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Analysis of the secondary and tertiary structures of Rous sarcoma virus RNA

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ABSTRACT

The secondary and tertiary structures of the 35S RNA of Rous Sarcoma virus have been investigated. T_1 RNase partial digests have been first resolved into their components by gel electrophoresis under non denaturing conditions and then each component analyzed further by various techniques. More than one hundred structured fragments have thus been isolated and shown to consist of several individual nucleotide sequences located far apart on the genome. On the basis of the results, a cloverleaf model for the structure of RSV 35S RNA is proposed that has implications for the various biological functions of this RNA.

INTRODUCTION

The genome of Rous sarcoma virus (RSV) is made up of two identical 35S RNA molecules associated in a 70 complex. The 35S RNA extracted from mature virions possesses features of both a mRNA and a template for reverse transcription : on one hand it is polyadenylated, capped and has the same chemical polarity (positive strand) as the polysome associated viral RNA found in infected cells and on the other hand it carries a $tRNA^{TRP}$ hybridized to a region one hundred nucleotides away from its 5' end, that serves as primer for reverse transcription (for a review see 1). Cells infected with RSV synthesize virus specific RNAs by transcription of the proviral DNA and these transcripts must serve both as genomes for the progeny and messengers RNAs for the synthesis of the various specific proteins, but the mechanism that allows discrimination of these two functions is unknown. Recently it has been shown that the subgenomic viral mRNAs have at their 5' end a segment of RNA corresponding to

that found on the 5' end of the genomic RNA (2, 3) indicating that splicing must occur at the level of proviral DNA transcription; it seems likely that the primary transcript is the genome length RNA and that it serves as a precursor to the spliced mRNAs since microinjection of 35S virion RNA into chick cell nuclei yields active mRNA for the envelope proteins (4). Immediately after budding from the host cell membrane progeny virus particles are found to contain the genomic RNA as a free 35S molecule that is neither associated with the primer $tRNA^{TRP}$ nor with another 35S as is found in the mature virion. The association of the two subunits and of the cellular $tRNA^{TRP}$ takes place in a maturation step following the budding of the virus particle and involves a complex series of events (for a model see 5).

Because of the role of RSV RNA in these various processes (maturation, reverse transcription, splicing and translation) a study of its structure is of particular relevance as it is expected to provide some information about the molecular basis for the specificity of these functions. Electron microscopy observations of RSV RNA under partially denaturing conditions have indicated that the molecule has a highly ordered structure (6) and have revealed the presence of a dimer linkage structure at the 5' end of the RNA of oncovirus (7). We have carried out (8) an analysis of RSV RNA structure based on the susceptibility of its molecule to various nucleases, and the results obtained revealed a large number of secondary structures along the 35S RNA molecule and indicated that the two subunits are intimately associated not only at their 5' ends but also via numerous other specific interactions to form the 70S RSV RNA complex.

In this paper we report a more detailed analysis of the secondary and tertiary structures of RSV 35S RNA based on the same strategy that Fiers and coworkers used with bacteriophage MS2 RNA (9). RSV 35S RNA is first digested partially with T_1 RNase and the digest resolved into its components by gel electrophoresis under non denaturing conditions; each fragment is then analyzed by various techniques and localized along the 35S RNA molecule on the basis of its T_1 RNase fingerprint. More than a

hundred of these structured fragments have been characterized and mapped on the viral genome. On the basis of these results we propose a cloverleaf model for the structure of RSV 35S RNA and discuss its implications for the various biological functions of this RNA.

MATERIALS AND METHODS

Cells and viruses. The Prague B t/s mutant (LA23) of RSV was grown in secondary cultures of chicken embryo fibroblasts as described before (10).

Isolation of virion nucleic acids. 70S RNA and 35S RNA unlabeled or labeled with $[^{32}P]$ orthophosphate or $[^{3}H]$ uridine were purified as previously described (10).

Enzymes. E.coli RNase III was purified as previously described and had a specific activity of 90,000 units per mg of enzyme at 37°C using $[^{3}H]$ poly r (A U) as substrate (11). Partially purified E.coli RNase IV was prepared according to the published procedure (12). A. oryzae T_1 RNase was obtained from Sankyo Inc.

Enzymatic degradation of RSV RNA. Labeled RSV 35S RNA (10^6 cpm/µg; about 5 x 10^5 cpm per assay) was digested with RNases III, IV, T_1 and S_1 nuclease in the following buffers at 37°C : RNase III, 50 mM Tris-HCl, pH 8, 100 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, and 0.1 mM EDTA. RNase IV, 0.1 M citrate buffer, pH 6.0, 5 mM EDTA. Reactions were stopped by adding 10 mM EDTA and 0.2 % SDS.

Partial degradation of $[^{32}P]$ 35S RNA with T_1 RNase. Purified $[^{32}P]$ 35S RNA (1.2 x 10^8 cpm; 10^6 cpm/µg) was digested with T_1 RNAse in 0.1 M Tris-HCl pH 8,0, 1 mM EDTA at 0°C for 30 min. The reaction was stopped with 1 % SDS and the mixture extracted twice with phenol saturated with 0.1 M Tris-HCl pH 8.0, and containing 10 mM EDTA, 1 % SDS and 1 % β-mercaptoethanol.

Purification of the partial degradation products of 35S RNA with T_1 RNase were purified by polyacrylamide gel electrophoresis (PAGE) (8 % acrylamide, 0.4 % bis-acrylamide in 75 mM Trisborate, EDTA). The components were detected by direct autoradiography,

then excised and eluted following the procedure described by Maxam (13).

