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Effects of the Open Reading Frames in the Rous Sarcoma Virus Leader RNA on Translation

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Three short open reading frames (ORFs) reside in the 5′ leader of Rous sarcoma virus (RSV) and are conserved in all avian sarcoma-leukosis retroviruses. Both extensions of the lengths of the ORFs and alterations in their initiation codons affect viral replication and gene expression. To determine whether the effects on viral replication were due to translational regulation mediated by the ORFs, we examined translation following mutation of the initiation and termination codons of each of the three ORFs. We found that the ORFs marginally enhanced downstream gene expression. Moreover, repression of downstream gene translation was proportional to the lengths of the elongated ORFs and depended on the initiation contexts of the AUG codons. Although the ORFs play a major role in viral activities, their effects on translation were relatively minor. Rather, the ORFs may affect the fate of unspliced avian retroviral RNA in chronically infected cells by participating in the sorting of viral RNA for either translation or encapsidation into virions.

All of the mRNAs from avian leukosis-sarcoma retroviruses have 5′ leader sequences that contain three short open reading frames (ORF1 to ORF3) that precede the retroviral *gag*, *gag-pol*, *env*, and oncogene coding regions (19, 45). The ORFs have lengths of 7, 16, and 9 codons, respectively, and their relative positions have been highly preserved within the conserved RNA secondary structure of the leader (9, 10, 17). However, their amino acid sequences are not conserved absolutely (5, 18, 22). In one model of translational control (39), the sequence context and distance from the 5′ cap suggest that the first AUG should be a favorable initiator of translation whereas the second and sometimes third AUG codons are expected to be relatively weak initiators.

This conservation prompted us to determine whether the three ORFs influence gene expression and/or viral activity. Earlier work in vitro (18, 39, 40) and in vivo (41) showed that ORF1 was a major ribosome-binding site which directed the synthesis of a seven-amino-acid peptide. This finding suggested that leaky scanning and/or an efficient reinitiation mechanism (24, 25, 37) operated to produce the major viral proteins. Site-directed mutations that altered ORFs at their initiation codons and/or in their lengths had profound effects on viral replication (31, 41). These results suggested that the ORFs exerted their effects through ribosome-mediated events. However, since the Rous sarcoma virus (RSV) leader RNA contains *cis*-acting domains responsible for viral replication, integration, and packaging as well as initiation of protein synthesis (1, 4, 6, 8–10, 20, 22, 34, 35, 47), the ORF mutations might have affected one or more biochemical mechanisms other than viral protein synthesis. We therefore initiated a study of the effects of the same ORF mutations only on translation.

There is precedent for regulation of gene expression by 5′ leader RNA ORFs. Multiple ORF-containing mRNAs include both viral RNAs that participate in highly regulated and complex life cycles and a number of cellular mRNAs that require highly regulated expression (3, 25, 36, 38). In general, ORFs in the leader sequences of mRNAs reduce translation (25). However, the AUG codon of the yeast transcriptional activator GCN4 is preceded by four short ORFs (21), two of which act as positive regulators of GCN4 protein synthesis (32), and an upstream ORF in another mRNA supports efficient translation of the downstream coding sequence (12).

In the accompanying report (31), we showed that mutagenesis of the AUG codons for ORF1 and ORF2 delayed the onset of viral replication while mutagenesis of ORF3 to enhance (FS3 and FS3TC3) or abolish (AUG3 and AUG123) its translation inhibited viral propagation. Elongation of ORF1 from 7 to 62 codons (TC1TC2) led to the accumulation of transformation-defective virus with a delayed onset of replication. In contrast, viruses with elongation of ORF1 to 30 codons (TC1), ORF2 from 16 to 48 codons (TC2), or ORF3 from nine to 64 codons (TC3), without any alterations in the AUG context, exhibited wild-type (WT) phenotypes. Since these effects on viral activities suggested a translational etiology, we either altered or eliminated the ORFs and examined the effects on translation of downstream genes. This was accomplished in two ways: (i) by using constructs in which the viral *gag*, *pol*, and *env* genes were replaced with the chloramphenicol acetyltransferase (*cat*) reporter gene, thus overcoming the complexity of the viral life cycle functions, and (ii) by examining the production of $Pr76^{gag}$ in cells transfected with mutated retroviral sequences. Our experiments indicate that downstream translation can be modulated by alteration of the lengths and sequences of the leader ORFs but that elimination does not appreciably raise or lower downstream translation.

