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Nonsense Codons within the Rous Sarcoma Virus *gag* Gene Decrease the Stability of Unspliced Viral RNA

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The intracellular accumulation of the unspliced RNA of Rous sarcoma virus was decreased when translation was prematurely terminated by the introduction of nonsense codons within its 5′ proximal gene, the *gag* gene. In contrast, the levels of spliced viral RNAs were not affected in our transient expression assays in chicken cells. Experiments using the transcription inhibitor dactinomycin showed that mutant unspliced RNAs were degraded more rapidly than wild-type RNA. Furthermore, mutant RNAs could be partially stabilized by coexpression of wild-type *gag* proteins in *trans*; however, intact *gag* proteins were not required to maintain the stability of RNAs which did not contain premature termination codons. Thus, termination codons seemed to destabilize the RNA not because of their effect on *gag* protein function but instead because they disrupted the process of translating the *gag* region of the RNA. Analysis of double-mutant constructs containing both deletions and termination codons within the *gag* gene also suggested that the stability of the unspliced RNA was affected by a *cis*-acting interaction between the RNA and ribosomes.

While the primary function of translation is to convert the coding information of mRNAs into protein, recent evidence suggests that translation may also affect the life span of some eukaryotic mRNAs. The accumulation of several mRNAs is altered if the coding region of these messages is not fully translated (3, 4, 7, 13, 14, 21, 27, 29, 33, 35). In some of these cases translation appears to be required for cytoplasmic degradation of the mRNAs (13, 14, 27, 29, 35), while in others premature termination of translation is associated instead with decreased RNA accumulation (3, 4, 7, 21, 33). In the case of the dihydrofolate reductase (*dhfr*) mRNA, it has been suggested that translation may be coupled to RNA splicing and nuclear transport (33).

We have found that accumulation of the unspliced RNA of the avian retrovirus Rous sarcoma virus (RSV) was also influenced by translation. The primary transcript of RSV contains four genes, 5′-*gag-pol-env-src*-3′ (Fig. 1). It resembles a cellular mRNA in being capped, polyadenylated, and internally methylated, but its fate is more complicated than that of a typical mRNA (for reviews, see references 9, 31, and 34). In the nucleus, a fraction of this transcript is alternatively spliced to form *env* and *src* mRNAs, while the rest is transported in an unspliced form to the cytoplasm. Here, the unspliced RNA is both packaged into virions as genomic RNA and used as mRNA for translation of the *gag* and *gag-pol* polyproteins. Translation terminates at nucleotide (nt) 2485 in most of the unspliced RSV mRNAs, producing a 76-kDa *gag* precursor polyprotein. This polyprotein is cleaved in the virion to produce the internal capsid structural proteins and the viral protease. About 5% of the ribosomes which translate *gag* read through its normal amber termination codon via a translational frameshift (16), producing a 180-kDa *gag-pol* protein, which is cleaved in the virion to yield the reverse transcriptase and integrase proteins.

Arrigo and Beemon (2) observed that frameshift mutations in the *gag* gene of RSV are associated with a decreased steady-state level of viral unspliced RNA, while levels of spliced RSV mRNAs are not affected. We have confirmed and extended this work using nonpermuted, infectious viral clones analyzed by transient expression in chicken embryo fibroblasts (CEFs). We found that unspliced RSV RNAs which contain premature nonsense codons in the *gag* gene accumulated to lower levels in the cell because they were degraded more rapidly than wild-type RNA. Mutant RNAs could be partially stabilized by coexpression of intact *gag* proteins in *trans* but were not stabilized by coexpression of *gag* proteins bearing a deletion of the nucleocapsid (NC) domain. Nevertheless, unspliced viral RNA containing the NC deletion but lacking a premature termination codon accumulated to wild-type levels within cells, suggesting that *gag* proteins were not required to stabilize RNAs lacking premature termination codons. Thus, it appeared that nonsense codons destabilized the RNA not because of their effect on *gag* protein function but because they disrupted the process of translating the *gag* gene. The phenotypes of mutant constructs containing in-frame deletions in combination with nonsense codons in *gag* suggested that RSV RNA contains *cis*-acting components which interact with the translational apparatus to influence the stability of the RNA.

MATERIALS AND METHODS

Construction of recombinant clones. pAPrC, a full-length, nonpermuted plasmid clone of the Prague C strain of RSV, was kindly provided by Meric and Spahr (26). The *Aat*II site in the pBR322 portion of this plasmid was removed by partial digestion with *Aat*II, digestion of the overhanging ends with T4 DNA polymerase, and religation. The resulting plasmid, pAPrCΔpBR, contains a unique *Aat*II site at nt 1258 in the *gag* gene of RSV. (All RSV sequence coordinates are from reference 34.) To mutate the *gag* gene, we first subcloned an *Aat*II-*Eco*RV fragment (nt 1258 to 3658) from pAPrCΔpBR into the same restriction sites of pBR322, creating the pAaRV subclone. Mutations in *gag* were first introduced into this subclone; the mutant *Aat*II-EcoRV fragments were then reinserted into the *Aat*II-EcoRV sites of pAPrCΔpBR to regenerate full-length viral constructs.

