Structural complexity of defective-interfering RNAs of Semliki Forest virus as revealed by analysis of complementary DNA

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ABSTRACT

   The 18S defective interfering RNA of Semliki Forest virus has been reverse transcribed to cDNA, which was shown to be heterogeneous by restriction enzyme analysis. After transformation to E.coli, using pBR322 as a vector, two clones, pKTH301 and pKTH309 with inserts of 1.7 kb and 2 kb, were characterized, respectively. The restriction maps of the two clones were different but suggested that both contained repeating units. At the 3' terminus, pKTH301 had preserved 106 nucleotides and pKTH309 102 nucleotides from the 3' end of the viral 42S genome. The conserved 3' terminal sequence was joined to a different sequence in the two clones, and these sequences were not derived from the region coding for the viral structural proteins. The DI RNAs represented by the two clones are generated from the viral 42S RNA by several non-continuous internal deletions, since the largest colinear regions with 42S RNA are 320 nucleotides in pKTH301, and 430 and 340 nucleotides in pKTH309. All these fragments had unique RNase T1 oligonucleotide fingerprints, suggesting that they were derived from different regions of 42S RNA.

INTRODUCTION

   The 42S RNA genome of Semliki Forest virus (SFV), an alphavirus, consists of about 13 000 nucleotides. The 5' end has a cap-structure $m^7$GpppAUG and the 3' end a poly(A) tract (1-3). Another positive-strand RNA is synthesized in SFV infected cells: the 26S RNA, which codes for the virion structural proteins is identical to the 3' third of 42S RNA and has a cap $m^7$GpppAUUG (3-6). The complete nucleotide sequence of 26S RNA has recently been determined (7,8). Both 42S and 26S positive strands are synthesized from 42S RNA negative strands (for review see 5,6).

   When the virus is passaged serially with undiluted inoculum, defective interfering (DI) particles are formed which strongly inhibit the replication of the standard virus (9,10) when replicating in a mixed infection together with standard virus. DI particles can be considered as deletion mutants of the standard virus (11-13).

   We have recently isolated DI RNAs after undiluted passages of SFV in BHK cells (14). The 18S DI RNAs have 3' terminal poly(A) and a heterogeneous 5'

terminal cap-structure $m^7GpppAU(AU)_{4-8}CAUG$ (15).

Here we describe the properties of double-stranded cDNA, which was obtained by reverse transcription of 18S DI RNA from the 11th undiluted passage of SFV in BHK cells. The cDNA was cloned in E.coli using pBR322 as a vector and two clones, pKTH301 and pKTH309 were compared. The complete nucleotide sequence of pKTH301 is reported elsewhere (16).


## MATERIALS AND METHODS

### Cells and virus

The origin and cultivation of BHK21 cells and the prototype strain of SFV has been described previously (17). SFV was passaged to generate DI particles (14) and progeny virus from the 10th undiluted passage, clarified by centrifugation at 10 000 g for 30 min and stored at $-40^0C$, was used as inoculum for production of DI RNA.

### Isolation of cytoplasmic RNAs

BHK21 cells in 1 liter roller bottles were infected with undiluted passage 10 virus (1 ml/$10^7$ cells) in MEM (Eagles minimum essential medium) supplemented with 0.2% bovine serum albumin (BSA). After 2 h adsorption at $37^0C$, the inoculum was removed and the cells were washed twice with Hank's balanced salt solution. MEM-BSA medium containing 20 mM Hepes pH 7.2 and 1 µg/ml of actinomycin D was added. At 3 h postinfection 500 µC/$7x10^7$ cells of $^3H$ uridine (29 Ci/mmol) was added and incubation continued for 7 h. The cytoplasmic RNAs were prepared as described previously (3,14). The 18S RNA was isolated from the cytoplasm in 15 to 30% (w/w) sucrose gradients made in 10 mM Tris-HCl, pH 7.4, 0.14 M NaCl, 1 mM EDTA (TNE) and 0.1% SDS. Centrifugation was in a Beckman SW27 rotor at 25 000 rpm for 15 h at $23^0C$. The 18S RNA peak was pooled, precipitated with 2.5 vol of ethanol and chromatographed on oligo(dT) cellulose (15).

The preparation of $^{32}P$-labeled cytoplasmic 42S and 26S RNA and of virion 42S RNA from purified SFV has been described previously (3,18).