MATERIALS AND METHODS

Construction of RSV-CAT recombinants. Except as noted, the SR-A(SF) strain of RSV DNA and the clones used were previously described (40). Plasmid DNAs were isolated from the *Escherichia coli* MV1190 (*recA*). We constructed (30) the pRSV-CAT series of 16 constructs containing WT and mutant RSV RNA leader sequences juxtaposed to the *cat*

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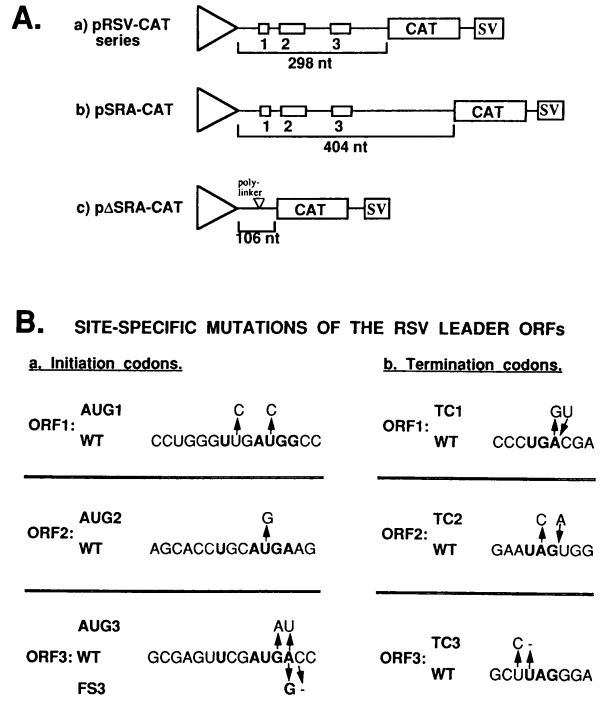

FIG. 1. CAT expression vectors. (A) Plasmid constructs are depicted linearized at the junction of the RSV LTR and the vector sequence. The RSV enhancer-promoter element is shown as a triangle; the leader ORF1 to ORF3 are shown as open small boxes; the *cat* coding sequences are shown as a large open rectangular labeled CAT; and the simian virus 40 intron and poly(A) sequences are labeled SV. The different construct segments are not in scale in order to emphasize the RSV leader. The length of the leader is shown under each map with brackets. The pRSV-CAT series (a) includes all of the test plasmids; pSRA-CAT (b) is identical to pWT-CAT of the pRSV-CAT series, with an extra 106 bp 3′ leader sequence between the leader's *Sst*I site and the *cat* gene; pΔSRA-CAT (c) replaces the leader sequence and ORF1 to ORF3 with the pUC polylinker. (B) Site-specific mutations of the RSV leader ORFs. The sequences of the initiation (a) and termination (b) codons and flanking nucleotides of the three RSV ORFs, sequences of the *gag* and *cat* initiation codons, and their respective site-specific mutations are listed. The AUG codons along with the −3 and +4 nucleotides relative to the A (+1) of the AUG and the termination codons (TC) are in bold letters. Arrows indicate nucleotide substitutions, insertions, and deletions (−).

reporter gene (14, 16). These plasmids all contain a transcriptionally active RSV(SR-A) long terminal repeat (LTR) and the 5′-proximal 260 bp of the 372-bp leader sequence. Each member of the pRSV-CAT series is named according to the mutation(s) in its leader (e.g., if WT, we call it pWT-CAT, and if its first AUG is altered, it is designated pAUG1-CAT; the ORF3 frameshift [31] is designated pFS3-CAT). Two additional CAT constructs were made (30). pSRA-CAT contains the full-length RSV (SR-A) leader of 372 bp, up to the AUG codon of the *gag* coding region; pΔSRA-CAT carries a deletion of most of the leader sequences in pSRA-CAT. Since the *cat* fragment from pRSV-CAT contains 39 bp of untranslated sequence upstream of the *cat* AUG codon (30), the total lengths of the chimeric RSV-CAT leaders were 73 nucleotides (nt) for the control pSRD construct, 298 nt for the pRSV-CAT series of 16 constructs, 404 nt for pSRA-CAT, and 106 nt for pΔSRA-CAT (Fig. 1A).

Site-specific mutagenesis of the RSV leader. The RSV leader-containing clones were mutagenized as previously described (41). Site-specific mutagenesis was performed by using *E. coli* CJ236 (13, 26). Single or multiple oligonucleotides (University of Minnesota Microchemical Facility) were used to obtain the leader mutants (Fig. 1B). Mutant clones were confirmed by DNA sequencing (U.S. Biochemical).

Cell culture and transient transfection for CAT assays. Quail QT6 cells were grown in Dulbecco modified Eagle medium (GIBCO-BRL) supplemented with 10% tryptose phosphate broth, 5% fetal bovine serum (HyClone Laboratories, Inc.), and 1% heat-inactivated chicken serum (GIBCO-BRL) at 37°C in a 5% CO_2 environment. Transfections were performed with DEAE-dextran-precipitated DNA (30). About 3×10^5 cells were plated per 100-mm-diameter plate 2 days before transfection; 16 μg of supercoiled plasmid DNA was routinely added to each plate, and three to four plates were transfected per construct. The DNA-DEAE-dextran precipitate stayed in culture in the presence of 3 ml of regular medium with 80 μM chloroquine (29) for 30 min. An equal volume of regular medium was added to dilute the coprecipitate for 3.5 h before it was aspirated and replaced by regular medium without any cell wash. The confluent plates were harvested 48 h posttransfection for assays.