Termination codons were introduced into the *gag* gene of

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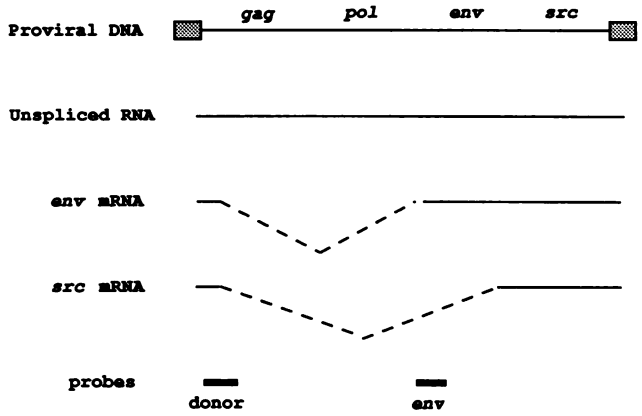

FIG. 1. RNAs produced by RSV. The unspliced transcript is 9312 nt in length. The *env* and *src* mRNAs utilize the same splice donor site (at nt 397) but different splice acceptor sites (*env*, nt 5078; *src*, nt 7054) (34). The locations of the two probes used for RNase protection assays are shown. ▨, LTRs.

the pAaRV subclone by digestion at unique restriction sites, blunting of the ends with either T4 DNA polymerase or Klenow enzyme, and religation with a 14-mer *Xba*I linker (New England BioLabs) containing amber stop codons in all three reading frames. Restriction sites used to generate these mutants were as follows: BT, *Bgl*II (nt 1631); StT, *Sst*II (nt 1806); SmT, *Sma*I (nt 1924); and RT, *Eco*RI (nt 2319). Control mutations (in-frame insertions of 10 to 18 bases) at these sites were created by using the same strategy but different linkers, as follows: *Bgl*II site and *Sst*II sites; 14-mer *Pst*I linker (U.S. Biochemical); and *Sma*I site, 12-mer *Cla*I linker (New England BioLabs).

In-frame deletions in *gag* were created as follows: ΔIFS (Δ1924–2319), digestion of pAaRV with *Sma*I and *Eco*RI, blunting of the *Eco*RI end with mung bean nuclease, and religation; ΔIFT (Δ1806–2319), digestion with *Sst*II and *Eco*RI, blunting of both ends with mung bean nuclease, and religation; and ΔIFB (Δ1631–2319), digestion with *Bgl*II and *Eco*RI, blunting of the ends with Klenow enzyme, and ligation with a 10-mer *Cla*I linker (New England BioLabs) to restore the reading frame. Constructs ΔIFS and ΔIFT were sequenced by the chemical method of Maxam and Gilbert (22).

Hybrid constructs SmTΔIFS, StTΔIFT, and BTΔIFB were constructed by ligation of the nonsense codon-containing *Xba*I linker described above onto pAaRV DNA, which had been digested to generate deletions as described above. Hybrid constructs StTΔIFS, BTΔIFS, and BTΔIFT were created by insertion of the nonsense codon-containing linker into the appropriate restriction sites of the previously made constructs ΔIFS and ΔIFT.

The remaining mutants were constructed directly in the context of the full-length viral plasmid. Ligation of a nonsense codon-containing *Spe*I linker (New England BioLabs) into the *Hpa*I site (nt 2734) created construct Hpa. Mutant AB was constructed by digestion of pAPrCΔpBR with *Aat*II, followed by generation of blunt ends with T4 DNA polymerase and religation. This created a 4-bp deletion which shifted translation into an alternate reading frame, in which two consecutive nonsense codons were encountered 8 bp after the deletion. A control mutation at this site was constructed by ligation of a 10-mer *Cla*I linker into the *Aat*II-cut, blunt-ended DNA. Constructs ABΔIFS and ABΔIFT were constructed identically to construct AB except that DNA from the deleted construct ΔIFS or ΔIFT was used as the starting material. Mutant fs532 was created by partial digestion of pAPrC DNA with *Bam*HI, generation of blunt ends with Klenow enzyme, and religation; this 4-bp insertion shifted translation into an alternate reading frame which contains a termination codon at nt 589. Mutant SmLB resulted from partial digestion of pAPrC with *Sma*I and ligation of a nonsense codon-containing 14-mer *Spe*I linker (New England BioLabs) into the *Sma*I site at nt 1924. This mutant is identical to mutant SmT except that it contains a different nonsense codon-containing linker. Mutant SmLA was constructed in the same way as SmLB; in this case a clone containing the linker inserted at the *Sma*I site at nt 524 was isolated. Mutant B+1, containing a 4-bp insertion at nt 1631, causes a frameshift into a reading frame containing a termination codon at nt 1668. This mutation was moved into pAPrC via an *Sst*II fragment (nt 543 to 1806) obtained from a pATV8K mutant B+1 construct previously described (2). Mutant fsΔIFS was created by using a two-step ligation: first, the *Aat*II (nt 1255)-to-*Eco*RV (nt 3658) fragment of ΔIFS was ligated to the *Eco*RV-*Aat*II fragment of wild-type pAPrC; then this 8.4-kb fragment was ligated to the 4.1-kb *Aat*II-to-*Aat*II fragment of fs532 to regenerate the full-length viral construct.

The *gag* expression vectors pHPgag and pHPgagΔIFT were constructed by digestion of either wild-type pAPrC or ΔIFT DNA with *Hpa*I and *Pvu*II, followed by ligation; this deleted nt 2734 to 8674. The *gag* expression vector pGag3.2 was constructed by deletion of sequences between the *Hpa*I (nt 2734) and *Bsu*36I (nt 8886) sites in pAPrCΔpBR. This was done by partial digestion with *Bsu*36I, generation of blunt ends with Klenow enzyme, complete digestion with *Hpa*I, and religation. Insertion of a nonsense codon-containing *Xba*I linker into the *Bgl*II site (nt 1631) of pGag3.2 created construct pGag3.2BT. Expression of RNA and protein from these expression plasmids was verified by RNase protection assays of transfected plasmids and by immunoprecipitation of [^{35}S]methionine-labeled proteins with anti-*gag* antiserum.

Cell culture and transfection. Secondary cultures of CEFs were grown in medium 199 (GIBCO) supplemented with 2% tryptose phosphate broth, 1% calf serum, 1% chicken serum, and penicillin-streptomycin (GIBCO). DEAE-dextran-mediated transfection was carried out as described previously (20), using a DEAE-dextran concentration of 200 μg/ml. DNA was incubated with the cells for 5 to 12 h in serum-free medium; subsequently, they were shocked with 10 to 20% dimethyl sulfoxide in *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-buffered saline for times ranging from 2 to 30 min, depending on the cell density. A DNA concentration of 8 μg/ml was used; for cotransfection experiments, 2 μg of full-length viral plasmid DNA per ml was mixed with 6 to 10 μg of pGag helper plasmid DNA per ml. RNA was harvested from the cells 36 to 48 h after the shock.