PAGE analysis of RNA fragments in 7M urea. This was carried out by gel electrophoresis (8 % acrylamide, 0.4 % bis-acrylamide, 75 mM Tris-borate pH 8,3, 3 mM EDTA) containing 7 M urea and run at 1 KV.

Fingerprint analysis of $[^{32}P]$ -labeled RSV RNA. Analysis of the products of complete T_1 digestion of RSV RNA was performed using two-dimensional polyacrylamide gel electrophoresis (14). The digestion products were located by direct autoradiography and the radioactivity in large T_1 oligonucleotides was quantitated as previously described (10).

Sequencing of RNA and of large T_1 oligonucleotides. RNA or T_1 oligonucleotides to be sequenced were first labeled at their 5' end with γ-$[^{32}P]$ ATP using T_4 polynucleotide kinase (P-L Biochemicals). The material was dissolved in 30 µl of a solution containing 50 mM Tris-HCl, pH 8, 10 mM $MgCl_2$, 5 % glycerol, 1 mM dithiothreitol, and 1.5 mM spermine. After 5 min at 37°C, the 30 µl reaction mixture was transferred to a tube containing dried γ-$[^{32}P]$ ATP (200 Ci/mmol), 1 unit of T_4 polynucleotide kinase was added and the reaction continued for a further 10 min. Reaction was stopped with 10 mM EDTA and 0.1 % SDS. RNA or oligonucleotides were purified by two-dimensional polyacrylamide gel electrophoresis.

To sequence 5' end-labeled RNA, we followed the method described by Donis-Keller et al., (15) to map adenines, guanines and pyrimidines in RNA, and we used both the "wandering-spot" analysis, Physarum I RNase, and pancreatic RNase to distinguish between uridines and cytidines (16-18). The partial cleavage products were separated on 12 % or 8 % polyacrylamide gel in 7 M urea and 75 mM Tris-borate EDTA, pH 8,3.

RESULTS

In our previous studies on RSV RNA structure, we analyzed the genomic RNA extracted from RSV Pr-B virions (8); however

preparations of RSV Pr-B are known to contain large amounts of td mutants (lacking three T_1 oligonucleotide markers in the src gene (19)). We have recently reported that a mutant of RSV Pr-B, temperature sensitive for transformation, LA23-B, does not yield td segregants even after several passages at high multiplicity in chick embryo fibroblasts, and that its genomic RNA appears to be stable as compared to that of RSV Pr-B (10). Therefore LA23-B has been used as the source of genomic RNA for the structural studies reported here; the characterization and the mapping of 44 T_1 oligonucleotide markers of LA23-B RNA have already been reported (10).

In order to compare the structure of this LA23-B RNA with that of RSV PrB, we analyzed its susceptibility to nucleases specific for structured RNA. $[^{32}P]$ 35S LA23-B RNA was digested with RNase III and RNase IV, subsequently digested with T_1 RNase and fingerprinted. The results obtained indicated that, as was the case for RSV PrB RNA, 35S RNA from LA23-B is very susceptible to RNases III and IV, nucleases that nick unpaired nucleotide sequences in the vicinity of double stranded RNA (11,12). The localization of secondary structures thus detected along the LA23-B genome was shown to be very similar to that reported for Pr-B RNA (8) (results not shown).

A. T_1 RNase partial digestion of $[^{32}P]$ 35S LA23-B RNA

T_1 RNase is an RNase that cleaves G-N bonds in single stranded RNA, but not in double stranded RNA and generally not in well structured RNA. In order to isolate structured regions of 35S RNA, $[^{32}P]$ labeled RNA was digested with T_1 RNase in 0.1 M Tris-HCl pH 7.5, 1 mM EDTA for 30 min at $0^{\circ}C$ and at RNA to enzyme weight ratios ranging from 25 to 600. The digestion products were phenol extracted and analyzed on a 8 % polyacrylamide gel under non denaturing conditions (Fig. 1). Structured RNA fragments ranged from 25 up to about 200, 300 and more than 600 nucleotides in length at RNA to nuclease ratios of 25, 50, 300 and 600 respectively.

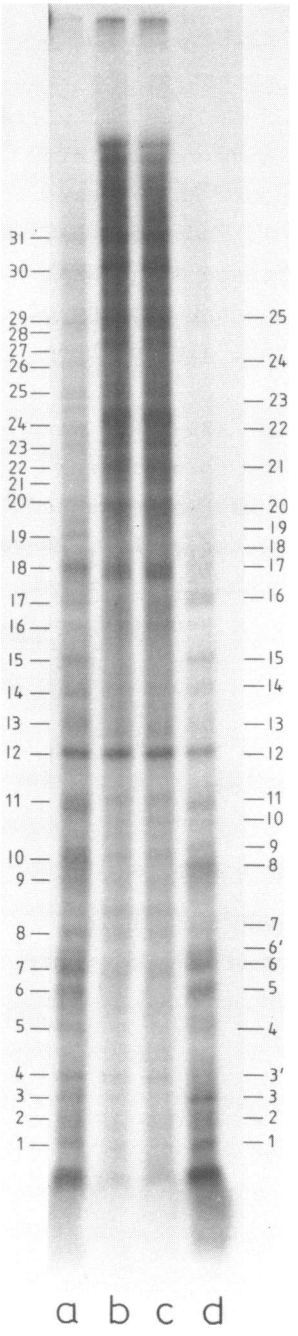


Figure 1 - Analysis by polyacrylamide gel electrophoresis of the products of partial nuclease digestion of RSV 35S RNA

$[^{32}P]$ 35S RNA was purified and digested with T_1 RNase as described in Methods. Ratios of RNA to T_1 RNase were 50(a), 300(c), 500(b) and 25(d). After two phenol extractions and an ethanol precipitation the digestion products were loaded on a polyacrylamide gel (8 % acrylamide, 0,4 % bis acrylamide and 75 mM Tris-borate, 3 mM EDTA pH 8,3). 20 x 37 x 0,5 cm; electrophoresis was at 800 V at room temperature and was stopped when the bromophenol blue reached bottom of the gel.

