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# Structure-function relationship of Rous sarcoma virus leader RNA

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ABSTRACT

Cells infected by RSV synthesize viral 35S RNA as well as subgenomic 28S and 22S RNAs coding for the Env and Src genes respectively. In addition, at least the 5' 101 nucleotides of the leader are also conserved and we have shown previously that this sequence contains a strong ribosome binding site (J.-L. Darlix et al., J. Virol. 29, 597). We now report the RNA sequence of Rous Sarcoma virus (RSV) leader RNA and propose a folding of this 5' untranslated region which brings the Cap, the initiation codon for Gag and the strong ribosome binding site close to each other. We also show that ribosomes protect a sequence just upstream from initiator AUG of Gag in vitro, and believed to interact with part of the strong ribosome binding site according to the folding proposed for the leader RNA.

## INTRODUCTION

Rous Sarcoma virus (RSV) RNA codes for four known genes which map in the order 5' to 3' : virus group specific antigens (Gag), viral DNA polymerase (Pol), envelope glyco-proteins (Env) and transforming proteins (Src). In addition two untranslated regions are found at the 5' (leader) and 3' (C region) ends of the viral genome (1,2).

In vitro translation of RSV RNA leads to the synthesis of Pr76K the precursor of virus specific antigens and of Pr180K that represents translation of the first two genes Gag and Pol (3). We have shown previously that ribosomes bind strongly and mainly to the 5' end sequence of RSV RNA (4). In addition binding of the ribosomes to that region is temperature dependent, sensitive to inhibitors of initiation of translation and involves the participation of methionyl tRNA; in this respect the binding of ribosomes


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to the 5' end of RSV RNA is similar to that found for other eucaryotic mRNA's. However, the AUG initiator for Gag gene translation is located at position 380 from the Cap (5). Nevertheless, inhibition of ribosome binding to this site or removal of the ribosome binding site are accompanied by an inhibition of the in vitro translation of Gag gene (4).

How then does initiation of translation operate ? How do the ribosomes bound at the 5' end of RSV RNA recognize the initiator AUG of Gag gene ? In order to answer to these questions, we have determined the RNA sequence of RSV leader and we propose a possible folding of this sequence where the 5' and 3' ends are tightly associated.

## 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 (4).

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 (4).

Enzymes. E.coli RNases III and H, were purified as previously described (6). 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 RNase III in the following buffer at 37°C : 50 mM Tris-HCl, pH 8, 100 mM NaCl, 5 mM Mg $Cl_2$, 1 mM DTT, and 0.1 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. 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 and Gilbert (8).

Sequencing of RNA and of large $T_1$ oligonucleotide. RNA or $T_1$ oligonucleotides to be sequenced were first labeled at their 5' end with $\gamma$- $^{32}P$ 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 $\gamma$- $^{32}P$ ATP (2,000 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., (9) to map adenines, guanines and pyrimidines in RNA, and we used both the "wandering-spot" analysis, Physarum M and pancreatic RNases to distinguish between uridines and cytidines (10). 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. Preparation and extraction of ribosome-protected RSV RNA fragments as well as in vitro protein synthesis were exactly as described before (4).

## RESULTS

### RNA sequence of RSV leader

The leader of RSV RNA was purified as described previously (5); briefly 35S RNA was cut by E.coli RNase H (6) in the presence of the pentadesoxynucleotide TCCAT, whose sequence is complementary to the 5' last nucleotides of Gag gene (4). The leader RNA was purified by polyacrylamide gel electrophoresis (PAGE) and partially digested with $T_1$ RNase in conditions described in Methods. Structured 5' $^{32}P$ RNA sequences of the leader were purified by 2 dimensional PAGE as described by De Wachter & Fiers (11) and obtained as sequencable RNA upon further purification by PAGE in 8 M urea. 5' $^{32}P$ structured RNAs located in the leader region were also obtained upon partial $T_1$ RNase digestion of RSV 35S RNA (7) and purified as described