Calcium phosphate transfection was carried out as described previously (19), using CEFs grown in Dulbecco modified Eagle medium (Flow Laboratories) supplemented with 2% calf serum. A 5.6-fold molar excess of helper plasmid to target plasmid DNA was used, and a total of 20 to 25 μg of DNA was applied to each 6-cm plate. Samples transfected without a helper plasmid were supplemented with pBR322 DNA up to a total of 25 μg. Cells were shocked and harvested in the same way as in DEAE-dextran transfections.

Actinomycin D (dactinomycin; Calbiochem) was used on cells at a final concentration of 1 μg/ml, which reduced

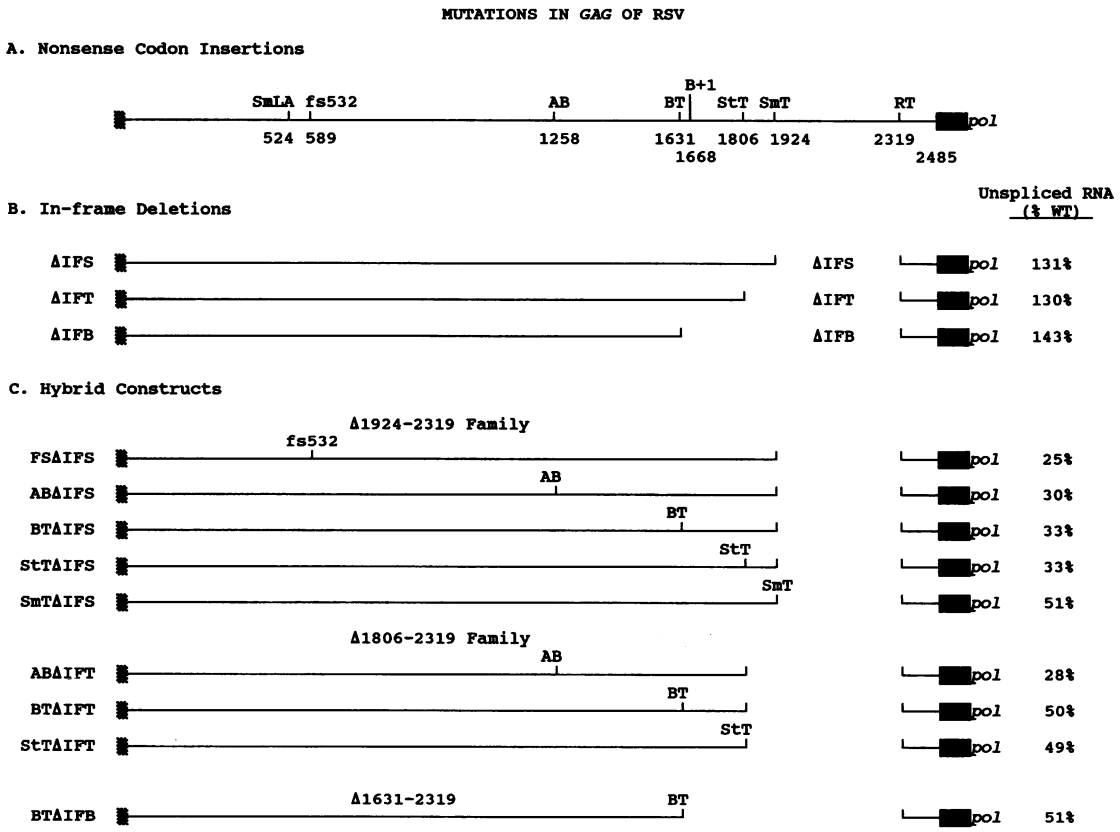

FIG. 2. Schematic diagrams of mutant constructs. Although only the *gag* gene is shown, all mutations are in the context of the full-length RSV genome in plasmid pAPrC (26). The column on the right shows the average amount of unspliced RNA produced by the mutants; see text and Table 1 for further details. ▩, Transcript of the 5′ viral LTR (RU_5); ■, *pol* gene; WT, wild type.

incorporation of [^{3}H]uridine (New England Nuclear) into trichloroacetic acid-precipitable material by >99% during an 8-h incubation period.

RNA isolation and mapping. Total cellular RNA was harvested with RNAzol (Cinna/Biotecx) according to the instructions of the manufacturer and as described previously (8). Transcription of antisense RNA probes and RNase protection analysis were carried out as previously described (2, 24). An equal fraction of a transfected plate of cells was used for each sample; typically, one-third to one-half of the RNA from a 6-cm plate was hybridized to ~500,000 dpm (0.02 pmol) of probe. The splice donor probe shown in Fig. 1 was a T7 transcript of an *Eco*RI (nt 9238)-to-*Bam*HI (nt 532) fragment of RSV cloned into pGEM-2 (Promega); this plasmid was linearized with *Bst*EII, yielding a transcript of 469 nt. Unspliced RNA protected 428 nt of the donor probe; spliced RNA protected 293 nt. The *env* probe (9a) was a T7 transcript of a *Bam*HI (nt 4715)-to-*Xho*I (nt 5259) fragment of RSV cloned into pGEM-2; this plasmid was linearized with *Dde*I, producing a 242-nt transcript which protected 213 nt of the unspliced RNA and 181 nt of the *env* mRNA. In some experiments a luciferase expression construct, pRSV/L (11), was used as a control for transfection efficiency. The luciferase probe was a T7 transcript of an *Eco*RI-to-*Xba*I fragment of luciferase cDNA cloned into pGEM-4; this plasmid was linearized with *Eco*RI, generating a 140-nt transcript which protected 99 nt of luciferase mRNA. The riboprobes were hybridized to total cellular RNA at 58°C; digestion conditions were 10 μg of RNase A per ml and 10 U of RNase T_1 (Calbiochem) per ml at 18°C for 1 h. Electrophoresis in the presence of 8 M urea was carried out on a 6% acrylamide gel (22) in experiments with the splice donor probe and on an 8% acrylamide gel when the *env* acceptor probe was used. A Molecular Dynamics PhosphorImager and densitometer and an LKB laser densitometer were used for quantification of RNA levels.