Exposure of the gel was for 10 min using XR5 kodak film. Bands were cut out and RNA fragments (see numbers) recovered as described in Methods.

B. Characterization and mapping of structured regions of 35S LA23-B RNA

Individual structured RNA fragments recovered from the neutral gel were characterized by polyacrylamide gel electrophoresis in 7 M urea to measure their respective length and by two dimensional gel electrophoresis to estimate their complexity.

The analysis by 7 M urea gel electrophoresis of individual structured RNA fragments obtained from a partial T_1 RNase digest at an RNA to enzyme ratio of 50 , is shown in Figure 2. Clearly each structured RNA fragment is composed of several different polynucleotides, and this is well demonstrated by the results of 2 dimensional PAGE analysis and by the T_1 fingerprint of 2 different structured RNA fragments generated upon partial digestion at an RNA to nuclease ratio of 25 (Fig. 3).Each fragment is made up of several RNA species, between 30 and 100 nucleotides long, as shown by PAGE in 7 M urea and confirmed by 2 dimensional PAGE, a technique that separates small RNAs according to their nucleotide composition in the first dimension, and to their length and structure in the second dimension. The results of two dimensional PAGE analysis suggest that the RNA species making up a structured RNA fragment have different sequences; this is in agreement with the T_1 fingerprint analysis which reveals the presence of several T_1 oligonucleotide markers in one RNA fragment. In fact T_1 oligonucleotides recovered in high yield are 2, 3, 10b and 20 for RNA 14 and 3, 30 and 36 for RNA 15 (Fig. 3c and d). Figure 4 reports the T_1 fingerprint analysis of structured RNAs generated upon partial digestion at an RNA to nuclease ratio of 50 (RNAs 13 to 16 with a mean chain length of 60 to 90 nucleotides; see Fig. 1a and 2). The results obtained show that each of these structured RNA fragment has several T_1 oligonucleotide markers, and this is true for most structured RNA fragments (see Table 1). Such data indicate that the structured RNA fragments are made up of several RNA species with different and non overlapping sequences, in agreement with partial sequence analysis of the major RNA species showing that they differ in their nucleotide

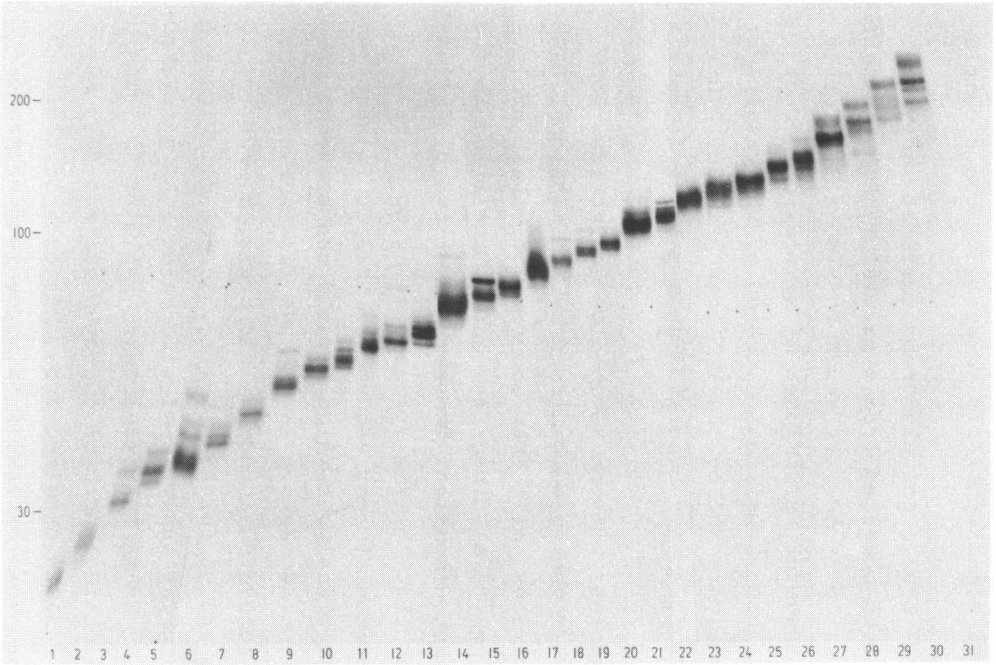


Figure 2 - Chain length analysis of RSV RNA fragments

$[^{32}P]$ RNA fragments generated upon partial nuclease digestion of RSV 35S RNA at a ratio of 50 (see Figure 1a) were recovered and dissolved in 30 µl of water. A 2 µl aliquot of every RNA fragment was heat denatured (30 sec, 100°C) in 6 µl of deionized formamide containing 0,25 % bromophenol blue and xylene cyanol. Polyacrylamide gel (8 % acrylamide, 0,4 % bis-acrylamide, 7 M urea, 75 mM Tris-borate EDTA - pH 8,3) was electrophoresed at 1.2 KV until the bromophenol blue reached bottom of the gel (35 x 37 x 0,04 cm). RSV RNA and DNA fragments of known chain length were used as markers.

Exposure was for 2 days using kodak XR5 film.

sequences (results not shown).

