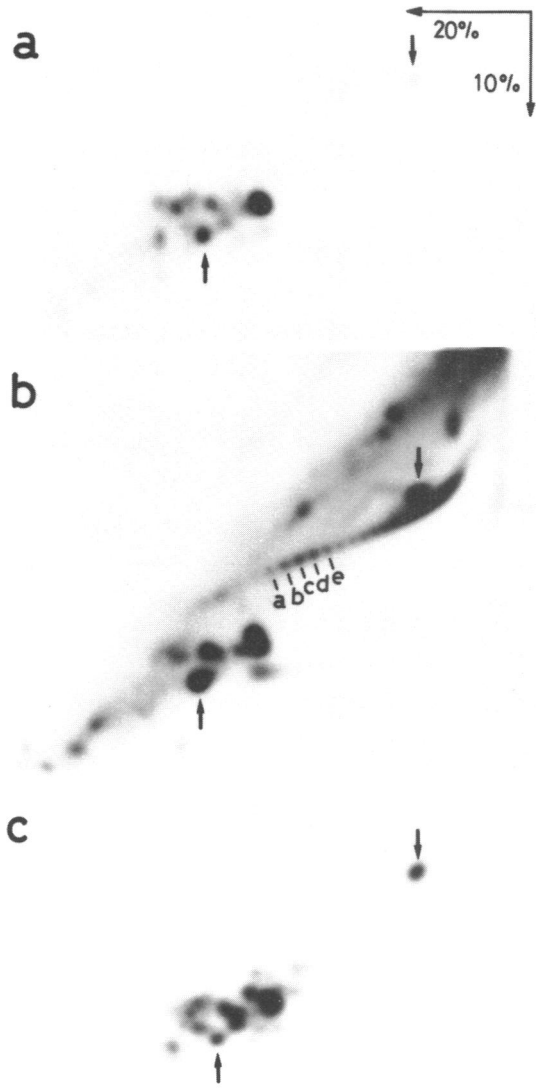

Figure 1. 2-D gel electrophoretic patterns of ^{32}P-labeled small RNAs from SFFV and SFFV-infected cells. (a) Free small RNAs in SFFV; (b) genome-associated small RNAs in SFFV; (c) cytoplasmic RNAs of 4 to 5S from SFFV-infected cells. Upward arrows indicate the position of $tRNA^{Pro}$, the primer for Mo-MuLV RNA-directed DNA synthesis in vitro. Downward arrows indicate the position of ribosomal 5S RNA. a through e in (b) indicate the positions of the series of RNAs from Mo-MuLV.

to that of Mo-MuLV, except for the series of RNAs which ranged from 4.5 to 5.5S. In the case of Mo-MuLV, there are five components in this series of RNAs (denoted as 16a through e in reference 15), whereas in SFFV there seem to be over thirty components, although larger RNAs were not separated from one another on the gel. Fingerprint analysis showed that the five components of Mo-MuLV correspond to the smallest five components of SFFV. Figure 2 shows the RNase T1 fingerprint and its schematic drawing of the RNAs of about 5S in the series of RNAs (the