### Synthesis of cDNA

For synthesis of the first strand the reaction mixture (100 µl) was: 50 mM Tris-HCl, pH 8.3, 60 mM KCl, 12 mM MgCl$_2$, 1 mM dithiotreitol (DTT) and 0.8 mM each of dCTP, dATP, and dTTP, 0.2 mM dGTP, 30-50 µCi of $(^{32}P)\alpha$-dGTP (about 400 Ci/mmol), 100-300 µg/ml of DI RNA as template and 5 µg/ml of oligo(dT$_{12-18}$) (PL Biochemicals) as a primer. The incubation was for 60 min at $42^0C$ with 400 U/ml of reverse transcriptase from avian myeloblastosis virus, a kind gift from Dr. J. Beard (Life Sciences Inc., Florida). The RNA template

was then hydrolyzed in 0.25 M NaOH at $50^0$C for 2 h followed by neutralization with HCl. The cDNA was chromatographed on Biogel P30 (BioRad) column and recovered by ethanol precipitation. The second strand was synthesized for 2 h with reverse transcriptase under the conditions mentioned above but omitting ($^{32}$P)α-dGTP. After Biogel P30 chromatography the cDNA was treated with 0.2 I.U. of S1 nuclease (Calbiochem) in 60 µl of 0.3 M NaCl, 30 mM Na-acetate, pH 4.5, 3 mM ZnCl$_2$ (S1 buffer) and 9-12S double-stranded cDNA was selected by centrifugation on a 15 to 30% (w/w) sucrose gradient for 30 h at 25 000 rpm in a SW27 rotor at $23^0$C as described above for the RNA. The DNA was precipitated with ethanol and stored at $-20^0$C.

## Cloning in E.coli

The 3' termini of the double stranded cDNA were elongated with dCTP to give homopolymeric tails in a 50 µl reaction mixture containing 0.14 M cacodylic acid, 30 mM Tris adjusted to pH 7.6 with KOH, 1 mM CoCl$_2$, 1 mM DTT, 0.13 mM $^3$H-labeled dCTP (4.7 Ci/mmol), 300 ng double stranded cDNA and 20 units terminal transferase (Bethesda Research Laboratories, USA) (19). pBR322 linearized with PstI was similarly tailed with dGTP. After 30 min incubation at $37^0$C, approximately 12 dC and 25 dG residues had been added, respectively. For molecular cloning the tailed cDNA and pBR322 were annealed in a molar ratio of 1:1 and used to transform E.coli strain χ1776 (kindly provided by R. Curtiss III) (20). Tetracyclin resistant, ampicillin sensitive colonies containing SFV specific sequences were detected by colony hybridization (21). Plasmid DNA was purified from bacteria treated for 5 h with 12.5 µg/ml chloramphenicol by scaling up the alkaline extraction method (22). After treatment with ribonuclease and pronase the DNA was purified by isopycnic banding in CsCl-ethidium bromide gradients followed by sedimentation in 15 to 30% sucrose gradients for 18 h as described above. For further experiments the plasmids were transformed (23) to E.coli HB101 (24). From this strain the plasmids were purified using the cleared lysate procedure (25) and banding in CsCl and sucrose gradients.

All work with recombinant DNA was performed in certified P2 conditions according to the instructions of the Finnish National Board of Health Committee for Recombinant DNA Research.

## Hybridizations

Single stranded cDNA/RNA. Single stranded $^{32}$P-labeled cDNA (less than 1 ng) was hybridized to 5 µg of nicked 42S RNA (or yeast RNA) in 100 µl of 5 x SSC (SSC = 0.15 M NaCl, 15 mM Na-citrate), 50% formamide for 4 h at $37^0$C. The

annealed material was precipitated with ethanol and digested with S1 nuclease
(0.25 I.U.) in 50 µl of S1 buffer. The amount of hybridized cDNA was deter-
mined as the TCA precipitable radioactivity.

Cloned DNA/RNA. Hybrid plasmids were cleaved with Bgl I restriction
enzyme and after phenol extraction precipitated with ethanol together with
$^{32}$P-labeled 18S DI RNA or 42S RNA. The precipitate was resuspended in 70%
formamide, 0.4 M NaCl, 0.1 M MOPS-NaOH, pH 7.5, 10 mM EDTA, 0.1% SDS and the
DNA denatured at 66°C for 10 min. The DNA/RNA hybrids were allowed to form at
52 to 56°C for 4 to 14 h (26). The samples were treated in either of two ways:
1) The hybridization mixture was cooled on ice, deionized glyoxal added to 1 M
and the preparation incubated at 11°C for 2 h (27). The samples were dialyzed
against 10 mM Tris-HCl, pH 7.4, 1 mM EDTA for 16 h and precipitated with etha-
nol. The precipitates were dissolved in S1 buffer and digested with various
amounts of S1 nuclease (0.1 to 1 I.U.) for 30 to 120 min at 37°C. 2) The
hybridization mixture was diluted with 15 vol of S1 buffer containing 20 µg/ml
sheared and 20 µg/ml sheared heat denatured calf thymus DNA and incubated with
0.5 I.U. S1 nuclease for 1 h at 37°C. The heteroduplexes were analyzed by gel
electrophoresis using double-stranded DNA as molecular weight markers.

