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Role of the open reading frames of Rous sarcoma virus leader RNA in translation and genome packaging

Olivier Donzé[1] and Pierre-François Spahr

Department of Molecular Biology, University of Geneva, 30 Quai Ernest Ansermet, 1211 Genève 4, Switzerland

[1]Corresponding author

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The Rous sarcoma virus (RSV) RNA leader sequence carries three open reading frames (uORFs) upstream of the AUG initiator of the gag gene. We studied, *in vivo*, the role of these uORFs by changing two or three nucleotides of the three AUGs or by deleting the first uORF. Our results show that (i) unlike most previously characterized uORFs, which decrease translation, the first uORF (AUG1) of RSV acts as an enhancer of translation, since absence of the first AUG decreased translation; AUG3 also modulates translation, probably by interfering with scanning ribosomes as described for other upstream ORFs, and mutation of AUG2 had no effect on translation. (ii) Mutation of each of the upstream AUGs lowered the infectivity of progeny virions. (iii) Unexpectedly, mutation of AUG1 and/or AUG3 dramatically reduced RNA packaging by 50-to 100-fold, unlike mutation of AUG2 which did not alter RNA packaging efficiency. Additional mutants in the vicinity of uORF1 and uORF3 were constructed in order to elucidate the mechanism by which uORFs affect RNA packaging: a translation model requiring uORFs 1 and 3, and involving ribosome pausing at AUG 3 is discussed.

Key words: ribosome scanning/Rous sarcoma virus/RNA packaging/secondary structure/translational control

Introduction

All eukaryotic mRNAs have at their 5′ terminus, preceding the initiator AUG, an untranslated sequence of variable length and composition. The mechanism of eukaryotic mRNA translation initiation has not been fully elucidated, but a likely model has been proposed, the so-called 'scanning hypothesis' (Kozak, 1989a). According to this model the initiation of mRNA translation involves first the binding of the 40S ribosomal subunit to the capped 5′ end of the untranslated sequence, followed by migration of the 40S subunit along the RNA until an initiator AUG is encountered. For efficient translation initiation this AUG should be in a good sequence 'context' (RccAUGG): the purine (R) at the −3 position being the most important in determining the strength of initiation together with, to a lesser extent, the G at the +4 position (Kozak, 1986). In the majority of eukaryotic mRNAs the 5′ proximal AUG is the initiator of the open reading frame (ORF) coding for the protein. However, a few cellular mRNAs and many viral mRNAs contain additional ORFs (uORFs) upstream of the major ORF (Kozak, 1989a). In several cases, it has been shown that these uORFs are involved in regulating the expression of the major protein product (Khalili *et al.*, 1987; Werner *et al.*, 1987; Geballe and Mocarsky, 1988; Kozak, 1989a, 1991 and references therein; Miller and Hinnebusch, 1989; Fütterer *et al.*, 1990; Waterhouse *et al.*, 1990; Arrick *et al.*, 1991; Schleiss *et al.*, 1991). For instance, the AUG initiator of translation of the yeast GCN4 gene product is preceded by four short uORFs which act as positive and negative regulators of GCN4 mRNA translation through a mechanism of translation reinitiation, involving both *cis* and *trans* components but not the peptides encoded by the uORFs (Hinnebusch, 1990). The yeast CPA1 gene encodes an enzyme of the arginine biosynthetic pathway whose expression is repressed by arginine: in this case, the peptide encoded by the uORF is involved in translational regulation of the CPA1 gene in the presence of arginine (Werner *et al.*, 1987).

Rous sarcoma virus (RSV) RNA belongs to the class of mRNAs containing uORFs. It has a 5′ untranslated leader sequence of 380 nucleotides, with three uORFs preceding the AUG initiation codon of the *gag*, *gag-pol* and *env* products. However, in contrast to most mRNAs, the 35S RSV RNA, in addition to serving as an mRNA, functions as a template for reverse transcription, as a precursor for splicing and as the genome of progeny virions (Perdue *et al.*, 1982; Varmus and Swanstrom, 1985; Varmus and Brown, 1989; Cobrinik *et al.*, 1988). Elements involved in each of these four functions are present in the RSV RNA leader sequence.

Earlier studies on the RSV leader showed that, *in vitro*, ribosomes bind upstream of the first uORF (Darlix *et al.*, 1979; Petersen and Hacket, 1985; Petersen *et al.*, 1984) and synthesize the heptapeptide encoded by this uORF (Hackett *et al.*, 1986). Using a complementation assay with the *env* protein, it was shown that deletion of the second and the third uORF in the leader did not significantly affect the translational efficiency of RSV RNA (Katz *et al.*, 1986). Two mutants with deletions of the proposed *psi* packaging signal [which spans from nucleotides 217 to 249 in the RSV leader (Linial and Miller, 1990)], including the third uORF, were defective in RSV packaging but translated the virus mRNA normally (Linial *et al.*, 1978; Koyama *et al.*, 1984). A deletion of the last two codons of the first uORF impaired reverse transcription but had no effect on translation (Cobrinik *et al.*, 1988) while deletion of the three uORFs slightly increased the *in vitro* synthesis of the *gag* precursor (Hensel *et al.*, 1989). Recently, while our work was in progress, it was reported that a single nucleotide change in the AUG of the first uORF altered virus production *in vivo* (Petersen *et al.*, 1989).

We have used site-directed mutagenesis to study *in vivo* the role of these elements in the virus life cycle, using a transient transfection assay in chicken embryo fibroblasts. We show that AUGs (or uORFs) can modulate RSV RNA translational efficiency; moreover, changes to the AUGs can

dramatically affect RSV RNA packaging, indicating that translation and packaging are intimately related.

Results

Introduction into the RSV genome of mutations to the AUGs of the leader

The 380 nucleotide leader of RSV contains three uORFs, 7, 16 and 9 codons in length, preceding the AUG that initiates the synthesis of the *gag, gag-pol* and *env* products (Figure 1a and b). To study the role of these AUGs, we mutated two or three nucleotides of the triplet AUG of each uORF in order to create a non-initiation codon (Figure 1c) (Peabody, 1989): the first AUG (position 41 relative to the 5′ cap) was replaced by an ACC codon (mutant pAM1); in pAM2, the second AUG (position 82) was mutated to UCA; the third AUG (position 197), which lies in a good initiation context (Kozak, 1989a) was changed to a UCA (mutant pAM3). Three other mutants completed the series: pAMUP, in which all three AUGs were mutated; pAM1M2,