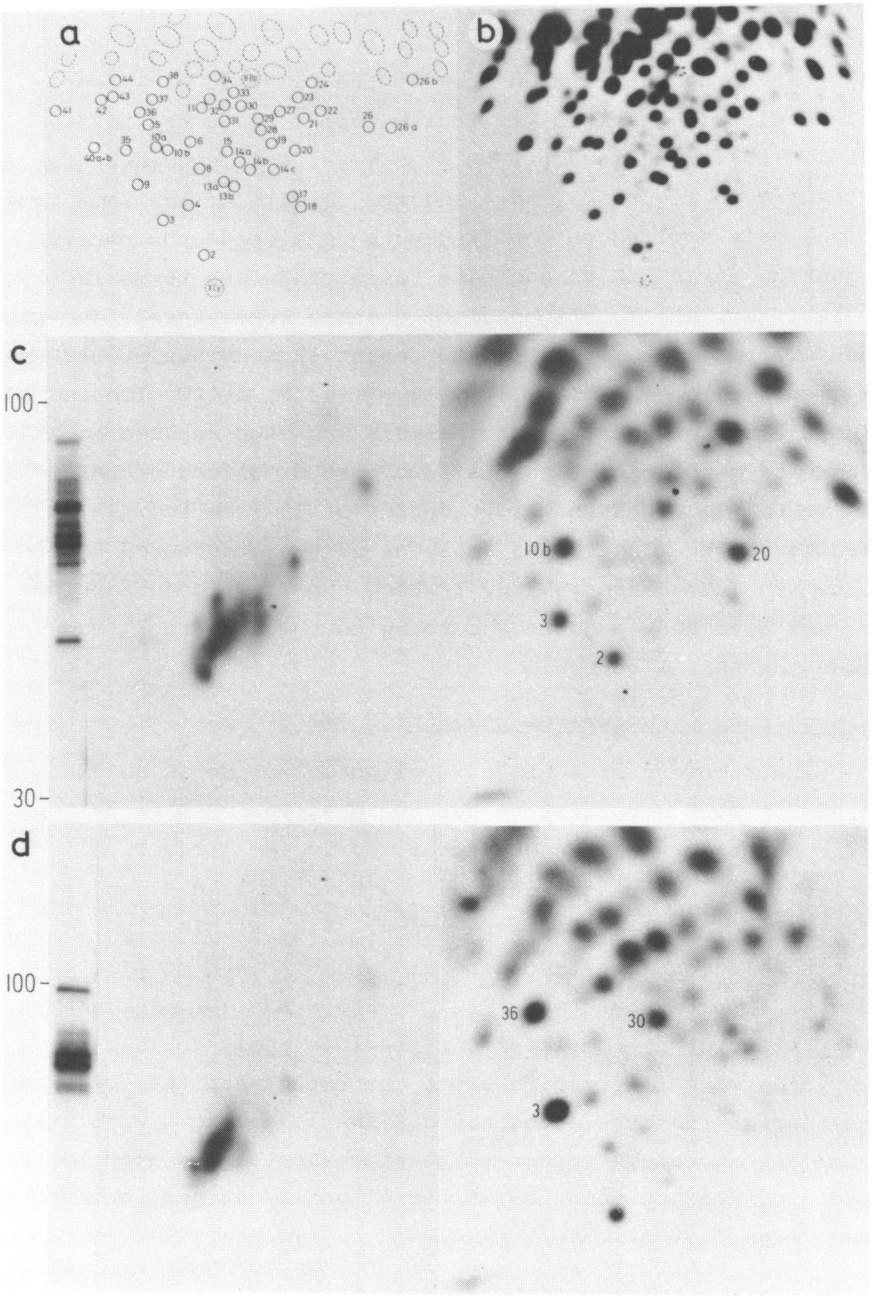
Mapping of the individual RNA species making up the structured RNA fragment was carried out by the fingerprint technique in order to detect in each species the presence of known T_1 oligonucleotide markers previously mapped along the LA23-B genome (10). The fingerprints of several RNA fragments are presented in figures 3 and 4 and the results of the same type of analysis applied to the fragments generated upon partial digestion at an RNA to nuclease ratio of 50 are summarized in Table 1. Not all T_1 oligonucleotides are represented, and among the 44 T_1 markers several were present in many structured RNA fragments, i.e. 40b, 14a, 14b, 17, 3, 4, 9, 2, 20, 10b and 40a (Table 1). The same type of analysis was also applied to RNA fragments generated by partial nuclease digestion of LA23-B 35S RNA at RNA to T_1 RNase weight ratios of 25 (see Fig. 3) and 100. Results of the fingerprint analyses of the structured RNA fragments are summarized in Fig. 5 where the T_1 oligonucleotide markers have been ordered according to their 5' to 3' mapping along the LA23-B genome.

C. Secondary structure of LA23-B 35S RNA

The recovery of a given T_1 oligonucleotide in an RNA structured fragment may first be due to an extensive base pairing in the nucleotide sequence characterized by the large T_1 oligonucleotide marker, and leading to a hairpin like structure. The results of the 3 sets of experiments (Fig. 5) clearly indicate that such secondary structures are abundant and confirm the results reported before for RSV Pr-B 35S RNA (8). Furthermore the present study completes our previous analysis (8), in providing more information about the extent and the location of secondary structures along the 35S RNA : sequences with a strong secondary structure appear to be approximately located in the middle of the gag gene and at the 5' end of the pol,env and src genes (see Fig. 5).