RESULTS

To test the effect of premature translation termination within the *gag* and *pol* genes of RSV on viral RNA metabolism, we used linker insertion mutagenesis to generate a set of mutants containing nonsense codons at various sites (Fig. 2A). All mutations were created within a full-length, nonpermuted plasmid clone of the Prague C strain of RSV called pAPrC (26). Since all mutations were within the intron of the spliced viral mRNAs, the mutant viruses produce spliced RNAs identical in sequence to those of the wild type. DEAE-dextran-mediated transfection was used to introduce the plasmids into secondary CEFs. Total RNA was harvested 2 days after transfection, and the amounts of spliced and unspliced viral RNAs were measured in RNase protection assays which distinguish between these RNA species. Most commonly, we used a probe spanning the common splice donor site for both *env* and *src* mRNAs; occasionally, a probe spanning the *env* splice acceptor site was used (Fig. 1).

Nonsense mutations in *gag* do not affect levels of spliced

TABLE 1. RNA phenotypes of mutant constructs

Construct	Termination codon at nt:	Mean amt of viral RNA produced (% of wild type ± SD)[a]		No. of assays
		Spliced	Unspliced[b]	
Wild type	2485	100	100	47
SmLA	524	83 ± 27	51 ± 5.3	5
fs532	589	99 ± 33	23 ± 5.5	9
AB	1258	106 ± 34	25 ± 5.9	10
BT	1631	86 ± 21	26 ± 5.3	9
B+1	1668	87 ± 26	21 ± 5.1	11
StT	1806	83 ± 23	19 ± 3.3	10
SmLB	1924	103 ± 46	24 ± 5.8	9
SmT	1924	79 ± 16	25 ± 3.4	6
RT	2319	121 ± 77	77 ± 15	13
Hpa (*pol*)	2734	115 ± 53	106 ± 18	6
ΔIFS	2485	83 ± 21	131 ± 18	5
ΔIFT	2485	101 ± 31	130 ± 28	9
ΔIFB	2485	88 ± 78	143 ± 38	3
fsΔIFS	589	74 ± 11	25 ± 1.5	5
ABΔIFS	1258	120 ± 37	30 ± .5	4
BTΔIFS	1631	99 ± 28	33 ± 6.0	7
StTΔIFS	1806	110 ± 22	33 ± 4.5	7
SmTΔIFS	1924	74 ± 30	53 ± 7.9	12
ABΔIFT	1258	122 ± 29	28 ± 4.1	5
BTΔIFT	1631	113 ± 38	50 ± 6.5	8
StTΔIFT	1806	110 ± 28	49 ± 6.1	9
BTΔIFB	1631	89 ± 49	51 ± 11	2

[a] Each transfection contained several wild-type samples for reference; the amounts of viral RNA produced by each mutant were quantitated relative to the amounts produced by the wild type in that particular transfection.

[b] Unspliced RNA levels were calculated after first normalizing the spliced band of the mutant to the spliced band of the wild type in each assay; i.e., this column lists the average unspliced/spliced ratio of the mutant versus that of the wild type.

viral RNA. After assaying each mutant many times and quantifying the amount of spliced RNA produced, we determined that nonsense mutations within the *gag* gene did not affect the total amount of spliced RNA produced (Table 1; Fig. 3). This result was substantiated by experiments in which viral DNAs were cotransfected with a second plasmid, pMyc23 (23), to control for transfection efficiency and RNA recovery (data not shown). This conclusion is consistent with earlier studies showing that disruption of *gag* proteins by frameshift mutations did not affect the amount of spliced RNA produced (2) and with work of Katz and Skalka showing that viral proteins were not required for splicing regulation at the *env* acceptor site (18). In contrast, specific *gag* deletions within nt 707 to 1006 do result in an increase in spliced RSV RNAs and define a *cis*-acting negative regulator of splicing (2, 23).

Since all mutants produced the same amount of identical, spliced RNAs, we used the spliced band as an internal control to normalize our measurement of the unspliced transcript. This allowed us to control for differences in transfection efficiency and resulted in much smaller standard deviations (Table 1). Therefore, when quantifying the amount of unspliced RNA produced by a construct, it is the ratio of the unspliced/spliced RNAs which we have presented (Table 1).

Accumulation of unspliced RNA is reduced in mutants bearing termination codons within *gag*. In contrast to the results with spliced RNA, the amount of unspliced RNA produced by our mutants was markedly decreased. Nonsense codons inserted at various sites within the first 75% of the *gag* gene (*gag* spans nt 380 to 2485) decreased unspliced RNA accumulation to 19 to 26% of wild-type levels (Fig. 3; Table 1). These RNase protection results were confirmed by Northern (RNA) analysis (data not shown). No polar effect was evident; nonsense codons at nt 589, 1258, 1631, 1806, and 1924 all produced similar low levels of unspliced RNA. Two exceptional cases were observed, however. The 3′-most mutant (RT), which has a termination codon only 165 nt from the end of *gag*, displayed a nearly normal phenotype, accumulating 77% of the wild-type level of unspliced RNA. The 5′-most mutant (SmLA), containing a nonsense codon 144 nt beyond the start of *gag* translation, was also anomalous, accumulating 51% as much unspliced RNA as the wild type. The latter phenotype may have resulted from internal reinitiation of translation: preliminary immunoprecipitation experiments suggested that SmLA might reinitiate translation at a methionine codon at nt 566 (data not shown).

