

Unspliced Rous Sarcoma Virus Genomic RNAs Are Translated and Subjected to Nonsense-Mediated mRNA Decay before Packaging

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Retroviruses package full-length, unspliced RNAs into progeny virions as dimerized RNA genomes. They also use unspliced RNAs as mRNAs to produce the *gag* and *pol* gene products. We asked whether a single Rous sarcoma virus (RSV) RNA can be translated and subsequently packaged or whether genomic packaging requires a nontranslated population of RNAs. We addressed this issue by utilizing the translation-dependent nonsense-mediated mRNA decay (NMD) pathway. NMD is the selective destruction of mRNAs bearing premature termination codons (PTCs). The pathway has been shown to be associated with splicing in higher eukaryotes. Here, we demonstrate that both translation and the cellular factor Upf1 are required for the decay of unspliced, PTC-bearing RSV RNA by the NMD pathway. To address the relationship between RNA translation and packaging, we examined virus produced in cells cotransfected with PTC-bearing retroviral clones and wild-type viral clones. We observed that PTC-bearing transcripts are packaged into viral particles at levels three- to fivefold less than those of control RNAs. Since PTC-mediated degradation requires translation, we conclude that RSV can package progeny virion particles using previously translated RNAs.

After reverse transcription and integration of their genomes, retroviruses use cellular machinery to produce new viral RNA and protein products (39, 46). Some of these viral RNAs are exported from the nucleus without first being spliced. Complex retroviruses export their unspliced RNA with the help of virus-encoded accessory proteins, such as Rev for human immunodeficiency virus type 1 (HIV-1) (28, 29). Simple retroviruses use *cis*-acting RNA elements, such as the constitutive transport element (CTE) for Mason-Pfizer monkey virus (9) or the direct repeats for Rous sarcoma virus (RSV) (37, 38), which recruit cellular export factors to the transcripts (8). After export, either these unspliced RNAs can be translated to produce Gag and Gag-Pol polyproteins (46) or they can be dimerized and packaged as genomes in progeny virions (6, 17).

Currently, there is no unified view regarding the source of retroviral RNAs that are packaged in virions. Levin and colleagues (21, 22) studied the effects of actinomycin D (actD) on Rauscher murine leukemia virus (MLV) full-length RNA metabolism. After 6 h of treatment with actD to inhibit RNA synthesis, MLV-infected cells still make viral proteins and bud viral particles. However, packaging of genomic RNA into these particles drops 10-fold, suggesting that the population of unspliced viral RNAs used for packaging has been depleted. Translation persists because the viral mRNA is stable in the cytoplasm. This suggests that MLV employs two functionally distinct pools of unspliced RNA.

In contrast to MLV, HIV-1 and HIV-2 continue to package genomic RNAs in infected cells after treatment with actD, apparently due to a single population of RNAs that can function interchangeably as mRNA and genomic virion RNA (10, 16). Further, neither active translation nor prior translation of the genomic RNA is a prerequisite for HIV-1 RNA packaging

(10, 16). However, HIV-2 encapsidates genomic RNAs cotranslationally, favoring the *cis* packaging of RNAs that have been translated (16).

Through actD treatment of cells containing radiolabeled RNAs, Stoltzfus et al. (44) determined the half-life of unspliced RSV RNAs to be 7.5 h both in the cytoplasm and in virions. Thus, they propose that RSV also employs a single population of RNAs to serve as genomic RNAs as well as mRNAs. In contrast, Sonstegard and Hackett (43) propose that ribosomes and the Gag precursor protein compete for binding to the third small open reading frame (uORF3) upstream of the *gag* gene. Translation of *gag* results in an increase in Gag protein concentration within the cell, eventually leading to its binding at uORF3, which contains part of the encapsidation signal, Ψ (1, 3, 24); this results in increased RNA packaging. In this model, polyribosome-bound RNAs are not substrates for packaging, and RSV's unspliced RNA is segregated into two functionally distinct pools (43).

To clarify the issue of whether translated RSV RNAs are packaged, we used a conserved cellular pathway known as nonsense-mediated mRNA decay (NMD). NMD is the selective destruction of mRNA transcripts bearing premature termination codons (PTCs) (25, 30, 32, 36). This pathway serves as a cellular quality control mechanism to ensure that mRNAs are processed properly. It has been estimated that 30% of all genetic disorders are caused by PTCs (13). The NMD pathway is directly coupled to the process of translation, and inhibition of translation blocks NMD (7, 12).