D. Tertiary structure of LA23-B 35S RNA

The characterization of the structured RNA fragments give


a
b
c
100
30
10b
20
3
2
d
100
36
30
3

also information on possible distant interactions between structured regions of the 35S RNA, i.e. on the tertiary structure of this RNA molecule. These RNA-RNA interactions are indicated by the fact that each RNA fragment is made up of several sequences characterized by several T_1 oligonucleotides, and by the fact that isolated unique sequences 100-120 nucleotides long and containing T_1 oligonucleotides 2 or 3 (see Fig. 4) are 2-4 times more susceptible to further degradation by T_1 RNase than when present in the starting structured RNA. Interactions that have been taken into account for their possible role in tertiary structure are those where a repetitive presence of the same set of T_1 oligonucleotides has been observed in several structured RNAs (see Fig. 5). Interactions are said to be of short range when 2 contiguous T_1 oligonucleotides were found to interact; these are (from 5' to 3' of the genome) : 40b and 14a, 3 and 36 and 20 and 10b (see Table 2). Other interactions are considered of long range and correspond to regions with T_1 oligonucleotide markers (in the 5' to 3' order) : (40b), (14b), 17-3-36) (2) (20-10b) (13b). These long range interactions are summarized in Table 2, where data of partial digestion of 35S RNA with T_1 RNase at RNA to enzyme ratios of 25, 50 and 100 have been compiled. Figure 6 illustrates a scheme of the possible interactions between different structured regions of 35S RNA.

Figure 3 - Characterization of RSV RNA fragments

$[^{32}P]$ RNA fragments obtained upon partial nuclease digestion at a ratio of 25 (see Figure 1d) were analyzed by PAGE in 7 M urea (as described in Methods (left), by 2 dimensional PAGE (1st dimension, 8 % acrylamide, 0,3 % bis-acrylamide in 25 mM citric acid pH 3,5, 6 M urea; 2nd dimension in 15 % acrylamide, 0,6 % bis-acrylamide in 40 mM Tris-citrate pH 8.0)(center) and by the T_1 RNase fingerprint technique (right). Chain length markers are the same as those described in Figure 2.

RNA fragments are 14(c) and 15(d) (see figure 1d). Exposures were for 2 days using kodak XR5 (left and center) and NS5T films (right).

T_1 oligonucleotides recovered in high yield are 2, 3, 10 b and 20 for RNA 14(c) and 3, 30 and 36 for RNA 15(d). Fingerprint of LA23-B 35S RNA(b) numbering (a) sequences and 5' to 3' order of the large T_1 oligonucleotides have been published (10).

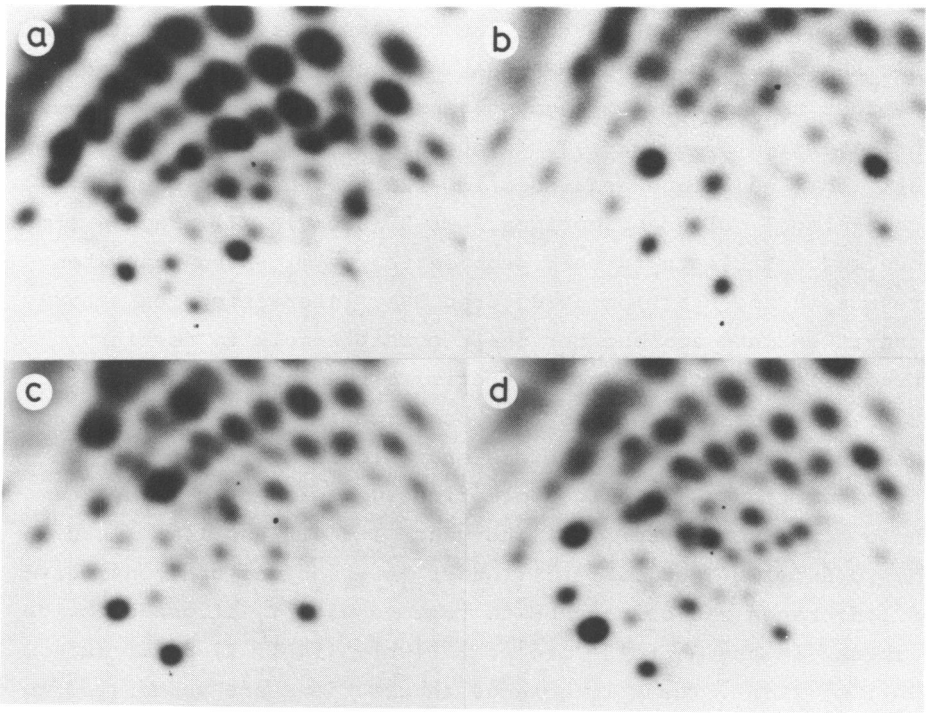


Figure 4 - T_1 fingerprint of RSV RNA fragments

RSV ^{32}P RNA fragments 13, 14, 15 and 16 (obtained upon partial nuclease digestion at a weight ratio of 50) were fingerprinted as described in Methods.

T_1 fingerprints of RNA fragments 13 to 16 are in the order (a) to (d). Exposures were for 2 days using kodak NS5T film. Spots corresponding to T_1 oligonucleotides present in good yield were cut out and counted using Cerenkov radiations; those include :

- for RNA No 13 : 3, 10b, 13b, 40, 21 and 32; for RNA No 14: 10b, 20 and in two and half fold lower yield : 2, 3, 8, 41; for RNA No 15 : 2, 3, 17 and in two fold lower yield : 30 and 36; for RNA No 16 : 3, 36 and in about two and half fold lower yield: 2, 9, 13b, 17 and 30.