in Methods. Both type of structured RNA sequences $^{32}P$ -labeled at the 5' end were sequenced using rapid sequencing methods (9, 10). Two RNA sequences are presented in figure 1 and they extend from position 158 to 207 ($RNA_1$) and from 311 to 371 ($RNA_2$). Position of the various RNA fragments is indicated by double arrows in figure 3. The 3' end of RSV leader RNA was determined by means of sequencing 3' - $^{32}P$ leader RNA. Part of RSV leader sequence was also determined by sequencing strong stop DNA (4) and cDNA's primed with d-(TCCAT). However, priming of cDNA synthesis did not occur at the 5' end of Gag gene but at position 140 and at the 5' end of P15 coding sequence. Consequently the 5' last 140 nucleotides of the leader were correctly determined by this method whereas the 3' end sequence corresponded in fact to that of P12 coding sequence (5). Accurate ordering of the RNA fragments between positions 217 to 311 was made possible by comparison with Pr-C RNA sequence obtained by D. Schwartz (personal communication) using viral cDNA transcripts. RNA sequence of the leader of Pr-B(LA23) is presented in figure 2, and it differs slightly from that of Pr-C, SR-A RNA (12) or Y73 RNA (13) determined using cDNA transcripts or cloned DNA, respectively. Moreover, we have frequently observed sequence microheterogeneities in RSV structured RNA's, and sometimes large differences from one clone of LA23 to another one (to be reported elsewhere), and these observations could account for the differences seen between the leader sequence of Pr-B (LA23), Pr-C, SR-A and Y73.

Proposed secondary structure of RSV leader RNA

Zuker and Stiegler have developed a program in order to fold RNA making use of the computer (14). To fold RSV leader RNA we have used this methodology taking also into account that G residues (or the next residue) susceptible to partial $T_1$ RNase digestion and the $tRNA^{tryp}$ primer binding site (pos. 102-118) should not be base paired.

A secondary structure of RSV leader RNA is proposed in figure 3 and this is a highly ordered structure reflecting the fact that a large number of partial $T_1$ RNase digests are located in this region. In addition $T_1$ oligonucleotides 6 and 35 located at the respective positions 9-24 and 274-289 are known