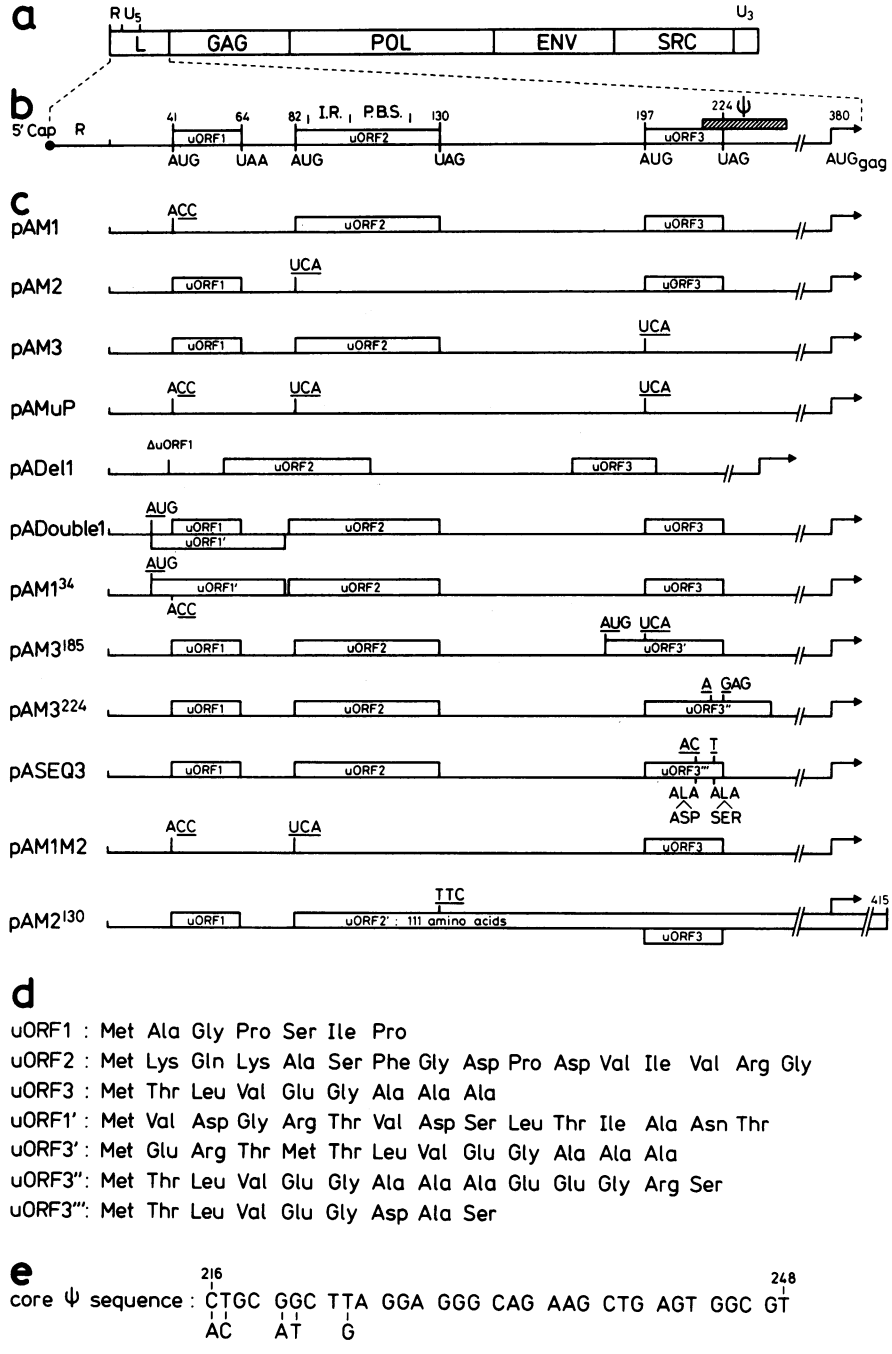

Fig. 1. (**a**) Genomic RSV pAPrC RNA. The regions encoding the *gag*, *pol*, *env* and *src* genes are shown in boxes. (R, terminal repeat; U5, unique sequence at the 5′ end; U3, unique sequence at the 3′ end). (**b**) Leader of RSV RNA with the three uORFs (shown as boxes). The numbers indicate the first and last nucleotides of the uORFs (IR, inverted repeat; PBS, primer-binding site for $tRNA^{TRP}$; Ψ: sequence involved in RNA packaging; AUG *gag*, initiation codon of the *gag* gene). (**c**) Schematic representation of RSV leader mutants. Underlined nucleotides shown for each mutant represent the sequence change. The designation uORF refers to uORFs encoding a different peptide. For each mutated leader sequence, the uORFs are drawn to scale and their location in the leader is as indicated. (**d**) Amino acid sequence of the peptides encoded by the uORFs designated as in c. (**e**) RSV core packaging *psi* sequence (Linial and Miller, 1990) and the nucleotides mutated (in $pAM3^{224}$ and pASEQ3).

lacking the two first AUGs and pADel1, in which the entire uORF1 was deleted (Figure 1c). Furthermore, from the data obtained with these mutants, various other virus mutants were constructed (Figure 1c) and these will be described in the appropriate sections below. All mutations were confirmed by DNA sequencing and the mutagenic fragments were cloned back into plasmid pAPrc, which contains a full-length copy of the wild-type RSV Prague C strain genome (Meric and Spahr, 1986). The effects of mutations within the leader were analyzed *in vivo* using a transient transfection assay in chicken embryo fibroblasts (CEF).

Effect of RSV leader mutations on in vivo translation

The role of the leader AUGs in translation *in vivo* was determined by two different approaches: analyzing the production of one of the viral proteins (Pr76, *gag* gene product) or investigating the effect of the leader on a reporter gene. In the first case, the amount of precursor Pr76 synthesized in transfected CEF was measured by immunoprecipitation followed by Western blot analysis using, in both steps, an anti-p27 [anti-capsid (CA)] antibody. Translation was normalized to the amount of mRNA using a denaturing Northern blot procedure (Figure 2); the amounts of 35S and 27S RSV RNAs were similar for each mutant, indicating that neither transcription nor splicing were affected by the leader mutations. In the reporter gene experiment, the leader sequences carrying the different mutations were fused to the firefly luciferase gene. The plasmids expressing the chimeric transcripts were transfected into CEFs and the luciferase activity in the cell extract was quantified by measuring the amount of emitted light in a luminometer; the luciferase activity was also normalized to mRNA levels. The translational efficiency of these chimeric constructs was also tested in a Quail cell line (QT35) and in RSV-transformed CEF with similar results to those obtained with CEF (data not shown). The experiments were performed more than 10 times using both approaches and similar results were obtained with both systems (Table I).

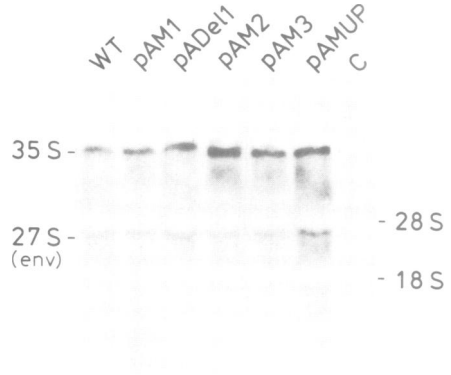

Fig. 2. Steady-state levels of viral RNA transcripts in transfected cells. The analysis was performed 60 h after transfection. Total RNA was extracted, electrophoresed in a denaturating agarose gel and analyzed by Northern blotting using a ^{32}P-labeled full-length pAPrC probe. Methylene blue staining of the filter after transfer was used to confirm the equivalence of RNA loading and transfer between samples and to determine the positions of the 18S and 28S rRNAs. Cells were transfected with the mutant DNA indicated above the lane. C, control: cells transfected with no DNA. 35S and 27S represent unspliced genomic RNA and spliced *env* mRNA respectively.