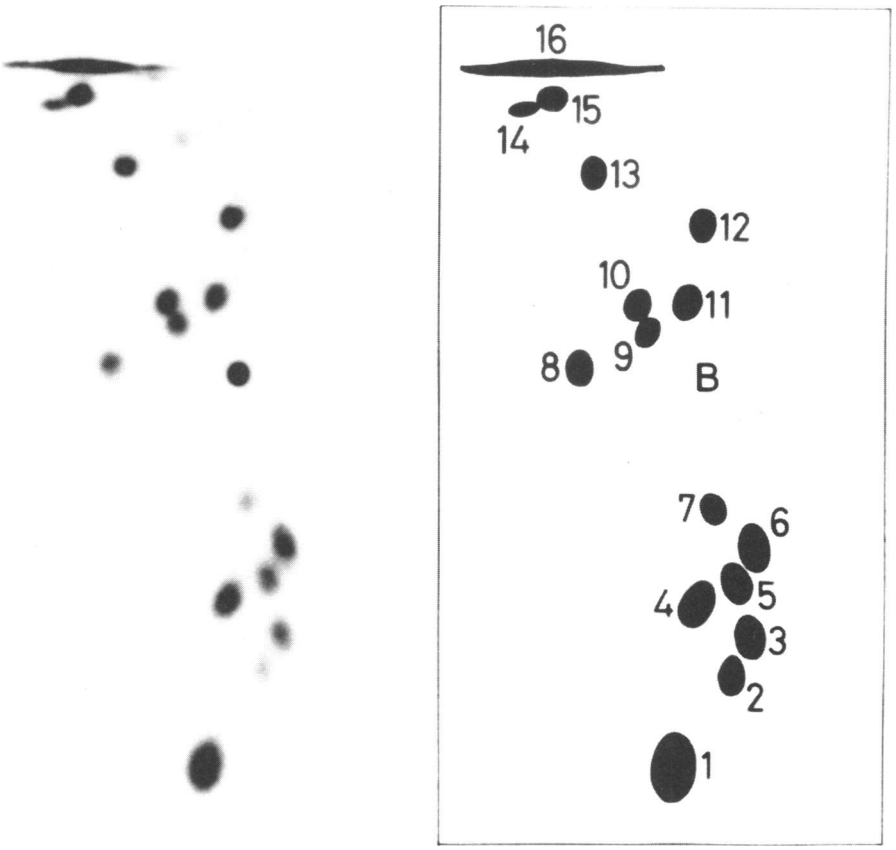

Figure 2. RNase T1 fingerprint of RNAs of about 5S and its schematic drawing. The first dimension, right to left, was electrophoresis on cellulose acetate in pyridine acetate (pH 3.5)-7M urea. The second dimension, from top to bottom, was electrophoresis on DEAE-cellulose in 7% formic acid. B denotes the position of the blue dye marker (xylene cyanol FF).

numbering system given in reference 15 is used). The fingerprint is identical to that of Mo-MuLV spot 16 RNAs, except for oligonucleotides 14 and 16. Preliminary experiments showed that oligonucleotide 14 is the 5'-terminal oligonucleotide, and that oligonucleotide 16 is the 3'-terminus of this RNA. The difference between oligonucleotide 14 in Mo-MuLV and SFFV must result from different modifications at their 5'-termini. Oligonucleotide 14 of each spot in the series of RNAs from SFFV had the same mobility, as shown in Figure 2. Peters et al. reported that the difference between the components in this series of RNAs from Mo-MuLV was that the larger RNAs had progressively more uridylic acid residues in oligonucleotide 16(15). This is also true in the case of SFFV RNAs: the only difference between the RNase T1 fingerprints of each component of this RNA series was in the mobility of oligonucleotide 16. In this figure, the sample RNA was a mixture of about ten RNAs of different lengths, and thus oligonucleotide 16 appeared as a streak. Analysis of oligonucleotide 16 of each RNA by digestion with pancreatic RNase showed that larger RNAs had progressively more uridylic acid residues and that the amounts of the other digestion products, Cp, A-Cp, and A-Up, were constant. The main portion of spot 16 in Figure 2 contained about 20 more uridylic acid residues than those in spot 16 of the smallest RNA.

Figures 3a and b are the pancreatic RNase fingerprints of the smallest RNA and the RNAs in the main portion (about 5S) of this series of RNAs, respectively. The two fingerprints are identical, except for the amount of uridylic acid. Comparison of the intensities of Up and other oligonucleotides shows that the larger RNA has more uridylic acid than the smaller one.

