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Effects of Alterations in the Leader Sequence of Rous Sarcoma Virus RNA on Initiation of Translation

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The 372-nucleotide leader sequence of Rous sarcoma virus RNA contains three conserved short open reading frames and other sequences responsible for a variety of life cycle functions. We have investigated several aspects of the leader RNA which may influence the translation of the major coding regions to which the leader is juxtaposed. We found that small perturbations of the leader length do not affect the binding and scanning of ribosomal subunits by more than about 10%, that the length and/or structure of the RSV RNA leader is near optimal for translation of the major coding regions of the viral RNA, that inclusion or deletion of open reading frames influences downstream initiation in a manner that is not strictly additive, and that reinitiation of translation at the *gag* gene is very efficient.

Four aspects of the leader RNA can affect translational efficiency: (i) the sequence of the 5′ end of the mRNA, which can affect the initial binding of 40S ribosomal subunits (3, 4, 11, 20), (ii) the presence of short open reading frames (ORFs) which may affect initiation at downstream initiation sites of major polypeptide-coding sequences (5, 12, 15, 18, 23, 27, 28, 30, 34, 35, 39, 40), (iii) sequence length (12, 13, 16, 17, 19) and/or features of the mRNA secondary structure (3, 19, 22, 29, 32), and (iv) certain sequences, which possibly act in cooperation with *trans*-acting factors, which may enhance or depress translation of downstream regions (1, 7, 13, 24, 28). The mRNAs of avian sarcoma/leukemia retroviruses have complex leader sequences that are relatively long and contain at least three short ORFs that are conserved with respect to length, position, and initiation AUG codon strength (6). We have shown that at least the two 5′-proximal ORFs of the Rous sarcoma virus (RSV) leader RNA are protected by ribosomes and translated in vitro (6, 34, 35) and in vivo (35a).

Synthesis of altered RSV mRNAs. In order to clarify the contributions of the length, the ORFs, and the structure of the leader sequences to translation of the major downstream coding regions on the multiply spliced RSV mRNAs, we examined the translational characteristics of a variety of altered RSV mRNAs. Because of the multifunctional nature of the RSV leader sequence in vivo, including initiation of translation and DNA synthesis, virion RNA packaging, and splicing, we examined the translational properties of the mRNAs in vitro. Since translation of RSV virion RNA in vitro accurately reflects translation in vivo (9, 34, 35a, 38), we could investigate the effects of alterations in the leader RNA sequence on translation without interference from other factors, such as *trans*-acting, RNA-binding proteins (e.g., *gag* proteins) that exist in virus-infected cells. Test plasmids were constructed that had altered leader sequences plus the first 92% of the RSV *gag* gene. With one exception, the test RSV RNAs were entirely composed of RSV RNA sequences up to the 11 5′-terminal nucleotides which were transcribed from the SP6 promoter. Altered RSV mRNAs were synthesized in vitro by using SP6 RNA polymerase (26) and were subsequently translated in a reticulocyte lysate (31, 34). Thus, the secondary structure of the test RSV mRNAs should be as near to that of the wild type as is possible.

All of the experimental RSV mRNAs, encoding the amino-terminal 647 amino acids of Pr76^{gag} (p70), were capped and synthesized (14; C. H. Hensel, Ph.D. thesis, University of Minnesota, St. Paul, 1988) from plasmids cleaved at the *Eco*RI site behind the poly(A) sequence. For reference, a *Sma*I-truncated pRSV-wt (wild type) template was transcribed to produce mRNA^{ref}, which encoded the amino-terminal 514 amino acids of Pr76^{gag} (p56). Because of cleavage at the upstream *Sma*I site, mRNA^{ref} was not polyadenylated. A summary of the leader sequences of all of the experimental mRNAs used in this study is shown in Fig. 1. The mRNAs range in size from 2.5 kilobases (+200+C) to 2.1 kilobases (Δ1-3).

Translational efficiencies of mutant RSV RNAs. The effects of alterations of the leader sequence on *gag* synthesis were assayed by adding equal amounts of the capped, test RNAs and mRNA^{ref} to the translational system and incubating the reactions at 30°C for 60 min. The proteins produced were separated by electrophoresis through polyacrylamide gels which were dried and exposed to X-ray film (Fig. 2). p70 synthesized from the test RNAs and p56 produced from mRNA^{ref} were excised from the gel and counted in a liquid scintillation counter. In order to avoid the effects of competition, the total concentration of mRNAs added was well below the saturation limit of the translational mix. The synthesis of RSV proteins was linearly dependent on message up to 12 ng of message per μl of lysate; only 2 ng of each test and the mRNA^{ref} were added per μl in the assays reported here. The ribosomes in the reticulocyte lysate were correctly recognizing the cap structure on the test mRNA, as shown by inhibition of translation by added cap analogs (Hensel, Ph.D. thesis).