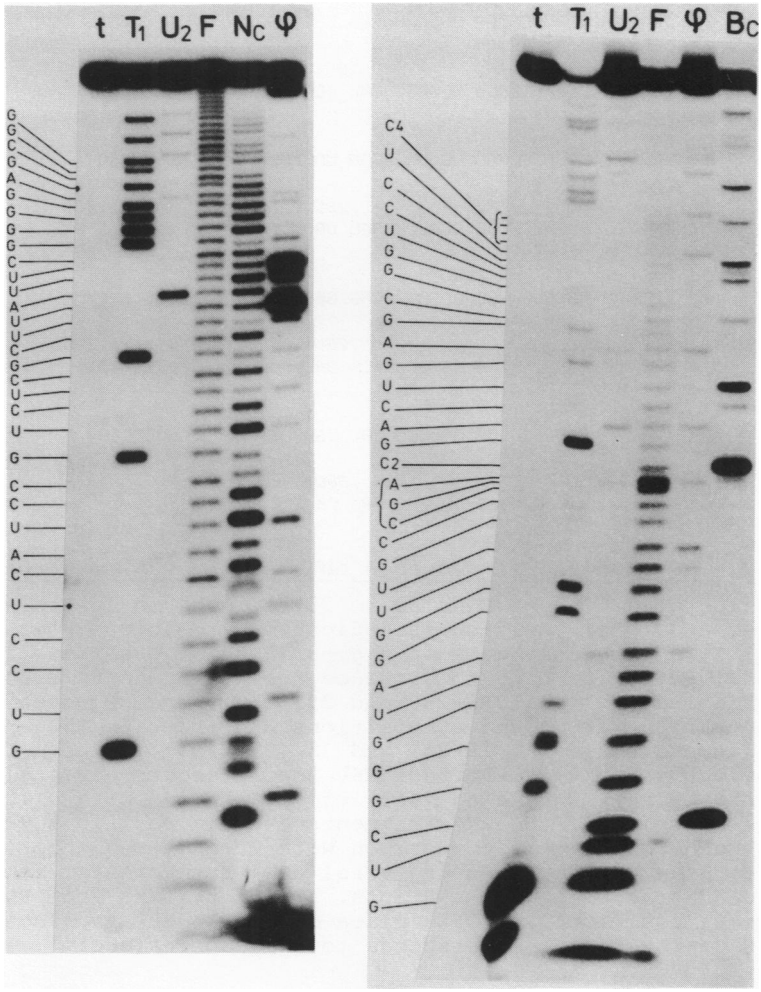


Figure 1 - Nucleotide sequence of two structured RNAs located in the leader

Partial $T_1$ RNase digestion, purification of the RNAs, 5'($P^{32}$) labelling and sequencing were as described in methods. The sequencing gels shown are 12% acrylamide, 0.6% bis-acrylamide in 7M urea and 75 mM Tris-borate, 1mM EDTA. t, $T_1$, $U_2$, F, Nc or Bc and Ø refer to control, $T_1$ RNase, $U_2$ RNase, formamide, Neurospora crassa or Bacillus aureus RNase and ØM RNase digestion, respectively. Autoradiography was for 2 days at -70°C using a screen. RNA 1 extends from position 158 to 207, and RNA 2 from 311 to 371 (see figure 3). Microheterogeneitics were found at positions 170 (U or C) and 177 (A or C) in $RNA_1$ and 345 (U or A) in $RNA_2$.

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          .           .20         .           .40         .
GGCCAUUUGA CCAUUCACCA CAUUGGUGUG CGCCUGGGUU GAUGGCCGGA

          .60         .           .80         .           .100
CCGAUGAUUC CCUGACGACU ACGAGCACAU GCAUGAAGCA GAAGGCUUCA

          .           .120        .           .140        .
UUUGGUGACC CCGACGUGAU UGUUAGGGAA UAGUGGAUGG CCAUAGACGG

          .160        .           .180        .           .200
CUCGGAGACC UCGUCCUCAC CCGUCUAGCU UAUUCGGGGA GCGGACGAUG

          .           .220        .           .240        .
ACCCUAGUAG AGGGGGCUGC GGCUUAGGAG GGCAGAAGCU GAGUGGCGUC

          .260        .           .280        .           .300
GGAGGGAGCU CUAUCGUCGG CGAGCUAACA UACCCUACCG AGAACACAGA

          .           .320        .           .340        .
GAGUCGUUGG AAGUCGGGUA GGUUGCCGAC CGACUGAGCG GUCCUCCCCA

          .360        .           .380
GGCGAGACCU UGGUCGCUCG GUGGAUCAAG CAUGGA
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Figure 2 - RSV leader RNA sequence obtained by RNA sequencing

Structured 5' $^{32}P$ RNAs 30 to 75 residues in length were sequenced and their position is indicated by double arrows (partial $T_1$ RNase cleavages) in fig. 3. RNAs at positions 208-266 and 217-266 were hard to sequence due to extensive stacking within A's and G's (pos 226-236 and 251-258), and sequence micro-heterogeneities were found at positions 235 and 243. Sequence of $T_1$ oligonucleotides 6 (9-25) and 35 (274-290) is known form our previous work (4-5). Part of RSV leader sequence was also determined by DNA sequencing (4-5) of cDNA primed with d(TCCAT). Accurate ordering of the RNA fragments between positions 217-311 was made possible by comparison with Pr-cRNA sequence obtained by D. Schwartz et al. using viral cDNA transcripts. Sequence of our cDNA transcript of Pr-B (pos. 1-140) differs from RNA sequence at position 121 (C in place of U). In the sequence given, G at position 1 corresponds to the cap residue.

to be susceptible to RNases III and IV (5, 15) that cut preferentially after unpaired U residues in a secondary structure (15) and indeed U's present in $T_1$ oligonucleotides 6 and 35 are unpaired in the proposed structure for RSV leader RNA. Several features of this secondary structure of RSV leader RNA appear to be of importance with respect to the ribosome binding at the 5' end of RSV RNA and the initiation of Gag gene translation :

1) Base pairings between most of the ribosome binding site (down to residue 52) and a 43 nucleotide sequence just preceding the AUG initiator of Gag put in a very close vicinity the Cap,