The NMD pathway is also associated with splicing (48, 49). Concurrent with the splicing event, a protein complex is deposited on the mRNA 20 to 24 nucleotides upstream of every exon junction (19). This complex, known as the exon junction complex (EJC), indicates the splicing history of each mRNA (20). During translation, ribosomes displace these complexes as they traverse the mRNA. When a ribosome encounters a termination codon located upstream of an EJC, the mRNA is

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degraded (25, 30). In support of the EJC-mediated model, naturally intronless genes, such as those for histone H4 and Hsp70, are immune to decay despite the presence of PTCs (31).

Earlier reports from our lab (2, 4, 5), which predate the proposed EJC-mediated model for NMD, demonstrate that nonsense codons at various positions in the *gag* gene of RSV lead to instability of the unspliced RNA in primary chicken embryo fibroblasts (CEFs). In light of recent findings linking NMD to splicing, this result is somewhat surprising, since unspliced RSV RNAs would have been predicted to be immune to decay. This investigation sought to determine if the PTC-induced instability was actually due to NMD. First, using a dominant-negative mutant of hUpf1 that abrogates NMD (45), we demonstrated the stabilization of RSV RNAs bearing PTCs. Secondly, inhibition of translation also led to stabilization of these RNAs. These results suggest that unspliced viral RNAs with PTCs are substrates for the NMD pathway.

We next examined whether translated RSV RNAs could subsequently be packaged. Analysis of packaged viral RNAs, produced in cells cotransfected with wild-type and mutant viral constructs, showed that PTC-bearing RNAs were packaged in progeny virions at a level three- to fivefold lower than that of RNAs bearing control codon insertions. This reduction in viral RNA levels is similar to that seen in cells as a result of NMD. If the packaged RNAs were not translated, they would not be subject to NMD, and no reduction in packaged levels would be expected. Since these PTC-bearing RNAs were subjected to NMD, and therefore at least one round of translation (7, 12), we conclude that RSV is able to package genomic RNAs that have previously been translated.

MATERIALS AND METHODS

Cell culture and transfection. Secondary cultures of CEFs were maintained in medium 199 (Gibco) supplemented with 2% tryptose phosphate broth (Sigma), 1% chick serum, 1% calf serum, and 1% penicillin-streptomycin (Gibco). Cells were grown at 39°C in the presence of 5% carbon dioxide. Transfections were performed using 200 µg of DEAE-dextran/ml in serum-free medium 199, as described previously (38), with cells that were approximately 80% confluent. Transfections done in 6-cm plates (see Fig. 1, 2, and 3) used 4 to 6 µg of DNA in 1 ml of medium 199 containing DEAE-dextran. Transfections done in 10-cm plates used 10 µg of DNA in 3 ml of medium 199 containing DEAE-dextran. Cells were incubated for 4 h at 39°C and then shocked with serum-free medium 199 containing 10% dimethyl sulfoxide for 2 min at room temperature. Cells were then washed twice with serum-free medium 199. Anisomycin (Calbiochem) was used to inhibit cellular translation at a final concentration of 100 µg/ml in serum-containing medium 199.

RNA isolation and RNase protection assays. Total cellular RNA was harvested 40 to 48 h posttransfection using RNA-Bee (Tel-Test) according to the manufacturer's instructions. The *gag* probe was generated in vitro using viral sequences under the control of a T7 promoter and incorporating [α -³²P]CTP (Perkin-Elmer). RNAs were precipitated and resuspended in 30 µl of 80% formamide hybridization solution (80% [vol/vol] deionized formamide, 40 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) [pH 6.7], 0.4 mM NaCl, 1 mM EDTA). The *gag* riboprobe (250,000 cpm) (see Fig. 1A) was added to each sample. RNAs were denatured at 95°C for 5 min and incubated for 16 to 20 h at 42°C. Three hundred microliters of RNase digestion buffer (10 mM Tris-HCl [pH 7.5], 300 mM NaCl, 5 mM EDTA) supplemented with 10 U of RNase T1/ml and 5 µg of RNase A/ml (both from Calbiochem) was added, followed by incubation at 37°C for 1 h. Digestion was halted by the addition of sodium dodecyl sulfate (SDS) and proteinase K (Roche) at final concentrations of 0.6% (vol/vol) and 0.14 mg/ml, respectively. After incubation at 37°C for 15 min, samples were extracted with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) and precipitated with ethanol. RNAs were resuspended in 95% formamide loading dye (95% [vol/vol] deionized formamide, 0.02% bromophe-

nil blue, 0.02% xylene cyanol), denatured for 5 min at 95°C, and incubated on ice for at least 2 min. Samples were loaded on a 6% acrylamide-8 M urea gel and electrophoresed. RNA levels were quantified using an InstantImager (Packard).