The RNAs can be divided into three groups, according to the type of modification of the leader sequence. Group I comprises RNAs whose leaders are either slightly shorter or slightly longer than in wild-type RSV RNA; they possess the

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A
                     100        200         300         400         500         600 nt
CLONE
RSV+200+C     1'     2'                      1   2           3                      4
RSV5'HP                         a       bc        def        3                      4
RSV-wt                      [----]       1   2           3                      4
RSVΔ1                                        2           3                      4
RSVΔ1-2                                                  3                      4
RSVΔ1-3                                                                         4

B

CLONE      SP6 element/                          RSV sequence
                                  ↓                                          1
RSV+25+C   GAATACAAGCT/CGATACAATAAACGCCATTTGACCATTCACCACATTGGTGTGCACCTGGGTTGATG..
RSV+24     GAATACAAGCT/CGATACAATAAACGCCATTTGACCATTCACCACATTGGTGTGCACCTGGGTTGATG..
RSV+15     GAATACAAGCT/---------AAACGCCATTTGACCATTCACCACATTGGTGTGCACCTGGGTTGATG..
RSV+13     GAATACAAGCT/-----------ACGCCATTTGACCATTCACCACATTGGTGTGCACCTGGGTTGATG..
RSV+13*    GAATACAAG--/---------AAACGCCATTTGACCATTCACCACATTGGTGTGCACCTGGGTTGATG..
RSV-wt     GAATACAAGCT/T------------------------TTCACCACATTGGTGTGCACCTGGGTTGATG..
RSV-5      GAATACAAGC-/----------------------------ACCACATTGGTGTGCACCTGGGTTGATG..
RSV-10     GAATACAAGCT/T-----------------------------------TGGTGTGCACCTGGGTTGATG..
RSV-15     GAATACAAGCT/T-----------------------------------------TGCACCTGGGTTGATG..
RSV-20     GAATACAAGCT/T-----------------------------------------------CTGGGTTGATG..

C

RSV sequence from ATG1 → ATG(gag):
 1                    ↓Δ1                  2                        ↓Δ1-2  ▼
ATGGCCGGACCGTTGATTCCCTGACGACTACGAGCACCTGCATGAAGCAGAAGGCTTCATTTGGTGACCCCGACGTGAT

AGTTAGGGAATAGTGGTCGGCCACAGACGGCGTGGCGATCCTGTCTCCATCCGTCTCGTCTATCGGGAGGCGAGTTCG
 3                                                                     ↓Δ1-3
ATGACCCTGGTGGAGGGGGCTGCGGCTTAGGGAGGCAGAAGCTGAGTACCGTCGGAGGGAGCTCCAGGGCCCGGAGCG