Gel electrophoresis
    Single stranded DNA was analyzed by electrophoresis on alkaline agarose
gels (28). The gels were neutralized for 1 h in 0.3 M Na-acetate, pH 4.8
before staining with ethidium bromide. Double stranded DNA was electrophoresed
on neutral agarose gels in E-buffer (29) or on 2.5-10% linear polyacrylamide
gradient gels (30). For autoradiography the agarose gels were dried onto DEAE
paper and polyacrylamide gels onto 3MM paper. Intensifying screens were used
when necessary. Fragments were eluted from agarose gel slices by electrophor-
esis into hydroxyapatite in dialysis bags (31) and from crushed polyacrylamide
by diffusion (32).

Restriction enzyme digestion
    Commercial restriction enzymes were used throughout. The digestions
were performed in 10 mM Tris-HCl, pH 7.6, 10 mM MgCl$_2$, 10 mM NaCl, 1 mM DTT
(BstNI, ClaI, HaeIII, HpaII, KpnI, MspI, SacII, TaqI), the same buffer with
50 mM NaCl (AvaI, BamHI, BglI, HindIII, HinfI, PstI, XbaI, XhoI) or with
100 mM NaCl (EcoRI) at the optimal temperatures.

End labeling and nucleotide sequence analysis
    5' terminal labeling of restriction fragments and nucleotide sequence
analysis were essentially as described by Maxam and Gilbert (32). 3' termini

were labeled in a "fill-in" reaction with the appropriate $(^{32}P)\alpha$-NTP (2000-3000 Ci/mmol) using 200 U/ml of reverse transcriptase in 50 μl of the buffer described above for cDNA synthesis. All isotopes were from the Radiochemical Centre, Amersham.

## Two-dimensional RNA fingerprinting

Nuclease S1 resistant $^{32}P$-labeled RNA fragments protected by pKTH301 or pKTH309 (not fixed with glyoxal) were eluted from polyacrylamide gel slices and purified on DEAE cellulose (33). 15 μg of yeast carrier RNA was added and the DNA/RNA hybrids (about 8000 cpm Cherenkov each) were melted at $100^\circ C$ for 3 min followed by quick cooling on ice. Five units of RNase T1 (Calbiochem) in 10 mM Tris-Hcl, pH 7.6, 1 mM EDTA was added and the samples were incubated at $37^\circ C$ for 15 min, followed by heating to $100^\circ C$ for 90 sec and a second incubation at $37^\circ C$ for 15 min. The samples were then applied to cellulose acetate strips (Schleicher & Schüll) and subjected to electrophoresis at 5000 V for 40 min at pH 3.5 (I dimension) (34). After transfer to polyethyleneimine thin-layer plates (Binkman Instruments) the oligonucleotides were chromatographed at $65^\circ C$ for 10 h using homomixture C digested for 3 min (II dimension) (34). Autoradiograms were prepared by exposing Kodak X-Omat L films at $-70^\circ C$ for 7 days using an intensifying screen.

## RESULTS

### Synthesis of cDNA from 18S RNA

The virus-specific RNAs were isolated from the cytoplasm of BHK cells, which had been infected with SFV from 10th undiluted passage. The 18S DI RNA obtained had an electrophoretic mobility in polyacrylamide gradient gels close to that of the ribosomal 18 S RNA marker, but gave several bands indicating heterogeneity of size and/or secondary structure (Fig. 1A).