The first AUG lies in a poor translation initiation context (UugAUGG) but is situated at a good consensus distance from the 5′ cap (~40 nucleotides; Figure 1b; Kozak, 1989a). Mutation of this codon to a non-initiation codon led to a 5-fold decrease in translation efficiency compared to the wild-type (cf. pAM1 and WT in Table I) suggesting that the first AUG or uORF1 acts as an enhancer of translation. The second AUG, whose position and sequence context (albeit a weak context) is highly conserved between avian retroviruses, does not appear to influence the scanning ribosomes since mutant pAM2 was translated as efficiently as the wild-type. Indeed, removal of the stop codon of uORF2 (position 130: pAM2^{130}, Figure 1c), which results in an uORF of 111 codons, terminating 11 codons downstream of the initiation codon of the *gag* gene, had little or no effect on Pr76^{gag} expression (Table I). Further, even in the absence of AUG1 the ribosomes failed to initiate at AUG2, since mutating AUG1 and the stop codon of uORF2 (BsM1M2^{130}: which should decrease translation if AUG2

Table I. Translation of the Pr76gag and the luciferase reporter genes

Virus mutant	Amount of PR76gag[a] (relative to WT)	Luciferase activity[b] (relative to WT)	Efficiency of virus particle production[c]	
			c.p.m.	(relative to WT)
WT	1.0	1.0	3000	1.0
pAM1	0.2	0.17	750	0.2
pAM2	1.1	1.0	2950	1.0
pAM3	1.8	2.0	5300	1.7
pAMUP	1.8	2.0	5200	1.8
pADel1	1.5	1.3	4300	1.4
pAM1^{34}	ND	0.9	–	
pADouble1	ND	1.0	–	
pAM1M2	1.0	1.0	2900	1.0
pAM2^{130}	ND	0.95	3000	1.0
BsM1M2^{130}	ND	0.3	–	

[a]To determine the levels of protein synthesis, cell lysates were immunoprecipitated, washed and the precipitated material was separated by gel electrophoresis as described in Materials and methods. Bands corresponding to the Pr76^{gag} were quantitated by scintillation counting. The results are expressed relative to the WT normalized to the amount of 35S mRNA (quantitated by hybridization of a denaturing Northern blot with a radiolabeled full-length RSV probe and densitometric scanning of the exposed film). The variation between independent transfections was < 10%.
[b]Levels of luciferase expression were normalized to WT and RNA levels. Luciferase activity was quantitated as described in Materials and methods. The leader sequences of the luciferase assay were cloned in the Bluescribe plasmid and thus designated BsWT, BSM1, BsM2, etc.
[c]Virus production was determined by endogenous reverse transcriptase activity and reverse transcriptase activity was measured by the dot-blot procedure, as described in Materials and methods.
ND, not determined.

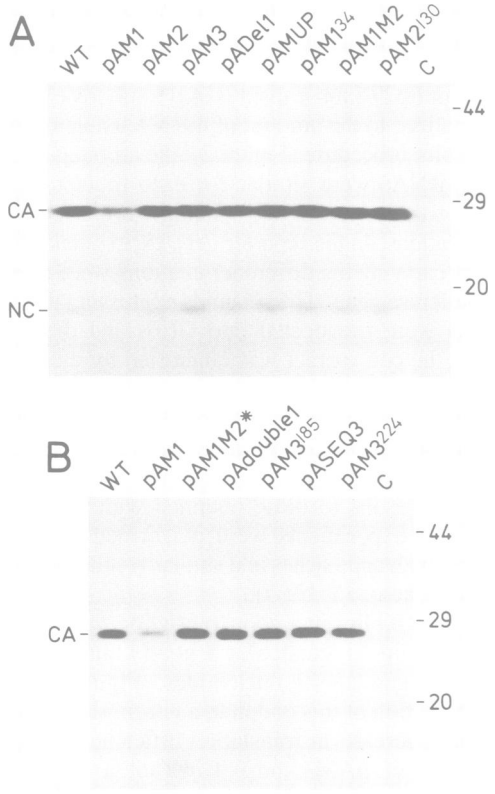

Fig. 3. Analysis of the virion *gag*-encoded proteins. Virions produced by the transfected cells were purified as described in Materials and methods. Viral proteins were resolved by SDS−PAGE and imunoblotted with polyclonal antibodies against RSV CA (p27) and NC (p12) and detected with ^{125}I-labeled protein A. The mutants are indicated above each lane (pAM1M2 and pAM1M2* are two independent clones). C, control. **A** and **B** represent two separate transfections.

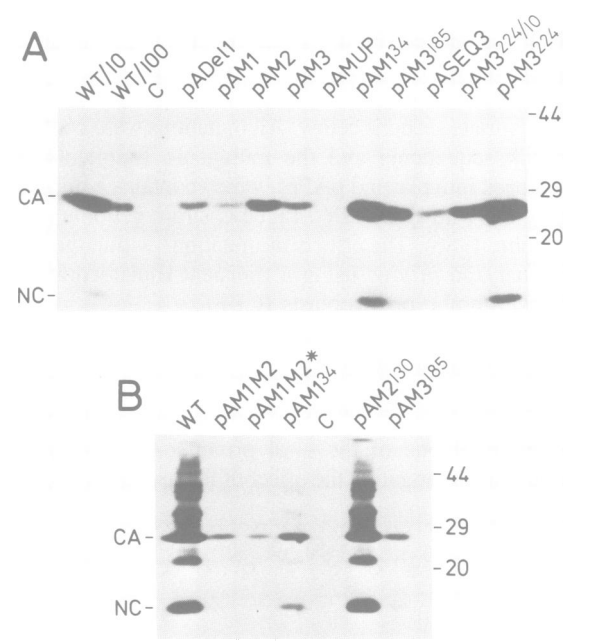

Fig. 4. Infectivity of the virus mutants. Chicken embryo fibroblasts were transfected with the plasmid DNAs as for a transient assay. The culture media were collected after 60 h and used to infect fresh cells (using the same number of particles, normalized to CA). After 6 days, the medium was analyzed for the presence of viral proteins by immunoblotting with anti-CA. Designation of lanes is as for Figure 3. WT1/10, WT1/100: infection with wild-type virus using 1/10 or 1/100 of the number of particles compared to the mutants respectively (similarly for pAM3^{224}/10). **A** and **B** represent two separate infections.

is recognized) resulted in almost the same phenotype as pAM1 (i.e. a 3-fold reduction in translation. Table I). The only initiation codon lying in a good sequence context (AcgAUGA), AUG3, influenced the process of translation: with mutant pAM3 we observed a 2-fold increase in translation compared with wild-type, showing that ribosomes which initiate at AUG3 fail to reinitiate at AUG*gag* (Table I). The mutation of all three upstream AUGs (pAMUP) had the same effect as mutation of the third (pAM3), namely a 2-fold increase in translation (Table I). If the effects of the mutations were additive, one would predict that this mutant (pAMUP) would show a decrease in translation, since it contains mutations in AUG1 (5-fold decrease), AUG2 (as WT) and AUG3 (2-fold increase). However, this discrepancy is accounted for by the translational phenotype of the mutant pAM1M2 (Figure 1c), which synthesized PR76 protein as efficiently as WT: thus, pAMUP can be defined as a pAM1M2 plus pAM3. Finally, deletion of the first uORF (pADel1) increased translation of the downstream product (1.4-fold in comparison to wild-type). This effect has also been observed *in vitro* (Hensel *et al.*, 1989).

Each mutant formed particles as demonstrated by the presence of capsid (p27), nucleocapsid (p12) or reverse transcriptase (RTase) activity in the culture medium. For each mutant, the number of particles released into the medium, as determined by RTase activity, correlated with the level of translation (Figure 3 and Table I).

Analysis of viral infectivity

Virus particles obtained by transfection were used to infect fresh CEF. Western blot analysis was performed directly on the cell culture supernatants both to equalize the amount of virus used for infection (Figure 3) and to monitor the appearance of progeny virus after infection (Figure 4 and Table II). The determination of virus infectivity was performed several times with similar results.

Mutation of the first AUG (pAM1) surprisingly impaired viral infectivity. pAM1 was $<1\%$ infectious, and transformed CEF with a delay of 2 weeks, by comparison to wild-type virus. In a parallel study, Petersen and colleagues also observed the same phenotype by changing the first AUG to ACC (Petersen *et al.*, 1989). Deletion of the first uORF (pADel1) also reduced virus infectivity to $1-5\%$ of wild-type: normal growth of the mutant resumed after 10 days (Table II). pAM2 showed a smaller decrease in viral infectivity and a $4-5$ day delay in the transformation of cells. pAM3 produced particles at a slower rate after infection (5% WT infectivity) with a delay in recovery of 1 week. Mutation of the first and second AUGs (pAM1M2) led to the same reduction in infectivity as pADel1 or pAM3. Finally, viral particles obtained by transfection of pAMUP were not infectious: no progeny particles could be detected, even after several weeks of growth.