ACTGACCCCTGCCGAGAACTCAGAGGGTCGTCGGAAGACGGAGAGTGAGCCCGACGACCACCCCAGGCACGTCTTTGG
                     4
TCGGCCTGCGGATCAAGCATGGAA.........
```

FIG. 1. 5′ ends encoded by the pRSV plasmids used in this study. 1, 2, 3, and 4 represent the four AUG codons found on wild-type RSV RNA. (A) Global comparison of test RNAs. The brackets at the 5′ end of RSV-wt indicate the range of 5′ termini of the clones shown in detail in panel B. 1′ and 2′ are additional copies of AUG1 and AUG2, respectively, on RSV+200+C RNA. a, b, c, d, e, and f are additional AUG codons on RSV-5′hp RNA not found on wild-type RSV RNA. nt, Nucleotide. (B) Detailed sequence comparison of group I mRNAs. The first 9 to 11 nucleotides of the mRNAs are transcribed from the SP6 promoter. The arrow indicates the normal 5′ end of RSV RNA; AUG1 is underlined. (C) Continuation of the common leader sequence of the RSV RNA. The sites where the Δ series RNAs are truncated are shown by arrows, and the positions of the three AUG codons of the leader ORFs are underlined and overlined. The arrowhead marks the position of the extra C found in RSV+25+C and RSV+200+C mRNAs. Constructions were performed as follows. First, an *Sst*I-*Eco*RI fragment containing the promoter-distal 111 nucleotides of the 371-nucleotide leader of the RSV RNA plus 92% of the *gag* gene was inserted downstream of the SP6 promoter to produce pRSVΔ1-3, which was used to construct pRSVΔ1 and pRSVΔ1-2 (which had either ORFs 2 and 3 or only ORF 3, respectively) by cloning *Hin*fI-*Sst*I or *Bst*EII-*Sst*I fragments from pSRA-5′ (35) into the *Hin*cII and *Sst*I sites of pRSVΔ1-3. A library of plasmids with additions to or deletions from the 5′ end of RSV RNA was constructed by cloning a BAL 31 nuclease-treated fragment from pSRA-5′ into pRSVΔ1; the extent of BAL 31 digestion was determined by nucleotide sequencing. Two members of this library were shown to have unusual structures. pRSV+200+C was the result of a three-fragment insertion which duplicated ORFs 1 and 2. pRSV-5′hp encoded an RNA containing seven upstream ORFs and an inverted repeat (of unknown origin) which would allow formation of an 81-nucleotide duplex region within the leader sequence. mRNAs RSV+13 and RSV+13* differed by two nucleotides at positions 10 and 11. pRSV-10, -15, -20, and -wt were constructed by using oligonucleotide-directed mutagenesis (41). M13 subclones containing the *Sph*I-*Sst*I fragment from pRSV-5 (for pRSV-10, -15, and -20) or pRSV+15 (for pRSV-wt) were used as templates for mutagenesis. All mutants were sequenced (25, 36). A 20-nucleotide oligo(dA) sequence was added at the end of the truncated gene to produce polyadenylated RNA transcripts (Hensel, Ph.D. thesis).

normal three ORFs in their leader sequences. Group II RNAs lack one or more of the upstream ORFs. Group III RNAs have leaders that are substantially longer than in the wild type and have extra ORFs. All studies used defined amounts of test and reference RNA as determined by autoradiography of the ^{3}H-labeled mRNAs. The efficiency of translation of a test mRNA was normalized to the efficiency of mRNA-wt as calculated from the equation efficiency = $[(^{35}S\ cpm_{test})/(^{35}S\ cpm_{ref})]/[(^{35}S\ cpm_{wt})/(^{35}S\ cpm_{ref})]$, where cpm is counts per minute. The translational efficiencies of group I mRNAs varied slightly, as shown in Table 1. Slight deletions in the leader had essentially no effect on downstream translation, whereas minor additional nucleotides at the 5′ end lowered translation by 20% or less. We conclude that wild-type RSV RNA has a length that is apparently optimal for translation of the *gag* gene.