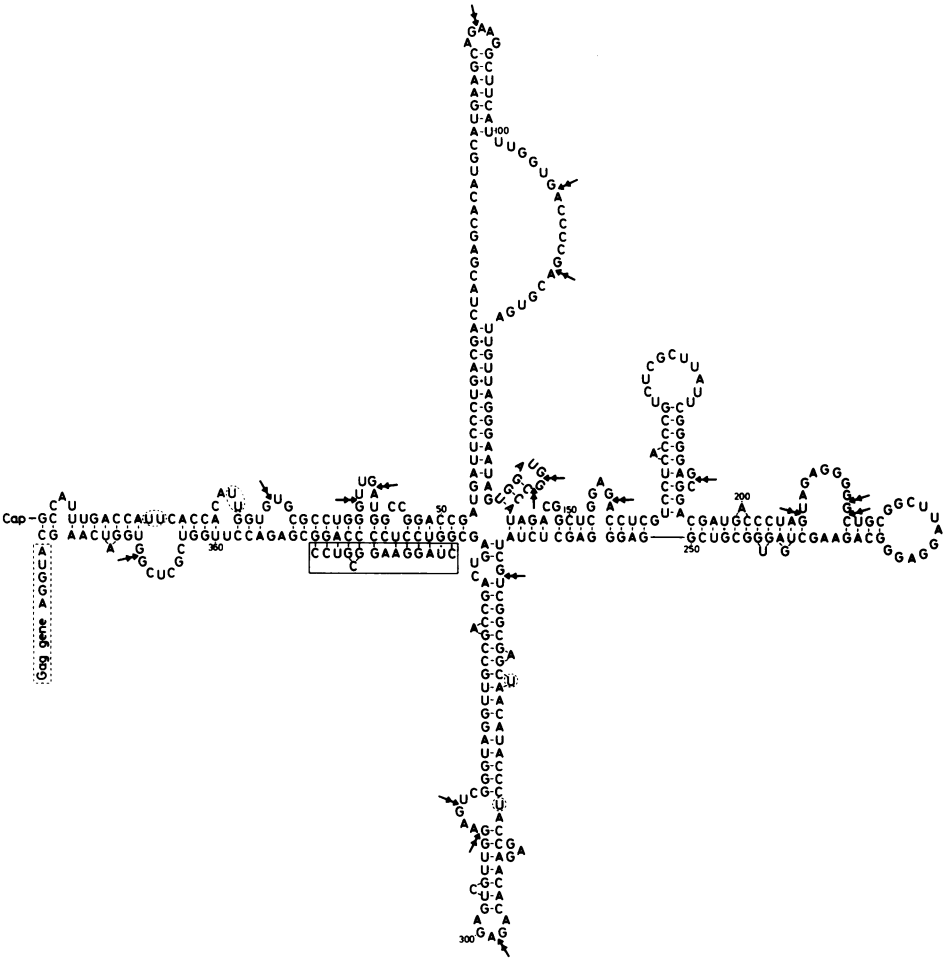


Figure 3 - Secondary structure model of RSV leader RNA

The secondary structure proposed was determined by computer analysis (14). The $tRNA^{tryP}$ binding site as well as G residues susceptible to partial $T_1$ RNase digestion (double arrows) were not allowed to base-pair. Optimal energy of this structure is estimated to be - 175 Kcal and that of the left arm containing the ribosome binding site is - 45 Kcal. U residues circled with a dotted line are part of $T_1$ oligonucleotides 6 and 35 susceptible to E. coli RNase III (see text). Sequence in the box is that of the 3' end of 18S rRNA.

the strong ribosome binding site of RSV RNA and the initiator codon for Gag gene.

2) The 3'-OH sequence of 18S rRNA is believed to be implicated in initiation of mRNA translation (16), and we have observed previously that from residues 4 to 13, 7 out of 10 nucleotides could form base pairs with the 3' end of 18S rRNA. More interesting is the possible interaction of residues 338 to 351 (thought to interact with part of the ribosome binding site, see fig. 3) with the 3' end of 18S rRNA since 12 out of 13 residues could form base pairs (see fig. 3).

Possible binding of ribosomes to sequences just upstream from initiator AUG of Gag

As reported before only the 5' end of RSV RNA interacts specifically with the ribosomes (4), since removal of the 5' last 101 nucleotides of RSV RNA results in the loss of specific binding of the ribosomes to RSV RNA as well as the absence of Pr76K synthesis in vitro (4). In agreement with this observation is the fact that addition of 7 mGMP inhibits both the binding of the ribosomes to the 5' end of RSV RNA and synthesis of Pr76K in vitro (4).

In view of probable interactions between the ribosome binding site and the 43 nucleotide sequence just upstream from initiator AUG of Gag (see fig. 3), we looked if among the RNA fragments protected by the ribosomes some could originate from the sequence potentially interacting with the ribosome binding site.