Virus purification. Viral particles were recovered from the culture media approximately 48 h after transfection, after first centrifuging at 2,000 rpm for 10 min in a Damon/IEC clinical centrifuge to pellet cellular debris. The supernatant was then divided into two equal aliquots and subjected to ultracentrifugation for 2 h at 4°C in a Beckman Ultracentrifuge, using an SW-41 rotor at 34,000 rpm. One of each pair of viral pellets was resuspended in RNA-Bee (Tel-Test) for RNA isolation. The other viral pellet was resuspended in Laemmli protein sample buffer (0.05 M Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 10% β -mercaptoethanol) and heated to 95°C for 2 min.

Western blot analysis. Cells were removed from the plates by incubation with trypsin (Gibco) and pelleted. Both total cellular proteins and virion proteins were subjected to SDS-polyacrylamide gel electrophoresis, using a 10% acrylamide resolving gel. After electrophoresis, proteins were transblotted to Hybond nitrocellulose (Amersham). The membrane was then blocked in phosphate-buffered saline-Tween (0.05%) with 5% dry milk for 1 h at room temperature. RSV Gag proteins were detected using polyclonal rabbit anti-avian myeloblastosis virus p19^{gag} serum, obtained from D. P. Bolognesi (Duke University), and ImmunoPure goat anti-rabbit immunoglobulin G (heavy plus light chains, horseradish peroxidase conjugated; Pierce). Blots were then visualized by autoradiography using the Enhanced Chemiluminescence reagent kit (Amersham).

RESULTS

Generation of a riboprobe that can discriminate between multiple species of mutated RSV RNAs. Earlier reports from our lab showed that unspliced RSV RNAs are destabilized when PTCs are present in the *gag* gene (4, 5). This work preceded reports that linked NMD to splicing (48, 49). Since naturally intronless cellular mRNAs are immune to NMD (31), we asked if the instability of the retroviral RNA was due to NMD or to another mechanism.

For this study, we used the Prague C strain of RSV. Mutants were created by inserting oligonucleotide linkers at nucleotide (nt) 1547 or 1924, both located within the *gag* gene, which has a natural termination codon at nt 2483. These oligonucleotide inserts contained either premature termination codons (1547T and 1924T) or control codons (1547C and 1924C), as described by Barker and Beemon (5). The inserts were placed into a viral vector, termed 10.8 (34), that contains a partial deletion of nucleocapsid (NC) (Fig. 1A). This deletion removes the zinc finger domains of NC and blocks the ability of the 10.8 Gag product to bind and package the retroviral RNA. This allows analysis of steady-state viral RNAs in the cell without the loss of RNAs due to packaging into progeny virions.

We generated a 770-nt ³²P-labeled antisense riboprobe that is complementary to nt 1494 to 2121 of wild-type RSV RNA (*gag* probe) (Fig. 1A). This probe spans both sites of oligonucleotide insertion and also the NC deletion site. To test this probe's ability to distinguish each of these RNAs from one another, CEFs were transiently transfected with retrovirus clones bearing oligonucleotide insertions that lacked PTCs. After 48 h, total cellular RNA was harvested and analyzed by RNase protection assay (RPA).

We found that the probe was able to discriminate the viral RNAs from one another due to probe cleavage at the site of oligonucleotide insertion as well as at the nucleocapsid deletion (Fig. 1B). The wild-type construct (RSV) yielded a protected product of 627 nt. The 10.8 and 1924C constructs yielded a major product of 430 nt (protecting from nt 1494 to 1924). The 1547C construct allowed probe cleavage at the NC deletion as well as cleavage at its unique oligonucleotide in-