The virus mutants (except pAMUP) analyzed in this study are only partially defective. The appearance of virus is unlikely to be the result of genetic reversion, since the progeny virus exhibited the same impaired growth phenotype as that of virus derived by transfection (data not shown).

Table II. Virus infectivity and virus RNA packaging

Virus mutants	Infectivity[a,b] D = 6 (relative to WT)	Delay in cell transformation[a,c]	RNA packaging[a,b] (relative to WT)
WT	**1.0**	**0**	**1.0**
pAM1	0.01	2 weeks	0.01–0.05
pADel1	0.01–0.05	10 days	0.01–0.05
pAM2	0.05–0.1	4–5 days	**1.0**
pAMUP	0	–[e]	0.01–0.05
pAM3	0.01–0.05	7–8 days	0.01–0.05
pAM1[34]	0.3	2–3 days	0.4–0.6
pADouble1	**1.0**	**0**	**1.0**
pAM3[185]	0.05–0.1	5–6 days	0.05–0.1
pASEQ3	0.01	–[e]	**1.0**
pAM3[224]	**1.0**	**0**	**1.0**
pAM1M2	0.01–0.05	ND	0.01–0.05
pAM2[130]	**1.0**	**0**	**1.0**

[a]Values are the average of at least 10 experiments with a standard deviation of no more than ±20%.
[b]6 days after infection, the medium was analyzed for the presence of viral proteins either by immunoblotting, or by reverse transcriptase activity (Dupraz *et al.*, 1990).
[c]The delay in the observation of cell transformation after infection. WT virus induced transformation occurred by day 6 after infection is designated 0.
[d]Virus genomic RNA packaging was determined by non-denaturing Northern blotting and quantitated by scintillation counting of the band representing viral RNA.
[e]not observed.
ND, not determined.
Numbers written in bold characters correspond to wild-type values.

Analysis of defective particles

Since the virus mutants displayed a considerable reduction in growth efficiency and yet transcription, splicing, translation and particle formation are not appreciably affected, it seemed likely that other steps in the replication cycle are affected. We further investigated each mutant by analyzing the viral RNA content of the virus particles. Nucleic acids were extracted from the virions produced by transfection and the RNA analyzed by a non-denaturing Northern blot procedure, conditions which preserve the tertiary structure of the 70S RNA dimer (Meric and Spahr, 1986). In all cases, the amount of RNA loaded was normalized to equivalent numbers of virions, as determined by Western blotting (Figure 3).

The mutant pAM1, which has only a two nucleotide change in an AUG codon, packaged <5% RNA by comparison with wild-type particles, as did the mutant pADel1 and pAM1M2 (Figure 5 and Table II). This result was unexpected in view of the position of the mutations up to 200 nucleotides upstream from the core packaging *psi* sequence required for efficient RSV RNA encapsidation, which spans from nucleotides 217 to 248 in RSV leader RNA (Linial and Miller, 1990). The mutation in pAM3 lies close to the packaging sequence (20 nucleotides upstream): the amount of viral RNA present in these particles was also much less than wild-type (<5%). This phenotype correlated well with the impaired viral growth observed for the mutants pAM1, pADel1, pAM1M2 and pAM3 (Figure 4); each mutant was 1–5% as infectious as wild-type virus. The pAMUP mutant was affected to the same extent as pAM1 or pAM3 (1–5% RNA packaging efficiency) but was not infectious. Viral RNA content was also analyzed by slot-blot hybridization with identical results (data not shown). Experiments on RNA packaging were performed more than 10 times yielding similar results each time.

In contrast to those mutants, the mutant of the second AUG of the RSV leader (pAM2) did package an equivalent amount of viral 70S RNA to wild-type (Figure 5), although its

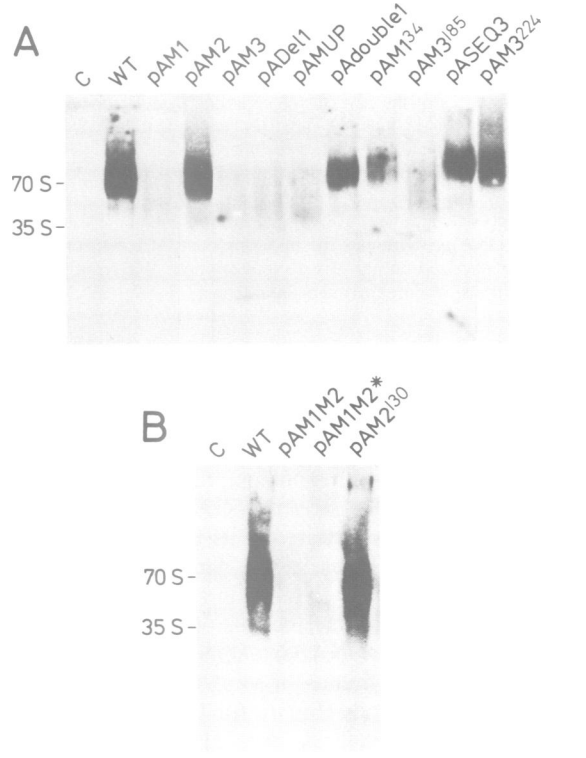

Fig. 5. Viral RNA content of the virions produced in a transient transfection assay. The virions were purified and viral RNA was extracted as described in Materials and methods. After size fractionation on a non-denaturing 0.8% agarose gel, the RNA was electrotransferred to a nylon membrane and hybridized with a probe specific for RSV RNA (plasmid pAPrc), ^{32}P-labeled by random primer extension. The RNA for each virus (wild-type and mutants) was extracted from an equivalent number of virions (normalized to CA protein, Figure 3). The viruses are indicated above the lanes as in Figure 3). 70S and 35S indicate the dimerized and monomeric RSV genomic RNA respectively. **A** and **B** represent two separate transfections.

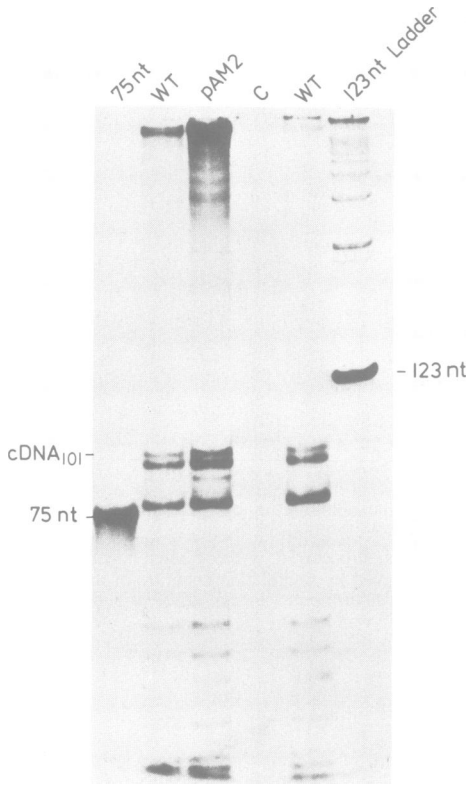

Fig. 6. Analysis of the reverse transcription product for wild-type virus and the pAM2 mutant. The 101 nucleotide cDNA was synthesized by incubation of permeabilized virions with [α-^{32}AP]dTTP and separated on a 8% non-denaturing polyacrylamide gel, as described in Materials and methods. 75 nt represents a ^{32}P-labeled 75 nt oligonucleotide and 123 nt ladder represents a ^{32}P-labeled 123 nt ladder. $cDNA_{101}$ represents the initial product (101 nucleotides in length) synthesized during reverse transcription. WT from two different transfection experiments are shown.