Group II RNAs, which had shorter leader sequences and lacked one or more of the upstream ORFs, showed increased translational efficiencies. However, deletion of the upstream ORFs did not have an additive effect on translational efficiency. The efficiency of RSVΔ1-2 RNA approximated that of RSVΔ1 RNA, and the efficiency of RSVΔ1-3 RNA was

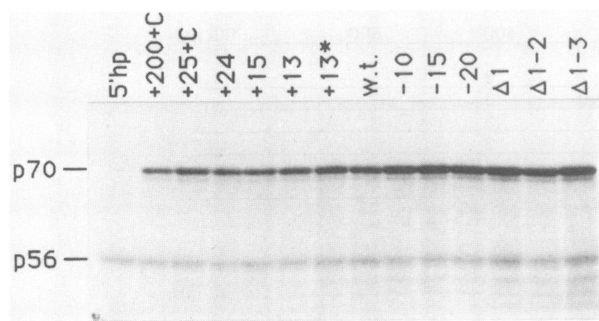

FIG. 2. Synthesis of p70 *gag* from test mRNAs and p56 *gag* from mRNA[ref]. Test mRNAs were synthesized from plasmids cleaved at the *Eco*RI site in the *gag* gene, while mRNA[ref] was synthesized from *Sma*I-cleaved pRSV-wt. A 1-ng sample of each RNA was added per μl of translational mix and incubated for 60 min at 30°C. Five microliters of each reaction was analyzed by electrophoresis (21, 34).

only slightly higher than that of RSVΔ1 RNA. Since RSVΔ1-3 mRNA lacked upstream ORFs, the lack of marked enhancement of translation of RSVΔ1-3 mRNA was surprising. Consequently, we measured the extent of ribosome binding at the third AUG codon in the RSVΔ1-2 mutant RNA by using the ribosome-binding-site-mapping procedure developed by Petersen et al. (35) for the study of initiation at AUG codons on the RSV leader RNA. The low level of ribosome binding at AUG3 was unchanged from that observed with the wild-type RNA (Fig. 3), despite its being the only upstream AUG codon and despite the similarity of the translationally important flanking sequences (17) around AUG2 and AUG3. AUG3 does not bind ribosomes efficiently, if at all, to virion RSV mRNA (34, 35) and RSV mRNA lacking AUG1 (35a).

Group III RNAs had major additions to the 5′ end. RSV-5′hp RNA contains a 81-nucleotide inverted repeat (Hensel, Ph.D. thesis) and thus potentially could form an intramolecular, double-stranded region possessing a ΔG of formation of −185 kcal/mol. This RNA is translated with only about 9% of the efficiency of RSV-wt RNA. Additionally, RSV-5′hp RNA contains seven ORFs upstream of the *gag* gene initiation codon. Thus, the observation that RSV-5′hp RNA translates even 9% as efficiently as RSV-wt RNA was surprising. The second group III RNA, RSV+200+C RNA, contains a direct repeat encompassing the first two ORFs found on RSV-wt RNA. RSV+200+C RNA thus possesses five upstream ORFs. This RNA translates with 71% of the efficiency of RSV-wt RNA. Either adding an extra copy each of ORFs 1 and 2 or increasing the length of the leader resulted in a decrease in p70 synthesis roughly equal to the increase in p70 synthesis seen with RSVΔ1-2, which lacked the first two AUG codons (Table 1).

TABLE 1. RNA translational efficiency

RNA group	Construct	Translational efficiency[a] ± SD
I	RSV+25+C	0.78 ± 0.05
	RSV+24	0.92 ± 0.09
	RSV+15	1.01 ± 0.08
	RSV+13	0.88 ± 0.05
	RSV+13*	0.79 ± 0.07
	RSV-wt	1.00
	RSV-10	0.98 ± 0.12
	RSV-15	1.04 ± 0.05
	RSV-20	0.96 ± 0.05
II	RSVΔ1	1.36 ± 0.13
	RSVΔ1-2	1.26 ± 0.03
	RSVΔ1-3	1.45 ± 0.18
III	RSV5′hp	0.09 ± 0.03
	RSV+200+C	0.71 ± 0.15

[a] Relative to that of the wild type (see text).

Translational reinitiation. A 40S ribosomal subunit may reach the initiation codon of p70 by two routes, both of which involve linear scanning. First, the subunit may simply bypass AUG codons whose flanking nucleotides confer a context that lowers the probability of initiation at the AUG codon (17); according to the Kozak rules, the first three AUG codons of RSV RNA have a moderate potential for initiation of translation, and the fourth AUG codon, where p70 synthesis was initiated, has a strong potential for initiation. The second means by which a ribosome or 40S subunit may initiate p70 synthesis is by first initiating at an upstream AUG codon, translating the short ORF, and then reinitiating downstream at the p70 start site (15).