Isolation and analysis of RNAs. Total cellular RNA was isolated from transfected quail cells 48 h posttransfection by single-step acid guanidinium thiocyanate-phenol-chloroform extraction (7) coupled with an LiCl wash (42). The integrity of the RNA was verified by visualization of ethidium bromide-stained RNAs in formaldehyde-agarose gels (2), and only samples that showed intact rRNA bands and an A_{260}/A_{280} greater than or equal to 1.85 were analyzed by nuclease S1 protection assays. For S1 analysis, we used a standard protocol (2) in which 50 or 100 μg of total RNA was mixed with 10^5 cpm of a 900-bp, uniformly ^{32}P-labeled, double-stranded DNA probe. Hybridizations were carried out in the presence of 80% formamide at 55°C for 16 h. Nuclease S1 reactions were done at 30°C with 2,000 U of enzyme (Bethesda Research Laboratories) for 90 min. Following termination of the reactions (2), samples were digested with proteinase K and ethanol precipitated. The protected fragments were resolved on a 1-mm-thick 6% polyacrylamide–8 M urea gel. Autoradiography of the dried gels was then performed at ambient temperature without intensifying screens for 12 to 36 h (X-Omat AR5 film; Eastman Kodak Co.).

The DNA probe was synthesized from a single-stranded DNA pUC119 phagemid template that carried a fragment of the RSV LTR-leader and one-sixth of *cat* coding sequences up to an internal *Pvu*II site (30). The RSV leader in the probe fragment represented WT sequences. Yeast tRNA and mock-transfected cellular RNA were used as negative controls, and an RSV-CAT RNA synthesized in vitro by T7 RNA polymerase from an expression plasmid was used as a positive control for the nuclease S1 assay. To standardize the amount of RNA used for each nuclease S1 experiment, a 425-bp probe from the chicken β-actin gene (23) was mixed with the RSV-CAT probe or with identical duplicate RNA samples by itself. This probe is complementary to the entire second exon of chicken β-actin gene and contains extra 3′ sequences from the third intron of the gene. A Bio-Rad model 620 video densitometer was used for quantitative analysis of bands on the autoradiograms.

CAT assays. Cell extracts were prepared by freeze-thawing cells 48 h posttransfection (27, 28). The protein content of the extracts was quantified by a Bradford assay (Bio-Rad