growth rate was also impaired (Figure 4). It is interesting to compare the properties of pAM2 with those of mutant S4C (see Figure 1C in Cobrinik *et al.*, 1991): the mutant S4C has two four-base complementary mutations in the U5-IR stem structure (maintaining the predicted secondary structure but altering its sequence, changing AUG2 to AUC). Like pAM2, S4C is impaired in its growth rate and shows no defect in viral RNA packaging. Since the pAM2 mutation lies close to the primer-binding site, we analyzed the synthesis of the initial intermediate of reverse transcription, a 101 nucleotide cDNA extending from the $tRNA^{TRP}$ primer to the 5′ terminus of viral RNA, as described in Materials and methods (Figure 6). No defect with respect to the $cDNA_{101}$ synthesis was apparent, showing that mutation of the AUG of the second uORF (containing the primer-binding site) did not affect this step of reverse transcription. The presence of smaller bands in Figure 6, in addition to the 101 base cDNA, probably represents premature termination of the reverse transcriptase under the conditions used (additional bands were consistently observed in the $cDNA_{101}$ synthesis experiment). One possible explanation for the growth defect of pAM2 is an effect on integration, since mutations in the region around AUG2 have been shown to result in an integration defect, altering the DNA sequence recognized by the IN (integrase) protein (Cobrinik *et al.*, 1991).

Taken together, these results indicate that AUG1 and -3 (or uORF1 and -3) play a fundamental role in the process of RNA packaging. To distinguish between the various possible mechanisms that can account for this observation, we introduced further mutations in the vicinity of uORF1 and -3 and tested them for effects on translation, infectivity and RNA packaging.

Mutations in the vicinity of open reading frames 1 and 3

uORF1 mutants. To study the packaging defect due to the mutation of AUG1 (or the deletion of uORF1) the mutant $pAM1^{34}$ was constructed in which the AUG1 at position 41 was changed to ACC (as in pAM1) and a new AUG was created at position 34, to replace a UGG (Figure 1c). In this case, translation of uORF1 would occur in a −1 frame relative to that of wild-type virus, producing a peptide of 15 amino acids, instead of the heptapeptide (Figure 1d: uORF1′): the AUG at position 34 is in an excellent context for translation [AccAUGG (Kozak, 1989a)]. This mutant was designed to test whether efficient packaging requires either the heptapeptide itself, a precise codon context and position, or a fixed uORF length. Another mutant was also constructed which contained AUG 34 in addition to the AUG 41 (pADouble1: Figure 1c). Both mutant DNAs were transfected in CEF and were compared with wild-type virus for various parameters. Both mutants formed normal amounts of virus particles relative to wild-type as detected by Western blotting using an anti-capsid antibody (Figure 3). Equivalent numbers of virions produced by transfection were then used to infect fresh CEF. The mutant pADouble1 infected cells with the same efficiency as wild-type, showing that the additional AUG 34 did not interfere with any step in viral replication (Table I). $pAM1^{34}$ was less infectious than wild-type, but its defect in viral growth was much less dramatic than for pAM1 (Figure 4 and Table II): it had a delay of 2−3 days in its ability to transform cells (in comparison to the 2 week delay of pAM1).

We investigated the amount of viral RNA packaged by $pAM1^{34}$ and pADouble1 using a non-denaturing Northern blot procedure. The addition of this new upstream AUG at position 34 restored viral RNA packaging to 40−60% of wild-type levels (cf. $pAM1^{34}$ and WT in Figure 5) which indicates that readdition of a single AUG codon within the RSV leader can restore RNA packaging by a factor of >20 (cf. pAM1 and $pAM1^{34}$; Figure 5). To study the effect of this AUG on translation, we used the chimeric construct containing the luciferase reporter gene. When the leader of RSV containing the AUG 34 was fused to the firefly luciferase gene, the efficiency of translation reached a wild-type level (Table I), indicating that efficient translation of a gene with an RSV leader requires an AUG close to the 5′-cap. The additional AUG 34 in the double mutant (pADouble1) did not interfere with the scanning ribosomes in that the efficiency of translation was similar to wild-type. These results also show that the wild-type phenotype, with respect to translation and packaging of viral RNA, is not dependent upon the synthesis of the heptapeptide product encoded by uORF1 and, moreover, that neither a precise codon context and position, nor a definite uORF length is required. Thus, for efficient translation and packaging, an uORF is crucial in the 5′ part of RSV leader.

uORF3 mutants. The role of uORF3 or AUG3 in viral RNA packaging was studied by an approach similar to that used for uORF1. Three additional mutants were constructed in the vicinity of or within uORF3 (Figure 1c). In pAM3^{185}, an AUG was introduced at position 185 of the leader replacing a GGG (a U → C mutation at position 183 was also introduced to create a *Nco*I restriction site), in addition to the pAM3 mutation. This new AUG 185 lies in a less favorable context for translation (UcgAUGA) than the wild-type AUG 197 (AcgAUGA). In this case, translation can proceed through uORF3 in the same frame as wild-type but producing a peptide of 13 amino acids instead of a nonapeptide (see Figure 1c and d: uORF3′). The role of the peptide itself was investigated by constructing the mutant pASEQ3 in which the alanines at positions seven and nine of the uORF3 nonapeptide, which are highly conserved between avian retroviral strains (Bizub *et al.*, 1984) were changed to asparagine and serine respectively (Figure 1d: uORF 3′′′). The wild-type uORF3 (nucleotides 198−223) terminates just at the boundary of the *psi* packaging sequence (nucleotides 217−248); a third mutant, pAM3^{224} was therefore constructed, in which the UAG stop codon at position 224 was mutated to a GAG (in addition to a G → A mutation at position 220, which has no effect on the peptide sequence). In this case (mutant pAM3^{224}), translation can proceed through the entire uORF3 and continue until a stop codon at position 239 (in the middle of the *psi* sequence) producing a 14mer polypeptide (Figure 1c and d: uORF3′′). It should be noted that the mutations in both pASEQ3 and pAM3^{224} include five nucleotide changes in the 5′ part of the *psi* sequence (Figure 1e).

The three mutant DNAs were transfected transiently into CEF and compared to wild-type virus. The three mutants released normal amounts of virions into the medium (Figure 3). By infecting fresh CEF with virions produced by transfection, we observed that only pAM3^{224} infected CEF as efficiently as WT (Figure 4). Mutation within the uORF3 (pASEQ3) dramatically impaired virus infectivity by comparison to wild-type virus, since only 1% of wild-type numbers of particles were detected in the medium after 6 days infection (cf. WT and pASEQ3 in Figure 4). The introduction of AUG 185 only partially restored the AUG3 function (Table II); indeed, virus production for this mutant was still impaired (Figure 4), but with a 5−6 day delay in cell transformation, in comparison to 1 week for pAM3.

The RNA content of particles produced by each mutant was analyzed as above. Normal amounts of RNA were packaged into particles produced by pAM3^{224} as expected from the infectivity of the particles (Figure 5). Mutation of the uORF3 product (pASEQ3) led, as described above, to impaired virus growth, but viral RNA content was similar to that of wild-type, quantitatively and qualitatively (all the RNA was matured as 70S RNA). These results suggest that the uORF3-encoded peptide is required for a step in the virus life cycle other than RNA packaging. Mutants pAM3^{224} and pASEQ3, in addition to demonstrating that the sequence and length of uORF3 are not important in RNA packaging, allow redefinition of the core packaging region which has previously been assigned to the sequence between nucleotides 217−248 (Linial and Miller, 1990): the two mutants together shorten this region by eight nucleotides and the packaging region, thus spans from nucleotides 225−248 (Figure 1E). In the last mutant affecting uORF3, pAM3^{185}, the presence of a new AUG did not restore efficient packaging, although slightly more RNA was packaged than in pAM3 (Figure 5 and Table II). This indicates that for AUG3, the phenotype cannot be restored completely simply by the addition of a new codon; this might be due either to a position effect [the new codon is situated 12 nucleotides upstream of the normal AUG (position 197)] or to the fact that this AUG is in a poor context for initiation of translation.