These results indicate that the members of this series of RNAs all contain a basic sequence, but have different numbers of uridylic acids at the 3'-terminus. Therefore, larger molecules contain poly(U) sequences at the 3'-end. This conclusion was confirmed by affinity chromatography on poly(A)-Sepharose. One of the smaller RNAs (spot b in Fig. 1b) and larger RNAs of about 5S were loaded on poly(A)-Sepharose columns. About 70% of the larger RNAs was retained in the column, whereas all the ^{32}P radioactivity of the smaller RNA passed directly through the

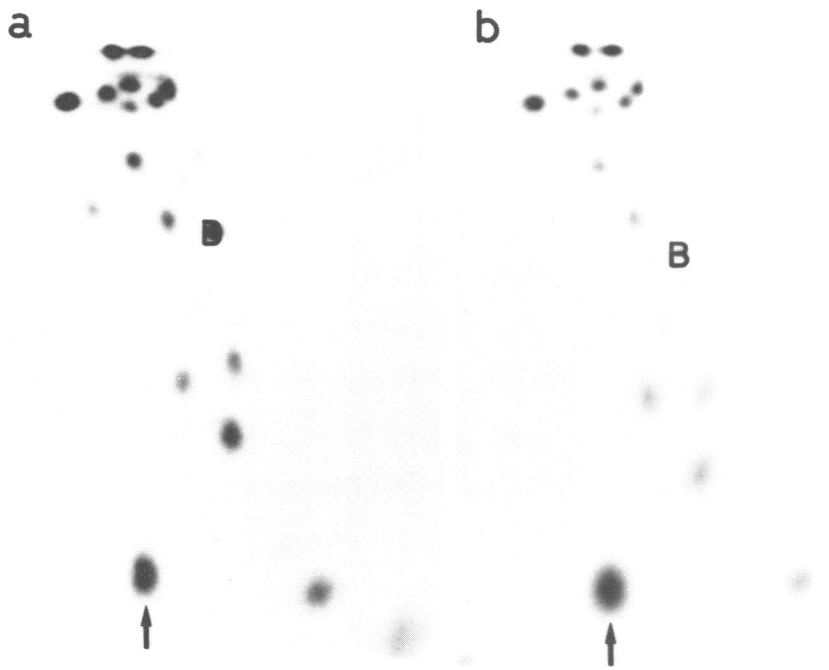

Figure 3. Pancreatic RNase fingerprints of smaller and larger RNAs. (a) One of the smallest RNAs (spot b in Fig. 1b); (b) mixture of larger RNAs of about 5S. Upward arrows indicate the position of Up.

column.

In Mo-MuLV, the series of RNAs bound weakly to genomic RNA and were released below 65°C, whereas $tRNA^{Pro}$, the primer for RNA-directed DNA synthesis, remained associated with genomic RNA at 65°C and was released by heating to 95°C (15). Figure 4 shows the results of 2-D gel analysis of small RNAs released by stepwise heat-denaturation of SFFV genomic RNA. The majority of this series of RNAs as well as several tRNAs and 5S RNAs were released from genomic RNA at 65°C (Fig. 4a). In contrast, $tRNA^{Pro}$ (possible primer) and the minority of this series of RNAs (5 to 5.5S) remained bound at 65°C and were released at 95°C (Fig. 4b). This result indicates that the majority of this series of RNAs were bound to genomic RNA of SFFV at the basic