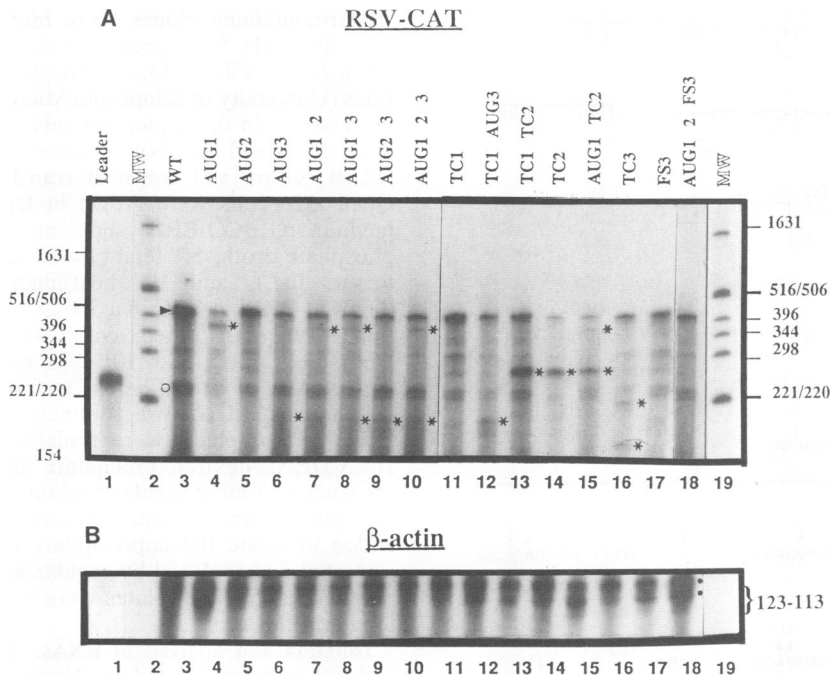

FIG. 2. RNA analysis by nuclease S1 protection assay. (A) Autoradiograms of 6% polyacrylamide–urea gels for resolution of nuclease S1-protected DNA fragments. Total RNA (50 μg) from QT6 cells transfected with the RSV-CAT constructs (lanes 3 to 18) was hybridized to the RSV probe. After digestion with nuclease S1, the predicted 412-nt fragment was detected (arrowhead at the left of lane 3 [WT]) along with smaller, barely visible, mutant-specific subfragments (labeled with asterisks to the right of appropriate lanes). The artifactual double band of 260 nt also detected in the mock transfection control is labeled by open circle at the left of lane 3. β-Actin mRNA hybridization (B) was the same as in RSV-CAT except that the RNAs were hybridized to the 425-nt β-actin probe. The doublet of bands is labeled with filled circles at the right of lane 18 (AUG12FS3), and the band sizes are shown in nucleotides at the right. Lane 1 (Leader) shows the positive nuclease S1 protection control of RSV-CAT RNA synthesized in vitro. Lanes 2 and 19 are ^{32}P-labeled molecular weight (MW) markers of pBR322 digested with *Hin*fI.

Laboratories). CAT enzyme reactions were run for 60 min at 37°C as described previously (15, 27, 28). To ensure that the assay conditions corresponded to the linear part of the response curve (28), pilot assays of serially diluted extracts were performed and compared with parallel assays with serial dilutions of commercial, purified enzyme (Sigma). The acetylated products of [^{14}C]chloramphenicol (Amersham) were resolved by thin-layer chromatography (TLC; TLC plates from J. T. Baker, Inc.), visualized by autoradiography at room temperature in the presence of an intensifying screen, and quantified by scintillation counting of the radioactive spots on the plates. The CAT activities were measured from each assay and expressed as picomoles of acetylated product per minute of reaction per milligram of protein extract. Routinely, a 2- to 5-μg (1/500 to 1/200 of a single plate's yield) extract from quail cells was used per assay and resulted in 50 to 65% acetylation for the same construct.

Immunoprecipitations of Pr76^{gag}. QT6 cells were seeded into 60-mm-diameter plates and were transfected with 20 μg of nonpermuted viral plasmid per plate by calcium phosphate-mediated transfection (reference 2, with modifications provided by the J. Wills laboratory). Transfected QT6 cells were maintained for 66 h in M199 (GIBCO-BRL) containing 10% tryptose phosphate broth, 1% chicken serum, and 5% fetal bovine serum. Monolayers were harvested and assayed at this time as described previously (31). The viral constructs were essentially as described in the accompanying report (31) except that the viral genes were flanked by two complete LTRs containing the same mutation (44).

RESULTS

In this study, we conducted a systematic, site-specific mutagenesis of each of the three RSV leader ORFs while preserving as much as possible the complex RNA structure of the leader. A series of 16 constructs with single or multiple mutations in the initiation and termination codons of the ORFs was introduced into mRNAs containing the RSV leader RNA juxtaposed to the *cat* reporter gene (Fig. 1). Effects of the mutations in the ORFs were quantified by using assays of *cat* gene expression following transfection of recipient cells with the DNA constructs. However, to ensure that the CAT enzymatic activities reflected translational efficiencies rather than differential transcription from the constructs, the levels of the RSV-CAT mRNAs were determined by a nuclease S1 protection assay.

Mutations in ORF1 to ORF3 do not affect RSV-CAT mRNA levels. Autoradiograms from a nuclease S1 protection assay of RNA from transfected QT6 cells are shown in Fig. 2A. The RSV probe hybridized to all of the 16 members of the pRSV-CAT series (lanes 3 to 18), forming a 412-bp hybrid. Since the probe carried the WT RSV leader sequence, nucleotide mismatches between the probe and those RNAs carrying ORF mutations were expected. Under the conditions of our experiment, we observed nuclease S1 cleavage at these internal mutation sites (faint bands designated by asterisks) only when two or more mismatched nucleotides occurred between the mRNA and the DNA probe (30). Hybridization of the probe to the in vitro-synthesized RNA spanning the RSV leader up to the *Sst*I site resulted in full

protection of the 260-nt leader RNA (lane 1), which was used as a positive control for the nuclease S1 assay.

A 425-bp chicken β-actin probe (30), which was expected to form a 123-bp nuclease S1-protected hybrid corresponding to the second exon of the gene (22), was used as an internal control to monitor the cellular RNA level loaded in each hybridization reaction. Nuclease S1 produced two major bands presumably due to the sequence divergence between chicken and quail β-actin genes (Fig. 2B). Although in some experiments there were slight variations in relative amounts of viral RNA synthesized from the mutant constructs, over the course of our experiments, the levels of mRNAs from the different constructs were similar, as judged by quantification by densitometry of the RSV-CAT and β-actin bands (30). We never observed any aberrant CAT transcripts resulting from multiple transcriptional initiation events or abnormal splicing. Accordingly, we concluded that the ORF mutations did not affect appreciably the RSV-CAT mRNA turnover in these experiments, and therefore the variations in CAT enzyme production should be due to altered translational efficiencies of the RSV-CAT mRNAs. These results on RNA stability are in agreement with a similar study conducted with another strain of RSV (10a).