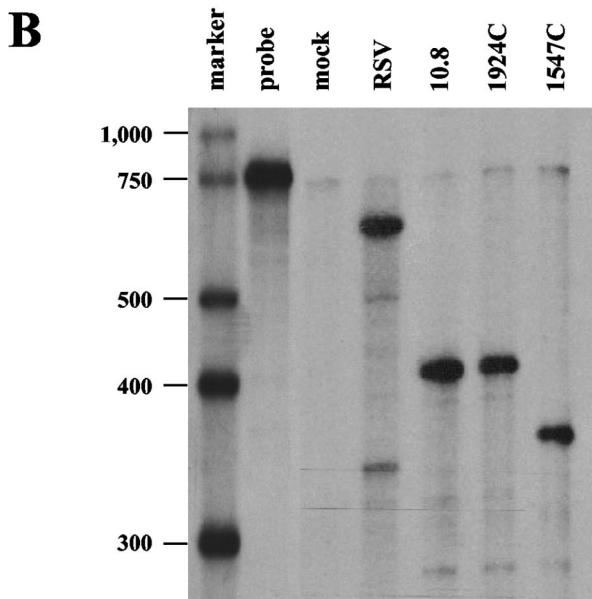
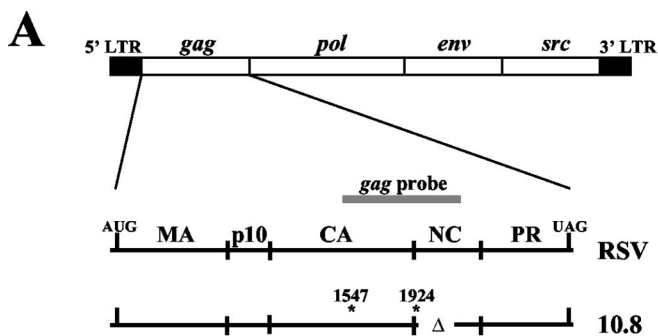


FIG. 1. A single antisense riboprobe complementary to RSV *gag* can differentiate between various mutant RNA species. (A) A detailed schematic of the integrated RSV genome with the *gag* region enlarged. The *gag* riboprobe (gray bar) spans nt 1494 to 2121. The deletion in nucleocapsid (nt 1924 to 2047) is indicated by Δ . Sites of oligonucleotide insertions are indicated by asterisks along with their nucleotide number. LTR, long terminal repeat; MA, matrix; CA, capsid; NC, nucleocapsid; PR, protease. (B) RNase protection assay of RNAs isolated from transiently transfected CEFs using the *gag* probe. 1924C and 1547C lanes represent CEFs transfected with retroviral constructs bearing linker insertions with control codons.

insertion site, yielding a major product of 387 nt (1547 to 1924). Since the constructs bearing PTCs (1547T and 1924T) have insertions at the same sites as their control codon counterparts, they generated the same-size products using this probe (data not shown), which was used in RPAs throughout this study.

Unspliced RSV RNAs bearing PTCs are substrates for the NMD pathway. We first wished to determine if Upf1, a vital cellular protein component of the NMD pathway (18, 26, 27), is involved in the instability of PTC-bearing RSV RNAs. To this end, we obtained a dominant-negative clone of human Upf1 (hUpf1) called R844C, which contains an arginine-to-cysteine mutation in its RNA helicase domain that abrogates NMD (45). CEFs were transiently cotransfected with wild-type

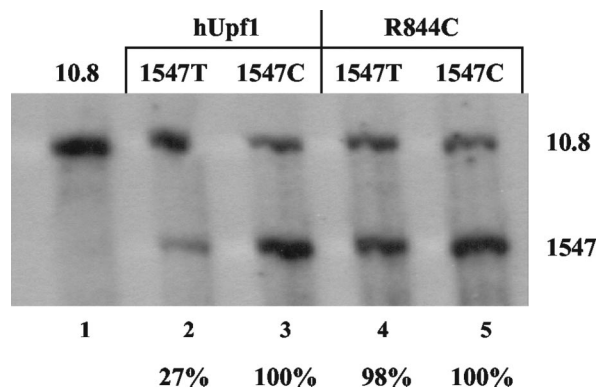


FIG. 2. The decay of unspliced RNAs bearing premature termination codons is dependent upon Upf1. CEFs were transiently transfected with equal amounts of three DNA constructs: 10.8 (as a loading control), the retroviral clones with a PTC (1547T) or control codon (1547C) insertion, and either wild-type hUpf1 or a dominant-negative mutant (R844C). CEFs were maintained for 48 h and then harvested for RNA. RNA analysis was done by RPA using the *gag* probe (shown in Fig. 1A). Percentages indicated represent RNA levels of 1547T RNAs normalized to the respective 1547C RNA (with either hUpf1 or R844C). Results are from five independently performed transfections.