Finally, in contrast to the effect observed with termination codons in *gag*, a nonsense codon introduced into the *pol* gene (at nt 2734; construct Hpa) yielded wild-type quantities of unspliced RNA (Fig. 3; Table 1). Wild-type levels of RNA were also observed with a second nonsense codon mutant in the *pol* gene (at nt 3658) and with a mutant in the *src* gene (at nt 7634) (data not shown).

The mutants described above contained deletions of up to 4 bases or additions of up to 18 bases at or near the site of nonsense codon insertion. It was possible, therefore, that the observed phenotype was unrelated to translation but was instead the result of disruption of RNA secondary structure.

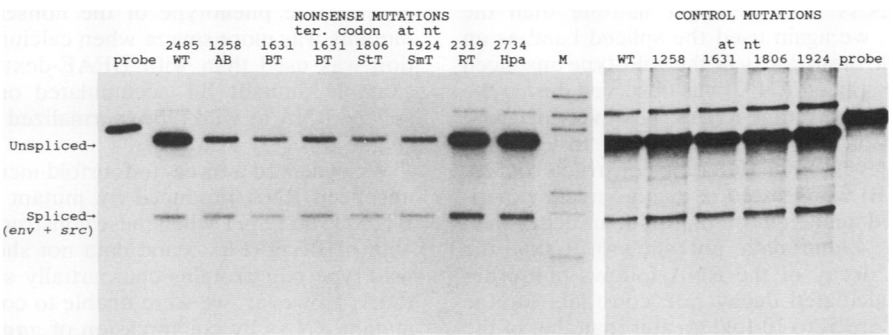

FIG. 3. Representative RNase protection assay of mutants containing nonsense codon insertions or control insertions in the *gag* gene. Mutant Hpa (nt 2734) is in the *pol* gene. A probe spanning the viral splice donor was used. The unspliced protected fragment is 428 nt; the spliced fragment is 293 nt. Lane M, RNA size markers (626, ~510, 469, 327, 313, 297, and 252 nt). WT, Wild type.

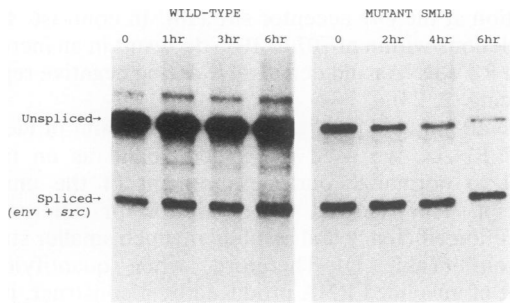

FIG. 4. Effect of dactinomycin incubation on viral RNAs, assayed by RNase protection. Times refer to incubation time with dactinomycin. Mutant SmLB contains a termination codon at nt 1924 in the *gag* gene. A probe spanning the viral splice donor site was used.

To test this possibility, we constructed a series of control linker insertion mutants. These mutants contained 10- to 18-base inserts at the same sites used to introduce linkers in mutants AB, BT, StT, and SmT, and they would be expected to perturb the RNA structure as much as the parallel nonsense codon insertions; however, the control linkers did not contain nonsense codons and did not alter the *gag* reading frame. The control constructs produced wild-type levels of unspliced and spliced RNA (Fig. 3). Therefore, the observed decrease in unspliced RNA levels with the nonsense codon insertion mutants appeared to be due to the interruption of translation.

Immunoprecipitation with anti-*gag* antibodies verified that the nonsense codon-containing mutants produced truncated *gag* precursor proteins of the expected sizes and that the control linker insertion mutants produced *gag* polyproteins indistinguishable in size from the wild type (data not shown).

Unspliced RNAs containing premature nonsense codons decayed more rapidly than wild-type RNAs. The decreased amount of unspliced RNA observed with mutants having premature nonsense codons could be due either to a lower rate of transcription or to a higher rate of RNA breakdown. Since these mutants all produced wild-type levels of spliced RNA species, it seemed unlikely that transcription was altered. Therefore, we hypothesized that excessive breakdown of the unspliced RNA might be occurring.

To test this, transfected cells were treated with 1 μg of dactinomycin per ml to inhibit transcription. This dose caused a >99% reduction in [^{3}H]uridine incorporation over the time course of the experiment (data not shown). Total RNA was harvested at various times after dactinomycin addition and analyzed by RNase protection (Fig. 4). Since the spliced viral RNAs have a longer half-life than the unspliced RNA (32), we again used the spliced band as an internal control. Little or no decay of the wild-type unspliced RNA, relative to the spliced RNA, was observed during the 6-h course of the assay. Mutant RNAs, however, decayed markedly within 2 h of drug addition, usually to less than 25% of their initial level. Four different constructs (fs532, AB, B+1, and SmLB) were tested in dactinomycin experiments, and all showed a much more rapid rate of decay than the wild type (Fig. 4 and data not shown). Using the assumption that the decay of the RNA follows first-order kinetics, we have calculated decay rate constants for the mutant RNAs which are 5- to 10-fold greater than that of the wild type. We propose, therefore, that the mutant RNAs accumulate to lower levels in the cell than the wild type because they are broken down faster.