DISCUSSION

Our previous studies on RSV RNA structure were performed on the 35S RNA subunit extracted from RSV Pr-B. Since preparations of Pr-B are known to contain relatively large amounts of

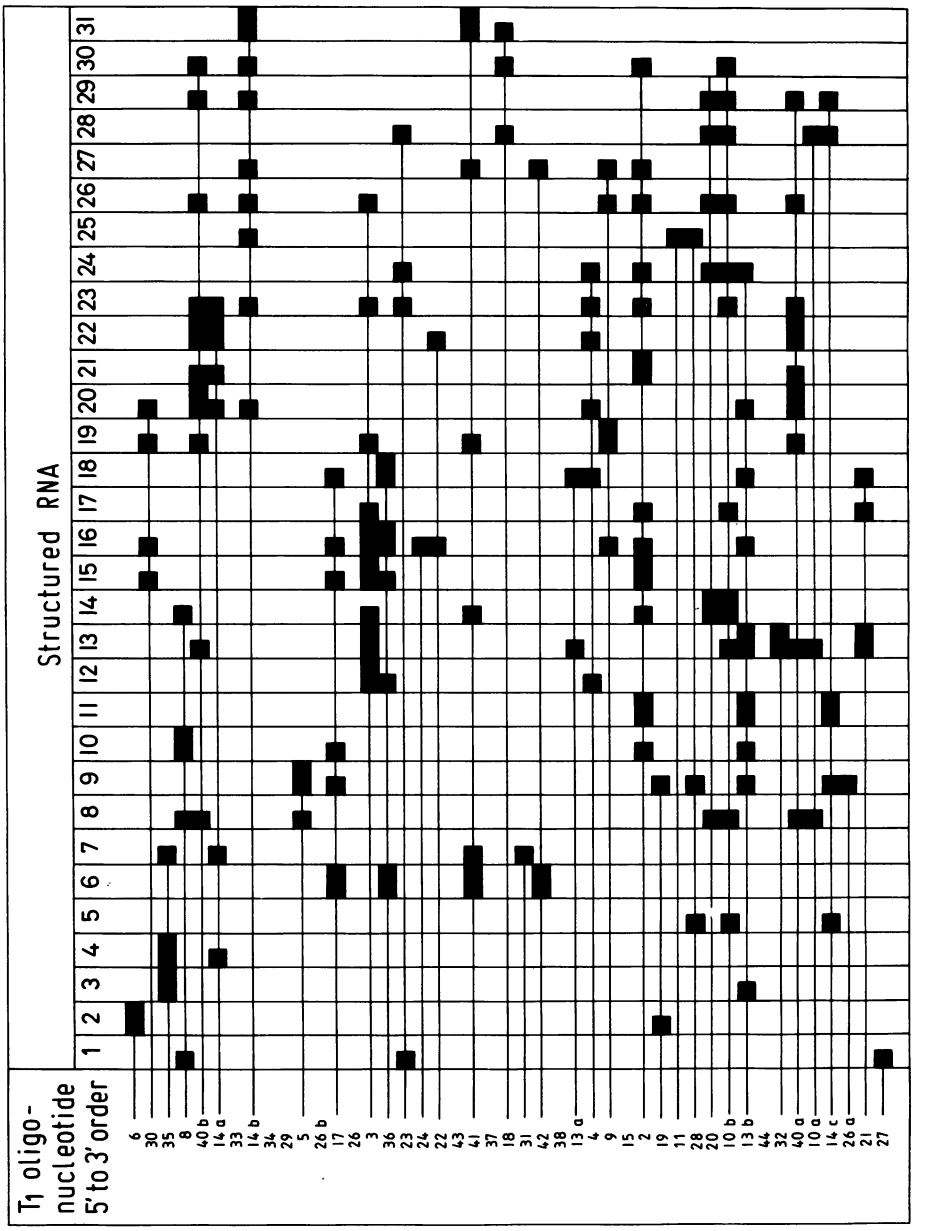

Structured RNA
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31
T1 oligo-nucleotide 5' to 3' order
6
30
35
8
40 b
14 a
33
14 b
34
29
5
26 b
17
26
3
36
23
24
22
43
41
37
18
31
42
38
13 a
4
9
15
2
19
11
28
20
10 b
13 b
44
32
40 a
10 a
14 c
26 a
21
27

Table 1 - Characterization of structured RNA fragments by the "fingerprint" technique

35S LA23-B RNA was digested with T_1 RNase at an RNA to enzyme ratio of 50 under conditions described in Methods. Structured RNA fragments (for numbering see Fig. 1 and 2) were purified by polyacrylamide gel electrophoresis and further fingerprinted as indicated in Methods. Spots corresponding to T_1 oligonucleotides were cut out and their radioactivity determined using Cerenkov radiations. Two boxes mean that T_1 oligonucleotide was recovered in high yield, and one that it was recovered in two to three fold lower yield.