The cDNA for 18S DI RNA was synthesized using oligo(dT)$_{12-18}$ primer and reverse transcriptase. The single-stranded, $^{32}P$-labeled cDNA contained species of 1.7 and 2 kb in length when analyzed on alkaline agarose gels (Fig. 1B). The labeled 1.7 and 2 kb cDNAs (5000 cpm, less than 1 ng) were hybridized with 42S RNA isolated from purified SFV. The 42S RNA protected the cDNA to about 95% from S1 digestion, while only about 3% of the cDNA remained acid precipitable when annealed in the presence of yeast RNA. The result was essentially the same for both the 1.7 and 2 kb materials and suggests that a vast majority of the cDNA was transcribed from virus-specific RNA. Since the 18 S RNA fraction must contain cellular polyadenylated RNAs as well, it may be that the affinity of reverse transcriptase is higher to the DI RNAs.
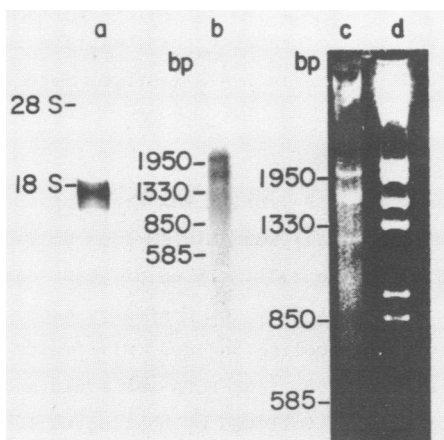
Figure 1. Lane a shows an autoradiograph of 18S DI RNA after electrophoresis on 2.5-10% polyacrylamide gel. 28S and 18S indicate the positions of ribosomal RNAs detected by staining. (b) Autoradiograph of single stranded cDNA reverse transcribed from 18S DI RNA on 1.0% alkaline agarose gel. Molecular weight markers are EcoRI/HindIII fragments of λ DNA (35). (c) Double stranded cDNA after S1 nuclease treatment on 1.2% agarose gel stained with ethidium bromide with (d) EcoRI/HindIII fragments of λ DNA as molecular weight markers.


Double-stranded DNA was synthesized after alkali treatment using reverse transcriptase. The S1 nuclease-digested product was purified by sucrose gradient centrifugation, and material sedimenting at about 10S was collected. It consisted mainly of 1.7 and 1.9 kb species, which could be seen by ethidium bromide staining (Fig. 1C) as well as by autoradiography. Since distinct size classes were not apparent in the 18S RNA fraction it is possible that the two size classes of cDNA resulted eg. from premature termination at strong stops close to the 5' end of the RNA.

Restriction enzyme analysis of double-stranded cDNA

The 1.7 and 1.9 kb bands of ^{32}P-labeled cDNA were eluted from agarose gel (10 to 20 ng of each) and digested with different restriction enzymes. 1 to 2 μg of pBR322 or λ DNA was mixed with each sample to control the completeness of the cleavage. The fragments were separated by gel electrophoresis, stained with ethidium bromide and autoradiographed (Fig. 2,3). The major radioactive bands were cut from the dried gel and quantitated (Table 1). If the double stranded cDNA would consist of a single molecular species, the sum of the restriction fragments should give 1.7 or 1.9 kb with all the enzymes used, and the fragments should be present in equimolar amounts. This is obvi-
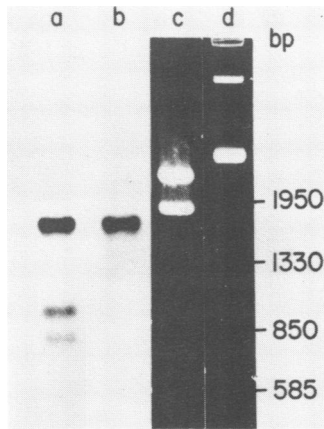
Figure 2. A mixture of $^{32}$P-labeled uncloned double stranded cDNA and unlabeled pBR322 digested with BglI restriction endonuclease after electrophoresis on 1.4% agarose gel. (a) Autoradiograph, (c) ethidium bromide staining of the same lane. (b,d) corresponding undigested control.

ously not the case. For example, BglI cleaved only one third of the cDNA in spite of complete digestion of the carrier DNA (Fig. 2, Table I), suggesting that most of the cDNA molecules are devoid of BglI sites. Similar indications of heterogeneity were obtained with other restriction enzymes (Fig. 3, Table 1). Interestingly, the pattern from 1.7 and 1.9 kb material were similar for reasons unknown at present.

Table 1. Relative molar yield[a] of restriction fragments derived from the 1.9 kb cDNA

| Fragment[b] | Enzyme | | | | |
|---|---|---|---|---|---|
| | MspI | PstI | HaeIII | HhaI | BglI |
| 1 | 0.8 | 0.4 | 2.6 | 1.7 | 6.6 |
| 2 | 1.4 | 1.6 | 7.0 | 2.5 | 2.9 |
| 3 | 1.3 | 1.8 | 3.5 | 18 | 3.2 |
| 4 | 2.4 | 3.5 | 20 | 13 | |
| 5 | 4.6 | 3.3 | 24 | 6.8 | |
| 6 | 2.7 | 4.2 | | | |
| 7 | 3.6 | 3.9 | | | |
| 8 | 2.6 | | | | |