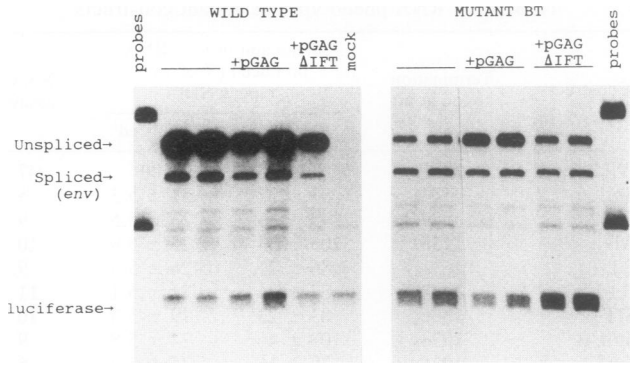

FIG. 5. Complementation of mutant RNAs with *gag* expression vectors. CEFs were transfected with viral constructs alone or cotransfected with the expression vector pHPGag or pHPGagΔIFT, using a calcium phosphate transfection procedure. Mutant BT contains a termination codon at nt 1631. An antisense probe spanning the *env* splice acceptor site was used for the RNase protection analysis. An RSV LTR-driven luciferase construct (pRSV/L) (11) was also transfected into all cells as a control for transfection efficiency and RNA recovery and assayed simultaneously with a luciferase riboprobe.

Complementation tests suggested that *gag* proteins can partially stabilize mutant unspliced RNAs. We investigated two possible hypotheses to explain the coupling that we observed between viral RNA stability and translation. First, since *gag* proteins bind to viral RNA (17), synthesis of a full-length *gag* product might be required to stabilize the RNA. Alternatively, the process of translating the *gag* region of the transcript might stabilize the RNA; for instance, traversing ribosomes might protect the RNA from nucleases.

The first possibility was addressed by expressing a mutant viral RNA in a cell along with intact *gag* proteins to see whether these proteins could affect the accumulation of the normally unstable RNA. We constructed plasmid pHPGag to express *gag* proteins in *trans*; this construct contains the *gag* gene flanked by RSV long terminal repeats (LTRs). Since it lacks the other viral genes, RNAs expressed by pHPGag could be easily distinguished from those transcribed from the full-length viral constructs. We cotransfected pHPGag into CEFs along with mutant viral constructs, using a calcium phosphate transfection protocol to increase the chance that any given transfected cell would pick up both types of plasmid (19, 30). RNA from such experiments was analyzed by RNase protection (Fig. 5). In general, the phenotype of the nonsense codon-containing mutants was more severe when calcium phosphate transfection was used than with DEAE-dextran transfection. For example, mutant BT accumulated only 8% as much unspliced RNA as wild type (normalized against spliced RNA) (Fig. 5).

We observed a three- to fourfold increase in the amount of unspliced RNA produced by mutant viral constructs AB, BT, StT, and SmT when these constructs were cotransfected with pHPGag (Fig. 5 and data not shown), suggesting that wild-type *gag* proteins can partially stabilize the unspliced RNA. However, we were unable to completely stabilize the mutant RNAs by coexpression of *gag* proteins, even when using a sixfold molar excess of pHPGag DNA. Cotransfection of pHPGag did not affect the level of wild-type RNAs (Fig. 5). We conducted similar cotransfection experiments

using DEAE-dextran transfection, employing pHPGag and a similar expression vector, pGag3.2. These experiments, which were assayed both by RNase protection and by Northern analysis, also showed an increase in the accumulation of mutant unspliced RNA upon coexpression of wild-type *gag* proteins; however, the effect averaged two- to threefold (data not shown).

To investigate the possibility that the enhanced RNA levels seen above might result from recombination between the *gag* expression vector and the mutant viral construct, we constructed a mutant *gag* expression vector bearing a termination codon at nt 1631 (pGag3.2BT). If recombination were occurring between the cotransfected DNAs, pGag3.2BT should be able to generate wild-type, stable RNAs when cotransfected with mutant viruses containing nonsense codons upstream of nt 1631. However, in cotransfection experiments using pGag3.2BT with viral mutant fs532, no complementation was observed (data not shown). Thus, it appeared that the increase in mutant RNA levels observed in cotransfection experiments was due to *gag* protein expression rather than to recombination.

Next, we tried to determine whether this RNA-stabilizing activity could be localized to a particular domain of the *gag* protein. The most obvious candidate appeared to be the NC domain for two reasons. First, the finding that termination codons introduced either 5′ to or within the NC-coding sequence destabilized the RNA, while a nonsense codon introduced 3′ of NC had little effect, suggested that this region might encode an important polypeptide. Second, the NC domain encodes an RNA-binding protein which has been shown to be essential for packaging of genomic RNA into virions (17, 25, 26). Therefore, the processes of stabilizing and packaging the RNA might be related and carried out by the same polypeptide.

This idea was tested by cotransfection experiments with a new *gag* expression vector, pHPgagΔIFT, which contains an in-frame deletion from nt 1806 to 2319. This deletion removes all of the NC-coding region (nt 1844 to 2111) as well as half of the protease domain (nt 2112 to 2485). This expression plasmid was cotransfected into CEFs along with the mutant viral constructs AB, BT, StT, and SmT, using calcium phosphate transfection. Cotransfection with pHPGagΔIFT did not cause an increase in the accumulation of unspliced RNA of any of these mutants (Fig. 5 and data not shown). This result suggested that the RNA-stabilizing activity of *gag* proteins might be encoded in the NC region. As the deletion encompassed more than the NC domain and also might disrupt the structure of the entire *gag* precursor, further experiments are necessary to verify this hypothesis.

In-frame deletion mutations suggested that *gag* proteins were not needed to maintain the stability of viral RNA lacking premature termination codons. The cotransfection experiments described above suggested that intact *gag* proteins (but not those with NC deletions) were able to stabilize mutant unspliced RNA. We next wanted to determine whether *gag* proteins were also necessary to stabilize viral RNAs lacking premature nonsense codons. If this were the case, the unspliced RNA produced by full-length viral constructs containing in-frame deletions of NC would be unstable.