hUpf1 or the R844C mutant and either the 1547T or 1547C viral construct. The 10.8 construct was also cotransfected for the purpose of normalization. After 48 h, total cellular RNA was harvested and analyzed by RPA (Fig. 2). When coexpressed with wild-type hUpf1, 1547T RNA levels were reduced to 27% of 1547C expression (Fig. 2, lanes 2 and 3), similar to the reduction seen previously without wild-type hUpf1 overexpression (4, 5). In contrast, the expression of 1547T was 98% of that of 1547C when both were expressed with R844C (Fig. 2, lanes 4 and 5). There was not a significant difference in the expression pattern of 1547C when it was expressed with either hUpf1 or R844C, demonstrating that the Upf1 mutant does not affect non-PTC-bearing RSV transcripts. These results implicate Upf1 involvement in the decay of unspliced retroviral RNAs bearing PTCs. Since NMD requires Upf1, we conclude that the instability is likely due to NMD.

To confirm and extend this result, we also examined the effect of translation inhibition on RSV RNA stability. Since translation is required for the NMD pathway (7, 12), its inhibition should lead to the stabilization of transcripts bearing PTCs. By measuring [³⁵S]methionine incorporation, we observed that 98% of all cellular translation was inhibited in CEFs treated with 100 μ g of anisomycin/ml for 1 h (data not shown). To test the effect of anisomycin treatment on expression of PTC-bearing viral RNAs, CEFs were transiently transfected with 1.5 μ g of wild-type RSV DNA (as a loading control) and 3 μ g of either the 1547T or 1547C viral DNA construct. Cells were maintained for 48 h and then treated with 100 μ g of anisomycin/ml. RNA was harvested 1 and 2 h post-drug treatment and assayed by RPA. Expression of the 1547T and 1547C RNAs relative to the RSV loading control was quantified. The untreated samples (0 h) were given a value of 1.0, and the expression of samples treated with anisomycin for 1 or 2 h was determined relative to the respective untreated samples (Fig. 3). Data were averaged from three experiments. While the anisomycin treatment did not affect the stability of