[a] The major bands in Fig. 2 and 3 were cut out and their radioactivity determined. The relative molar yield was calculated as CPM in band per estimated size in bp.
[b] Fragment number refers to the Figure 3 so that the largest fragment indicated by an > is referred to as 1, next is 2 and so on.
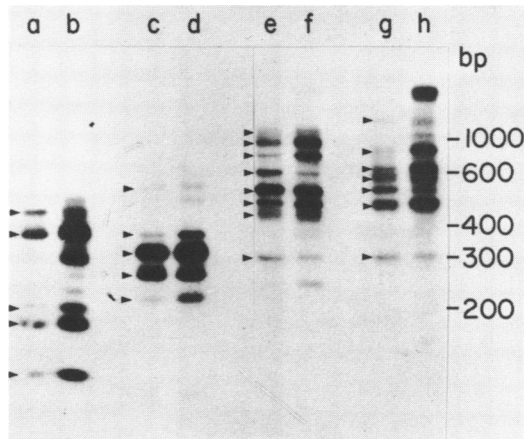
Figure 3. Electrophoretic analysis of fragments of uncloned ^{32}P-labeled 1.9 kb (a,c,e,g) and 1.7 kb (b,d,f,h) double stranded cDNA after digestion with restriction endonucleases: (a,b) HaeIII, (c,d) HhaI, (e,f) MspI, (g,h) PstI. The molecular weight scale was calculated from respective restriction fragments of pBR322, which was used in each digestion as internal marker (36). For arrow heads see Table 1.

## Cloning of cDNA

The double-stranded cDNA sedimenting at about 10S was tailed with dC using terminal transferase, and the PstI-linearized pBR322 was tailed with dG. After transformation of E.coli with the annealed recombinant plasmids, colonies were tested for the presence of SFV specific sequences by hybridization and the size of the hybrid plasmids was determined by gel electrophoresis. Two clones with different restriction patterns were selected for further characterization. These clones, pKTH301 and pKTH309, had inserts of about 1.7 kb and 2 kb respectively.

## Colinearity of inserts in pKTH301 and pKTH309 with 18S DI RNA

[32]P-labeled 18S DI RNA was hybridized with unlabeled, BglI digested recombinant plasmids pKTH301 and pKTH309 or with pBR322, under conditions which favor DNA/RNA hybrid formation (26). BglI releases the insert together with 1500 bp of flanking sequences as these cDNAs did not have the BglI cleavage site (see below). The hybrids were treated with S1 nuclease, with and without prior glyoxal fixation, and analyzed on agarose gels. Fixation of the single strands reduced the background in the autoradiographs but did not otherwise affect the results. For estimation of size it was assumed that DNA/RNA hybrids migrate like double-stranded DNA (37). As shown in Fig. 4, a dominant 1.7 kb
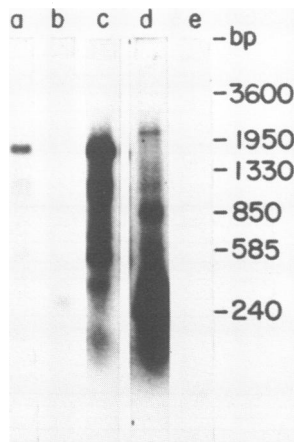
**Figure 4.** Size of ³²P-labeled 18S DI RNA/pKTH301 (a,c), /pKTH309 (b,d), /pBR322 (e) hybrids after S1 nuclease treatment, determined by electrophoresis on 1.4% agarose gel.  Exposure for 4 h (a,b) and for 2 days (c,d,e).

band was obtained for pKTH301/18S RNA hybrids, whereas for pKTH309/18S RNA a faint 2 kb band was seen together with a dominant band of 250 bp.  We interpret these results to mean that both clones had an insert which was colinear with one particular species among the 18S DI RNA population. The RNA complementary to pKTH301 is fairly common, whereas that complementary to pKTH309 is rare in the 18S DI RNA population.  These results confirm that faithful cDNA copies were synthesized by the reverse transcriptase. The presence of the dominant 250 bp band could be explained if we assume that a stretch of this length in pKTH309 is colinear with a nucleotide sequence present in several different DI RNA molecules.