Upstream ORFs marginally enhance downstream gene expression. To measure the translational efficiencies of the mRNAs, we assayed CAT activities in lysates from transfected cells. These experiments were repeated an average of five times each. A representative autoradiogram of a TLC plate with CAT assays from transfected QT6 cells with several RSV-CAT constructs is shown in Fig. 3. The values of CAT activity from the constructs were normalized relative to that of pWT-CAT and are reported in Fig. 4 and 5. We mutated each AUG codon in the RSV leader, and the effects of single, double, and triple mutations on CAT expression were tested (Fig. 4). Losses of either ORF1 (AUG1) or ORF2 (AUG12 and AUG2) had marginal effects on CAT expression, but loss of ORF3 (AUG3) reduced synthesis of CAT by 50%. The results of the AUG1 and AUG2 mutations are in accord with effects seen on viral replication rates (31). However, the AUG3 mutants, which were unable to propagate, and the AUG12 mutant, whose replication was markedly repressed, had only slightly reduced translational competencies.

Elongated ORFs reduced downstream translation. Our previous findings indicated that extensions of the leader ORFs could delay and/or reduce virus production (31). Accordingly, we examined the effects of the lengths of the upstream ORFs on downstream gene translation (Fig. 5). Additionally, the frameshift FS3 mutation, which elongated ORF3 to 29 codons and put its AUG codon in a more favorable translational context, was tested either alone (AUG12FS3) or following ORF1 and ORF2 (FS3).

Lengthening either ORF1 from 7 to 30 codons (TC1) or ORF2 (TC2) from 16 to 48 codons reduced CAT activity by about 50%. In contrast, lengthening the translationally silent ORF3 from 9 to 29 codons (TC3) had little effect on CAT expression. However, when the AUG codon of ORF3 was altered to a more favorable context for translational initiation (FS3 mutants), translation of the downstream CAT gene was reduced by 70%. Fusion of all three ORFs into a single ORF of 62 codons reduced CAT expression 70%. The relative CAT activity from the FS3 and AUG12FS3 mutants indicated that by increasing the translational potential of ORF3, the extension of ORF3 reduced downstream translation (compare TC3 with the FS mutants). Thus, the observed decreases of downstream translation upon elongation of

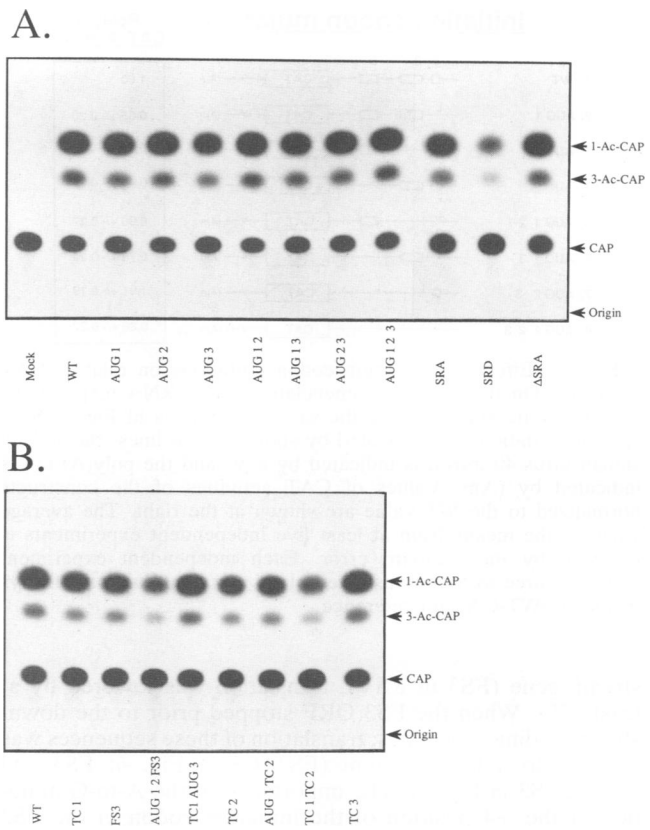

FIG. 3. CAT assays. Shown are autoradiograms of TLC plates used for CAT assays of transfected cell extracts. Five micrograms of cell protein extract was allowed to react in the presence of 0.1 μCi (1.75 nmol) of [^{14}C]chloramphenicol and 80 nmol acetyl coenzyme A at 37°C for 1 h and used for chromatography after organic-phase extraction. Each sample is labeled according to the transfected construct. The positions of chloramphenicol (CAP) and the CAT products 3-acetylchloramphenicol (3-Ac-CAP) and 1-acetylchloramphenicol (1-Ac-CAP) are shown at the right. (A) CAT activities from the AUG mutants and different RSV LTRs; (B) CAT activities from the TC mutants.

leader ORFs correlate with their lengths and the strengths of their initiation codons; the stronger the initiation codon context, the more pronounced the translational effect.