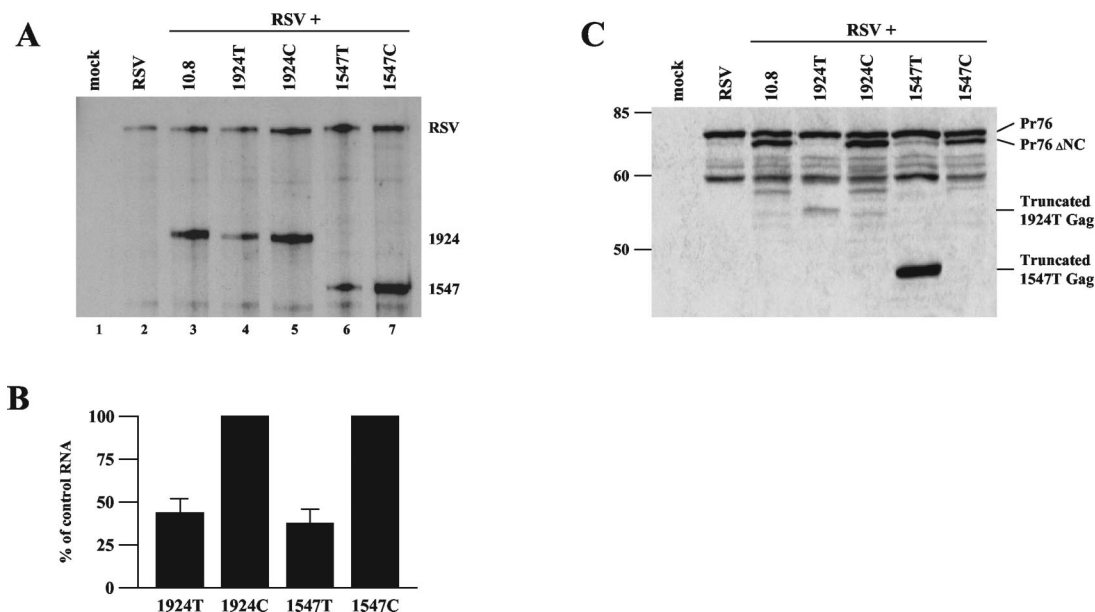


FIG. 5. Decay of unspliced retroviral transcripts with nonsense codons occurs in the presence of wild-type Gag. CEFs in 10-cm plates were transiently transfected with equivalent amounts (5 μ g) of the wild-type and mutant RSV clones indicated. (A) Total cellular RNA was harvested and analyzed by RPA using the *gag* probe. (B) Bar graph quantification of 1924T and 1547T RNA levels normalized to their control codon counterpart, set at 100%. Results are from six independently performed transfections. (C) Western blot analysis of intracellular viral proteins. Cells were trypsinized, resuspended in 1 \times Laemmli buffer, and used for SDS-polyacrylamide gel electrophoresis. Antibodies were targeted against the viral MA protein.

body against the matrix (MA) protein, which is at the N terminus of the Gag polyprotein (Fig. 5C). We found that Pr76^{gag}, the Gag polyprotein precursor, is produced in all samples, while a slightly smaller Gag product (Pr76 Δ NC) is made in the 10.8 samples, including 1547C and 1924C, with only trace amounts in 1547T and 1924T. A minor amount of the 1924T truncated protein product was detected, consistent with the decay of its corresponding mRNA. Interestingly, the truncated product from 1547T was consistently expressed at a much higher level than the other Gag proteins. This is puzzling, since the amount of protein produced does not correlate with the reduced 1547T mRNA expression level seen (Fig. 5A). This smaller truncated product may not be suitable for use in the assembly of progeny virions since it does not contain a full capsid (CA) domain, and thus, the 1547T Gag precursor may simply accumulate in the cell. In contrast, the 1924T products, which terminate after the CA domain, may be assembled into hybrid particles with wild-type Gag proteins.

These results confirm that the NMD pathway, even in the presence of wild-type Gag protein, selectively degrades unspliced viral transcripts bearing PTCs. Having established that the NMD effect was not suppressed with wild-type Gag expression, we can now examine the effects of PTCs on viral packaging.

RSV genomic RNAs are sensitive to NMD, indicating prior translation has occurred. The virion particles present in the supernatant from the previous experiment (Fig. 5) were purified for analysis of both viral RNA and protein levels. The supernatant was split into two equal portions. Viral particles in each were pelleted by ultracentrifugation. One half was harvested for RNA, which was then analyzed by RPA using the *gag* riboprobe (Fig. 6A). NC deletion 10.8 RNAs were packaged efficiently into viral particles, indicating no preference for

cis packaging (Fig. 6B, lane 3). To test for potential differences in virus production, the other half was used for Western blot analysis, employing antibodies directed against MA (Fig. 6C). No appreciable difference in viral MA levels produced from the different constructs was observed by use of Western blotting (Fig. 6C).

Further, 1547T and 1924T RNAs were packaged in virions 19 and 30% as often as their non-PTC-bearing counterparts 1547C and 1924C (Fig. 6A, compare lanes 6 and 7 and lanes 4 and 5). These results are similar to the relative amounts of PTC-containing and -lacking RNAs observed within the cell (Fig. 5). The reduced amount of packaging of PTC-bearing RNAs relative to that of their control codon counterparts suggests that most of the genomic RNAs that RSV packages are first translated and then packaged as depicted in the model in Fig. 4A.

Some (19 to 30%) of the PTC-bearing viral RNAs were not degraded by the NMD pathway and were packaged into progeny virion particles. It is possible that this RNA was never translated, and that is why it was not degraded. If so, these RNAs represent the amount of genomic RNA that RSV packages without translation. Therefore, the remaining 70 to 80% of the non-PTC-bearing RNAs that are packaged represent RNAs that have been translated and subsequently packaged as genomes. We therefore conclude that most of the genomic RNAs that RSV packages have been translated previously, at least once. However, a small fraction of the genomic RNAs could be transcribed and packaged directly without being first translated.