Restriction maps of pKTH301 and pKTH309

        The restriction maps were constructed using double digestions with two enzymes at a time, and by partial cleavage (33) of material end-labeled at the HindIII sites close to  one end of the  inserts.    The orientation of the insert in pKTH301 was determined by Southern blot hybridization of PstI and PstI/HindIII fragments to ³²P-labeled 26S RNA.  The two clones had clearly different restriction maps (Fig. 5).  Comparison of these with the map of 26S RNA (7,8) suggests that the 3' end with the HindIII sites is conserved in all three RNAs, but then the maps differ from each other before the AvaI site of 26S RNA.  No further region of the 26S RNA can be found in the cloned DI RNAs on the basis of the restriction maps.  In fact, all 26S RNA sequences except
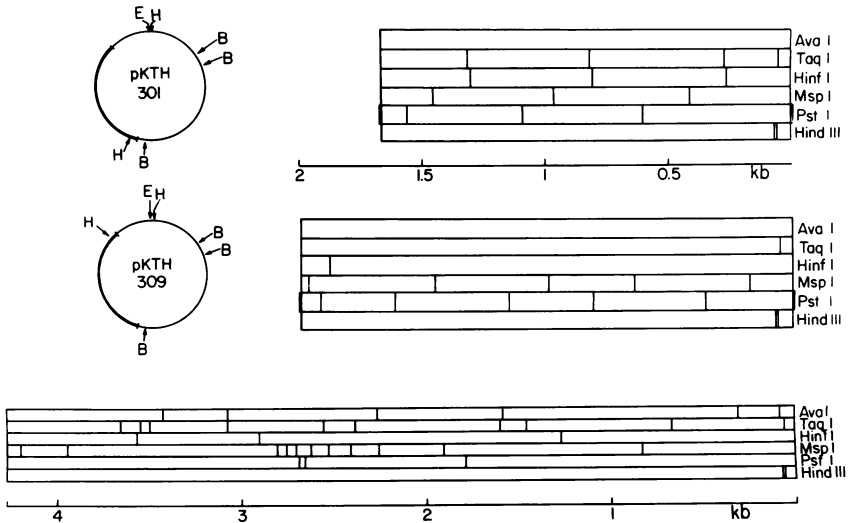
**Figure 5.** Restriction endonuclease maps of pKTH301, pKTH309 and cDNA of 26S RNA (7,8) (bottom). Note the difference of scales. E = EcoRI, H = HindIII, B = BglI.

the 3' terminal conserved region are missing from pKTH301, as revealed by nucleotide sequencing (16).

A striking feature for both restriction maps is the symmetrical distribution of the cleavage sites of certain enzyme pairs, like PstI/HinfI, HinfI/TaqI in pKTH301, and PstI/MspI in pKTH309. For pKTH301 this reflects a structure consisting of repeating units, and it may well be that pKTH309 also consists of repeats.

Regions of 42S RNA present in the clones

The length of the conserved 3' terminal sequence in pKTH309 was determined by nucleotide sequencing from the HindIII site(s). The clone pKTH309 apparently has 102 poly(A)-preceding nucleotides identical with the 3' end of the viral 42S RNA (8,36), which is 4 nucleotides less than for pKTH301 (Fig. 6). The preserved 3' end is joined to different sequences in the two clones, and these sequences cannot be found in the 26S RNA. Thus also pKTH309 must have a deletion of at least 4200 nucleotides.

To determine the colinearity of the clones with 42S RNA, the BglI-digested pKTH301 and pKTH309 were hybridized to ^{32}P-labeled virion 42S RNA, followed by treatment with varying concentrations of S1 nuclease, and the RNA/DNA hybrids were separated on polyacrylamide gels (Fig. 7). The 42S RNA frag-

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                                                      |HindIII·   |HindIII    |TaqI
AUGUCUACGC ACAAACUCAU AUUGACACAU UAAUUGGCAA UAAUUGGAAG CUUACAUAAG CUUAAUUCGA 301

AUUAAGGUCC UAUCAAACAU AUUGACACAU UAAUUGGCAA UAAUUGGAAG CUUACAUAAG CUUAAUUCGA 309
     |AvaI
GGAAACUCGG GGCAACUCAU AUUGACACAU UAAUUGGCAA UAAUUGGAAG CUUACAUAAG CUUAAUUCGA 42S


CGAAUAAUUG GAUUUUUAUU UUAUUUUGCA AUUGGUUUUU AAUAUUUCCA      3'                301

CGAAUAAUUG GAUUUUUAUU UUAUUUUGCA AUUGGUUUUU AAUAUUUCCA₆     3'                309

CGAAUAAUUG GAUUUUUAUU UUAUUUUGCA AUUGGUUUUU AAUAUUUCCAₙ     3'                42S
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Figure 6. Nucleotide sequence of the 3' ends of DI RNAs corresponding to pKTH301 and pKTH309 as compared with the 3' end of 42S RNA genome (8,38). The nucleotides preceding the common sequence are underlined.

ments protected by pKTH301 were 590 and 320 nucleotides long, but the larger fragment was less intense and disappeared when the concentration of S1 nuclease was increased. The most heavily labeled 320 bp fragment was resistant even to high enzyme concentrations. Thus we conclude that it was strictly colinear with some part of the 42S RNA, whereas the larger fragment may represent some type of minor mismatching. Similar protection experiment with pKTH309 yielded two major, 430 and 340 bp, hybrids and a less dominant 500 bp fragment which again may represent minor mismatching.