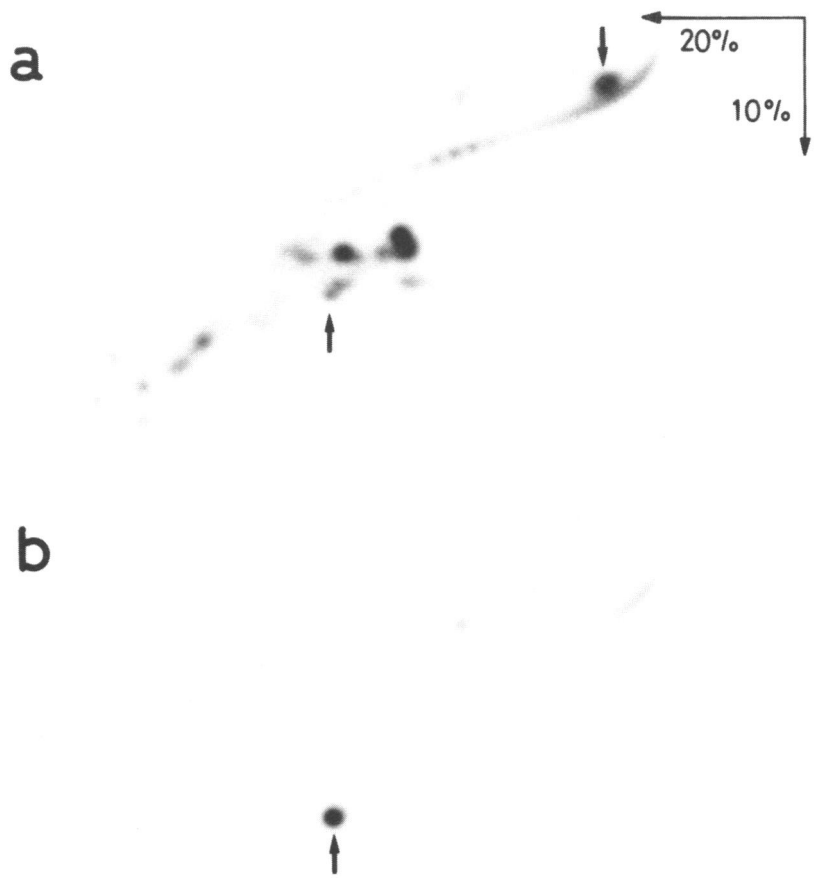

Figure 4. Stepwise heat denaturation of SFFV genomic RNA. Uniformly ^{32}P-labeled SFFV 60~70S RNA was heated to 65°C and then to 95°C in 10mM Tris-HCl (pH 7.5), 10mM NaCl, 1mM EDTA (12,15) and analyzed by 2-D gel electrophoresis (15,19). (a) Small RNAs released below 65°C; (b) small RNAs released between 65 and 95°C. Arrows as in Fig. 1.

sequence and that the minority of larger RNAs were also bound at the 3'-end as poly(A)-poly(U) hybrids.

Finally, the molar content of the series of RNAs in the 32S subunit of genomic RNA was calculated from the amount of ^{32}P,

determined by counting Cerenkov radiations of each spot (15). Assuming that the average lengths of 32S subunits and the series of RNAs were 7,000 and 120 nucleotides, respectively, it was calculated that 85 ± 5% of the 32S subunits were associated with one of the series of RNAs.

Analysis of low molecular weight RNAs from SFFV-infected cells. For examination of the distribution of this series of RNAs in SFFV infected cells, ^{32}P-labeled cells were separated into nuclear and cytoplasmic fractions. RNA was prepared from each fraction and further fractionated by poly(U)-Sepharose column chromatography. The resulting nuclear and cytoplasmic poly(A)-containing RNAs were then analyzed by 2-D gel electrophoresis. Figure 5a shows the 2-D gel pattern of small RNAs released at 95°C from cytoplasmic poly(A)-containing RNA. In contrast to the composition of the series of RNAs associated with the viral genome (Fig. 1b), smaller RNAs (4.5S) were abundant (80%) in the series of RNAs associated with cytoplasmic poly(A)-containing RNA. Moreover, several 4S RNAs which were different from tRNA species released from viral RNA, were also obtained. Figure 5b shows the pattern of small RNAs released from nuclear poly(A)-containing RNA. Only four to five spots of 4.5S RNAs were found on the gel. One of these spots (correspond to spot d in Fig. 1b) was eluted from each gel, and digested with RNase T1. The RNase T1 fingerprints of these two samples were identical (Fig. 6), and were also indistinguishable from that of the viral spot d.