DISCUSSION

This study focuses on two distinct events that occur within the cell regarding the metabolism of retroviral RNA. Our first finding is that unspliced RSV transcripts are subject to NMD,

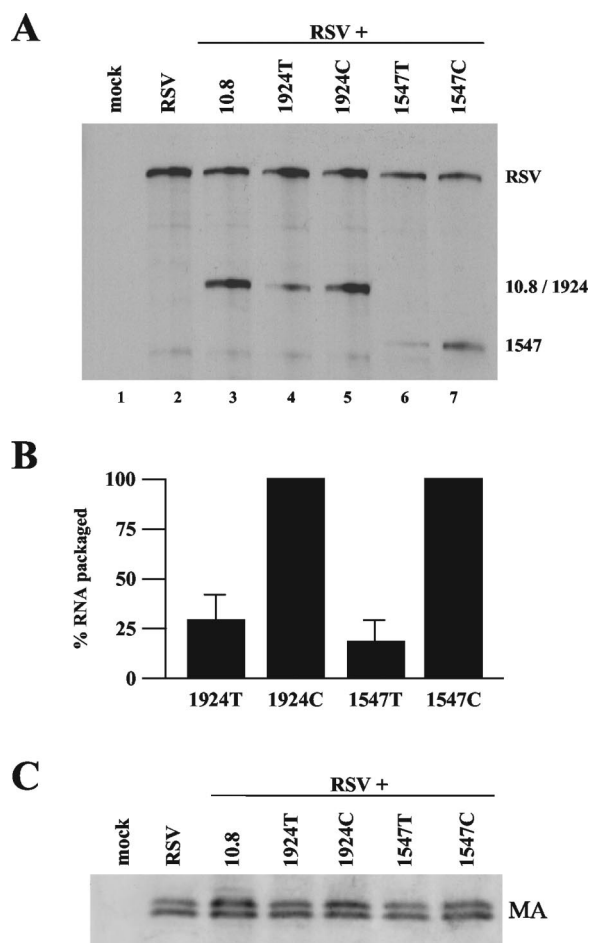


FIG. 6. RSV packages genomic RNAs that have been translated. (A) A representative RPA, using the *gag* probe, of RNA extracted from pelleted viral particles from the medium of the CEFs transfected as described for Fig. 5. (B) Bar graph quantification of RPA data from six independent experiments. 1924T and 1547T RNA levels were normalized to their control codon counterpart, set at 100%. Results are from six independently performed transfections. (C) Western blot analysis of viral particles, using antibodies targeted against the viral MA protein.

a process that was previously reported to require a splicing event in higher eukaryotes (48, 49). The second is that RSV uses the same unspliced RNAs for both translation and packaging. Since PTC-bearing RNAs are not packaged as frequently as non-PTC-bearing RNAs, we conclude that most packaged RNAs are first translated.

RSV unspliced RNA undergoes nonsense-mediated mRNA decay. The first half of the study examines the susceptibility of unspliced retroviral RNAs to NMD. We demonstrated that the decay requires Upf1, a protein known to be essential for NMD (18, 26, 27). Additionally, we showed that this decay was dependent upon translation, another required event in the NMD pathway (7, 12). This evidence is consistent with the NMD pathway being responsible for the decay of RSV RNA. However, it remains to be understood how this retroviral RNA is vulnerable to this decay, since NMD is thought to require a splicing event (31, 48, 49).

In mammalian systems, naturally intronless genes are im-

mune to NMD, and NMD is restored when an actively spliced intron is inserted into the gene (31). Further, splicing results in the deposition of an EJC upstream of every splice junction (19, 20). Translating ribosomes subsequently displace EJCs. The failure of the ribosome to remove EJCs bound to the transcript because of premature termination activates NMD (32). Analogously, it was previously reported that a PTC 165 nt upstream of the natural termination codon for the RSV *gag* gene caused decay while one only 30 nt upstream did not (5). If EJC components (or some other unknown NMD-promoting complex) are recruited to the unspliced RNA messages, at least one complex is likely located within this 135-nt region upstream of the natural termination codon.

Alternatively, the virus may utilize a novel pathway that does not require splicing but does require the activity of Upf1. Gatfield et al. (15) recently demonstrated that unspliced messages in *Drosophila* undergo NMD; further, this decay requires all three Upf proteins but does not require EJC components. Intronless HEXA minigenes transfected into Chinese hamster ovary cells are also susceptible to NMD (40). It was also found that PTCs within the *src* gene of RSV, introduced by frameshift mutations, caused down-regulation of *src* mRNA (42). This is particularly interesting since the *src* coding region, including its initiating AUG, lies completely downstream of the splice junction. These examples, as well as our data regarding unspliced RSV RNA, indicate that NMD may function by utilizing more than one specific recognition mechanism. The idea that Upf1, but not the EJC proteins, is involved is possible given that Upf1 has separable roles in the cell (33). Since RSV uses an unknown nuclear export pathway for unspliced RNAs (38), NMD-promoting factors that are recognized by Upf1 may be added during export rather than during splicing. It will be of interest to determine the composition and location of this NMD-promoting complex, as well as how Upf1 interacts with it.