The protected ^{32}P-labeled RNA fragments were digested with T1 ribonuclease and the oligonucleotides were separated. The fingerprints of the 320-nucleotide fragment protected by pKTH301, and of the 430- and 340-nucleotide fragments protected by pKTH309 are shown in Fig. 8, together with the fingerprint of the total ^{32}P-labeled 18S DI RNA. All the protected fragments showed different oligonucleotide patterns, indicating that they were derived from
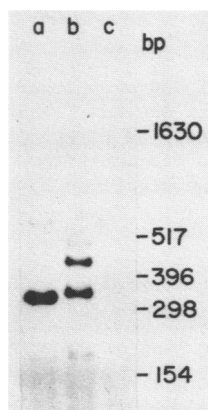
Figure 7. Size of ^{32}P-labeled 42S RNA/ pKTH301 (a), /pKTH309 (b), and /pBR322 hybrids after S1 nuclease treatment as determined by electrophoresis on 2.5-10% polyacrylamide gradient gel. HinfI fragments of pBR322 were used as molecular weight markers (36).
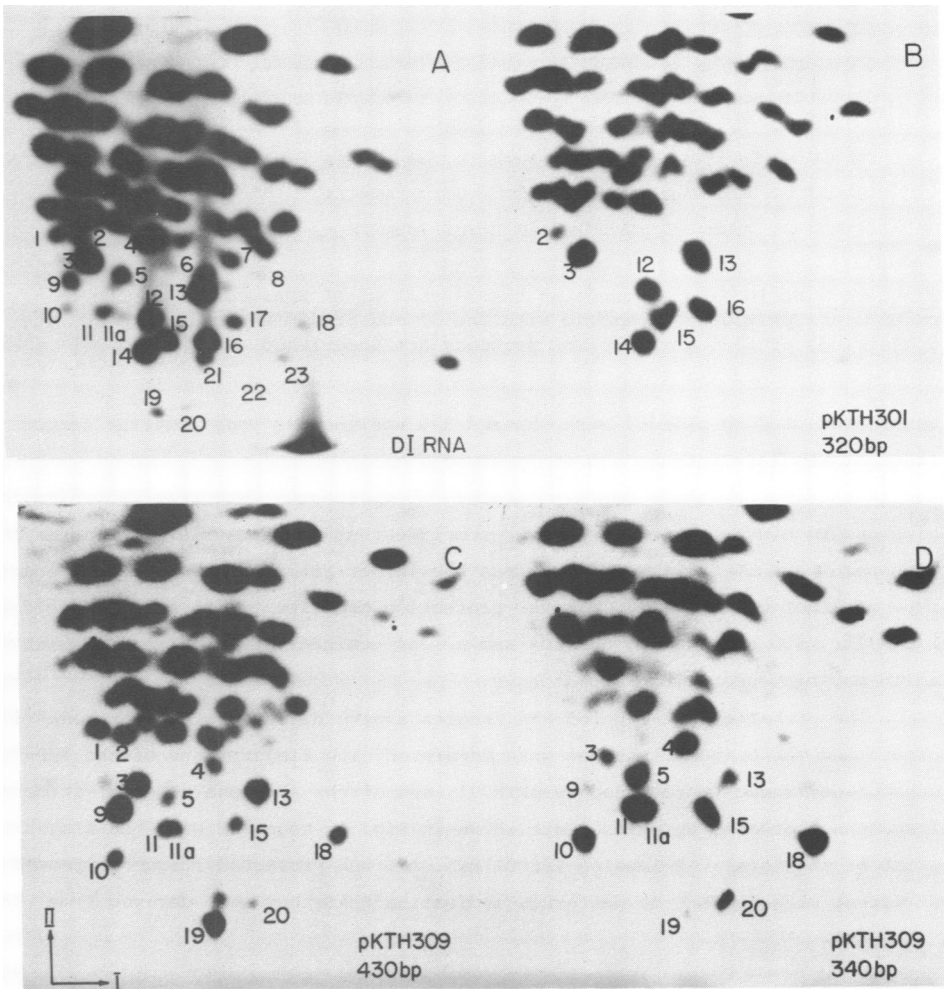
<u>Figure 8.</u>  Two-dimensional separation by electrophoresis (I) and thin-layer
chromatography (II) of RNase T1 oligonucleotides derived from 42S RNA fragments
protected by pKTH301 and pKTH309.  A: fingerprint of the total 18S DI RNA popu-
lation.  B: the 320 bp fragment from pKTH301.  C: the 430 bp fragment from
pKTH309.  D: the 340 bp fragment of pKTH309.  The long oligonucleotides have
been numbered arbitrarily.