To test this idea, we constructed three in-frame deletions covering the critical region (Fig. 2B). All possess the same 3′ boundary (nt 2319), but they extend in the 5′ direction to either nt 1924 (ΔIFS), nt 1806 (ΔIFT), or nt 1631 (ΔIFB). When transfected into CEFs, these mutants all produced wild-type levels of spliced RNA (Table 1). This result was verified in experiments in which pMyc23 was cotransfected and assayed as a control for transfection efficiency. Surprisingly, these in-frame deletion mutants reproducibly accumulated even greater levels of unspliced RNA than did the wild type (Table 1; Fig. 6, lane 7). This increased cellular accumulation of the unspliced RNA (about 30 to 40% over wild-type levels) may result from an inability to package the unspliced RNA into virions in the absence of NC (25, 26). This result demonstrated that the portion of the *gag* protein essential for stabilizing mutant RNAs was not needed to maintain the stability of RNAs which can be translated to the end of *gag*. Thus, it appeared that synthesis of truncated *gag* proteins might not be the primary cause of the RNA instability of the nonsense codon insertion mutants, prompting consideration of the alternate possibility that the process of translating the *gag* region of the RNA affected the stability of the unspliced RNA.

Mutant RNAs were stabilized by deletion of sequences downstream of the nonsense codon. To further investigate the possibility that *cis*-acting viral sequences were involved in RNA stabilization, the three in-frame deletion mutations shown in Fig. 2B were combined with various termination codon insertion mutations to create the hybrid viral constructs shown in Fig. 2C. These were assayed in transient transfection experiments in CEFs, and the results are summarized in Table 1. The relative phenotypes exhibited by the different constructs in each separate transfection precisely paralleled the average phenotypes presented in the table. Two representative RNase protection assays using some of these mutants are shown in Fig. 6.

Examination of the phenotypes of these constructs revealed two patterns. First, constructs containing a deletion extending from the point of nonsense codon insertion to nt 2320 accumulated two to three times as much unspliced RNA as constructs containing the same nonsense codon without the deletion. For example, nonsense codon mutant StT (Fig. 6, lane 6) produced 19% of wild-type levels of unspliced RNA, while the hybrid construct StTΔIFT (Fig. 6, lane 5) produced 49% of wild-type levels. Similar results were seen by comparison of the pairs of constructs BT versus BTΔIFB and Smt versus SmtΔIFS (Fig. 2). Both members of these pairs terminated translation at the same point, producing identical truncated *gag* proteins, yet had different RNA stabilities. These results suggested that a *cis*-acting RNA component was affecting the stability of the unspliced RNA.

A second pattern was evident upon examination of families of constructs containing the same deletion in combination with nonsense codons at various positions upstream of the deletion. For example, the deletion from nt 1806 to 2319 in ΔIFT was able to stabilize a termination codon at the brink of the deletion (nt 1806) (StTΔIFT; Fig. 6, lane 5) as well as one 170 nt upstream of it (BTΔIFT; Fig. 6, lane 4). However, when the nonsense codon was moved 550 nt upstream (ABΔIFT; Fig. 6, lane 3), the deletion lost its stabilizing effect. A similar result was observed with the Δ1924–2319 family of constructs. Thus, the deletion only stabilized the construct whose nonsense codon was located at the brink of the deletion (Fig. 6, lane 13), while the phenotype of a construct with an upstream nonsense codon was unaffected by the deletion (Fig. 6, lane 12). In summary, deletion of downstream sequences was able to stabilize, by a factor of two- to threefold, termination codons located at the 5′ edge of the deletion; however, nonsense codons located several hundred bases upstream of the deletion were unaffected.

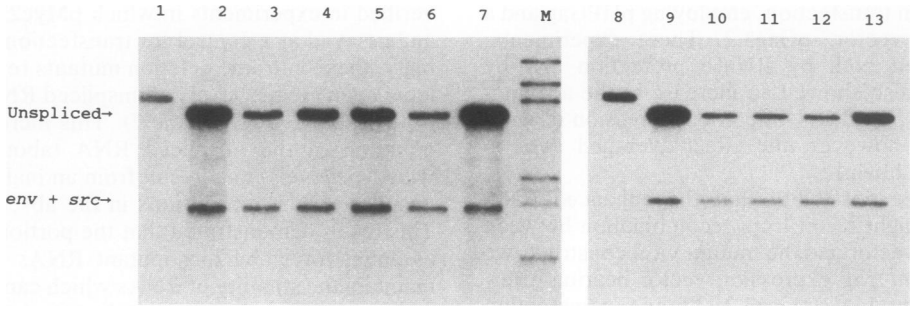

FIG. 6. RNase protection analyses of CEFs transfected with nonsense codon insertion mutants, an in-frame deletion mutant, and several hybrid mutants. A probe spanning the viral splice donor site was used. Lanes: 1, probe; 2, wild type; 3, ABΔIFT; 4, BTΔIFT; 5, StTΔIFT; 6, StT; 7, ΔIFT; 8, probe; 9, wild type; 10, fs532; 11 and 12, fsΔIFS; 13, SmTΔIFS; M, RNA size markers (626, ~510, 469, 327, 313, 297, and 252 nt).

DISCUSSION

Our results suggest that the stability of the unspliced RNA of RSV is coupled to the process of translating the *gag* gene of this RNA. We found that mutant unspliced RSV RNAs containing premature nonsense codons in *gag* accumulated to lower levels than did the wild-type RNA; this reduced accumulation could be attributed to an increase in the decay rate of the mutant RNAs. However, nonsense codons elsewhere in the 9.5-kb RSV transcript did not affect the stability of the RNA. Similarly, nonsense codons within the *gag* gene of human immunodeficiency virus have been associated with decreased levels of unspliced viral RNA (1). In contrast, frameshift mutations resulting in premature translation termination within the *gag* gene of Moloney murine sarcoma virus ts110 do not affect viral RNA stability (10). While we also found that *gag* proteins could partially stabilize mutant RSV RNAs when expressed in *trans*, this interaction was not needed to maintain the stability of viral RNAs lacking premature termination codons. Therefore, premature nonsense codons appear to destabilize the RNA not because of their effect on *gag* protein function but instead because they disrupt translation of the *gag* region of the RNA.