Discussion

We have characterized the role of the three upstream open reading frames present in the leader of RSV. The conservation of these elements among all avian strains in position, length of coding sequence and strength of initiation potential (Bizub *et al.*, 1984) suggests that the virus requires these uORFs for its viability (Petersen *et al.*, 1989). Indeed, the effect of mutation of the leader AUGs was striking.

uORFs and translation

The first uORF, close to the 5′-cap site, acts unexpectedly as an enhancer of translation, since mutation of the AUG to ACC (pAM1) led to a 5-fold decrease in translation (Table I). Usually, removing an uORF or an AUG upstream of the main ORF results in an increase in translation of the downstream product, since 40S ribosomes can directly reach the initiation codon without interference (Kozak, 1989a). Few exceptions are known where absence of an upstream AUG causes decreased translation of the downstream product. The leader of cauliflower mosaic virus RNA contains several open reading frames upstream of the major ORFs: removal of some of these uORFs led to a decrease, while removal of others resulted in an increase, in translational efficiency (Fütterer and Hohn, 1991; Fütterer *et al.*, 1990). The mechanism by which these elements act is still obscure. In the yeast *Saccharomyces cerevisiae*, GCN4 gene expression is regulated by amino acid availability through a translational control mechanism involving four short uORFs in the leader of GCN4 mRNA: mutation of the first and/or second uORF AUGs is partially inhibitory for GCN4 expression in amino acid-starved cells. This was explained by a reinitiation mechanism in which ribosomes must translate uORF1 (or uORF2) in order to scan past the following uORFs and initiate at the GCN4 start site. Absence of the first AUG allows ribosomes to initiate at uORF3 and -4, and, after completing translation of these uORFs, the majority of these ribosomes are unable to reinitiate at the AUG of GCN4 (Abastado *et al.*, 1991). In polio virus, Pelletier *et al.* (1988) observed a decrease in translation after mutating the AUG7 of the RNA leader. This case, however, is peculiar since polio virus initiates translation by internal ribosome binding. Moreover, it was shown that this AUG is involved in the binding of a factor (p52) and that the observed decrease in translation was probably due to impaired binding of this factor to the region of AUG7 (Meerovitch *et al.*, 1989).

In RSV, AUG1, although in a weak context by the rules of Kozak (Kozak, 1986), is the predominant ribosome-binding site of RSV mRNA resulting, at least *in vitro*, in the synthesis of a heptapeptide (Petersen and Hackett, 1985; Hackett *et al.*, 1986). Sequence comparison of the first uORF of different avian retrovirus strains (Hackett *et al.*, 1992) showed that the stop codon is either UGA or UAA, suggesting a role for this uORF in translation. The same comparison of uORFs 2 and 3 showed that the stop codon

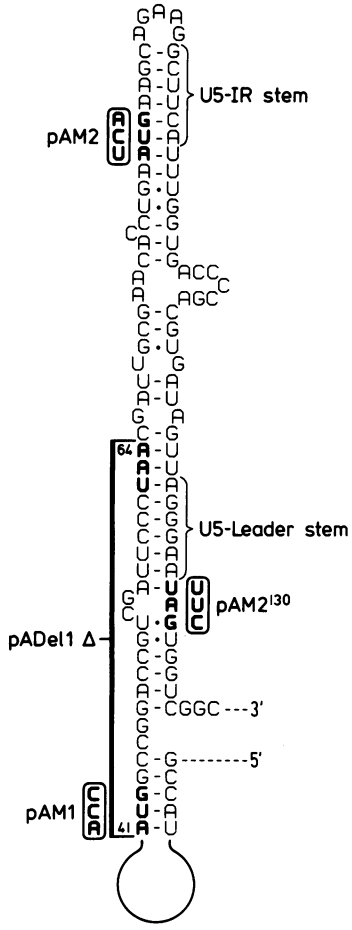

Fig. 7. Predicted secondary structure of the first 140 nucleotides of RSV (Pr-C) RNA leader. Numbers indicate nucleotides from the viral RNA 5'-end. The initiation and stop codons of the uORF1 and -2 are shown in bold characters; sequence substitutions of the mutants pAM1, pAM2 and pAM2^{130} are shown in boxes; the pADel1 sequence deletion is indicated by the bar. U5-IR and U5-leader are structures shown by Cobrinik *et al.* (1991) to be involved in reverse transcription and in integration. The lowest calculated ΔG for this structure is −40 kcal/mol.

is UAG for both uORFs in all avian strains, suggesting a role in RNA structure in addition to a possible role in translation [mutation of either of these stop codons had no effect on virus phenotype (pAM2^{130} and pAM3^{224}: Figure 4)]. The uORFs 1 and 2 are localized in a region of the leader with a complex secondary structure (Cobrinik *et al.*, 1988, 1991; Aiyar *et al.*, 1992; Hackett *et al.*, 1992). This region, called the primer-binding loop (PBL), contains, in addition to the two first uORFs, the tRNA^{TRP} primer-binding site and two motifs involved in reverse trancription (Figure 7; U5-IR and U5-leader stem; Cobrinik *et al.*, 1991). This structure appears to be conserved between different retrovirus species such as MMTV, MuLV, SNV and HIV (Cobrinik *et al.*, 1988). These motifs form a stable stem−loop structure [ΔG = −40 kcal/mol, according to the algorithm for RNA secondary structure (Jaeger *et al.*, 1989)] which should be partially inhibitory to scanning ribosomes [scanning 40S ribosomal subunits are stopped by secondary structures with ΔGs > −30 kcal/mol (Kozak, 1989b)]. We propose that RSV has avoided this 'translation reverse transcription' interference by encoding an uORF in this secondary-structure-rich region to maintain the 5' proximal sequence in an open configuration, enabling ribosomes to scan the entire RNA leader up to the *gag* initiation codon. Our results support such a model: mutation of the first AUG reduced translation by a factor of five (Table I) suggesting that, in the absence of uORF1, the PBL hairpin is not melted by the uORF1 translating 80S ribosomes, partially blocking scanning ribosomes. Thus, uORF1 is required for translational enhancement only when the inhibitory stem−loop is present: indeed in the mutant pADel1, where the uORF1 is deleted and the structure is strongly destabilized (ΔG = −23 kcal/mol; Figure 1c and Figure 7), translation of *gag* was enhanced 7-fold compared to pAM1 (Table I). Moreover, destabilization of the PBL structure by changing a few nucleotides should restore translation in the absence of the uORF1. Indeed, when AUG1 is mutated and AUG2 [which is involved in the U5-IR stem; Figure 7 (Cobrinik *et al.*, 1988)] is mutated to UCA (mutant pAM1M2), the PBL is destablized by ~7 kcal/mol (ΔG = −32 kcal/mol): this is sufficient to allow scanning ribosomes to melt the structure and explains why this double mutation does not affect translation efficiency. Parkin and colleagues (Parkin *et al.*, 1988) showed that changing four nucleotides in the first 60 nucleotides of the TAR element of HIV, a similar hairpin structure in the virus leader RNA, decreased ΔG of the structure by 5 kcal/mol (from −36 to −31 kcal/mol) and, subsequently, increased translation 5-fold. In this case, it appears that decreasing the stability of an inhibitory secondary structure by only 5 kcal/mol is sufficient to relieve translational inhibition. In conclusion, the presence of an uORF in the PBL is required for efficient translation and the product encoded by the minicistron is not required since mutant pAM1^{34}, having the AUG1 displaced to nucleotide 34 and translating a totally different peptide, showed a translation efficiency similar to wild-type. Moreover, the product of translation, Pr76gag, does not play a role in the pAM1 translational decrease, since similar results were obtained with a reporter gene (Table I).

uORFs and packaging

The most striking effect observed in this study of mutation of the uORF AUGs was the unexpected defect in viral RNA packaging. Changing only two or three nucleotides of AUG1 and/or AUG3 led to a 50- to 100-fold decrease in the amount of RNA packaged (Figure 5 and Table II). The observation that mutation of one of these AUGs (pAM1, pADel1 and pAM3) or both (pAMUP) led to the same packaging impairment, suggests that both AUGs act co-operatively.