In these cases, poly(A)-containing RNA was purified by affinity chromatography on poly(U)-Sepharose. If many small RNAs terminating in poly(U) are bound to the poly(A) sequence of poly(A)-containing RNA, this complex may not be retained on the column. This possibility was tested by analyzing the flow-through fractions from the poly(U)-Sepharose column by 2-D gel electrophoresis. Considerable amounts of the series of RNAs (about 5-fold more radioactivity than in the bound fractions) were obtained in the flow-through fractions of nuclear and cytoplasmic poly(A)-containing RNA, but no significant amounts of larger poly(U)-containing molecules were recovered in this fraction. It is still unknown whether these smaller molecules

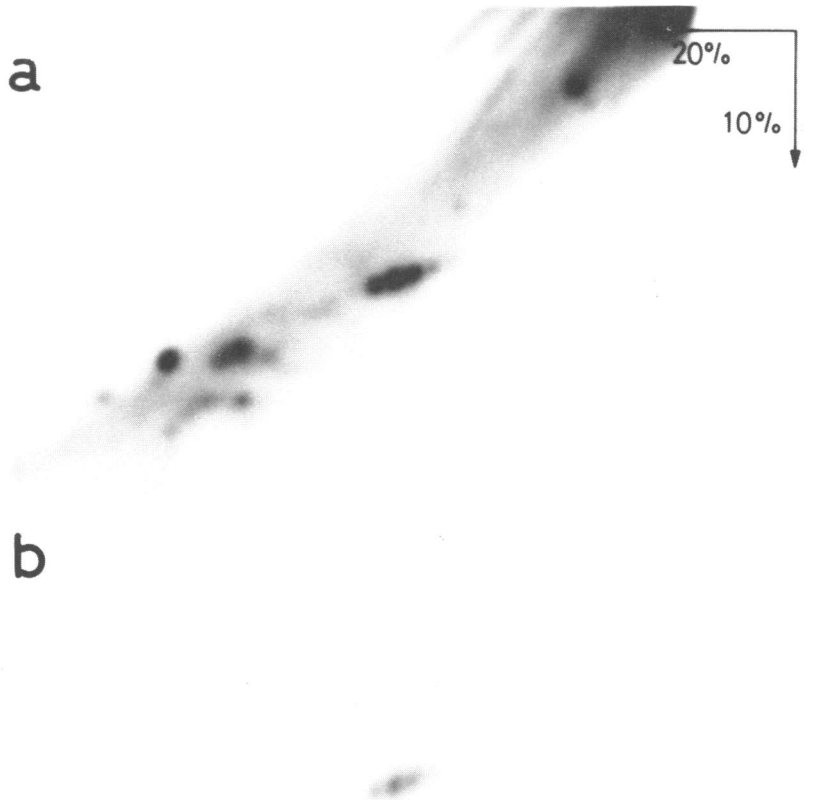

Figure 5. 2-D gel electrophoretic patterns of ^{32}P-labeled small RNAs released from cellular poly(A)-containing RNAs. (a) Small RNAs released from cytoplasmic poly(A)-containing RNA; (b) small RNAs released from nuclear poly(A)-containing RNA. These autoradiograms were obtained from the RNAs of cells labeled for 4 hours. Essentially the same patterns were obtained for RNAs of cells labeled for 24 hours.

are in a free form or are associated with other RNAs. The series of RNAs are adsorbed very tightly to DEAE-Sephadex and are not eluted with 1M NaCl (F. Harada, unpublished data), which