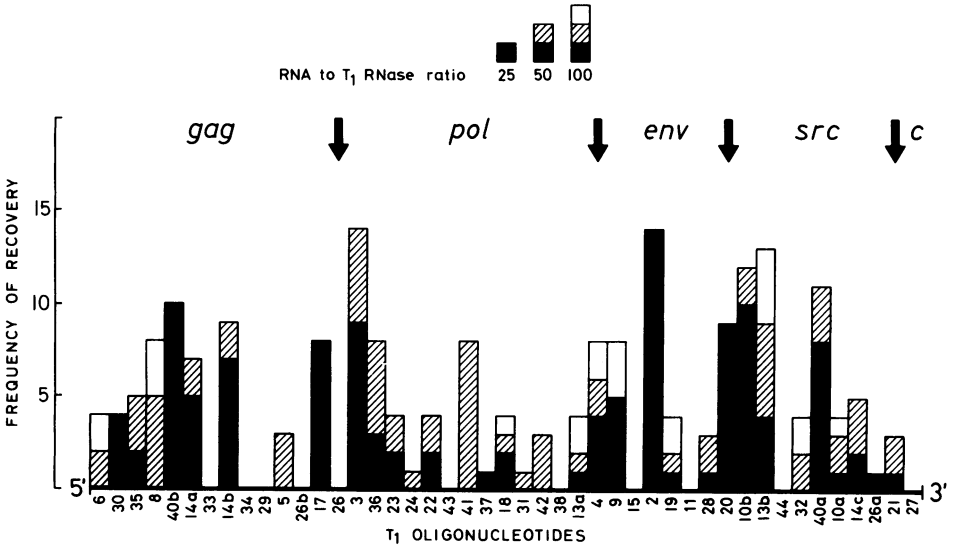


Figure 5 - T_1 oligonucleotide mapping of RSV RNA structures

RNA fragments recovered upon partial digestion of 35S RNA at RNA to nuclease ratios of 25, 50 (see Table 1) and 100 were mapped by means of the T_1 fingerprint technique. Frequency of recovery of the T_1 oligonucleotides correspond to the number of times that a given T_1 oligonucleotide was found in the structured RNA fragments. 5' to 3' order of the T_1 oligonucleotides has been published (10). Arrows point to the regions assumed to be in between the genes according to the size of the viral proteins (1, 24), to the size of the viral genes as estimated by electron microscopy (see ref. 7 for a review) and to our own unpublished work for the size of the gag and src genes. Positions of the arrows must be considered as indicative and could be in error by 1-2 T_1 oligonucleotide markers.

Table 2 - Possible interactions between regions of 35S RNA

Structured region with T_1 oligonucleotide (5' to 3' order)	Interactions with regions with T_1 oligonucleotide
40b	14b ; 3 ; 2 ; 20 (5,5,5) (2,4,5) (6,4,6) (4,6,6)
14b	40b ; 2 ; 10b ; 20 (4,4,6) (3,4,6) (3,3,5)
17	36 ; 2 ; 13b (2,4,6) (3,3,4) (2,4,4)
3	40b ; 2 ; 10b (3,6,6) (3,5,6)
2	40b ; 14b ; 17 ; 3 10b ; 20 ; 9 (7,6,6) (8,4,6) (3,3,4)
10b-20	40b ; 14b ; 3 ; 2

Numbers in parentheses refer to the times that the T_1 oligonucleotides were found in the same structured RNA species. Order of these numbers corresponds to partial degradation carried out at RNA to enzyme ratios of 25 (first), 50 (second, see Table 2) and 100 (third).

td mutants (19) and since we have found that a t/s mutant of Pr-B, LA23, did not contain td mutants we have used the RNA extracted from the latter for the studies reported here. This genomic RNA of LA23-B has been characterized (10) and represents a homogeneous population of molecules and is thus a more suitable material for structure studies.

LA23-B 35S RNA was first treated with E.coli RNases III and IV as was done previously for Pr-B RNA (8), and the results

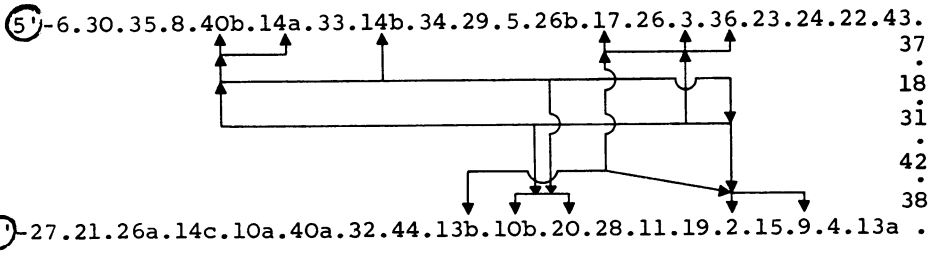


Figure 6 - Interactions between structured regions along 35S RNA

Arrows point to regions of interactions and straight lines connect regions that are thought to interact (see Table 3). Order of the T_1 oligonucleotides is known from previous studies (10).

obtained confirmed the abundance and mapping of RNA secondary structures (8). As a further step in the elucidation of RSV 35S RNA structure, T_1 RNase partial digests were analyzed and the nuclease resistant fragments characterized, a strategy developed by Fiers et al. to analyze MS2 RNA sequence and structure (9) and applied by Ross and Brimacombe to determine interacting sequences in ribosomal RNA (20). It should be pointed out that the amount of T_1 RNase needed for a partial digestion of 35S RNA in order to obtain RNA fragments varying in chain length from 30 to 250 was 5 times higher than that used with MS2 RNA (9); since 70 to 80 % of the nucleotide sequences of MS2-RNA are believed to be involved in base pairing (21), this demonstrates the extremely tight structure of LA23-B 35S RNA.

Upon partial digestion of 35S RNA with T_1 RNase at three different RNA to enzyme ratios (25, 50 and 100) more than 100 RNA fragments have been purified by polyacrylamide gel electrophoresis in non denaturing conditions. The sequence complexity of the structured RNA fragments and their mapping on the LA23-B genome was determined by PAGE in denaturing conditions and by the T_1 fingerprint analysis. Since 44 large T_1 oligonucleotides have been mapped along the 10,000 nucleotide long LA23-B genome, a T_1 oligonucleotide should be the marker for a sequence of 225 nucleotides, on the average. Although T_1 oligonucleotides are evidently not evenly distributed along the RNA, preliminary