This conclusion was supported by examination of hybrid constructs in which deletions immediately downstream of termination codons resulted in increased RNA stability, whereas RNAs bearing termination codons hundreds of nucleotides upstream of the deletion were not stabilized. One possible interpretation of this finding is that the deleted region of *gag* contains nuclease-sensitive sites, which could be protected from nucleases by translating ribosomes. Since deletions were able to stabilize only RNA with termination codons near the 5′ brink of the deletion, there may be multiple nuclease-sensitive sites within *gag*. This hypothesis is similar to that proposed to explain hyperlabile RNA resulting from nonsense codons in bacterial operons (15, 28). An alternative hypothesis is that deletions may stabilize the unspliced RNA merely by bringing the artificially introduced termination codons closer to the natural *gag* stop codon. Stable viral RNA may require ribosomal progression to a point close to the normal *gag* stop codon, possibly to interact with some structure or protein. Comparison of phenotypes of some of our double mutants slightly favors the first of these hypotheses. In construct BTΔIFT, the effect of the nonsense codon is suppressed twofold by a deletion which puts it 330 nt upstream of the normal *gag* terminator. However, in construct StTΔIFS, the inserted nonsense codon is brought to within 280 nt of the *gag* terminator without significant amelioration of the effect of the nonsense codon. This suggests that particular sequences in *gag* may be of more importance in determining RNA stability than the distance between the nonsense codon and the *gag* stop codon.

As translation takes place in the cytoplasm, it seems likely that coupling between viral RNA translation and stability might also be a cytoplasmic event. The continual presence of ribosomes traversing *gag* RNA could sterically shield putative nuclease-sensitive sites from attack. Alternatively, the passage of ribosomes over *gag* might alter the RNA secondary structure (or its pattern of bound proteins) so as to dismantle nuclease target sites. Premature termination codons which halt the ribosomal progression could render *gag* sequences downstream of the translated region accessible to nucleases. *gag* proteins expressed in *trans* may bind the free viral RNA, thereby providing an alternative form of protection.

Alternatively, the link between translation and accumulation of the unsliced RSV RNA may occur either at the level of RNA transport from the nucleus to the cytoplasm (translational translocation) or by some sort of nuclear scanning of the RNA for the presence of nonsense codons. Both of these possible models were originally proposed by Urlaub et al. (33) to explain the effects of nonsense codons in *dhfr* RNA and have been supported by work of Cheng et al. (7) with triosephosphate isomerase (TPI) RNA. There is a striking similarity between the phenotype of our RSV nonsense mutants and that of similar mutations in the *dhfr* and TPI RNAs (7, 33). In all three cases, termination codons located early in the gene cause a reduced accumulation of the mRNA to about 10 to 20% of the wild-type level, while those located near the normal stop codon (in the final exon or the 3′ end of the penultimate exon in *dhfr* and TPI) do not (7, 33). Also, in RSV and TPI, certain deletions which bring nonsense codons closer to the normal termination codon suppress the effect of the nonsense codon. In both the *dhfr* mRNA and the RSV unspliced RNA, there are long 3′ untranslated regions, which are presumably stabilized by a translation-independent mechanism.

There are, however, differences between RSV and these two cellular RNAs. While termination codons did not decrease the stability of the mature *dhfr* and TPI mRNAs, they substantially reduced the half-life of the RSV unspliced RNA, measured in all three cases by use of dactinomcin (7, 33). This difference might be related to the fact that the *dhfr* and TPI assays measure the stability of the mature spliced mRNA, presumably located almost exclusively in the cytoplasm, while our assays of RSV unspliced RNA measure the

decay of the whole population of unspliced RNA, including primary transcripts in the nucleus and mRNA and genomic RNA in the cytoplasm. If the majority of mutant viral RNAs were located in the nucleus at the time the cells were harvested, then excessive nuclear degradation might underlie the decreased mutant RNA half-life that we observed when assaying total RNA.

Another difference between RSV unspliced RNA and these two cellular RNAs should be noted, however. Since the effect of a nonsense codon seems to depend on its position relative to an intron in the case of *dhfr* and TPI RNAs, a coupling between translation and splicing has been proposed (7, 33). Although the RSV RNA affected by nonsense codons is transported to the cytoplasm without being spliced (9, 31, 34), its transport still might be coupled to translation in some way. Recent evidence suggests that the unspliced RSV RNA may interact nonproductively with the splicing machinery (12, 23), so it is possible that interaction of the RNA with ribosomes facilitates its dissociation from spliceosomes. Alternatively, ribosomes may need to progress to near the normal RSV *gag* stop codon in order to pull an RNA structure (such as a putative pseudoknot at the *gag-pol* junction [6]) through the nuclear pore. Analysis of the mutant and wild-type RSV RNAs present in the nucleus and cytoplasm should help distinguish between the possible mechanisms by which termination codons might destabilize the unspliced RNA.

The finding that *gag* proteins can stabilize nonsense codon-containing RNAs within the cell is interesting, even though it does not appear to be the primary cause of the mutant RNA instability. It is not surprising to find that *gag* proteins and the viral RNA interact within the cell, since their interaction appears to be responsible for packaging genomic RNA into virions. Since the NC protein is essential for packaging RNA (25, 26), it is also not surprising that its deletion abolishes the interaction. However, it is unexpected that coexpression of *gag* proteins should increase the intracellular accumulation of mutant unspliced RNAs. The traditional model of assembly of C-type retroviruses (5, 9) suggests that interaction of the unspliced RNA with the *gag* precursor occurs as the viral particle assembles at the plasma membrane. If this were the case, coexpressed *gag* proteins would be expected to package and export the mutant RNAs from the cell, thus further decreasing their accumulation. The ability of *gag* proteins to have precisely the opposite effect suggests that they may instead interact with the unspliced viral RNA at some stage prior to assembly of viral particles at the plasma membrane. It is possible, for instance, that *gag* proteins help to export the RNA from the nucleus or protect it from cytoplasmic degradation.

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