Different models can be proposed to explain the phenotype observed. Firstly, an RNA structure might be required to allow recognition of an RNA-binding moiety: this model requires, in our case, that two nucleotide changes at different positions (AUG1 and AUG3: Figure 1b) in the RSV leader sequence are sufficient to disturb the putative structure. However, it was reported that deletion of 21 nucleotides near the first uORF (leaving the uORF almost intact) impaired reverse trancription, but did not affect packaging (Cobrinik *et al.*, 1988) suggesting that the RNA secondary structure in the vicinity of uORF1 is not crucial for packaging. This model also requires that the introduction of a new AUG at position 34 is sufficient to restore the normal structure, since pAM1^{34} packages an almost normal level of genomic RNA. A second model is that the products of uORFs 1 and 3 are involved in RNA packaging. This is not the case for uORF1,

since in pAM1[34] the peptide encoded would be translated in a −1 reading frame relative to the wild-type sequence, producing a different 15 amino acid peptide. Moreover, the heptapeptide amino acid sequence encoded by the first minicistron is not highly conserved (Hackett *et al.*, 1986). The third uORF product is highly conserved in amino acid composition (Bizub *et al.*, 1984) but does not apparently play a role in RNA encapsidation because mutant pASEQ3, which has two conserved uORF3 amino acids mutated, and mutant pAM3[224], which synthesizes a 14 amino acid peptide containing the nine amino acids encoded by uORF3, plus two glutamic acids, an arginine, a glycine and a serine, produced virions containing normal amounts of RNA. Moreover, complementation assays using plasmids providing the first and/or the third peptides in *trans* failed to restore packaging to the deficient mutants (data not shown). A third model postulating a protein-binding site at AUG1 is incompatible with the data obtained for pAM1[34], but the data do not exclude the possibility of a protein binding at AUG3 (although the phenotype of mutant pAM3[185], showing a slight increase in RNA packaging, does not support such a model).

Since RSV 35S RNA serves both as a messenger RNA and as the genome of progeny particles, it follows that there must be discrimination between these two roles late in infection, possibly by interference between translation and packaging, as has been shown for hepatitis B virus (Nassal *et al.*, 1990). Our results support such a translation interference and we propose the following model: the first and third uORFs are translated but the second is not [our data confirm that uORF1 and 3, but not uORF2, interfere with translation (Table I: pAM1, pAM1[34], pAM3, pAM2[130] and BsM1M2[130])] and these two events are essential to allow efficient RNA packaging. We postulate that ribosomes, after translating the uORF1, terminate at the stop codon of uORF1 (position 64) and reinitiate at AUG3, which lies 130 nucleotides downstream of the uORF1 stop codon [an ideal distance for efficient reinitiation according to Kozak's study: the efficiency of reinitiation at a downstream AUG improves as its distance from the upstream ORF increases, 79 nucleotides being a minimum distance for efficient reinitiation (Kozak, 1987)]. This reinitiation model would imply that the key step in a translational interference model would be the pausing of ribosomes at AUG3. It has been shown that ribosomes pause during translation at various positions on mRNA, including at the initiator AUG and at the termination codon (Wolin and Walter, 1988). Since the *psi* packaging sequence is downstream of AUG3, interaction with the *gag* protein, resulting in packaging, can only occur if the *psi* sequence is clear of ribosomes: pausing would, thus, increase the probability of a *gag* protein binding to the *psi* sequence. This ribosome pausing model implies that packaging would not be influenced by 80S ribosomes translating uORF3: this is, indeed, shown by the phenotype of pAM3[224], in which the uORF3 terminates at the end of the *psi* sequence (Figure 1c) without impairing packaging. Further, a new AUG replacing the mutated AUG3 would be expected to restore packaging: for mutant pAM3[135], in which a new AUG (position 185) is created, packaging was restored, albeit partially (which may be explained by the weak context for translation of this AUG). Thus, AUG3 seems to be used as a barrier to pause the scanning 40S ribosomes: but what is the role of the first AUG in packaging? As mentioned above, translation of uORF1 appears to prevent the recognition of AUG2. However, in virus mutants, where we would expect initiation to occur at AUG2 (pAM1 and pADel1), ribosomes would terminate at the stop codon position 130 (Figure 1c). This codon is only 67 nucleotides upstream of AUG3, which is too close for efficient reinitiation (Kozak, 1987), preventing the pausing at AUG3; the scanning ribosomes, thus, continuously cover the packaging region, decreasing the probability of its recognition by the *gag* protein. This model would predict that a mutant lacking the first as well as the second AUG would package normal amounts of viral RNA, since ribosomes would then pause at AUG3. However, this was not observed for mutant pAM1M2 (Figures 1c and 5). Therefore, efficient packaging of viral RNA appears to require the translation of an uORF in the 5′ proximal part of the RSV leader RNA: the position of this uORF does not appear to be important (see pAM1[34]) except, probably, that it should be sufficiently distant from AUG3 to allow reinitiation of translation. The mechanism by which this element, situated 200 nucleotides from the *psi* sequence, acts remains to be elucidated.

In conclusion, we have shown in this study that RSV uses uORFs not only in translation regulation, but also in packaging of retroviral RNA during virus assembly. These data highlight the intimate relationship between translation and RNA recognition that occurs for a positive strand RNA virus. Other retroviruses, such as murine retroviruses also have AUGs and uORFs in the leader sequence: it would be of interest to see if these viruses also use these elements in packaging.

Materials and methods

Cell culture

Chicken embryo fibroblasts prepared from Spafas eggs (Gs$^-$ and Chf$^-$: Norwich, Connecticut, USA) were grown in Dulbecco's-modified Eagle medium containing 5% fetal calf serum (GIBCO Laboratories, Grand Island, NY) at 41°C in an atmosphere supplemented with 5% CO_2.

Bacterial strains

Escherichia coli DH5alpha, MV1190 and CJ236 were grown according to the instructions of the mutagenesis kit (Biorad). *E.coli* DH5alpha was rendered competent as previously described (Sambrook *et al.*, 1989). Plasmid DNAs were purified from either small or large bacterial cultures by the alkaline lysis method and were further purified for transfection by equilibrium density gradient centrifugation in cesium chloride−ethidium bromide (Sambrook *et al.*, 1989).

Cloned DNAs

Plasmid pAPrc has already been described (Méric and Spahr, 1986): it contains a non-permutated copy of the provirus RSV Prague C strain. Plasmid pAsPrc is a *SalI*−*Eco*RV subclone of pAPrc in pBR322 containing the entire leader and *gag* sequences. All the mutations were constructed in pBS*lead*, a 1167 bp *Sph*I fragment of pAsPrc cloned in the phagemid vector Bluescribe (−) (Stratagene, San Diego).