A MODEL STRUCTURE FOR RSV 35S RNA

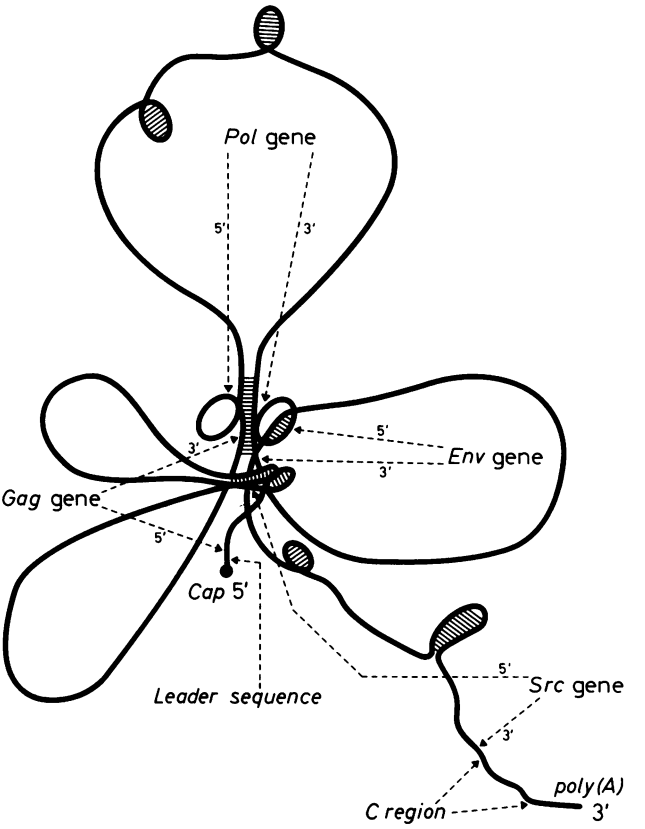


Figure 7 - Proposed model for the structure of 35S RNA

Positions of the 5' and 3' ends of the viral genes are only indicative on this model, but roughly correspond to strongly structured regions of the 35S RNA genome as indicated by the data presented in figures 5 and 6 (see also ref. 1 and 7).

data on the sequences of unique fragments purified from the structured RNAs 1 to 22 (see Fig. 1) indicate that a sequence of up to 120 nucleotides in length has only one T_1 marker. Consequently a structured RNA fragment carrying several T_1 markers can probably be made up of several unique sequences with chain length of 120 nucleotides each. Structured sequences thus

mapped by their respective T_1 oligonucleotides are illustrated in Figure 5 where regions of strong secondary structure corresponding to a high frequency of recovery of a T_1 oligonucleotide are characterized by the T_1 markers 40b-14a, 14b, 17, 3, 4-9, 2, 20-10b and 40a equivalent to 1 300 nucleotides in length. Weaker secondary structures are also observed, such as the one with T_1 oligonucleotide 6, and correspond to 22 T_1 markers equivalent to 2 600 nucleotides in length (see Fig. 5). Once more this emphasizes the extraordinary high level of secondary structure of the 35S RNA of Rous sarcoma virus.

Preliminary data from partial digestion experiments carried out with 70S RNA indicate similar results with, however, a high background level of every T_1 oligonucleotides in the fingerprints of the purified RNA fragments. Only one major difference was observed and this concerns the high recovery of T_1 marker No 8 present in a fragment 38 nucleotides long. This finding agrees with our previous data suggesting that T_1 oligonucleotide 8 with the sequence $A_4C_2UCUACU_2CUA_2CUA_2G$ (10) is involved in the dimer linkage structure (7).

Characterization of the RNA fragments also gives valuable information on possible interactions between structured regions of LA23-B 35S RNA i.e. on its tertiary structure. Suggestive interactions are indicated by the following criteria : (1) the interacting sequences are present in the same fragment purified by PAGE in non denaturing conditions, (2) the interacting sequences are reproducibly found in at least 3 different RNA fragments generated by partial digestion of 35S RNA at more than one RNA to nuclease ratio. Based on these criteria most of the RNA-RNA interactions appear to map close to the 5' end and in the middle of the Gag gene, near the 5' and 3' ends of the Pol and Env genes and near the 5' end of the Src gene (Fig. 6). These results can be illustrated in a model for the structure for LA23-B RNA 35S RNA (Fig. 7).

Several features of this cloverleaf like model desserve comments :

a) the center of the model is characterized by several T_1 oligonucleotide markers for sequences mapping close to the 5' end of the RNA molecule, near the 5' end of the Pol, Env and Src genes and near the 3' end of the Gag, Pol and Env genes. Such interactions should bring together sequences that map far away from each other, and thus might be important in the splicing process that gives rise to subgenomic 27S and 21S RNA that serve as messengers for the Env and Src proteins (2).

b) among the several T_1 oligonucleotides of the center of the cloverleaf like model, one finds number 2 that is known to be well structured (its sequence being $UCUCUC_4A_2CUA_2UAU_2ACUGG$; see ref. 10). This region was shown to bind a small single stranded DNA (22) and upon u.v. irradiation of the virus the viral protein p.19 was shown to bind to T_1 oligonucleotides 2 and to a lesser extend to 3, 13b and 14a (unpublished observations). This core protein is at the NH_2 terminus of the viral Gag precursor and is possibly involved in viral maturation (1,5).

c) the very 5' end of the 35S RNA molecule does not seem to be involved in tertiary structures although it was shown to have a secondary structure (8). Indeed T_1 oligonucleotide 6 was found in RNA fragment 2 (see Table 1) being part of a sequence 28 nucleotides long which agrees with the secondary structure presented in ref. 8. This finding correlates well with the fact that the 5' end sequence is accessible to viral DNA polymerase for the initiation of cDNA synthesis and might account for the observation that in vitro the major ribosome binding site on RSV RNA lies in that region (22,23).

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