different regions of 42S RNA. Note, however, that the two fragments derived

from pKTH309 appear to be cross-contaminated. Several of the long oligonucleo-

tides from the 320 bp fragment of pKTH301 could easily be recognized in the

fingerprint of 18S DI RNA. Many of the large oligonucleotides derived from the

430 and 340 bp fragments of pKTH309 could also be traced in the DI RNA finger-print as faint spots (Fig. 8). This result is in good agreement with the idea that DI RNA species equivalent to pKTH309 represents a minor species in the 18S DI RNA population.

DISCUSSION

In this study we have analyzed the cDNA transcribed from cytoplasmic 18S DI RNAs generated after 11 undiluted passages of Semliki Forest virus in BHK cells. This RNA species is about 1/6 of the size of the viral 42S genome, and it appears already after 4 transfers with undiluted inoculum and remains the dominant cytoplasmic RNA for 17 passages. The 18S RNA is packaged into virus particles with an efficiency about 1/10 of that of 42S RNA, but it is pre-served during serial transfers by its ability to replicate much more efficiently than 42S RNA (14).

The 18S DI RNA species generated in our laboratory consists of a hetero-geneous population according to several criteria: 1) The cap-containing T1 oligonucleotides are heterogeneous (15). 2) Numerous T1 oligonucleotides from 18S DI RNA are present in varying nonequimolar amounts (15,16). 3) The double stranded cDNA (1.7 kb and 1.9 kb) yielded fragments in nonequimolar amounts with several restriction enzymes (Table 1). For example, two-thirds of the cDNA molecules were lacking the unique cleavage site for BglI. 4) The two cloned cDNAs, pKTH301 and pKTH309, have different restriction enzyme maps and apparently consist of different sequences (see below).

Hybridization of two clones (pKTH301 and pKTH309) to 18S DI RNA followed by treatment with S1 nuclease showed that the clones were colinear with the RNA and not artefacts by reverse transcriptase. The different restriction enzyme maps of the two clones suggested that with the exception of the 3' terminus, they have different primary structures. However, both maps showed cleavage symmetry for certain pairs of restriction enzymes (Fig. 5). Since the nucleotide sequence of the insert in pKTH301 is known (16), this symmetry has become interpretable. The molecule consists of 3 linear repeats of 484 nucleo-tides. One of the repeats has an "insert" of 60 nucleotides. Restriction sites within the repeat give the symmetry in the restriction map. Similar explanation for the symmetry of pKTH309 map is very likely, but remains to be shown by nucleotide sequencing.

Previously characterized DI RNAs of SFV and Sindbis virus have been suggested to be generated by continuous internal deletions of variable size, with preserved genomic 5' and 3' termini (for review see 6,12,13). If our 18S

DI RNAs would be similar, hybridization of pKTH301 and pKTH309 to 42S RNA genome should have given large DNA/RNA hybrids after S1 nuclease treatment. This was not the case with either of the clones. Only a 320 bp long hybrid was obtained with pKTH301 whereas two fragments of 430 bp and 340 bp were obtained from the hybridization with pKTH309. Analysis of T1 oligonucleotides of these protected fragments revealed that they all were different. It is thus evident that pKTH301 and pKTH309 are built up of relatively short sequences which differ from each other. pKTH309 may contain two different subunits, 430 and 340 nucleotides long, derived from two different regions of 42S RNA at least a few nucleotides apart. The repeating unit of 484 nucleotides in pKTH301 must be assembled from at least two subunits, one of which is 320 bp long.

Further undiluted passages have generated larger DI RNAs (24S and 33S), which have less complex oligonucleotide fingerprints and apparently consist of 5 to 10 copies of a repeating unit structurally related to that in pKTH301 (14).

A common feature to all DI RNAs isolated in our laboratory and to those previously described for SFV and Sindbis virus (12,13) is a poly(A) stretch at the 3' end. In pKTH301 this is preceded by 106 nucleotides which are identical to those in 42S RNA (8,38), and in pKTH309 the conserved sequence is 4 nucleotides shorter. The common 3' sequence is joined to a different sequence in each of the two clones, and these sequences are not found in the 26S RNA. Clearly, more information on the location of the repeat units in 42S RNA and on the primary structure of different DI RNAs is required in order to understand how they are generated.

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