To construct the plasmid carrying the luciferase gene (Bs*luc*), the *Bsm*−*Bam*HI fragment (with the ends filled by the Klenow fragment of DNA polymerase I) from pRSV*luc* (de Wet *et al.*, 1987) containing the luciferase gene was cloned by blunt end ligation into Bs*leadMin* to replace the *Hin*dIII fragment encoding the *gag* gene (the *Hin*dIII ends were similarly filled by Klenow prior to ligation) Bs*leadMin* carries the 1157 bp *Sph*I fragment of pAsPrc in which the AUG initiator of the *gag* gene at position 380 has been mutated to TTC in order to create a *Hin*dIII site at the beginning of the *gag* gene (Oertle *et al.*, 1992). Bs*luc*, thus, has the RSV leader fused to the luciferase gene, plus 20 nucleotides of the luciferase leader in between. A 440 nucleotide *pst*I fragment from each Bs*lead* mutant was cloned into the corresponding site of Bs*luc* to replace the sequence derived from BS*leadMin*.

Site-directed mutagenesis

The following oligonucleotides were synthesized on an Applied Biosystems 381A DNA synthesizer and purified as previously described (Lo *et al.*, 1984):

AUG1 (pos. 41): 3′-CGTGGACCCAACTGGCGGCCTGGCAGC-5′
AUG2 (pos. 82): 3′-CGCTTGTGGACTAGTTTCGTCTTCCGA-5′
AUG3 (pos. 197):3′-CCCCTCGCCTGCAGTTGGGATCATCTC-5′
Del1: 3′-CCACACGTGGACCCAACGCTAACGCTTGTGGAC-5′
$M1^{34}$: 3′-AACCACACGTGGTACCAACTGGCGGCCTGGCAGCTA-5′
Double1: 3′-AACCACACGTGGTACCAACTACCGGCC-5′
$M2^{130}$: 3′-CACTATCAATCCCTTAAGACCAGCCGGTGTCTG-5′
$M3^{185}$: 3′-AGAGCGAATAGGTACCTCGCCTGCAGTTGGGAT-CATCTC-5′
SEQ3: 3′-CATCTCCCCCTGCGCAGAATCCTCCCGTCT-5′
$M3^{224}$: 3′-CTCCCCCGACGTCGACTCCTCCCGTCTTCG-5′
M1M2:3′-CGTGGACCCAACTGGCGGCCTGGCAGCTAA-GGGATTGCTAACGCTTGTGGACTAGTTTCGTCTTCC-5′

All the mutants were constructed as described previously (Kunkel, 1985) with some modifications. Briefly, single-stranded uracil-containing template DNA was obtained by introducing the phagemid pBS*lead* into *E.coli* CJ-*236* (dut^-, ung^-) and infection with helper-phage M13K07 as described previously (Vieira and Messing, 1987). The synthesis of the mutagenic strand was performed according to the mutagenesis kit instructions (Biorad). The resulting double-stranded DNA was introduced into *E.coli* DH5alpha (dut^+, ung^+, $RecA^-$) by the $CaCl_2$ transformation protocol and the bacteria were grown on LB + ampicillin (100 μg/ml) plates.

The introduction of the mutation was confirmed by the dideoxy-chain termination method of DNA sequencing using T7 polymerase (Pharmacia) primed by a synthetic oligonucleotide complementary to the 3′ end of the RSV leader. The mutated fragments were cloned back into pAsPrc using the *SphI* sites and then the *SalI*–*Eco*RV fragment was introduced into pAPrc.

Transfection and infection

Chicken embryo fibroblasts, either freshly prepared or kept frozen in the presence of 15% glycerol, were used after two to seven passages. Transfection of recombinant plasmids and infection by virus were performed as described previously (Méric *et al.*, 1988).

Protein analysis

Viral proteins produced by the transfected or infected cells were analyzed by immunoprecipitation and immunoblotting with polyclonal antibodies against RSV CA (p27) and NC (p12), as described previously (Méric and Spahr, 1986). Exogenous templates reverse transcriptase activity in virions purified from the medium of transfected cells was measured as described previously (Méric *et al.*, 1988).

Luciferase assay

Each 10 cm plate of transfected CEF was washed three times in phosphate-buffered saline without Ca^{2+} and the cells were harvested in 500 μl of lysis buffer (1% Triton X-100, 25 mM glycylglycine, pH 7.8, 15 mM $MgSO_4$, 4 mM EGTA, 1 mM DTT). Cell debris were pelleted by centrifugation in a microcentrifuge for 5 min at 4°C. A 50 μl aliquot of extract was added to 350 μl of assay buffer (25 mM glycylglycine, pH 7.8, 15 mM KPO_4, pH 7.8, 15 mM $MgSO_4$, 4 mM EGTA, 5 mM ATP, 1 mM DTT) in a small test-tube. The reaction was initiated by the injection of 20 μl of 1 mM luciferin and the emitted light was quantitated in a Bioorbit luminometer.

Purification of RNA

The viral RNA of virions produced after transfection was purified and analyzed by a non-denaturing Northern blot procedure as previously described (Khandjian and Méric, 1986; Dupraz *et al.*, 1990).

Total cellular RNA was purified from subconfluent cultures by lysis in guanidinium thiocyanate, followed by centrifugation through cesium chloride as described previously (Sambrook *et al.*, 1989). The RNA pellet was dissolved in STE (1% SDS, 10 mM Tris–HCl pH 7.4, 1 mM EDTA) and precipitated twice with ethanol in the presence of 0.3 M Na-Acetate, before analysis on a 1.2% denaturing agarose–TBE gel. The RNA was electrotransferred onto a GeneScreen nylon membrane (New England Nuclear Corp., USA) in 25 mM sodium phosphate buffer (pH 6.5). The wet nylon membrane was UV irradiated for 2 min at a distance of 5 cm from two germicide lamps (15 W each), prehybridized and hybridized with either a RSV-specific probe or Bsluciferase, as described previously (Khandjian, 1986). The RNA was quantified either by scanning densitometry of the autoradiography or by 'Cerenkov' counting of the filter.

Analysis of the reverse transcription product in permeabilized virions

Cell culture media from two successive 24 h incubations were collected and centrifuged at 7000 *g* for 10 min at 4°C. The supernatants were sedimented through a 3 ml cushion of 20% sucrose in 25 mM Tris–Cl pH 7.5, 50 mM NaCl and 1 mM EDTA pH 8.0 (NTE) for 2 h at 100 000 *g* and 4°C in a Beckman SW28 rotor. The virus pellet was resuspended in 3 ml of NTE and resedimented through a 1 ml cushion of sucrose in NTE at 150 000 *g* and 4°C in a Beckman SW60 rotor. The virus was resuspended in 25 μl of 100 mM Tris–Cl pH 8.3, 100 mM NaCl and 1 mM EDTA and 50 μl of strong stop cocktail was added (50 mM NaCl, 50 mM Tris–Cl pH 8.3, 5 mM $MgCl_2$, 1 mM DTT, 100 μM dGTP, 100 μM dCTP, 100 μM dATP, 5 μM dTTP, 10 μCi[α-^{32}P]dTTP, 0.005% NP40 and 100 μg/ml actinomycin-D) and incubated for 2 h at 42°C. The reaction was stopped by the addition of EDTA pH 8.0 to 0.4 mM, SDS to 1% and 10 μl proteinase K (10 mg/ml) and incubated for 5 min at 37°C. The mixture was extracted with phenol and then with chloroform–isoamyl alcohol (24/1). The DNA was purified by ethanol precipitation in the presence of 0.2 M NaCl and 10 μg yeast tRNA carrier and then denatured for 2 min at 90°C prior to electrophoresis in a non-denaturing polyacrylamide gel (8%).

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