In this study, and in most others, the effect of NMD is not absolute; PTC-bearing transcripts are not fully turned over (5, 26, 31, 40). There are several possible reasons for this. One possibility is that a fraction of the RNAs may not be translated, allowing them to escape decay. Second, the cellular site of NMD seems to vary from transcript to transcript. Some reports indicate that NMD occurs in association with the nucleus (23), while others indicate cytoplasmic NMD (5, 40). If NMD occurs in the cytoplasm, the examination of total cellular RNA levels will include nuclear RNA fractions that have yet to be subjected to NMD. Interestingly, it was reported that some RNAs bearing PTCs accumulate at the site of transcription in the nucleus (35). Third, the NMD pathway may be inefficient, allowing some PTC-bearing transcripts it detects to escape decay.

Genomic RSV RNAs are translated prior to packaging. The second half of this report employed the translational dependency of NMD to investigate whether retroviral genomic RNAs are translated prior to packaging. PTC-bearing RNAs were packaged into progeny virions at levels three- to fivefold less than those of non-PTC-bearing RNAs, indicating that the genomic RNAs were subjected to the NMD pathway and were therefore translated prior to packaging. Consistent with a previous report (44), we conclude that the RNAs used to package RSV virions come from the same pool of RNAs that are

translated to synthesize the structural and enzymatic viral proteins. Observations supporting this same idea have been described for HIV-1 (10, 11, 14). It should be noted that this conclusion is not universal and does not apply to all retroviruses, since MLV packages from two distinctly segregated pools (14, 21, 22).

In contrast to our findings, Sonstegard and Hackett (43) propose that RSV RNA is segregated into two populations, based on competition between Pr76^{gag} and ribosome binding to uORF3. However, a more recent report consistent with our findings dispels the relationship between packaging and translation. Banks and Linial (3) report that the translation of uORF3 plays a minimal role in packaging. They propose that the uORF3 initiation codon is critical for packaging but not for translation.

Parent and colleagues (41) provide evidence depicting a transient localization of the Gag precursor protein to the nucleus, suggesting that nuclear Gag-RNA interactions may exist in transformed quail cells. They report that blocking Gag entry into the nucleus interferes with proper packaging of genomic dimers. Since RSV RNAs are subject to NMD (this study) in the cytoplasmic fraction of CEFs (5), we suggest that they are translated before packaging. Primary CEFs may support viral packaging and localize Gag differently than transformed quail cells. Based on our results, we believe that unspliced RNAs are first exported to the cytoplasm, translated, and then made available for packaging. However, it is possible that Gag localization in the nucleus could result in direct packaging, thereby shielding the RNA from translational machineries.

Our study also provides some insight into *cis* versus *trans* packaging. Our study used packaging-deficient viral clones bearing NC deletions. These RNAs encode Gag proteins incapable of binding and packaging viral RNAs. However, coexpression of wild-type clones in CEFs yielded viral progeny with RNAs containing the NC deletion, demonstrating that *trans* packaging for RSV is very efficient (Fig. 6B). Kaye and Lever (16) present a contrary model for HIV-2, suggesting that RNAs are packaged cotranslationally, with *cis* packaging being favored.

Our evidence provides viral strategies that may serve to promote the production of error-free infectious particles. Since the retroviral reverse transcriptase is error prone (47), the introduction of mutations, including PTCs, into the unspliced transcripts is probable. Through the NMD pathway, the virus confers a selective advantage to RNAs without PTCs. As a result, this will increase the concentration of Gag proteins that become structural proteins in progeny virions. Second, requiring genomic viral RNAs to be translated prior to packaging provides a quality control mechanism for the virus to help promote successful infection. By packaging RNAs that have been translated and therefore subjected to NMD, the virus promotes the selection of genomic RNAs lacking gag PTCs that will be used to infect new host cells.

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