Alteration of AUG3 inhibits viral protein synthesis. Except for the AUG12 and AUG3 constructs, the results for the CAT assays parallel the results seen in the long-term transfection-infection studies (31). Since the CAT constructs had truncated leader sequences between ORF3 and the downstream initiation codon for CAT, we examined the intrinsic translational efficiencies of several of the RSV mutant RNAs that were examined in the accompanying report (31). Figure 6 shows the transient expression of Pr76^{gag} in transfected QT6 cells. Whereas Pr76^{gag} synthesis was greatly reduced 2 weeks after infection with the AUG3 mutant viruses (Fig. 8 of reference 31), 66 h after transfection, the AUG3 mutant permitted synthesis of Pr76^{gag}, in support of the CAT results (Fig. 4). However, the FS3 mutation, which inactivated the virus and inhibited Pr76^{gag} synthesis both transiently (Fig. 6) and over the long term (31), reduced CAT activity by only about 70%. This discrepancy is most easily explained by the difference in the lengths of the FS3 ORFs in the viral and CAT constructs. When the FS3 ORF overlapped the down-

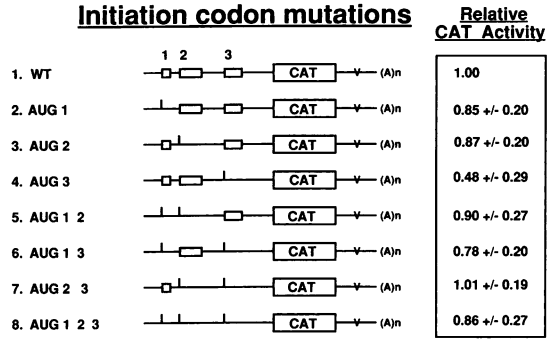

FIG. 4. Effects of initiation codon mutations on relative CAT activities. On the left, the nomenclature and mRNA maps of the constructs are shown, using the same symbols as in Fig. 1. Site-specific mutations are indicated by short vertical lines, the spliced simian virus 40 intron is indicated by a V, and the poly(A) tail is indicated by (A)n. Values of CAT activities of the constructs normalized to the WT value are shown at the right. The average value of the means from at least five independent experiments is followed by the standard error. Each independent experiment included three to four transfected plates per construct and always included pWT-CAT for reference.

stream gene (FS3 in RSV), translation was lowered by at least 95%. When the FS3 ORF stopped prior to the downstream coding sequences, translation of these sequences was reduced to a lesser extent (FS3TC3 in Fig. 6; FS3 and AUG12FS3 in Fig. 5). The importance of the A-to-G mutation at the +4 position of the initiation codon in the FS3 mutation is emphasized by the finding that $Pr76^{gag}$ is synthesized in the FS3R mutant but not in the FS3 mutant (Fig. 6); the FS3R and FS3 mutants have equivalent elongated ORFs, but the normal AUG codon context is maintained in the FS3R mutant rather than in the enhanced context as in the FS3 mutant. The FS3 mutation appears to influence downstream translation by variation in the AUG context and/or alteration in RNA secondary structure rather than as a consequence of changes in the amino acid sequence of the peptide encoded by ORF3 (31).

The RSV leader lowers downstream translation. As noted above, 106 nt of RSV leader sequence which directly precede the *gag* AUG codon were deleted in the RSV-CAT constructs. pSRA-CAT, which contains the full-size RSV leader with WT ORF sequences, was constructed (30) to

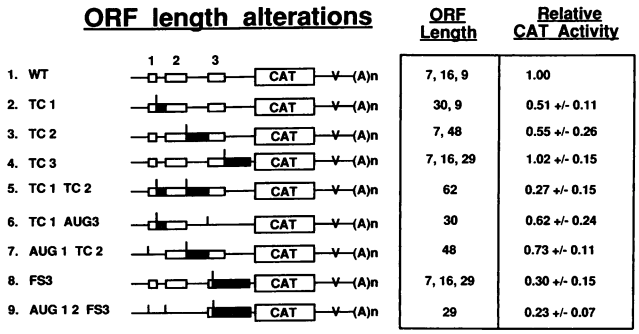

FIG. 5. Influence of ORF length alterations on relative CAT activity. Details are as for Fig. 4 except that the translated sequences between WT ORFs in the elongated ORF mutants are depicted by solid boxes and the table on the right includes the length (in codons) of each ORF in each construct.