Our combined observations are consistent with the following conclusions. First, alterations in the leader sequence of RSV RNA can modify the translational efficiency of RSV RNA in a noncompetitive environment. Second, assuming that the 9% synthesis of p70 from mRNA RSV-5′hp was due to either internal initiation (2, 10, 33) at the *gag* initiation codon or to a few ribosomes that managed to penetrate the major barrier of secondary structure in the leader RNA, perhaps by hopping (8) rather than by unwinding of the very strong stem by cap-binding protein (37), at least 90% of the synthesis of p70 appears to be due to scanning ribosomes. Third, either the length or structure of the RSV RNA leader is near optimal for translation of the major coding regions. Additions or deletions of 20 nt or less to the leader affected translation, perhaps at the level of ribosomal subunit binding, by no more than 20%. Fourth, all of the short ORFs (21 to 48 nt) lower the efficiency of downstream translation by about 20%, but these effects are not additive. The effects may be due either to addition of a translational site or to alterations in the length and/or structure of the leader RNA. Elimination of a single leader ORF has relatively little effect on *gag* synthesis. Fifth, assuming a relatively high utilization of ORFs 1 and 2 (34, 35, 35a), reinitiation at the *gag* AUG codon is very efficient, since omission of all three leader ORFs increased synthesis only 45% over wild-type RSV RNA. The translational efficiencies reported here were not affected by differential stabilities of the test mRNAs (J. Westerlund et al., manuscript in preparation). In general, our data suggest that the combination of leader length, number of short ORFs, and RNA secondary structure (except for the artificial 5′hp RNA) does not grossly affect initiation at the *gag* AUG initiation codon by more than 40% in vitro. From other studies in vivo, we conclude that translation of mRNAs such as RSV RNA probably is regulated by *trans*-acting factors that interact with sequences in the RNA leader (34, 35a; A. Moustakas et al., in preparation).

Why are the three short ORFs maintained in the leader RNA sequences of RSV and all other avian leukosis/sarcoma retroviruses? The chemical diversity of the products of ORF 1 (6) from various avian retroviruses and the apparent low activity of ORF 3 on RSV RNA (34, 35a) suggest that any direct function of their encoded peptides is unlikely. Presum-

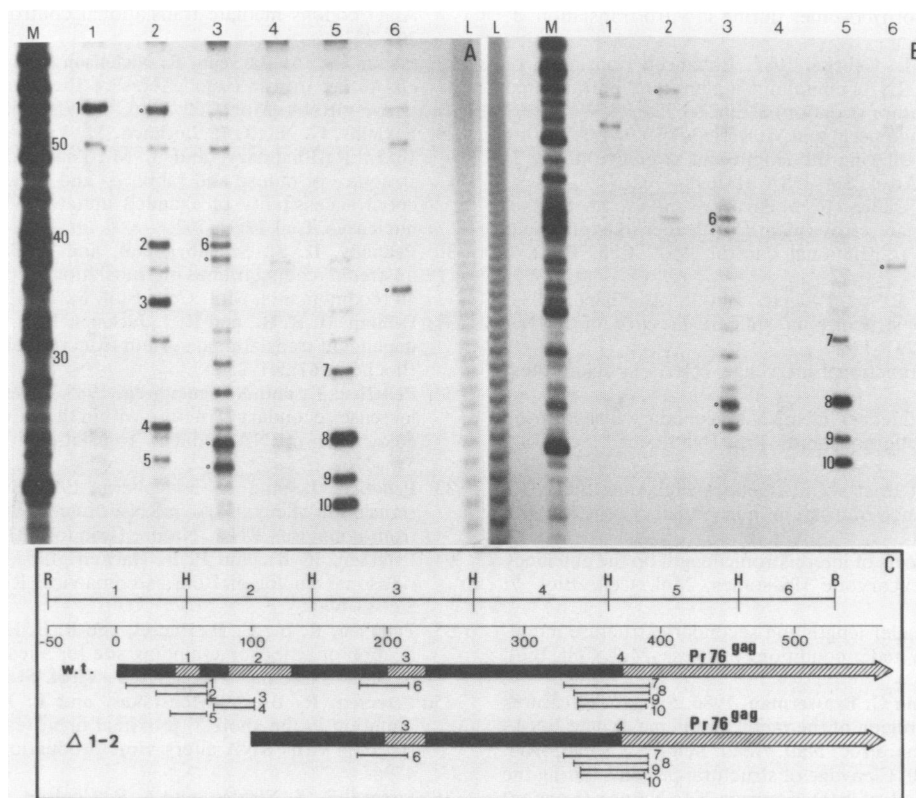

FIG. 3. Ribosome protection of RSV RNAs (34, 35), using sparsomycin in the translational systems containing either RSV-wt (A) or RSVΔ1-2 (B). Lanes 1 to 6 show the distribution on a 16.5% polyacrylamide gel of ribosome-protected fragments selected by the pSRA5′ subclones 1 to 6 of single-stranded DNA fragments shown in panel C. Identified fragments are numbered and located on the maps of the 5′ ends of the RSV RNAs in panel C. M, Marker fragments from ribosomal and transfer RNA; sizes (in nucleotides) are identified on the gel in panel A; L, poly(A) ladder markers; ○, non-RSV bands which served as markers for placement of other bands. (C) Map of the ribosome-protected fragments which were eluted and identified by partial RNase T1 digestion (34, 35a). ▨, ORFs in the RNA leader (■) and the *gag*-coding region. B, *Bam*HI; H, *Hae*III. The scale between the subclone map and the RNA maps indicates the distances in bases from the 5′ end of RSV-wt. All major bands (except band 6) selected by subclone 3 in lanes 3 of panels A and B are non-RSV RNA; presumably they are derived from G+C-rich rRNA, since subclone 3 is 65% G+C.

ably the ORFs are maintained because either the primary sequences or their translation is biologically important. Tests of these possibilities using constructs composed of modified RSV leader sequences with marker genes are in progress.

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