Several experiments were devised :

a) RNA fragments protected by the ribosomes against $T_1$ RNase in the presence or absence of 0,5 mM 7 mGMP were further digested by panc. α $T_1$ RNases and analyzed by paper electrophoresis at pH 3,5. In addition to G, C and U residues, AC, AU, Cap-GC, AG and AAG were detected (characterized by paper electrophoresis at pH 3,5 after total alkaline digestion). Addition of 0,5 mM 7 mGMP resulted in a 2,5 fold decrease of all spots including AG and AAG.

b) RSV RNA $P^{32}$ - labeled in vivo was heat denatured, poly $A^+$ RNA was purified by chromatography on oligo d(T)

cellulose, and sized by sedimentation on a sucrose gradient (as reported in ref. 4). 7 polyA$^+$ RNA species ranging from 15 to 35S were selected and reticulocyte ribosomes were bound to the different RNA species - $^{32}P$ - RNA fragments protected by the ribosomes using the 7 different RNAs were purified and analyzed by PAGE in 8 M urea. 35S RNA was the sole RNA species to give rise to well defined sizes of ribosome - protected RNA fragments (as shown in fig. 4a). Addition of 0,5 mM 7 mGMP strongly reduces these RNA bands (see ref. 4).

c) The RNAs of well defined sizes protected by the ribosomes (see fig. 4a) were recovered, extensively digested with $T_1$ RNase and analysed. In addition to $T_1$ oligonucleotide 6 (16 nucleotide long) already well characterized (see ref. 4 and fig. 5), two other $T_1$ oligonucleotides 9 and 10 residues are present. These two oligonucleotides were recovered, completely digested with panc RNase and analysed by paper electrophoresis at pH 3,5. $T_1$ oligonucleotide 9 residues was shown to contain the cap group, and the one 10 residues in length contained C and U residues and AG (characterized by paper electrophoresis upon total alkaline digestion).

d) The various RNA fragments protected by the ribosomes were also completely digested with $T_1$ and panc RNases, and analysed by paper electrophoresis at pH 3,5. They contained G, U and C residues and also AG, AAG and Cap-GC.

e) Ribosomes bound at the 5' end of RSV RNA should move as soon as translation starts, and then protection of the large $T_1$ oligonucleotides should disappear. Experiment was done (see fig. 5) and following translation for 3 min *in vitro* protection of $T_1$ oligonucleotides 16 residues (No 6), 10 and 9 residues in length decreases drastically.

In conclusion ribosomes bind to the 5' end of RSV RNA and protect defined sequences against nuclease digestion as shown before (4). Protection is sensitive to the competitive inhibitor of translation initiation, 7 mGMP (see also ref. 4) and is drastically reduced as translation takes place. In addition to these

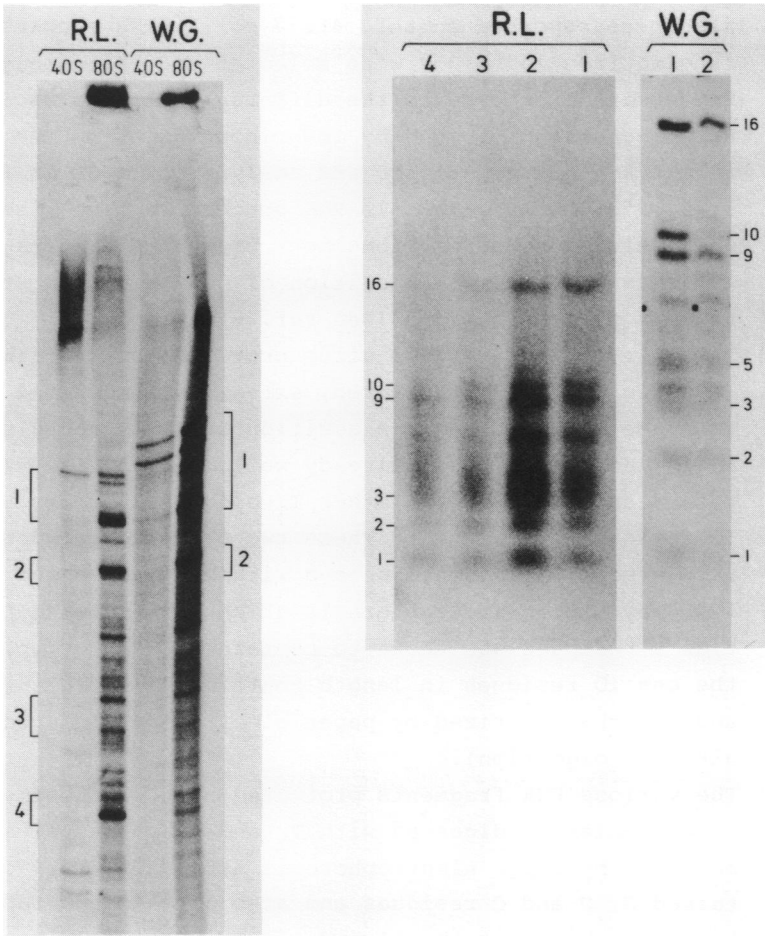


Figure 4 - Analysis of RSV RNA sequences protected by reticulocyte and wheat germ ribosomes

Binding of ribosomes to $^{32}P$ RSV 35S RNA and purification of the RNA fragments protected against $T_1$ RNase digestion were as described previously (4).