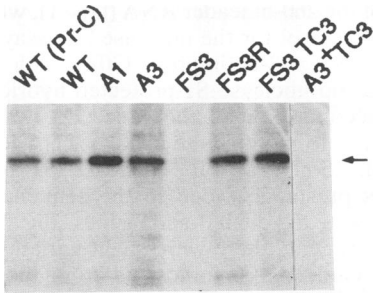

FIG. 6. Analysis of viral protein in cells infected with mutant viruses. Cell lysates were obtained from [^{35}S]Met-labeled cells transfected 66 h earlier with the indicated viral constructs. Proteins were immunoprecipitated with an anti-RSV antiserum as described previously (31) and separated on 10% polyacrylamide gels. The viruses are labeled above the lanes; PrC is the Prague C (clone ATV8R) strain of RSV. A molecular weight standard was run in each gel (not shown). The position of $Pr76^{gag}$ is noted by the arrow at the right.

determine whether this extra, ORF-less sequence influenced downstream translation. Inclusion of the last 106 nt of leader in front of the CAT gene did not affect enzyme expression (Fig. 7).

The results shown in Fig. 4 suggested that elimination of leader ORFs by AUG codon mutagenesis had little effect except in the case of AUG3. These results were unexpected since leader ORFs generally reduce downstream translation. Consequently, we examined the effect of deleting most of the leader sequences. pΔSRA-CAT, which carries only a short 5′-proximal stretch of the RSV leader and lacks ORF1 to ORF3, resulted in a mild increase of CAT expression (Fig. 7). Compared with the ORF-less mutant AUG123, which had a 15% decrease in translational activity (Fig. 4), the leaderless mutant showed an approximate 30% increase in CAT expression. Thus, addition of the RSV leader with mutations in all three of its AUG codons upstream from the *cat* ORF resulted in a relative decrease in CAT protein synthesis, presumably as a result of the complex structure of the leader RNA (17). Reincorporation of all three short ORFs in the leader (WT) resulted in an increase in CAT synthesis which ameliorated much of the difference. We conclude that three components of the RSV leader RNA, secondary (and tertiary) structure, length, and ORFs, exert positive and negative effects on CAT translation that balance each other.

The CAT construct (15) which served as the donor of the CAT and simian virus 40 sequences for our recombinant

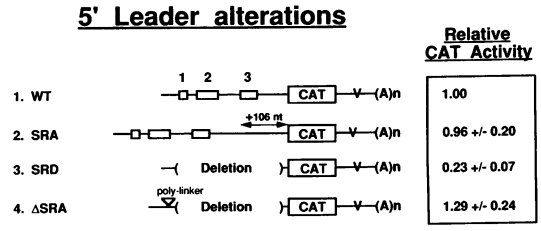

FIG. 7. Effects of leader length alterations on CAT activity. Details are as for Fig. 4. WT is the unmutated leader fragment from the RSV-CAT series (see Fig. 1). The position of the extra 106 nt of the leader in the SRA clone is indicated by the double-arrowed line, and the deletions are drawn as gaps between parentheses. In mutant ΔSRA, the polylinker insertion from the pUC vector is shown.

constructs was derived from the SR-D strain of RSV in which most of the RSV leader sequences, including the upstream ORFs, were deleted. When we compared this construct (Fig. 7, SRD) with our series of RSV-CAT constructs, we found a four- to fivefold decrease in CAT activity relative to the WT (SRA) and a sixfold decrease relative to ΔSRA. This surprising difference was due to three nucleotide changes, at positions (relative to the transcriptional initiation site) −209, −82, and −33 (an insertion), which are in defined, *cis*-acting, transcriptional regulatory sites in the RSV LTR, and is strictly transcriptional, as indicated by RNA assays (30).

DISCUSSION

We have exploited the simplicity and high efficiency of expression of the CAT assay system to study the effects of mutations that were known to reduce retroviral replication (31) on the intrinsic translational efficiency of the mRNAs. Since the mutations that we made in the RSV leader ORFs did not appreciably affect transcription and RNA processing, we conclude that the observed effects on *cat* gene expression were due to modulation of the rate of translational initiation by ribosomes moving along the RSV leader RNA sequence. In essence, ORF1 to ORF3 in the RSV leader RNA have a moderately positive effect on *cat* gene expression. This conclusion was unexpected given the common hypothesis that upstream ORFs generally attenuate downstream translation (25). Our data for the AUG1 mutant showed an average decrease of 30% in CAT activity. In previous work in which the effect of the same mutant in an RSV proviral construct was tested (41), we demonstrated that this mutation slowed viral propagation. The AUG2 mutation had an equivalent effect.