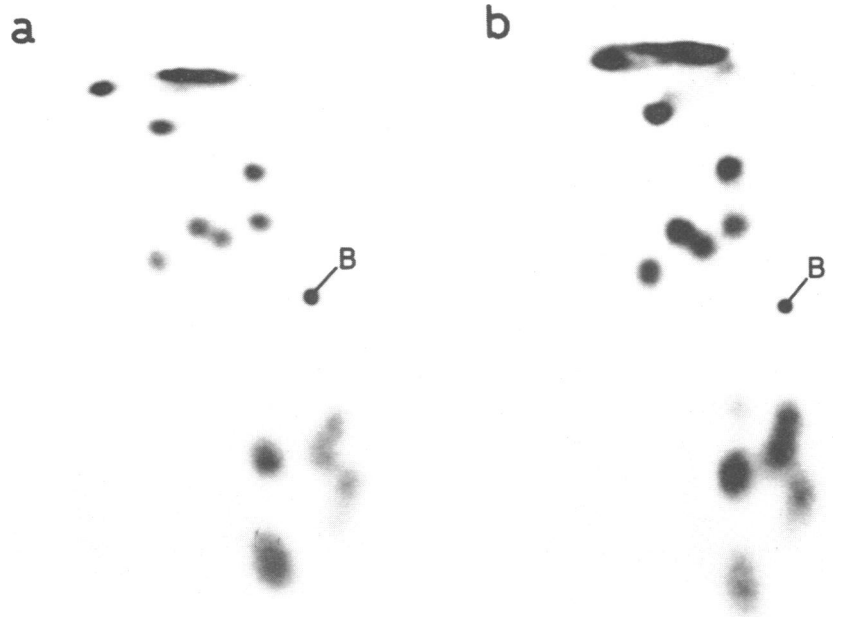

Figure 6. RNase T1 fingerprints of small RNAs released from cellular poly(A)-containing RNAs. Small RNAs corresponding to spot d of Fig. 1b were eluted from the gels (Fig. 5), and digested with RNase T1. (a) Spot d from cytoplasmic poly(A)-containing RNA; (b) spot d from nuclear poly(A)-containing RNA.

would explain why this series of RNAs was not obtained from the sample in Figure 1c purified on DEAE-Sephadex (27).

DISCUSSION

In this work we isolated a new species of small RNAs from SFFV virions and SFFV-infected cells. These molecules were associated with the genomic RNA of SFFV and the cytoplasmic and nuclear poly(A)-containing RNAs of infected cells. The series of RNAs is composed of more than thirty components ranging from 4.5 to 5.5S on gel electrophoresis. Genomic RNA of virions and

cytoplasmic poly(A)-containing RNAs were associated with all members of this series of RNAs. The main component of this series of RNAs in viral genomic RNA are about 5S, whereas 80% of the components in cytoplasmic poly(A)-containing RNA are 4.5S. In contrast, nuclear poly(A)-containing RNA is associated with only 4.5S RNAs. The nucleotide sequences of these molecules from the three different sources are identical except for their 3'-termini, larger RNAs having progressively more uridylic acid residues at the 3'-end.

From these findings we suggest that newly synthesized nuclear poly(A)-containing RNA is associated with 4.5S RNA in the nucleus, and then matures to cytoplasmic poly(A)-containing RNA and is transported to the cytoplasm. In the cytoplasm, the 3'-terminus of some of the 4.5S RNA associated with cytoplasmic poly(A)-containing RNA is combined with uridylic acid residues by poly(U)-polymerase (28-32). Then the complex containing genomic RNA is packaged into virus particles. The lack of uniformity at the 3'-termini of these small RNAs associated with nuclear poly(A)-containing RNA may be a result of incomplete termination of transcription or post-transcriptional cleavage by some nuclease. It should be noted that these molecules also exist in uninfected mouse cells (manuscript in preparation).

Since the series of RNAs is associated with nuclear, cytoplasmic, and viral poly(A)-containing RNAs, it could have one or more of such functions as participation in splicing and joining of nuclear poly(A)-containing RNA, transportation of mature cytoplasmic poly(A)-containing RNA from the nucleus to the cytoplasm, packaging of viral genomic RNA into virions, translational control of mRNA, transcriptional control by reverse transcriptase, and protection of poly(A)-containing RNA from nuclease attack. Determinations of the nucleotide sequences of these molecules and their binding site on poly(A)-containing RNA should provide information on their function(s).

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