$^{32}P$ RNA fragments were analyzed on a 20 % polyacrylamide gel in 7M urea (see methods). R.L. is reticulocyte lysate; W.G. is wheat germ extract; 40S and 80S refer to 40S and 80S ribosomes. Top RNA bands in 1 are 55-60 nucleotides long. $^{32}P$-RNA fragments protected by 80S ribosomes of reticulocyte lysate (R.L. 1 to 4) or wheat germ (W.G. 1 and 2) were recovered by diffusion (see methods) extensively digested with $T_1$ RNase and analyzed on a 25 % polyacrylamide gel in 7M urea. Numbers on the site of each analysis refer to the number of residues by refe-

rence with $T_1$ oligonucleotide markers and 16, 10 and 9 correspond to $T_1$ oligonucleotide 6, the one located just upstream from Gag initiation codon and the Cap containing $T_1$ oligonucleotide, respectively. These 3 $T_1$ oligos were recovered, extensively digested with panc-RNase and analyzed on a Wh 3MM paper at pH 3.5. Only $T_1$ oligonucleotide 10 residues in length was shown to contain 1 AG.

sequences, experiments described above suggest that ribosomes protect also sequences located just upstream from initiator AUG of Gag gene; these sequences are characterized by AG, AAG and $T_1$ oligonucleotide 10 nucleotide long (see figures 2 and 3).

## DISCUSSION

We report here RNA sequence data on RSV leader RNA that differ only slightly from those obtained by sequencing Pr-C cDNAs (Schwartz et al., personal communication) or cloned Y73 DNA (13), but somewhat more from the DNA sequence published by Swanstrom et al. (12) where 10 nucleotides are missing as compared to Pr-B leader RNA (outside 5' and 3' sequences, see fig. 3).

Taking advantage of the computer program of Zuker and Stiegler (14) for folding RNA, we propose a secondary structure for RSV leader RNA. G residues susceptible to partial $T_1$ RNase digestion as well as the binding site for primer $tRNA^{tryp}$ were not allowed to base-pair. Similarly the sequence AUGGA at the beginning of Gag gene is not base-paired since it should be accessible to d(TCCAT) and RNase H (5). The "cross-like" secondary structure proposed for RSV leader RNA deserves several comments :

(i) The cap, the initiation codon for Gag gene and the strong ribosome binding site are adjacent to each other and constitute the left arm of the model. 80S ribosomes bind to the 5' end of RSV RNA would then ignore the remaining part of the leader sequence and especially the $tRNA^{tryp}$ binding site during translation of Gag gene. Accordingly presence of primer $tRNA^{tryp}$ on RSV RNA does not affect synthesis of Pr76K, the Gag precursor, in vivo (P.F. Spahr, unpublished data).

(ii) The upper arm is the site where viral DNA polymerase initiates reverse transcription, by elongation of the $tRNA^{tryp}$