Surprisingly, mutation of the AUG of ORF3 showed the most pronounced negative effect on *cat* gene expression (Fig. 4). The negative effect of AUG3 did not persist in the double mutants (AUG13 and AUG23) or in the triple, ORF-less, mutant (Fig. 4). Ribosome binding experiments (40) indicated that 70 to 80% of the 40S subunits bind at the first two AUG codons to initiate translation but not at the third. Supporting the hypothesis that ORF3 is not normally translated at a high rate are the results from the ORF elongation studies; in these studies, ORF3 elongation in the TC3 mutant did not affect either translation (Fig. 4) or viral propagation (31) whereas the FS3 mutants did affect protein synthesis, as discussed below. Consequently, the effect of the AUG3 mutation probably reflects a subtle alteration in some parameter affecting translation, such as RNA secondary structure (17, 31). The same alteration in the nucleotide sequence of the leader RNA apparently affects another viral process, e.g., packaging, that results in defective viral propagation. Similar results resulting from two mutations at positions 375 and 376 of the leader RNA of ev-1 virus have been observed (17, 22). The small reduction in translation for the AUG3 mutant is not sufficient to explain the absence of viral propagation in AUG3 viral mutants (31). The synthesis of $Pr76^{gag}$ in cells transiently transfected with the AUG3 virus mutant (Fig. 6) support our finding that the AUG3 mutation does not grossly affect downstream translation. Our results differ from those reported by Donzé and Spahr (10a), who found, using similar but not identical mutants in the PrC strain of RSV, that the AUG1 genotype (pAM1) reduced translation 80% and that mutants lacking ORF3 (pAM3 and pAMuP) had twofold increases in translation of downstream coding sequences. Although we initially expected to find, and therefore sought, such dramatic changes in translation, we have never reproducibly observed such effects in mutant leader RNAs. In sum, our results emphasize the complexity of translational control in eukaryotic mRNAs containing ORFs in extended leader sequences. In these cases, translation cannot always be reconciled with simple models (reviewed in references 25, 43, and 46).

The ORF elongation mutants (Fig. 5) were designed to test the importance of the ORF length. Elongation of ORF1 (TC1 and TC1TC2) showed the greatest effects (50 to 70% reduction) and were independent of ORF3 (TC1AUG3, 40% reduction); this finding supports the hypothesis (31) that elongated ORFs decrease the number of leaky scanning ribosomal subunits because ribosomes which translate longer ORFs reinitiate at the next downstream AUG codon less efficiently. ORF2 elongation (TC2 and AUG1TC2) showed a negative effect similar to that of ORF1 elongation. In contrast to the major effect of the AUG3 mutation, the elongation of ORF3 (TC3) had no effect on *cat* expression, probably because of the ORF's weak ability to initiate translation. These results from the TC mutants do not correspond with results obtained in another study in which elongated ORFs in the RSV leader sequence (mutants pA-Double1, $pAM1^{34}$, and $pAM2^{130}$) did not have any significant effects on downstream translation (10a). Since the constructs used in that study were subtly different from ours for both AUG and TC mutants, we cannot definitively identify the reasons for the difference in the results obtained in that study and those that we have repeatedly observed.

Constructs containing a frameshift mutation in ORF3 (FS3) resulted in a pronounced negative effect on CAT activity (Fig. 4). This effect was enhanced when the two upstream ORFs were absent (AUG12FS3). These results, in contrast to those for the TC3 mutant, were probably due to the improvement of the AUG codon of ORF3 for translational initiation (Fig. 1) as a result of change in codon context and/or surrounding RNA secondary structure. This interpretation is further supported by the devastating effects of the FS3 mutation in vivo (31, 43) and the results with the FS3 and $AUG3^{+}$ mutants used to study effects of ORFs on viral replication. The possibility that the amino acid sequence of the encoded peptide is involved in the effect is unlikely because (i) the ORF3 amino acid sequence is not conserved among the different avian retroviruses (5, 18) and (ii) the FS3R mutation, which encodes a different peptide but does not appreciably affect synthesis of $Pr76^{gag}$ (Fig. 6), does not support a role of the ORF3 peptide in virus propagation (31). Thus, our results do not support the proposed role for the ORF3 peptide in viral replication (10a).

In summary, the mutations described here indicate that the three short ORFs of the RSV leader RNA play only a minor role in regulating downstream gene translation. However, those mutations that have pronounced effects on *cat* gene translation also have the greatest effects on viral propagation (31). But not all of the mutations that altered ORF1 to ORF3 and caused distinct retroviral phenotypes appreciably affected CAT and transient $Pr76^{gag}$ synthesis, possibly because ORF1 to ORF3 affect processes other than translation (e.g., RNA packaging), as has been observed with ORFs in the leader RNA of hepatitis viruses (11, 33), and/or because the ORFs have small effects on viral protein synthesis that lead to enhanced effects on viral replication (10a, 31). We are currently investigating whether the profound effects on viral propagation by the ORF mutations are due to a reduction in efficiency of encapsidation of viral RNA during virion formation.

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