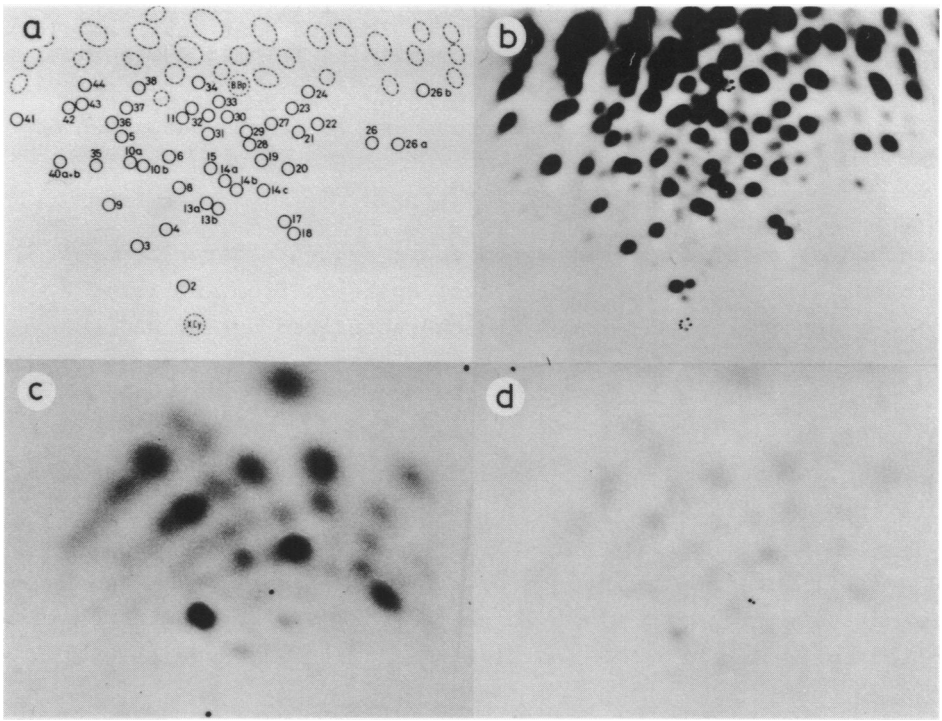


Figure 5 - Binding of the ribosomes at the 5' end of RSV RNA during in vitro translation

Conditions of ribosomes binding to $^{32}P$ 35S RNA and purification of the ribosome protected $^{32}P$ RSV RNA fragments were as described in details in ref. 4. $T_1$ fingerprint analyses were done according to De Wachter and Fiers (11). a - Schematic representation of the $T_1$ fingerprint of $^{32}P$ -LA23 RNA shown in b. c - $T_1$ fingerprint of the viral RNA fragments protected by ribosomes from the reticulocyte lysate. d - As in c except that translation of RSV RNA occurred during 3 min after which diphteria toxin and sparsomycin were added. Dots correspond to the dye markers. Autoradiography was for 2 to 4 days using Kodak NS5T films.

hybridized to residues 102-118 of RSV leader RNA (17) (see fig. 3). (iii) The right arm of the model contains several loops and that located at the end is rich in G residues which appear to be resistant to partial $T_1$ RNase digestion (pos 220-230). A large number of RNA-RNA interactions were shown to occur in RSV 35S RNA (7,15) and one of them should take place between residues 200-280 and 2500-2700 (see ref. 7 and Darlix, unpu-

blished data). The loop located at the end of the right arm might participate in this RNA-RNA interaction.

The bottom arm of the model does not appear to be implicated in protein or RNA interactions as yet known.

The ribosome binding site includes sequences located at the 5' end of the leader and very probably other ones just preceding the initiator AUG of Gag gene, all located in the same RNA structure domain. This ribosome binding site resembles other ones also located in well structured RNA domains in MS2, R17 and Qβ in prokaryotes (18, 19) and β-globin, STNV and BMV in eukaryotes (20-22). The 3' end of 18S rRNA is believed to interact with the 5' non coding region of mRNAs and participate in some fashion in the initiation of protein synthesis (16). We have previously indicated a possible interaction between a sequence close to the Cap and the 3' end of 18S r-RNA (4), however a more favorable interaction (12 base-pairs with 8 G-C; see fig.3) would involve the $T_1$ oligonucleotide 10 residues in length located 30 nucleotides upstream from Gag initiation codon and probably recognized by the ribosomes. However initiator AUG of Gag gene appears to be just outside the ribosome binding site, i.e. the sequences protected by the ribosomes against $T_1$ RNase digestion. Nevertheless, inhibition of ribosome binding to this 5' end of RSV RNA is accompanied by an inhibition of the in vitro translation of Gag gene. For example, 7 mGMP inhibits binding of the ribosomes (see result, section 2, ref. 4) probably by compering out the cap binding protein (16, 23, 24); preliminary results show that the cap binding proteins and elongation factor 1 can be cross-linked to the ribosome binding site as in the case of reovirus mRNA (16). In addition, as soon as translation starts ribosomes move and initiate Pr76K synthesis at the AUG closest to the cap (see fig. 3). How ribosomal components and initiation factors participate in the initiation process is not known and this is presently under investigation.

Recently it has been suggested that Env and Src mRNAs have the same leader as 35S mRNA (25) indicating that translation initiation of Gag, Env and Src genes should proceed in a similar fashion.

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